University of Ljubljana

Faculty of Electrical Engineering

Eva Pirc

High voltage pulse generation for electroporation based technologies and therapies

DOCTORAL DISSERTATION

Ljubljana, 2020

University of Ljubljana

Faculty of Electrical Engineering

Eva Pirc

High voltage pulse generation for electroporation based technologies and therapies

DOCTORAL DISSERTATION

Mentor: Matej Reberšek, Ph.D.

Ljubljana, 2020

Univerza v Ljubljani

Fakulteta za elektrotehniko

Eva Pirc

Generiranje visokonapetostnih pulzov za na elektroporaciji temelječe tehnologije in terapije

DOKTORSKA DISERTACIJA

Mentor: doc. dr. Matej Reberšek

Ljubljana, 2020

"It always seems impossible until it's done!"
Nelson Mandela

PREFACE

The presented Ph.D. thesis is a result of expert review, mathematical, system and statistical analysis, experimental investigations, development, prototyping related to a versatile electroporation device and electronic emulator and economical evaluation of electrochemotherapy. The work was carried out during the Ph.D. study period at Laboratory of Biocybernetics, Faculty of Electrical Engineering, University of Ljubljana, Slovenia. The results of the research work are presented in eight papers, which were published, accepted for publication, submitted, or in preparation for submission to international scientific journals and one book chapter.

Book chapter — E. Pirc, M. Reberšek and D. Miklavčič, **"Dosimetry in Electroporation-Based Technologies and Treatments"**, in *Dosimetry in Bioelectromagnetic*, Markov MS (Ed.), CRC Press, Boca Raton, Taylor & Francis Group, pp. 233-268, 2017.

Paper 1: — J. Dermol-Černe, E. Pirc and D. Miklavčič, **"Mechanistic view of skin electroporation – models and dosimetry for successful applications: an expert review"**, *Expert Opinion on Drug Delivery*, submitted.

Paper 2: — E. Pirc, M. Reberšek and D. Miklavčič, **"Functional requirements and quality assurance necessary for the successful incorporation of electroporation-based therapies into clinical practice"**, *Journal of Medical Devices*, vol. 14, no. 1, Mar. 2020.

Paper 3: — A. Cvetkoska, E. Pirc, M. Reberšek, R. Magjarevi adn D. Miklavčič, **"Towards standardization of electroporation devices and protocols"**, *IEEE Instrumentation Measurement Magazine*, accepted for publication.

Paper 4: E. Pirc, D. Miklavčič, and M. Reberšek, **"Electronic emulator of biological load during electroporation"**, *IEEE Transactions on Biomedical Engineering*, in preparation for submission.

Paper 5: E. Pirc, D. Miklavčič, and M. Reberšek, **"Nanosecond Pulse Electroporator with Silicon Carbide MOSFETs, Development and Evaluation"**, *IEEE Transactions on Biomedical Engineering*, vol.66, no.12, pp: 3526-3533, Dec. 2019.

Paper 6: E. Pirc, D. Miklavčič, and M. Reberšek, **"High frequency and high voltage asymmetricbipolar pulse generator for electroporation based technologies and therapies"**, *Biomedical Engineering Online*, in preparation for submission.

Paper 7: E. Pirc, L. Pecchia, M. Reberšek, G. Serša, M. Snoj, A. Grošelj and D. Miklvavčič, **"Study design of a medical device premarket assessment: a case study on electrochemotherapy"**, *Zdravniški Vestnik*, vol. 87, no. 1–2, Mar. 2018.

Paper 8: E. Pirc, C. Federici, M. Bošnjak, B. Perić, M. Reberšek, L. Pecchia, N. Glumac, M. Čemažar, M. Snoj, G. Serša and D. Miklavčič, **"Early cost-effectiveness analysis of electrochemotherapy as a prospect treatment modality for skin melanoma"**, *Clinical Therapeutics*, in preparation for submission.

Acknowledgements

I would like to thank all my co-workers from the Laboratory of Biocybernetics at the University of Ljubljana, especially the head of the laboratory at the time of my Ph.D., prof. dr. Damijan Miklavčič and my Ph.D. mentor doc. dr. Matej Reberšek, for support, guidance and the opportunity to meet the scientific world. Thank you Peter, Matej K., Janja, Lea, Bor, Samo, Saša, Tina and Aleksandra for stimulating discussions, comments and help during my Ph.D..

I would also like to thank dr. Leandro Pecchia and Carlo Federici from the University of Warwick, for sharing their knowledge and enabling the work done on the field of health technology assessment.

I would also like to thank dr. Maša Bošnjak, dr. Katja Uršič and other members of the Institute of Oncology Ljubljana from the Department of Experimental Oncology, who helped with the data collection for cost-effectiveness analysis of electrochemotherapy and performed the *in vivo* experiments of high-frequency electrochemotherapy.

At last, thank you, my family, for support, encouragement, and understanding during my long studies. Thank you, Miha for always standing by my side and all the help regarding 3-D printing and technical executions.

This work has been supported by the Slovenian Research Agency (ARRS) under a Junior Research grant and by research core funding (P2-0249, funding for Junior Researchers to Eva Pirc). Experiments were performed within the Infrastructure Program: Network of research infrastructure centers at the University of Ljubljana (MRIC UL IP-0510). Part of the work was supported by COST Action BM1309 (COST EMF-MED) with the Short Term Scientific Mission (ECOST-STSM-BM1309–110416–073660), performed at the University of Warwick. And in LABEX Sigma-Lim – XLIM, Faculté des Sciences et Techniques ABEX Sigma-Lim – XLIM, Faculté des Sciences et Techniques, Limoges, France in the scope of the ARRS bilateral project Comparison of Electroporation Using Classical and Nanosecond Electric Pulses on Excitable and Non-excitable Cells and Tissues, BI-FR/16-17-PROTEUS. The research was conducted in the scope of the European Associated Laboratory for Pulsed Electric Field Applications in Biology and Medicine (LEA EBAM).

Table of Contents

Abstract

The first observations of the effects of pulsed electric fields on biological material date more than 250 years back. But in the past two decades applications of electroporation emerged in medicine, food processing, and biotechnology. The phenomenon of electroporation leads to increased cell membrane permeability. Due to exposure to high voltage electric pulses, the cell membrane becomes permeable to molecules, which otherwise can not cross the membrane. A pulsed power generator termed as electroporator and electrodes are used to expose cells to a pulsed electric field. Electroporator generates electrical pulses with a specific shape, amplitude, duration, number, repetition rate and sequence (bursts of pulses). By applying electrical pulses to the electrodes, which are in direct contact with the tissue, an electric field is generated in tissue with specific distribution and intensity.

In order to properly understand the current situation and trends of development on the electroporation field, a thesis begins with a review of commercially available devices, their characteristics, limitations, and weaknesses. Also, expert opinion about the electroporation devices used in skin electroporation was provided. The electroporation device manufacturers are currently hindering the development of electroporation field by concealing the output pulse parameters of their devices, designing them in a way to disable output pulse measuring, specifying characteristics which device cannot deliver, and making devices that do not warn the user when pulse delivery fault occurs. The quality of the pulse delivery was evaluated, by checking if the delivered pulses were adequately addressed and measured in the electroporation studies, with the focus on the field of nanosecond electroporation, where the delivery and measuring are the most challenging. Additionally, recommendations for standardization, mainly focusing on the evaluation of electroporators proper or improper operation were proposed and electronic emulator of the biological load during electroporation, which enables constant and sustainable testing and unbiased comparison of different electroporators operation, was developed.

Secondly, we developed a high-frequency high voltage generator by using and improving the latest pulse generator designs. The developed 4 kV pulse generator, generates high voltage pulses with minimized switching time between positive and

negative pulse. The minimal pulse duration is 200 ns and maximal repetition rate 2 MHz, it is also able to generate asymmetrical bipolar pulses. The developed device was tested in first *in vivo* high-frequency electrochemotherapy (HF-ECT), in which HF-ECT with bleomycin and cisplatin was proved to be as effective as well established "classical" ECT with bleomycin and cisplatin.

Third, the cost-effectiveness of electrochemotherapy of stage IV and IIIc skin melanoma was calculated for patients treated at the Institute of Oncology in Ljubljana using the Cliniporator device and associated electrodes. The probability of ECT (with hospitalization) being cost-effective for the patient with stage IV and IIIc skin melanoma is just above 50 %, which implies the prices of the device and electrodes should be reduced for successful implementation into clinical practice. However, if patients have bleeding lesions the ECT can be assumed cost-effective (probability rises to 0.97).

Keywords: electroporation, standardization, electroporator design, cost-effectoveness analysis.

Razširjen povzetek v slovenskem jeziku

Seznam slik

Seznam uporabljenih simbolov

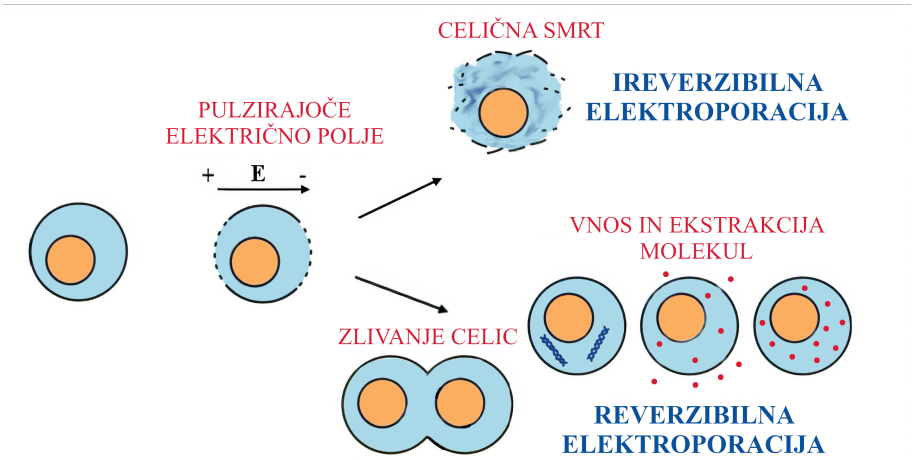
V doktorski disertaciji so uporabljene naslednje veličine in simboli:

Veličina / oznaka		**Enota**	
Ime	Simbol	Ime	Simbol
čas	t	sekunda	s
frekvenca	f	hertz	Hz
jakost električnega polja	E	-	V/m
tok	I	amper	A
napetost	U	volt	V
transmembranska napetost	V_{TN}	volt	V
dvižni čas	t_R	sekunda	s
čas padanja	t_F	sekunda	s
čas med polperiodama	t_{IPD}	sekunda	s
amplituda	A	-	-
časovna konstanta	τ	sekunda	s
dolžina pulza pri polovični amplitudi	t_{FWHM}	sekunda	s
impedanca bremena	Z_L	ohm	Ω
upornost	R	ohm	Ω
kapacitivnost	C	farad	F
induktivnost	L	henry	H
dolžina pulza	t_p	sekunda	s
napetost	V	volt	V
dielektričnost	ε	-	F/m
magnetna permeabilnost	μ	-	H/m

Ključne besede: elektroporacija, standardizacija, razvoj elektroporatorjev, analiza stroškovne učinkovitosti.

I Uvod

Elektroporacija je "temeljna" tehnologija [1], ki se je že dobro uveljavila v medicini [2], živilski industriji [3] in genski transfekciji. Tehnologija veliko obeta tudi na drugih področjih, kot sta proizvodnja biomase [4] in transdermalni vnos učinkovin v telo [5]. Ko celico izpostavimo visokonapetostnim električnim pulzom, se prepustnost celične membrane poveča. Ločimo med reverzibilno in ireverzibilno elektroporacijo: če se celična membrana po izpostavljenosti električnim pulzom povrne v prvotno stanje oziroma ohrani svoje fiziološko delovanje, gre za reverzibilno elektroporacijo; v primeru, ko se membrana ne povrne v prvotno stanje, se celična vsebina razlije in celica odmre, elektroporacija je takrat ireverzibilna [6, 7]. Elektroporacijo izkoriščamo za vnos večjih in manjših molekul v celico, ki v normalnih razmerah težko ali ne morejo prehajati skozi membrano, za ekstrakcijo posameznih snovi iz celice ter fuzijo oziroma zlivanje celic in povzročitev celične smrti (Slika I.1).



Slika I.1: Simbolični prikaz različnih načinov uporabe elektroporacije. Ko celico izpostavimo pulzirajočemu električnemu polju, se celična membrana polarizira, s tem pa se zviša njena prepustnost. Elektroporacija je lahko reverzibilna (vnos ali ekstrakcija molekul in zlivanje celic) ali ireverzibilna (celična smrt) [8].

Elektrokemoterapija je aplikacija, ki združuje vnos kemoterapevtika (v celice) z elektroporacijo, poveča se učinkovitost protitumorskega delovanja, odmerek kemoterapevtika pa je zato manjši in lokalno omejen [9]. Genska elektrotransfekcija omogoča nevirusni način vnosa DNA in se vse bolj uveljavlja na področju genskih cepiv in terapij [10]. Ireverzibilna elektroporacija (krajše IRE) se v medicini izkorišča

za netermično ablacijo malignega ali normalnega tkiva, celična smrt je v primeru ustrezne uporabe IRE posledica permeabilizacije celične membrane in ne povečanja temperature tkiva [11]. V biotehnologiji elektroporacijo poznajo pod imenom polzirajoče električno polje *(ang.: Pulsed Electric Field (PEF))* in jo izkoriščamo za ekstrakcijo posameznih snovi iz celice, prezervacijo živil (PEF ohrani barvo in okus živila ter ohranja nivo antioksidantov), podaljšanje roka uporabe predvsem tekočih živil [12] in kot tretma, ki olajša rezrez živil (za razrez živila, predhodno tretiranega s PEF, je potrebno manj sile, obenem je rez gladkejši; noži imajo daljšo življenjsko dobo, porabi se manj energije in tako npr. krompirček pri cvrtju vsrka veliko manj olja, kar pomeni dodaten prihranek).

I.I Elektroporacija kot fenomen

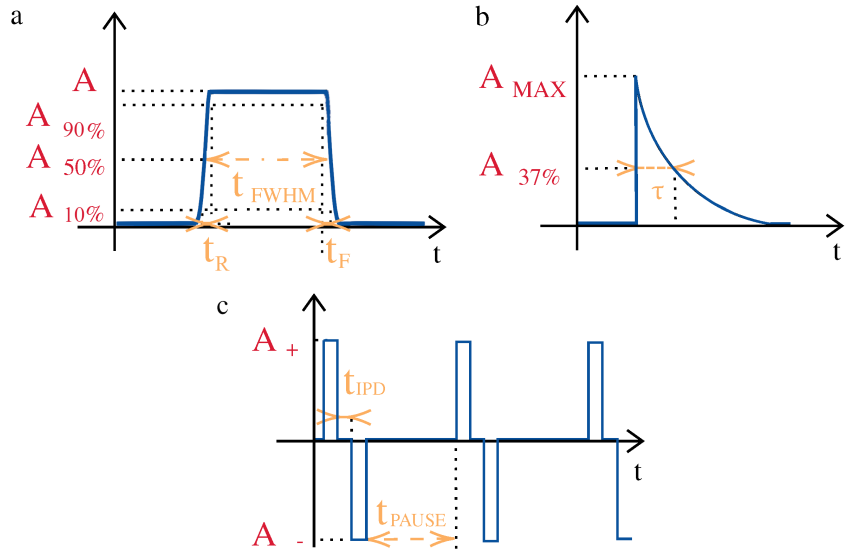
Elektroporacija je fenomen, pri katerem se zaradi izpostavljenosti pulzirajočemu električnemu polju celici poveča permeabilnost in prevodnost celične membrane. Vsaka biološka celica je obdana s celično membrano, ki jo sestavljajo predvsem lipidi, in sicer fosfolipidi, glikolipidi in holesterol. Ko lipide obdamo z vodno raztopino, zaradi svojih dielektričnih lastnosti, spontano izoblikujejo lipidni dvosloj, skozi katerega večje molekule, voda in njene raztopine ne morejo prosto prehajati [13]. Poleg lipidov se v membrani nahajajo tudi druge molekule, ki omogočajo selektivni transport v celico in iz nje. V zadnjih letih se je pojavilo kar nekaj teoretičnih razlag elektroporacije. Med vsemi se je najbolj uveljavila in se zdi najbolj verjetna tista teorija, ki temelji na izoblikovanju vodnih por v celični membrani. Ko celico izpostavimo visokonapetostnim električnim pulzom, se na membrani inducira napetost, ki sproži reorganizacijo celične membrane in formiranje hidrofilnih por. S tem je omogočen ionski in molekulski transport molekulam, ki drugače ne morejo prosto prehajati skozi membrano [14]. Predlagana teorija še ni bila eksperimentalno dokazana, vendar molekularna dinamika nudi prepričljivo potrditev [15].

Za lažje razumevanje procesa elektroporacije celico modeliramo kot elektrolit (prevodni medij), ki je obdan z dielektrično plastjo. Dielektrična plast predstavlja lipidni dvosloj, ki ima lastnosti izolatorja. Vsaka celica ima mirovni transmembranski potencial med -90 in -40 mV, ki je posledica negativno nabite citoplazme [16]. Celico izpostavimo električnemu polju tako, da preko elektrod na celično suspenzijo ali tkivo dovedemo električne pulze ustrezne jakosti in trajanja. Zaradi nizke prevodnosti membrane se električno polje skoncentrira predvsem v celični membrani, ki se polarizira, na njen pa se vzpostavi transmembranska napetost V_{TN}, ki je vsota mirovnega potenciala in pritisnjene napetosti. Napetost V_{TN}

je pogojena z obliko celice ter položajem oziroma orientacijo elektrod. Časovna konstanta celične membrane je v milisekundnem območju [17], torej, ko celico izpostavimo krajšim pulzom (nanosekundno območje), se celična membrana obnaša kot kondenzator. Posledično se pritisnjena napetost pojavi tudi v notranjosti celice, kar pomeni, da jo lahko izkoriščamo za znotrajcelično manipulacijo [18].

I.II Dozimetrija pri elektroporaciji

Lokalno električno polje,je tisto polje, ki ga celica "čuti" in je poglavitni razlog za elektroporacijo celične membrane. Porazdelitev električnega polja in intenziteta sta pogojeni s tipom aplikatorja oziroma s geometrijo elektrod in karakteristiko dovedenih električnih pulzov. Zato se za različne aplikacije uporablja različne oblike, amplitude, trajanja, ponavljalne frekvence in sekvence električnih pulzov. Ustrezen opis elektroporacijskih pulzov je ključen za pravilno poročanje in razumevanje elektroporacijskih študij [8].



Slika I.2: Opis kvadratnih (a), eksponentno padajočih (b) in bipolarnih (c) elektroporacijskih pulzov.

Eksponentno padajoče pulze definiramo z maksimalno vrednostjo A_{MAX} in časovno konstanto τ. Vrednost časovne konstante je pogojena s karakteristiko izhodne stopnje in je definirana kot čas, v katerem amplituda pade na 37 % A_{MAX} (Slika I.2b). Kvadratne pulze opišemo z amplitudo (tj. tista vrednost, ki se najbolje približa vsem točkam v visokemu nivoju) in dolžino pulza. Dolžina pulza pa

je definirana kot FWHM *(ang.: Full Width at Half Maximum)*. FWHM je čas, ki preteče, medtem ko amplituda doseže 50 % vrednosti v fazi vklopa in ponovno pade na 50 % A_{MAX} v fazi izklopa. Pri elektroporacijskih pulzih ponavadi podamo še dvižni čas t_R *(ang.: rise time)* in čas padanja t_F *(ang.: fall time)*. Dvižni čas je definiran kot čas, pri katerem pulz naraste od 10 % do 90 % amplitude. Za čas padanja pulza pa velja ravno obratno- amplituda pade od 90 % do 10 % amplitude (Slika I.2a).

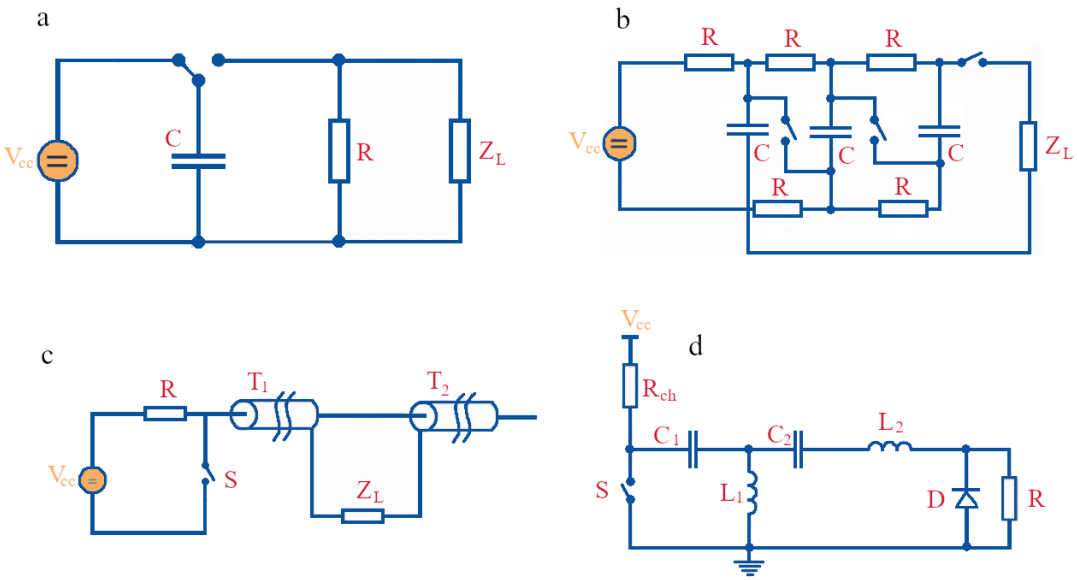
V zadnjem času se na področju elektroporacije vse bolj uveljavlja uporaba bipolarnih pulzov, zato ker področje občutljivosti, preklica in vzbujalnih učinkov elektroporacijskih pulzov še ni dovolj dobro raziskano. Učinek občutljivosti je definiran kot dvig oziroma upad vnosa molekul v celico v primeru apliciranja dvakratne namesto enojne polovične doze. Pri uporabi krajših pulzov, dolgih nekaj sto nanosekund, so odkrili, da pride do preklica bioloških učinkov [19, 20, 21, 22]. Efekt preklica je definiran kot upad vnosa v celico, ko to izpostavimo enemu monopolarnemu pulzu, ki mu skoraj strnjeno sledi drugi monopolarni pulz nasprotne polaritete (torej gre za en bipolarni pulz), v primerjavi z enim samim monopolarnim pulzom. Vsako polperiodo bipolarnega pulza opišemo enako kot kvadratni elektroporacijski pulz, čas med polperiodoma imenujemo t_{IPD} *(ang: Inter Pulse Delay)*, pavzo med dvema bipolarnima pulzoma pa opredelimo s t_{PAUSE}.

Posebej zasnovane naprave, ki se uporabljajo za generiranje elektroporacijskih električnih pulzov, imenujemo elektroporatorji. Ker se električne karakteristike bioloških bremen močno razlikujejo, poleg tega se tudi njihova prevodnost zaradi elektroporacije med dovajanjem pulzov spreminja, razvoj elektroporatorjev proizvajalcem predstavlja velik izziv [23, 24, 8].

I.II.I Generiranje električnih pulzov

Elektroporator je naprava, ki je zgrajena iz visokonapetostnega pulznega generatorja, merilnega sistema in zaščite. Obstaja več vrst elektroporatorjev, ki jih ločimo glede na karakteristike generiranih električnih pulzov in namen uporabe. Generiranje eksponentnega pulza je precej enostavno, potrebujemo le kondenzator in vir električne napetosti (Slika I.3a). Kondenzator priključimo na želeno napetost, ga napolnimo in s tem določimo maksimalno amplitudo pulza. Nato odklopimo vir in priključimo breme Z_L, skozenj se kondenzator začne prazniti. Vsa energija, ki jo želimo prenesti na breme, mora biti že pred preklopom shranjena v kondenzatorju. Napetost na kondenzatorju pada eksponentno, kondenzator se tako izprazni v času 5τ, vendar ne smemo pozabiti, da se impedanca biološkega bremena med

dovajanjem pulza niža, kar pomeni, da se spreminja vrednost τ. Torej je dolžina generiranega pulza odvisna od karakteristik posameznega biološkega bremena. Za zmanjšanje omenjenega vpliva dodamo bremenu še soupor, ki pa mora imeti (vsaj 10-krat) nižjo vrednost od bremena Z_L. Vendar ne smemo pozabiti, da se na dodanem uporu lahko porabi tudi do 90 % energije. V kolikor želimo generirati kvadratne pulze, potrebujemo večje kondenzatorje, ki jih nato le delno izpraznimo. Problem lahko rešimo z vzporedno vezavo večih kondenzatorjev in pripadajočih preduporov - tako sestavimo Marxov generator (Slika I.3b). Vsi kondenzatorji se tako hkrati napolnijo skozi upore in ob sklenitvi stikala hkrati izpraznijo skozi breme Z_L. Vsa stikala morajo preklopiti hkrati, na breme se tako prenese maksimalna dovedena napetost, pomnožena s številom kondenzatorjev. Vlogo stikalnih elementov pri elektroporatorjih v večini prevzamejo močnostna MOSFET ali IGBT stikala. Paziti moramo, da je padec napetosti na stikalu pri prenosu čim manjši, enako velja tudi za breme, kar pomeni, da mora biti kapacitivnost kondenzatorja čim večja. Polnjenje kondenzatorja z veliko kapacitivnostjo pa je dolgotrajno, kar nas dodatno omejuje, saj novega pulza ne moremo generirati, dokler kondenzator ni povsem poln. [8, 23, 25].



Slika I.3: Štirje osnovni koncepti vezij za generiranje elektroporacijskih pulzov.

Za generiranje krajših pulzov v nanosekundnem območju se uporablja drugačne rešitve, kot sta Blumline generator (Slika I.2c) in generator z diodno odpiralnim stikalom (Slika I.2d). Kot pri ostalih vezavah tudi tukaj obravnavamo delovanje

v dveh fazah. V fazi polnjenja se skozi upor R polni valovna linija, ki se nato po preklopu izprazni preko bremena Z_L. Impedanca valovodne linije Z_0 mora biti enaka oziroma čim bolj podobna impedanci bremena Z_L. Z_0 je enaka $(L/C)^{0.5}$, kjer je C kapacitivnost in L induktivnost, na 1 m dolžine valovodne linije. Dolžina pulza (t_p) je tako enaka $2l(\varepsilon\mu)^{0.5}$, kjer l predstavlja dolžino linije, ε dielektričnost in μ magnetno permeabilnost izolatorja v valovodni liniji. Amplituda izhodnega pulza je enaka napajalni napetosti ($V_{izh} = V_{vh}$). Glavna slabost Blumline generatorja je nizka ponavljalna frekvenca pulzov (do 10 kHz, ker se generator polni preko nizkoprevodnega upora) in konstantna dolžina pulza, ki je odvisna od dolžine valovodne linije. V kolikor pa vežemo dve stikali na koncu valovodnih linij, lahko nadgradimo Blumlein-ov generator v Blumleinov generator z variabilno dolžino pulza [26].

Generatorji z diodnim odpiralnim stikalom *(ang.: Diode Opening Switch - DOS)*, imajo zelo hiter cikel polnjenja in praznjenja, kar pomeni, da omogoča generiranje večjega števila pulzov v krajšem času. Pulz oblikuje s pomočjo polprevodniške diode, ki ima dolgo, a na koncu naglo povratno okrevanje. Gre za ne prav pogosto uporabljeno polprevodniško diodo, ki jo uporabimo kot nabojno krmiljeno stikalo *(ang.: charge controlled switch)*. Njena posebnost je, da je sposobna zelo hitro preklopiti in delovati pri relativno velikih močeh [27, 28, 29].

I.II.II Dovajanje električnih pulzov, merjenje in standardizacija

Pri elektroporaciji je za zagotavljanje kakovosti poskusov in terapij ključnega pomena merjenje. A v zadnjem času se povečuje število neuspešnih ponovitev objavljenih študij [30]. Opisi uporabljene opreme in metod so velikokrat pomanjkljivi ali napačni. V številnih prispevkih s področja elektroporacije raziskovalci ne poročajo dovolj podrobno, da bi lahko druge skupine ponovile ali nadgradile njihove študije [18, 31]. Eden izmed glavnih problemov je, da raziskovalci ne nadzorujejo dovajanja pulzov in posledično o meritvah pogosto sploh ne poročajo. Na trgu pa je na voljo vse več elektroporacijskih naprav, večnamenskih ali zasnovanih za posebne aplikacije. Zdi se, da bolj kot raste trg, oziroma se povpraševanje na trgu povečuje, manj izvemo o napravah s strani proizvajalcev. Nekatere so namerno izdelane tako, da ne omogočajo merjenja. Na področju genske elektrotransfekcije, kjer se najpogosteje uporabljajo vnaprej programirani postopki elektroporacije, smo že dosegli kritično točko. Raziskovalci pogosto ne poznajo niti osnovnih parametrov električnih pulzov, kot so oblika, dolžina, ponavljalnja frekvenca pulza, še manj pa je znanega o jakosti električnega polja. Raziskovalci poznajo samo zaporedno številko izbranega programa, ki je nameščen na njihovo napravo. Torej je prehajanje med

napravami različnih proizvajalcev skoraj nemogoče. Omejevanje ključnih informacij o karakteristikah električnih pulzov ovira razvoj novega znanja, onemogoča primerjavo rezultatov ali protokolov ter zavira celoten razvoj področja genske elektrotransfekcije. Kakovostno elektroporacijo je mogoče zagotoviti le z ustreznimi meritvami, saj elektroporatorji ne delujejo vedno v skladu s tehničnimi specifikacijami. Na tem področju še ni nikakršne regulative, ki bi zagotavljala in urejala kakovost elektroporacijskih naprav.

Merjenje električnih pulzov in ovrednotenje generiranega električnega polja predstavlja poseben izziv pri uporabi zelo kratkih nanosekundnih (od 1 do 20 ns) elektroporacijskih pulzov zaradi končne hitrosti potovanja pulzov (20 cm/ns) in s tem povezanih odbojev pulzov. Nanosekundni elektroporatorji so običajno sestavljeni iz generatorja nanosekundnih pulzov in valovodne linije ali mreže za oblikovanje pulzov *(ang.: Pulse Forming Network (PFN))* in sistema za dovajanje elektroporacijskih pulzov, kot so različne komore ali elektrode [32, 33, 18]. Nanosekundni pulzi potujejo približno 20 cm/ns v koaksialnem kablu. Torej je potrebno pri uporabi nanosekundnih pulzov vedno upoštevati, da se pulzi ob spremembi impedance odbijejo oziroma se njihova moč v prenosni liniji zniža. Skratka, ko ujemanje impedance ni zagotovljeno, so odboji prisotni in odvisni od bremena. Če je impedanca bremena večja, so odboji pozitivni in se prištevajo k amplitudi. V primeru nižje impedance bremena se amplituda zaradi odbojev zniža. Pulzi lahko zaradi odbojev postanejo bipolarni, kar pa lahko privede do efekta preklica biološkega učinka [18].

I.III Uporaba elektroporacije v medicini

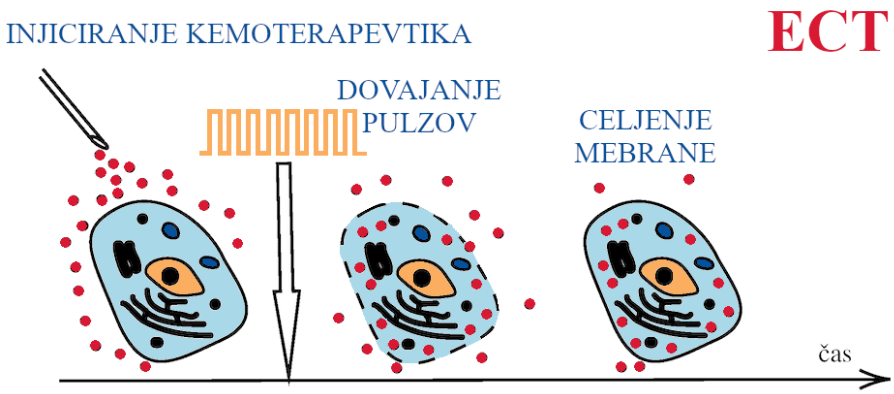
Ker je tkivo zelo nehomogen prevodnik, pri *ex vivo* ali *in vivo* elektroporaciji tkiv električnega polja ne obravnavamo več kot homogenega. Nekateri biološki materiali so anizotropni, zato je potrebno upoštevati tudi smer električnega polja. Nehomogenost tkiv se razlikuje od tkiva do tkiva in je odvisna od frekvence (pri višjih frekvencah je nižja). Ker se med elektroporacijo spremeni prevodnost biološkega bremena, se sočasno spreminja tudi porazdelitev električnega polja [34]. Tumorji imajo zaradi celične nekroze večinoma višjo vsebnost vode [35]. Pri elektroporaciji tkiva moramo torej dobro poznati značilnosti posameznega tkiva, najpogosteje in najenostavneje tkivo opišemo s specifično prevodnostjo in relativno dielektričnostjo. Vsiljena napetost se porazdeli po tkivu, največji padec napetosti pa je na najmanj prevodnem delu tkiva, ki je v primeru elektroporoacije z neinvazivnimi elektrodami ponavadi koža, saj je njena prevodnost kar 10–100-krat nižja od prevodnosti tkiva, ki se nahaja pod njo. Zato je potrebno dodatno pozornost posvetiti neželenemu rezis-

tivnemu gretju, ki lahko v primeru prevelikega padca napetosti poškoduje zdrave celice [36, 37]. Za določitev lokalne porazdelitve električnega polja znotraj tumorjev ali tkiva se zato uporabljajo različne numerične metode [38, 39]. V primeru elektroporacije globoko ležečih tumorjev in tumorjev v notranjih organih, ki so obdani s tkivi, ki imajo različne električne lastnosti, je potrebno terapijo vnaprej individualizirano začrtati [40]. Posamezni tumorji se prav tako razlikujejo po obliki, velikosti in lokaciji. Vsak tumor se zato analizira individualno in na podlagi analize optimizira položaj igelnih elektrod ter karakteristiko dovedenih električnih pulzov [39, 41, 42]. Terapija bo uspešna le, če bo celoten tumor izpostavljen ustreznemu električnemu polju [35, 43], kar pa je trenutno mogoče zagotoviti s pomočjo numeričnega modeliranja distribucije električnega polja [44]. Takšni izračuni električnega polja temeljijo na podlagi realnih vhodnih podatkov. V določenih primerih se uporablja tudi slikovno vodeno vstavljanje elektrod [38, 45, 46]. V primeru ireverzibilne elektroporacije pa je pri načrtovanju terapije potrebno upoštevati tudi izračune povečanja temperature [47]. Tudi pri *in vivo* elektroporaciji je za zagotavljanje kakovosti terapije potrebno meriti in s tem nadzorovati dovedene električne pulze. Večinoma merimo napetost in tok dovedenih pulzov, v raziskovalne namene pa se uporablja tudi elektroimpedančna tomografija (EIT), vendar nam nobena od teh meritev ne poda informacije o porazdelitvi električnega polja znotraj tkiva. Medtem ko MR-EIT (magnetnoresonančna električnaimpedančna tomografija) omogoča rekonstrukcijo porazdelitve električnega polja z uporabo MR slikanja in numeričnih algoritmov, tako lahko izmerimo porazdelitve gostote električnega toka in električne prevodnosti med elektroporacijo [48]. Generiranje homogenega električnega polja znotraj heterogene strukture tkiv še vedno predstavlja velik izziv. Porazdelitev električnega polja je predvidoma možno izboljšati z uporabo visokonapetostnih, visokofrekvenčnih, bipolarnih električnih pulzov.

I.III.I Elektrokemoterapija

Elektrokemotrapija ali krajše ECT *(ang.: Electrochemotherapy (ECT))*, vodilna aplikacija v medicini na področju elektroporacije, je antitumorska terapija, pri kateri celice oziroma tkivo izpostavimo visokonapetostnim električnim pulzom, ki permeabilizirajo celično membrano in s tem omogočijo vnos že prej vbrizganega (intratumorsko ali intravenozno) kemoterapevtika (bleomicin ali cisplatin) v celico in s tem zvišajo njuno toksičnost [49, 50] (Slika I.4). Permeabilizirane celice so v primeru ustrezne porazdelitve električnega polja večinoma tumorske celice. Prav tako je potrebno za uspešno terapijo zagotoviti dovolj visoko koncentracijo

vbrizganega kemoterapevtika v tumorju [51]. ECT je zelo učinkovita terapija, saj z enim samim tretmajem doseže med 50 in 70 % popolnih odzivov in 80 % objektivnih odzivov [52]. ECT je torej primerljiva, če ne celo bolj učinkovita kot standardne terapije kožnega raka [53]. V večini se uporablja v skladu s standardnim operativnim postopkom *(ang.: Standard Operating Procedures (SOP))* za zdravljenje kožnih in podkožnih tumorjev oziroma metastaz, ki je bil vzpostavljen leta 2006 [54]. Ker SOP predpisuje priporočila le za obravnavo manjših kožnih tumorjev ($<$ 3 cm premera), je bil leta 2018 nadgrajen. Posodobitev vsebuje smernice za zdravljenje primarnih in metastatskih tumorjev kože tudi večjih od 3 cm [55]. Britanski nacionalni inštitut za odličnost v zdravstvu in oskrbi *(ang.: National Institute for Health and Care Excellence (NICE))* je prepoznal ECT kot sestavni del multidisciplinarnega zdravljenja bolnikov s kožnimi metastazami, ki niso kožnega izvora, in melanomom (IPG 446, http://www.nice.org.uk/guidance/ipg446). Prav tako je v slovenskih smernicah za zdravljenje kožnega melanoma ECT eden od možnih načinov zdravljenja pri recidivih na ekstremitetah, kjer ni možna preprosta ekscizija ($>$ 3 - 5 zasevkov), ali pri zelo hitro ponavljajočih se recidivih (prej kot v 3 - 6 mesecih) [56]. V zadnjih letih pa se vse več pozornosti posveča tudi uporabi ECT za zdravljenje globoko ležečih tumorjev, predvsem metastaz v jetrih, zlasti kadar se nahajajo v bližini večjih žil in zato posledično niso primerne za odstranitev v sklopu kirurškega posega [9].

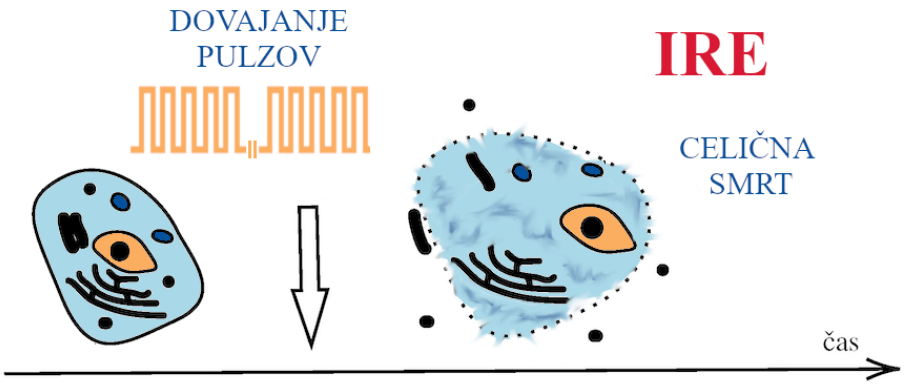


Slika I.4: ECT: Po injiciranju kemoterapevtika (8 - 28 minut) dovedemo elektroporacijske pulze, ki elektroporirajo celično membrano in s tem omogočijo transport kemoterapevtika v celico, kar poveča toksičnost in učinkovitost terapije.

Parametri električnih pulzov, optimizirani *in vitro*, se preslikajo tudi v optimalne

parametre *in vivo*. Trenutno je pod imenom "klasični" elektroporacijski pulzi definirana sekvenca osmih monopolarnih pravokotnih pulzov, dolžina vsakega pulza je 100 μs, ponavljalna frekvenca pa 1 Hz ali 5 kHz. Prednosti višje ponavljalne frekvence so krajše trajanje elektroporacije, občutek samo ene aplikacije električnih impulzov in enkratno krčenja mišic. Ker se mišično krčenje pojavi le enkrat, se izognemo tudi neželenemu premiku elektrod med samo sekvenco. Prav tako bolniki poročajo, da je bolečina v primeru 5 kHz manjša kot pri 1 Hz [57, 58]. Amplituda napetosti je najpogosteje nekje med 200 in 1000 V. Odvisna je od elektrod in ciljnega tkiva, zagotoviti pa mora, da je električno polje med elektrodama višje od 400 V/cm. ECT skoraj nima stranskih učinkov, po terapiji se lahko pojavi lokalna bolečina, ki zbledi v nekaj dneh. Do večjih zapletov pa lahko pride, če dovedeni elektroporacijski pulzi zmotijo ritem srčne mišice. Pri elektroporaciji kože je to skoraj nemogoče oziroma je zelo malo verjetno, vendar se verjetnost poveča pri tretiranju globoko ležečih tumorjev, ki se lahko nahajajo tudi v bližini srca. V kolikor je amplituda dovedenih električnih pulzov višja od mejne vrednosti fibrilacije ali če so električni pulzi dovedeni med pozno atrijsko ali ventrikularno sistolo, lahko zmotijo srčni ritem. Rizično območje za ventrikle je v bližini T-vala, za atrije pa v bližini S-vala [59]. Zato je za zmanjšanje tveganja dovajanje elektroporacijskih pulzov sinhronizirano s srčnim ritmom [60].

I.III.II Ireverzibilna elektroporacija



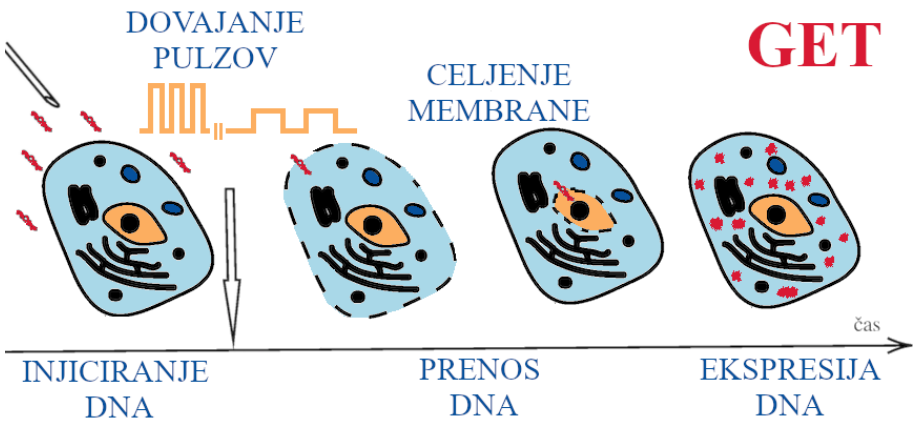
Slika I.5: Pri IRE celici dovedemo elektroporacijske pulze, ki povzročijo celično smrt, domnevno odmrejo zaradi izgube homeostaze.

Ireverzibilna elektroporacija (krajše IRE) se uporablja za "netermično" ablacijo tkiv in tumorjev. Pri IRE celice domnevno odmrejo zaradi izgube homeostaze (Slika I.5) in ne zaradi dviga temperature [61], kljub temu pa se lahko temperatura v bližini

elektrod z uporabo višjih napetosti in večjega števila pulzov znatno poviša [62, 63]. Pri IRE se srečamo s skoraj enakimi izzivi kot pri ECT, za uspešno terapijo mora biti celotno tumorsko tkivo izpostavljeno ustrezni jakosti električnega polja, ki mora biti višja, kot je prag ireverzibilne elektroporacije. Poleg tega je treba paziti, da je elektroporacija zdravega tkiva čim manjša, saj se s tem izognemo nepotrebnim termičnim poškodbam. Uporablja se predvsem v kliničnih študijah za zdravljenje globoko ležečih tumorjev, operativno ali perkutano v jetrih, trebušni slinavki, ledvicah, pljučih in drugih organih [11]. IRE nima SOP, različne raziskovalne skupine uporabljajo različne protokole, ki se od vrste tumorjev in stopnje rakavega obolenja nekoliko razlikujejo. Za vsak posamezen tumor je potrebno izdelati individualni načrt zdravljenja. IRE je lahko tudi minimalno invaziven v kombinaciji z ultrazvokom, računalniško tomografsko usmeritvijo ali slikanjem z magnetno resonanco [64]. Pri IRE je varnost še pomembnejša kot pri ECT, saj se ponavadi tretira veliko več tkiva (tudi do 50 cm^3), hkrati je število dovedenih pulzov veliko večje (ponavadi dovedemo vsaj 90 pulzov) [65]. Za prečkanje praga IRE mora biti električna poljska jakost veliko višja kot pri ECT, tipično imajo pulzi amplitude tudi do 3000 V in dosegajo tokove tudi do 50 A. Zaradi višjih električnih polj in operacije, ki je lahko blizu srca, morajo biti elektroporacijski pulzi sinhronizirani z refraktarno dobo srčnega ritma [66]. Čas terapije je v primerjavi z referenčnimi postopki izjemno kratek - traja le nekaj minut. Dejanski čas je mogoče izračunati iz števila apliciranih pulzov in povprečnega srčnega utripa. "Klasična" IRE temelji na uporabi monopolarnih električnih pulzov, ki jih običajno spremlja signifikantna mišična kontrakcija, zato se za zmanjšanje te uporablja živčno-mišična zaviralna sredstva, prav tako so pacienti v splošni anasteziji. Toda pred kratkim so v seriji večih študij avtorji pokazali, da se lahko z uporabo visokofrekvenčnih bipolarnih sekvenc pulzov, t. i. H-FIRE *(ang.: High-Frequency IrReversible Electroporation)*, izognemo neželeni mišični kontrakciji. Arena et al. so z uporabo H-FIRE pulzov ponavljalne frekvence 250 ali 500 kHz dosegli enake rezultate ablacije in natančnosti kot pri "klasični" IRE [67].

I.III.III Genska elektrotransfekcija

Genska elektrotransfekcija je obetavna nevirusna metoda vnosa genov [68]. Uporablja se za zdravljenje rakavih in drugih genskih obolenj [69] ter za DNK cepljenje [70]. *In vitro* pa se električni impulzi pogosto uporabljajo za transfekcijo bakterijskih in evkariontskih celic [71]. Metoda vnosa genov z elektroporacijo se uspešno uporablja že od leta 1982, vendar so natančni molekularni mehanizmi



Slika I.6: Najprej se vbrizga DNK, nato (0,5-2 minute) dovede elektroporacijske pulze, ki sprožijo interakcijo med permeablizirano celično membrano in plazmidno DNK. Tvori se DNK-membranski kompleks, DNK se s še neznanim postopkom prenese v citoplazmo in nato v jedro. V primeru uspešne transfekcije pride do ekspresije transfeciranih genov.

prenosa DNK še vedno neznanka [68, 72]. Do prenosa DNK lahko pride le v primeru reverzibilne elektroporacije, saj mrtve celice ne morejo izražati transfeciranih genov. Osnovni koncept ostaja enak, najprej se lokalno vbrizga DNK, nato pa dovede elektroporacijske pulze (to so ponavadi daljši pulzi v milisekundnem območju, nižjih napetosti (nekaj 100 V)), kar sproži interakcijo med permeabilizirano celično membrano in plazmidno DNK. Tvori se DNK-membranski kompleks, DNK se s še neznanim postopkom prenese v citoplazmo in nato v jedro. V primeru uspešne transfekcije pride do ekspresije transfeciranih genov (Slika I.6) [73]. V sklopu nekaterih študij so raziskovalci pokazali, da se genska transfekcija poveča, če dovedemo kratke visokonapetostne in dolge nizkonapetostne pulze v sklopu ene sekvence. Visokonapetostni pulzi so ključni za permeabilizacijo celične membrane in tvorbo pore, medtem ko nizkonapetostni pulzi elektroforetsko vlečejo negativno nabito DNK v celico. V eni od študij predlagajo uporabo osmih visokonapetostnih pulzov dolžine 100 μs z amplitudo 1300 V/cm, ki jim sledi en daljši 100 ms dolg nizkonapetostni pulz 100 V/cm [74], kasneje pa je skupina prof. Miklavčiča pokazala, da kratki visokonapetostni pulzi niso samo ključni, ampak tudi zadostni za uspešen vnos DNK v primeru optimalne koncentracije plazmidne DNK. Vendar, kljub temu mislijo, da je elektroforetska sila nizkonapetostnih pulzov ključna pri *in vivo* aplikacijah, saj smo omejeni s koncentracijo plazmida [68]. Parametri elektroporacijskih pulzov so odvisni od vrste antigena tumorja in ciljnih tkiv, ciljne celice v specifičnem tkivu pa so različne [72]. Različne raziskovalne skupine

uporabljajo različne pulze, v nekaterih študijah so pokazali, da sprememba smeri električnega polja oziroma orientacije dovajanja pulzov poveča ekspresijo, saj do vnosa DNK pride le v delu membrane, ki je obrnjen proti katodi [75, 73]. V zadnjih nekaj letih pa se na področju genske elektrotransfekcije vse več pozornosti posveča tudi nanosekundni elektroporaciji [76]. Z večjimi pulzi dosežemo večjo ekspresijo, a hkrati manjše preživetje, medtem ko je pri nižjih pulzih preživetje višje, ekspresija pa nižja.

I.IV Vrednotenje novih tehnologij v medicini

Svetovna zdravstvena organizacija (WHO) definira vrednotenje novih tehnologij v medicini *(ang.: Health Technology Assessment (HTA))* tako: "HTA se nanaša na sistematično vrednotenje lastnosti, učinkov in/ali vplivov zdravstvene tehnologije. Je multidisciplinarni postopek za vrednotenje družbenih, ekonomskih, organizacijskih in etičnih vprašanj zdravstvene intervencije ali inovativne tehnologije. Glavni namen ocenjevanja je informiranje strategov in odločevalcev." [77]. Ocenjuje oziroma evalvira se sisteme, splošno opremo, instrumente, strojno in programsko opremo ter postopke, standarde, norme, kadrovske veščine, strokovno znanje, zdravila, javne zdravstvene programe ipd. HTA se trenutno uporablja večinoma za farmacevtske izdelke, vendar se predvsem v tujini počasi uveljavlja tudi na področju biomedicinskih naprav, za kar pa je potrebno obstoječe metode nekoliko modificirati [78]. Nova zdravila se lahko vpelje v klinično prakso, ko proizvajalci pridobijo zadovoljive dokaze o njihovi učinkovitosti, medtem ko je za uporabo medicinske naprave potrebno dokazati le, da je za uporabo varna, ne pa tudi učinkovita. Rezultat HTA zagotavlja informacije o stroških/ekonomski učinkovitosti, prihrankih, uspešnosti, varnosti, etičnih in družbenih vplivih in izboljšavah zdravljenja/zdravil. Če je analiza uspešno zaključena, mora odgovoriti na vprašanje, ali resnično potrebujemo to tehnologijo in zakaj. Formalni dokaz HTA je mogoče najti v sistematičnih pregledih, metaanalizah in kliničnih študijah [79]. S pomočjo odločitvene multikriterijske analize lahko na podlagi kakovosti prilagojenih življenjskih let *(ang.: Quality Adjusted Life Years (QALY))* ocenimo stroškovno učinkovitost nove terapije ali tehnologije. Ena od pogosto uporabljenih analiz je analiza stroškovne učinkovitosti *(ang.: Cost-Effectiveness Analysis (CEA))*; njen osnovni koncept in načela so predstavljeni v naslednjem podpoglavju. V zadnjih letih pa se vse več pozornosti posveča zgodnjemu vrednotenju novih tehnologij v medicini (eHTA) *(ang.: early Health Technology Assessment (eHTA))*, ki zajema primere z omejenimi kliničnimi dokazi in na podlagi

teh napoveduje s pomočjo statističnih metod [80, 81]. Stroški, vključeni v eHTA, se ocenjujejo za najslabši primer (najvišji strošek), negotovost pa se količinsko določi s pomočjo statističnih tehnik. Rezultat eHTA obvešča nosilce odločanja o tveganju in priložnostih, pri čemer tveganje predstavlja potencialni strošek, priložnost pa potencialno učinkovitost. eHTA predvideva, da bo HTA narejena, ko bo na voljo zadostna količina podatkov. Njen glavni doprinos pa je, da omogoča hitrejšo implementacijo medicinskih pripomočkov ali naprav, ki lahko potencialno rešijo življenje ali znatno izboljšajo kakovost življenja, v klinično prakso.

I.IV.I Stroškovna analiza v medicini

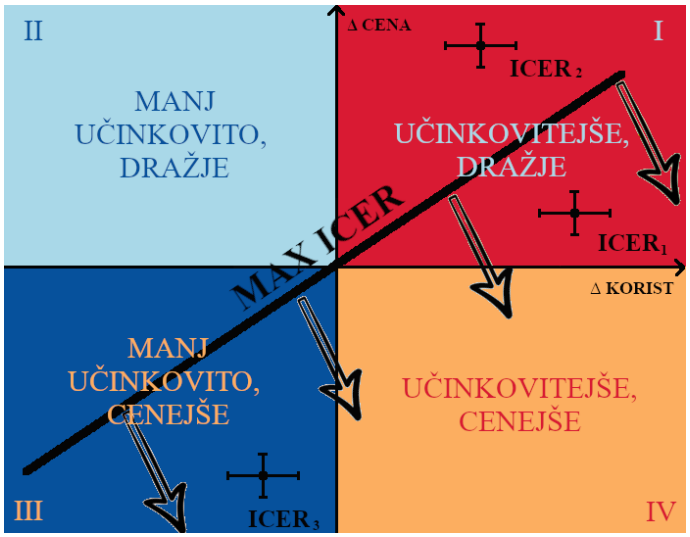
Trenutno je na voljo več metod vrednotenja tehnologij in terapij v medicini, ena teh je tudi analiza stroškovne učinkovitosti (CEA),ki meri inkrementalno vrednost stroškovnega vložka, potrebnega za umestitev nove tehnologije, in vpliv nove tehnologije na zdravje bolnikov, slednje je definirano s kakovostjo življenja [82]. Pri CEA predvidevamo, da se ljudje v svojem življenju gibljejo skozi različna zdravstvena stanja, vsakemu od teh stanj pa je pripisana posebna vrednost - kakovost življenja *(ang.: Quality of Life (QoL))*. QoL opisuje kakovost vsakodnevnega življenja posameznika, vključno s socialnimi, čustvenimi in fizičnimi vidiki. Za oceno kakovosti življenja se uporabljajo indikatorji zdravstvene oskrbe, ki nam povedo, kako zdravstvena oskrba vpliva na paciente, imenovani zdravstvenemu stanju prilagojena življenjska leta *(ang.: Health Adjusted life Years (HAYs))*. Eno od meril je "kakovostni prilagojena življenjska leta" *(ang.: Quality Adjusted Life Years (QALY))*, to je splošen indeks (ni specifičen za bolezensko stanje) in združuje dolžino življenja s kakovostjo življenja. QALY se uporablja pri analizah stroškovne učinkovitosti za določitev inkrementalnega razmerja stroškov (nova tehnologija ali terapija v primerjavi z referenčno) in pridobljenega QALY. Indeks je definiran kot utežena življenjska leta, oziroma čas s kakovostjo življenja pridobljeno skozi ta čas [83]. QoL je normaliziran na standardizirano lestvico in ima vrednost med 0,0 (smrt) in 1,0 (popolno zdravje). Nekateri avtorji navajajo, da je mogoče doseči tudi negativne vrednosti, ki opisujejo stanja, slabša od smrti (npr. demenco ali komo pogosto štejemo za enaki ali slabši od smrti [84]). Orodje (vprašalnik) za ocenjevanje EuroQol (EQ-5D), je standardiziran instrument, ki se uporablja kot merilo zdravstvenih rezultatov, je široko uporabljeno in prevedeno v večino jezikov [85, 86]. Zdravstveno stanje se meri v petih dimenzijah: mobilnost (sposobnost hoje); samooskrba (sposobnost pranja ali oblačenja); običajne dejavnosti (kot so "delo, študij, gospodinjska opravila, družinske ali zasebne dejavnosti");

bolečina/nelagodje; tesnoba/depresija. Anketiranci ocenjujejo vsako dimenzijo; obstajata dve različici s tristopenjsko (EQ-5D-3L-vsako dimenzijo ocenjuje s tremi vrednostmi: "0" - ni težav; "1" - nekaj težav; "2" - veliko težav) in petstopenjsko lestvico (EQ-5D-5L). Na podlagi samoocene bolnikovih telesnih, socialnih in kognitivnih sposobnostih se izračuna končna vrednost indeksa. EQ-5D-3L torej definira 243 (3^5) potencialnih zdravstvenih stanj, ki skupaj z dvema dodatnima stanjema, enim za smrt in enim za nezavest, skupno definira 245 zdravstvenih stanj. Primer: eno leto popolnega zdravja je vredno 1 QALY, vsako leto nepopolnega zdravja pa manj kot 1 QALY, odvisno od bremena bolezni, ki ga čuti bolnik [87]. Tako bo intervencija, ki ustvari šest dodatnih let zdravstvenega stanja vrednosti 0,65 QALY, ustvarila več QALY kot primerljiva tehnologija, ki v zdravstvenem stanju ocenjenih na 0,5 QALY ohrani bolnika 7 let.

Rezultat CEA predstavimo z inkrementalnim razmerjem stroška *(ang.: Incremental Cost Ratio (ICER))*. ICER je opredeljen kot dodatni strošek na dodatno korist, ki se meri s QALY. Ker ima QALY normalno porazdelitev in ima vsota dveh normalnih spremenljivk tudi normalno porazdelitev, ICER lahko izračunamo kot razmerje dveh spremenljivk [88] s pomočjo naslednje enačbe *(Enačba I.1)*, kjer zdravljenje B predstavlja zlati standard oz. referenčno merilo in zdravljenje X novo terapijo (npr. elektrokemoterapijo):

$$ICER = \frac{cena_{terapijaX} - cena_{terapijaB}}{korist_{terapijaX} - korist_{terapijaB}} \tag{I.1}$$

ICER za lažje razumevanje prikažemo kot distribucijo vzorčne populacije v grafu stroškovne učinkovitosti (Slika I.7), v katerem ponazorimo razmerje med inkrementalnimi stopnjami koristi in skupnim, dodatnim stroškom potrebnega za zagotavljanje želenega izida. Vsak od kvadrantov koordinatnega sistema predstavlja en možen izid, če je nova terapija X bolj učinkovita in cenejša v primerjavi z referenčno terapijo B, se nahajamo v četrtem kvadrantu, kar seveda pomeni, da je vredna implementacije v klinično prakso. Vendar se rezultati CEA novih terapij pogosteje nahajajo v I. kvadrantu, torej je nova terapija X bolj učinkovita in hkrati dražja. V tem primeru se je potrebno odločiti, ali pridobljena korist upraviči dodaten strošek [89]. Maksimalen ICER *(ang.: Maximum acceptable ceiling ratio (max ICER))* je mejna oziroma maksimalna vrednost, ki jo je odločevalec pripravljen plačati za en pridobljen QALY. V grafu stroškovne učinkovitosti jo ponazorimo kot mejno premico, če se ICER nahaja v ravnini pod premico, potem je nova terapija X stroškovno učinkovita in je sprejeta, v nasprotnem primeru pa zavrnjena zaradi stroškovne



Slika I.7: Graf stroškovne učinkovitosti; terapiji 1 in 3, ki ležita v ravnini pod premico MAX ICER, sta stroškovno učinkoviti, medtem ko je terapija 2 stroškovno neučinkovita.

neučinkovitosti [90]. Maksimalen ICER določi odločevalec, WHO pa predlaga, da se mejno vredneost določi v skladu z BDP posamezne države.

II Namen

Namen te doktorske disertacije je precej vsestranski, združuje tri različne tematike s skupno glavno temo, tj. elektroporacija. Glavna tema prvega doprinosa k znanosti je pregled in predstavitev delovanja in uporabe na trgu razpoložljivih komercialnih elektroporacijskih naprav. Pri testiranju smo zaznali očitno potrebo po razvoju elektronskega emulatorja biološkega bremena. Zato se je namen razširil še na razvoj elektronskega vezja, ki se na elektroporacijske pulze odzove enako kot biološko breme. Poleg tega smo se zaradi pregleda poglobili tudi v merjenje dovedenih elektroporacijskih pulzov, to je nujno za zagotavljanje kakovosti elektroporacije. S tem se splošni namen prvega doprinosa k znanosti razširi na področje dozimetrije v elektroporaciji. Med drugim smo se osredotočili tudi na merjenje nanosekundnih pulzov, ki je med najbolj zahtevnimi na področju elektroporacije. Zaključimo, da je standardizacija na področju elektroporacije nujna. Elektroporacija je vsestranska tehnologija, zato bi bilo za vsako aplikacijo ali podpodročje potrebno določiti poseben standard. Kljub temu se lahko za elektroporatorje na splošno definira nekatere minimalne osnovne tehnične zahteve in dovoljena odstopanja. Namen je bil tako tudi napisati priporočila za standardizacijo elektroporacijskih naprav.

Namen drugega doprinosa k znanosti je bil razviti nov vsestranski visokofrekvenčni, visokonapetostni elektroporator. Nedavne raziskave na področju uporabe visokofrekvenčnih pulzov v medicini t. i. H-FIRE *(ang.: High-Frequency IrReversible Electroporation)* so vzbudile zanimanje za raziskovanje učinka občutljivosti in preklica bioloških učinkov. Učinek občutljivosti je definiran kot dvig oziroma upad vnosa molekul v celico v primeru apliciranja dvakratne namesto enojne polovične doze elektroporacijskih pulzov. Pri uporabi krajših bipolarnih pulzov (dolžine nekaj sto nanosekund), so raziskovalci odkrili, da pride do preklica bioloških učinkov [19, 20, 21, 22]. Efekt preklica je definiran kot upad vnosa v celico, ko to izpostavimo enemu monopolarnemu pulzu, ki mu skoraj strnjeno sledi drugi monopolarni pulz nasprotne polaritete (torej gre za en bipolarni pulz), v primerjavi z enim samim monopolarnim pulzom [91]. Eden od poglavitnih izzivov na področju elektroporacije še vedno ostaja enakomerna elektroporacija heterogene strukture tkiv – uporaba visokofrekvenčnih bipolarnih pulzov bi lahko rešila tudi ta problem [92]. Za študijo vseh zgoraj opisanih efektov (tudi *in vivo*) potrebujemo pulzni generator, ki bo generiral bipolarne pulze nastavljive amplitude vse do 4 kV, ob tem bo morala biti

najvišja pavza med posameznimi pulzi in dolžina pozitivnega in negativnega pulza 1 μs. Visokofrekvenčne pravokotne pulze je najlažje generirati tako, da s pomočjo silicijevih tranzistorjev vklapljamo in izklapljamo naelektrene kondenzatorje. Glede na popis in pregled elektroporatorjev [8, 93, 94] ter ekonomski oceni [95, 96], ki sta bili do sedaj narejeni, sem prišla do zaključka, da obstajajo zanimiva področja, ki jih je še potrebno raziskati. Razvoj bipolarnega elektroporatorja z maksimalno napetostjo 4 kV in minimalnim časom trajanja pulza 1 μs omogoča raziskavo bioloških učinkov zelo visokih in hkrati kratkih električnih pulzov tako *in vitro* kot *in vivo*. Predvidevamo, da je lahko glavna prednost teh specifičnih pulzov zmanjšanje krčenja mišic in bolečine, ki ju povzroča električno polje [97, 67, 98]. Trenutno za zmanjšanje mišične kontrakcije pred terapijo pacientom vbrizgajo relaksatorje mišic, vendar je potrebno med samim tretmajem za vzdrževanje nevromuskularne blokade spremljati živčno-mišično delovanje. [99, 100]. Poročajo, da je mejna vrednost, ki sproži kontrakcijo mišic, dvakrat nižja kot mejna vrednost elektroporacije [98]. Prav tako poročajo, da bipolarni pulzi [101] in H-FIRE pulzi [67] zmanjšajo kontrakcijo mišic. Pokazali so tudi, da IRE lahko izvedemo tudi pri dolžini pulza 1 μs, in sicer s povečanjem števila pulzov in povečanjem električne poljske jakosti [67]. Glede na nove raziskave [102] se namen razširi tudi na *in vivo* poskuse z razvito napravo. V prvi visokofrekvenčni elektrokemoterapiji (HF-ECT) *in vivo* ocenimo učinkovitost terapije in mišične kontrakcije, ki so prisotne med dovajanjem pulzov.

Namen tretjega doprinosa k znanosti je opredelitev proračuna za razvoj nove klinične elektroporacijske naprave. Zato se v zadnjem delu osredotočamo na vrednotenje tehnologij v zdravstvu, natančneje na analizo stroškovne učinkovitosti. Nekaj stroškovnih analiz elektroporacije je že narejenih [9, 96], vendar zaradi pomanjkanja podatkov niso najbolj objektivne, saj ne vključujejo podatkov o dvigu kakovosti življenja. Osredotočamo se na elektrokemoterapijo kožnega melanoma in bazalnoceličnega karcinoma (BCC), saj se terapiji na teh dveh vrstah rakavega obolenja izvajata najdlje in imata dobre rezultate.

III Rezultati in razprava

V sklopu te doktorske disertacije so predstavljeni trije izvirni prispevki k znanosti:

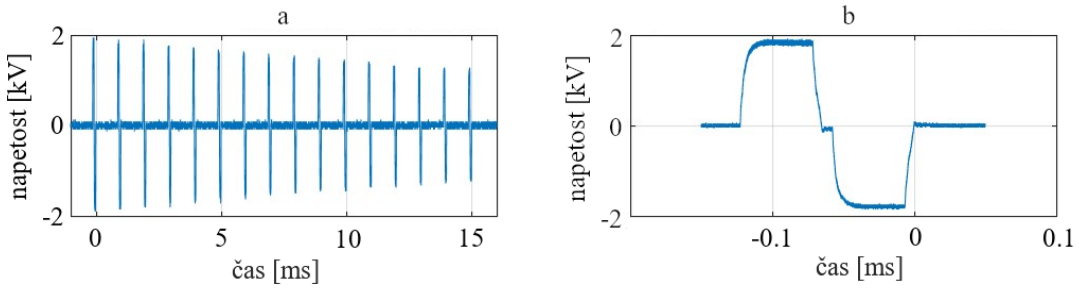
1. Ocene elektroporatorjev in predlog standardizacije elektroporatorjev kot samostojnih medicinskih naprav.
2. Razvoj novega bipolarnega visokofrekvenčnega visokonapetostnega pulznega generatorja - elektroporatorja.
3. Ocena cenovne učinkovitosti elektrokemoterapije v terapiji bazalnoceličnega karcinoma in kožnih metastaz melanoma.

Rezultati in razprava so sestavljeni iz osmih znanstvenih člankov in enega poglavja iz knjige. V sklopu razširjenega slovenskega povzetka so povzeti rezultati vsakega od prispevkov. Prvi prispevek k znanosti je sestavljen iz knjižnega poglavja, ki je izšlo leta 2017, in petih znanstvenih člankov. Članek 1 in 4 sta v recenzijskem postopku, članek 2 in 3 sta že sprejeta v objavo, medtem ko je članek 5 že objavljen. Drugi prispevek k znanosti je predstavljen v članku 6, ki je v recenzijskem postopku, tretji prispevek k znanosti pa je sestavljen iz dveh člankov, članek 7 je bil objavljen leta 2019, članek 8 pa je v postopku oddaje v recenzijo.

III.I Dozimetrija pri elektroporaciji

Merjenje je nujno za kvalitetno elektroporacijo, saj se lahko le s pomočjo meritev prepričamo, da smo elektroporacijske pulze res dovedli in da naprava deluje v skladu sprednastavljenimi vrednostmi. Težave nastopijo predvsem takrat, ko naprave niso sposobne generirati tistega, kar obljubljajo v tehničnih specifikacijah. Če so biološka bremena bolj prevodna, se ponavadi zaplete, ker naprave niso zmožne generirati tako visokega toka ali nimajo dovolj velike zaloge energije in posledično dovedeni pulzi ne ustrezajo prednastavljenim karakteristikam. Tipična prevodnost suspenzije v elektroporacijski kiveti je nekje med 100 in 50 Ω, vendar se lahko v primeru uporabe bolj prevodnega medija spusti tudi pod 10 Ω. Velike variacije karakteristik bioloških bremen predstavljajo ogromen izziv pri načrtovanju in razvoju elektroporacijskih naprav ter rezultirajo v različnih rešitvah. Prav tako je potrebno za izboljšanje ponovljivosti že objavljenih študij

zelo podrobno poročati o vseh karakteristikah uporabljenih električnih pulzov. Priporočamo, da raziskovalci v svoja poročila vključijo vsaj dve meritvi, eno ki natančno prikazuje en sam pulz in drugo pri večji časovni skali, ki zajema celotno sekvenco pulzov (Slika III.1). V kolikor grafična predstavitev meritve ni mogoča, je potrebno električne pulze ustrezno besedno opisati (v pomoč je slika I.2).



Slika III.1: Priporočamo, da raziskovalci v svoja poročila vključijo vsaj dve meritvi uporabljenih elektroporacijskih pulzov: a) slika, ki zajema celotno sekvenco dovedenih pulzov; b) slika, ki natančno prikazuje en sam pulz, v kolikor to ni mogoče, je potrebno električne pulze ustrezno besedno opisati.

Najlažji način merjenja elektroporacijskih pulzov je merjenje napetosti in toka s pomočjo osciloskopa. Vendar je potrebno izbrati ustrezne sonde in osciloskop (maksimalna amplituda, pasovna širina, časa vzpona ...). Pri večini aplikacij se uporabljajo mikro- ali milisekundni pulzi, zato so primerni že osciloskopi in sonde z nekaj MHz pasovne širine. Merjenje je bolj zahtevno, ko dosežemo nanosekundno področje, kjer so potrebne GHz pasovne širine in hitri časi vzpona ter prav tako visoke, nekaj deset kilovoltne, maksimalne napetosti. Paziti je potrebno na parazitne induktivnosti in kapacitivnosti ter odboje signala, zato morajo biti sonde nameščene čim bližje elektrod in brez kakršnihkoli dodatnih priključnih žic. Poudariti je potrebno, da se pasovna širina sond konča pri –3 dB, kar pomeni, da ima sonda na koncu pasovne širine napako 29 %. Napaka 29 % je sprejemljiva za elektroniko, vendar pa ima lahko amplituda, ki odstopa za 29 %, drugačen biološki učinek [103, 104].
V sklopu te doktorske disertacije sta predstavljena dva pregleda elektroporatorjev, prvi je predstavljen v knjižnem poglavju (Table 12.1. in 12.2, priloženi na strani 76-83) in zajema pregled na trgu dostopnih komercialnih elektroporatorjev ter elektrod in njihovih tehničnih specifikacij, ki jih podajajo proizvajalci. Kontaktirali smo 25 proizvajalcev, vendar jih je bilo le 13 pripravljenih sodelovati, ostali pa se niso odzvali ali niso želeli razkriti parametrov pulzov, ki jih generirajo njihove naprave. Nekateri so nas obvestili, da so karakteristike električnih pulzov predmet njihove

intelektualne lastnine. Z rastjo elektroporacijskega trga na žalost vse več proizvajalcev prikriva parametre izhodnih impulzov svojih naprav. Pri drugem pregledu, ki je objavljen v tabeli članka 1 (Table 1 in 2, priloženi na straneh 111-121), pa smo predstavili elektroporatorje, ki se najpogosteje uporabljajo pri elektroporaciji kože, in različne tipe elektrod. V tabeli je pripisano tudi strokovno mnenje, v sklopu katerega komentiramo naprave na podlagi zbranih tehničnih specifikacij in testiranj, ki smo jih opravili v sklopu te doktorske disertacije.

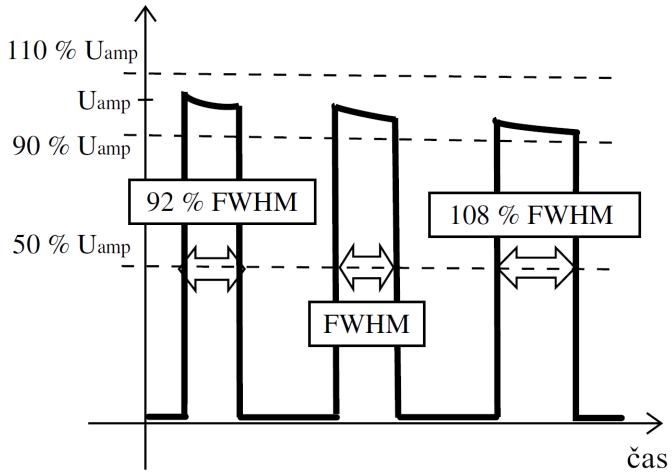
III.II Predlogi za standardizacijo

Standard za elektroporatorje bi moral definirati naslednje zahteve:

- največjo dovoljeno odstopanje oziroma toleranco generiranih pulzov v primerjavi s prednastavljenimi vrednostmi pri obremenitvi in brez obremenitve;
- protokol testiranja elektroporatorja, ki oceni, če naprava deluje v skladu s tolerancami;
- preizkusna bremena za posamezne aplikacije;
- navodila za pripravo tehnične dokumentacije naprave (ki se lahko glede na aplikacijo razlikujejo), specifikacije morajo biti navedene skupaj s pogoji, pod katerimi jih naprava doseže;
- predpulz, če se uporablja (amplituda, oblika, trajanje in pavza med predpulzom in pred nastavljeno sekvenco elektroporacijskih pulzov);
- kako zagotavljati varnost naprave, kot so galvanska ločitev, omejitve toka, energije in napetosti, opozorila, v primeru, če je bilo dovajanje pulzov prekinjeno ali pa je prišlo do okvare;
- največja dovoljena odstopanja oziroma tolerance geometrije elektrod in elektroporacijskih kivet.

V sklopu doktorske disertacije smo določilo sprejemljive tolerance in predlagali rešitev, ki bi omogočila nepristransko testiranje in oceno elektroporacijskih naprav. Predlagamo, da proizvajalci naprav določijo aplikacije, za katere je njihova naprava izdelana, in nato navedejo tehnične specifikacije in tolerance v skladu s tipičnim bremenom za aplikacije, npr. napetost: 200-1000 V (za bremena z impedanco večjo od 50 Ω in za pulze do 1 ms ($N_{MAX} = 10$); 200-2000 V (za bremena, večja od 100

Ω, in za trajanje pulzov do 100 μs ($N_{MAX} = 10$)); kjer je N_{MAX} največje število pulzov. Na podlagi pregleda literature [105, 106, 107, 108], naših izkušenj in glede na trenutno stanje tehnologije smo opredelili tolerance amplitude napetosti in trajanja pulza. Z razpoložljivimi elektronskimi komponentami in obstoječimi rešitvami vezij je zahteve mogoče enostavno izpolniti. Objavljene permeabilizacijske funkcije in preživetja celic [105, 106, 107, 108] kažejo, da lahko več kot 15 % odstopanje povzroči bistveno drugačen učinek elektroporacije, vendar v primeru *in vivo* elektroporacije lahko pride do dodatnih napak, ki so posledica mišičnega krčenja med dovajanjem pulzov, vstavitve elektrod in drugih okoliščin, ki skupno napako zlahka pripeljejo na 15 %. Zato menimo, da morajo proizvajalci slediti najsodobnejšim trendom in priporočamo, da je toleranca amplitude napetosti 10 %, torej izmerjena amplituda napetosti ne sme biti nižja od 90 % ali višja od 110 % prednastavljene napetosti. Kar seveda pomeni, da mora biti tudi napetost od prvega do zadnjega impulza v tem območju. Trajanje pulza se definira kot FWHM odstopanje od prednastavljene vrednosti in ne sme biti večje od 8 % [106, 107], kot je prikazano na sliki (Slika III.2).



Slika III.2: Amplituda napetosti ne sme biti nižja od 90 % ali višja od 110 % prednastavljene napetosti. Kar pomeni, da mora biti tudi padec napetosti med prvim in zadnjim pulzom pri največjem številu impulzov v tem območju. Odstopanje trajanja pulza od vnaprej nastavljene vrednosti ne sme biti večje od 8 %.

Predlagane tolerance morajo biti izpolnjene od maksimalnih pa vse do minimalnih nastavitev, te so: največja amplituda pulza, največja dolžina pulza, največje število pulzov in minimalna ponavljalna frekvenca pulzov, ko kot breme uporabimo tipično breme aplikacije, za katero je naprava narejena. Predlagamo, da se sestavi seznam aplikacij in za vsako razvije tipičen emulator biološkega bremena.

Postopek razvoja emulatorja smo podobno opisali v članku 4. Poleg tega je treba standardizirati tudi elektrode in določiti dovoljena odstopanja razdalj med elektrodama, ustrezne materiale, v primeru večkratne uporabe pa tudi določiti sterilizacijske postopke in vzdrževanje. Klinični elektroporator se mora nenehno samotestirati in kalibrirati. Odstopanja, določena v sklopu tega doktorata se nanašajo na "klasično" elektroporacijo in niso ekvivalentna za nanosekundno elektroporacijo.

III.II.I Emulator biološkega bremena

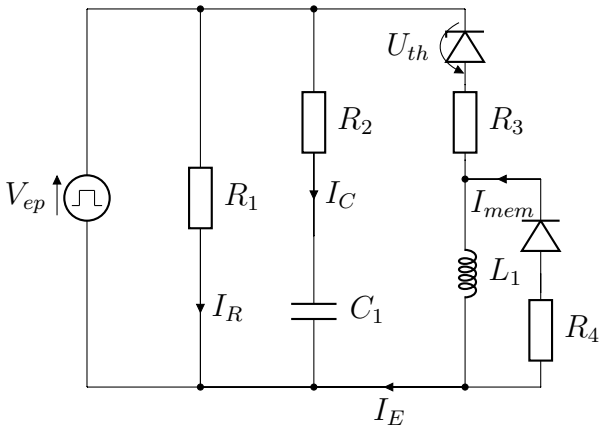
Razvili smo analogno elektronsko vezje, ki ima impedanco ekvivalentno govejim jetrom med "klasično" (ne nanosekundno) elektroporacijo z igelnimi elektrodami. Analizirali in parametrizirali smo natančne meritve toka in napetosti med elektroporacijo tkiva govejih jeter *ex vivo* tako, da smo poiskali enačbo *(Enačba III.1)* za tok v odvisnosti od časa t in števila pulzov N, ki je primerna za opis analognega elektronskega vezja (spominski tok I_{mem} se izračuna s pomočjo enačbe III.2, končni tok pa s enačbo III.3). Z metodo najmanjših kvadratov smo poiskali vrednosti posameznih elementov v ekvivalentnem vezju (Slika III.3). Predlagano vezje oziroma emulator biološkega bremena smo simulirali, razvili in ovrednotili (Slika III.4).

$$I(t,N) = \begin{cases} \frac{U_{amp}}{R_1} + \frac{U_{amp}}{R_2} * e^{-(t-(N-1)*T_{per})/\tau_1}, & U_{amp} < U_{th} \\ \frac{U_{amp}}{R_1} + \frac{U_{amp}}{R_2} * e^{-(t-(N-1)*T_{per})/\tau_1} + (\frac{U_{amp}-U_{th}}{R_3} - I_{mem}(N))* & \\ *(1 - e^{-(t-(N-1)*T_{per})/\tau_2}) + I_{mem}(N), & U_{amp} > U_{th} \end{cases} \tag{III.1}$$

$$I_{mem}(N) = I_{M0}(N) * e^{-T_{pause}/\tau_3} \tag{III.2}$$

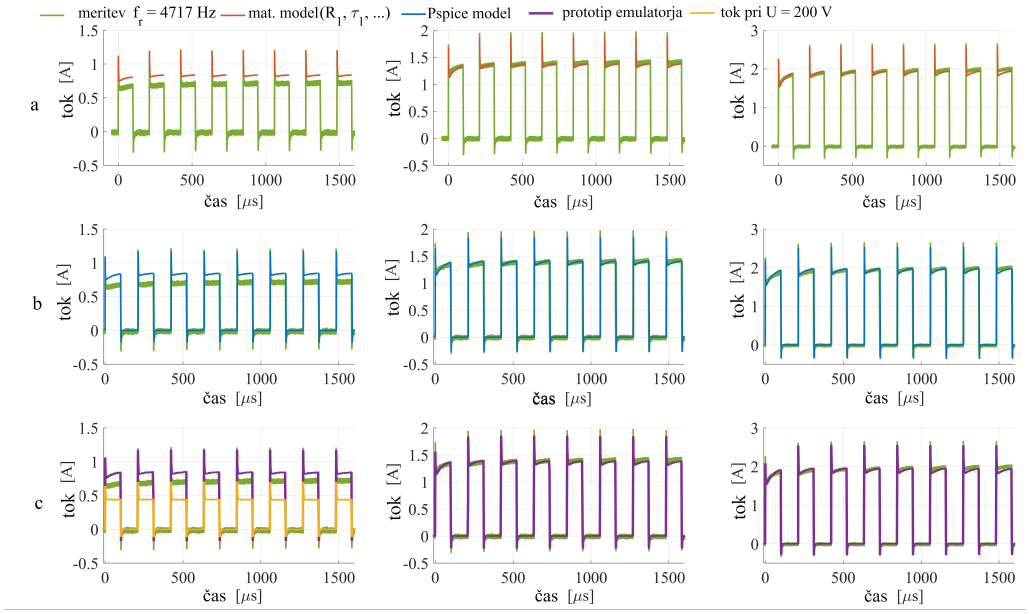
$$I_{M0}(N) = \begin{cases} I((N-1)*T_{per} - T_{pause}), & N > 1 \\ 0, & N = 1 \end{cases} \tag{III.3}$$

Predlagani elektronski emulator biološkega bremena natančno simulira tok med elektroporacijo. Predlagana rešitev je potrditev koncepta z ogromno potenciala. Za vsako biološko breme je torej potrebno pridobiti natančne meritve napetosti in toka in nato razviti model, v skladu s postopki, ki so opisani v članku 4. Z vidika standardizacije elektroporacijskih naprav bi bilo smiselno vsako komponento v predlaganem vezju nadomestiti s komponento nastavljive vrednosti. Posamezne kondenzatorje z nizom kondenzatorjev z različnimi vrednostmi in prav tako Zener diodo z Zener diodami z različnimi prebojnimi napetostmi, s tem bi definirali en vsestranski emulator za posamezno aplikacijo. Glavna prednost predstavljenega procesa



Slika III.3: Shema vezja elektronskega emulatorja elektroporacijskega bremena.

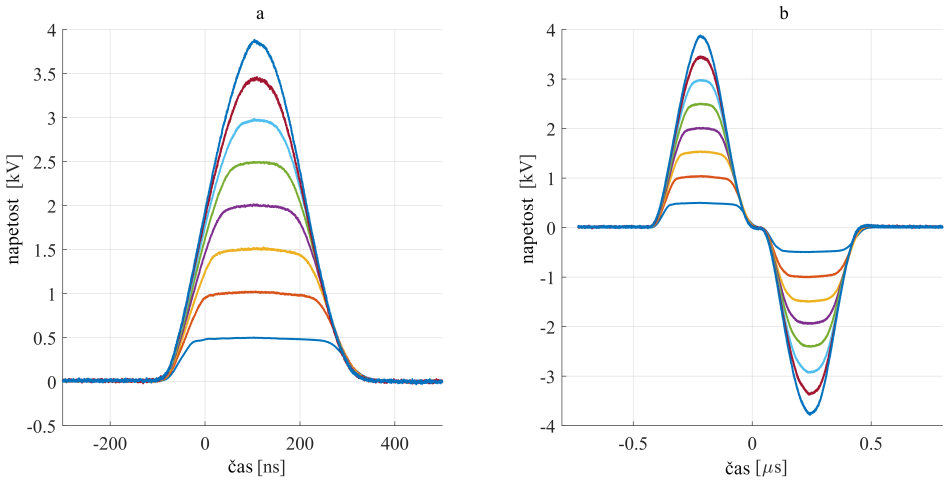
oziroma koncepta emulatorja je, da omogoča trajnostno, ponovljivo in nepristransko testiranje ter primerjanje delovanja različnih elektroporacijskih naprav in predstavlja prvi korak proti standardizaciji elektroporatorjev.



Slika III.4: Povprečje petih meritev toka pri ponavljalni frekvenci 4717 Hz (zelena), (a) numerično modeliran tok s pomočjo programskega okolja Matlab (oranžna, prva vrstica), (b) modeliran tok s pomočjo programskega okolja PSpice (modra, druga vrstica) in (c) izmerjen tok skozi prototipa (vijolična, tretja vrstica). Rumena krivulja v tretji vrstici je dodatna meritev toka prototipa emulatorja pri napetosti 200 V, s tem želimo pokazati odziv, ko je napetost nižja od pragovne napetosti. V prvem stolpcu je predstavljen tok pri 500 V, v drugem 750 V in tretjem pri 1000 V.

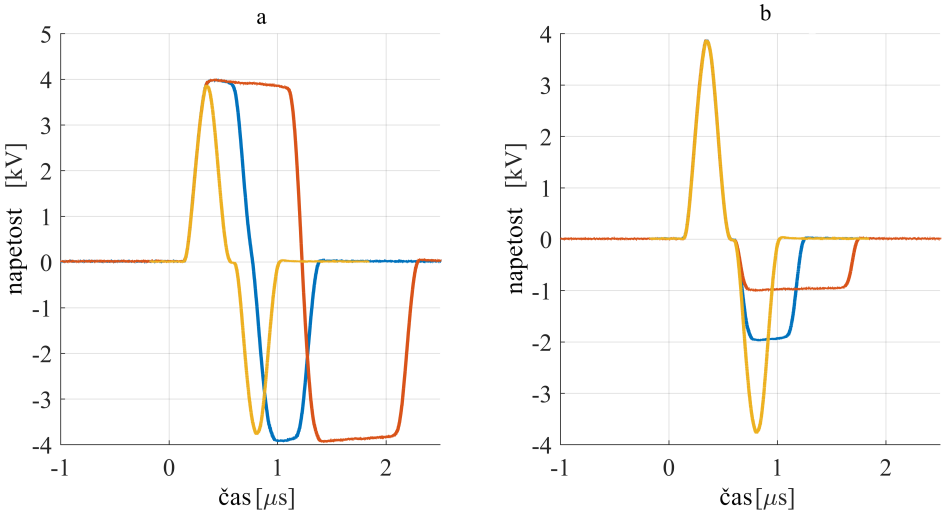
III.III Razvoj novega visokofrekvenčnega visokonapetostnega pulznega generatorja

Razvito napravo smo najprej preizkusili na 80 Ω uporu in nato še *in vivo* na miših pri prvi visokofrekvenčni elektrokemoterapiji *in vivo*. Meritev generiranih monopolarnih in bipolarnih pulzov z minimalnim trajanjem pulza je prikazana na sliki (Slika III.5). Začetna polnilna napetost kondenzatorja je bila nastavljena na 500 V, nato pa smo jo dvignili vse do 4 kV s korakom po 500 V. Zato, da smo dosegli želenih 4 kV, smo bili prisiljeni povečati širino pulza prožilnega signala na 260 ns, kar pomeni, da se minimalno trajanje izhodnega pulza elektroporatorja poveča iz predvidenih 100 ns na 200 ns. Na sliki (Slika III.5b) je prikazan bipolarni pulz, zakasnitev med prožilnim signalom pozitivne in negativne polperiode je bila nastavljena na 200 ns. Poleg simetričnega generiranja pulzov različnih dolžin (Slika III.6a) naprava omogoča tudi generiranje asimetričnih bipolarnih pulzov, ki so prikazani na sliki (Slika III.6b). Širino pulza pozitivne in negativne polperiode je mogoče, (vsakega posebej oziroma neodvisno) spreminjati od 200 ns pa vse do 1 ms (Slika III.6b). Enako velja tudi za amplitudo pulza (Slika III.6b), ki jo lahko spreminjamo na območju od 0 do 4 kV.



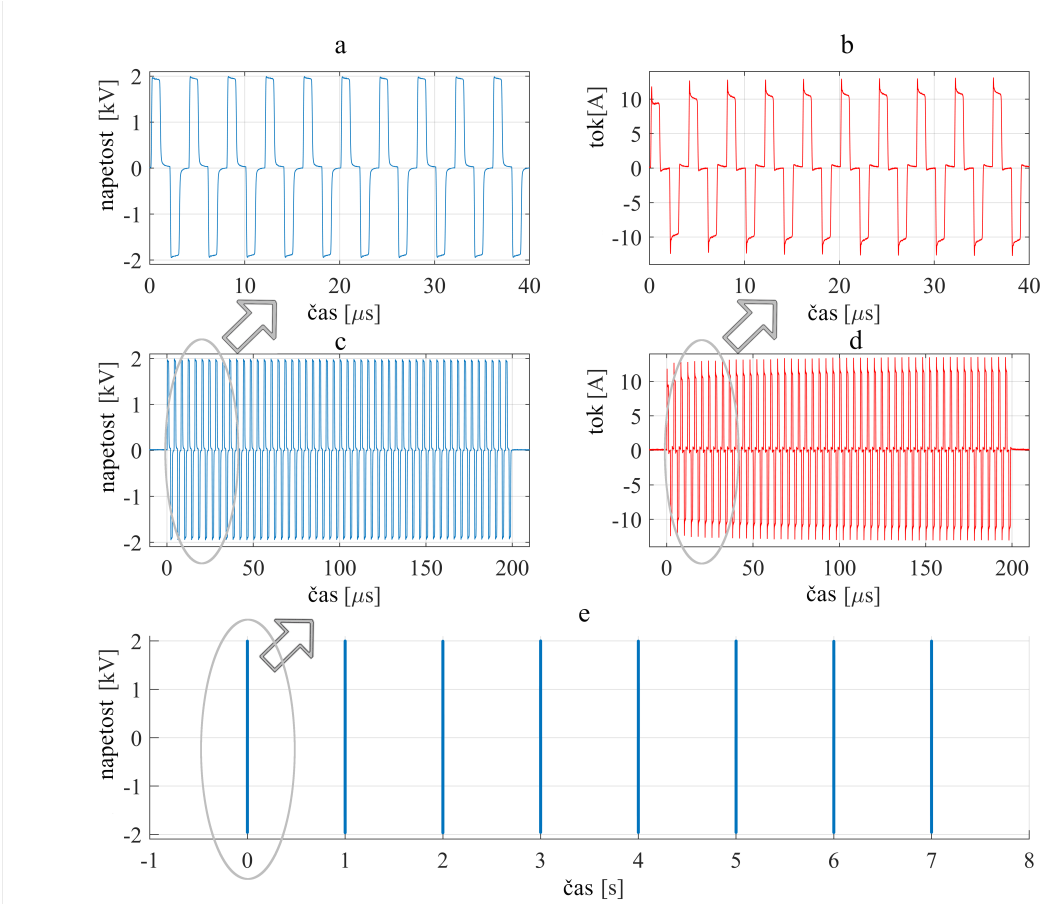
Slika III.5: Meritev monopolarnega (a) in bipolarnega (b) pulza, generiranega na 80 Ω uporu. Začetna polnilna napetost kondenzatorja je bila nastavljena na 500 V, nato pa smo jo dvignili vse do 4 kV s korakom po 500 V.

Izvedli smo tudi *in vivo* meritve toka in napetosti pulzov, uporabljenih pri visokofrekvenčni elektrokemoterapiji na miši, prikazane so na Sliki III.7. Naprava tudi pri *in vivo* pogojih deluje v skladu s pričakovanji. V sklopu *in vivo* poskusov



Slika III.6: a) Slika prikazuje generiranje simetričnih bipolarnih pulzov različnih dolžin. Polnilna napetost kondenzatorja je bila nastavljena na 4 kV, prožitveni pulz pa je bil nastavljen na 260, 400 in 800 ns za obe polperiodi. b) Slika prikazuje asimetrično generiranje bipolarnih pulzov ekvivalentnega naboja. Amplituda pozitivne polperiode in trajanje so bili nastavljeni na 4 kV 200 ns, trajanje prožilnega signala negativne polperiode pa na 260 ns, 400 ns in 800 ns in hkrati polnilna napetost negativne polperiode na 4 kV, 2 kV in 1 kV.

smo primerjali "klasično" elektrokemoterapijo z visokofrekvenčno elektrokemoterapijo. Preko ploščatih elektrod (razdalja med elektrodama je 6 mm) smo v primeru "klasične" elektrokemoterapije dovedli osem monopolarnih pulzov dolžine 100 μs, ponavljalne frekvence 1 Hz in amplitude 780 V, pri visokofrekvenčni elektrokemoterapiji pa smo en monopolarni pulz nadomestili s sekvenco bipolarnih pulzov 1-1-1-1 μs (trajanje pozitivne polperiode - pavza med polperiodama - trajanje negativne polperiode - pavza med bipolarnimi impulzi), ponavljalna frekvenca sekvenc je enaka kot ponavljalna frekvenca pulzov pri "klasični" elektrokemoterapiji, amplitudo napetosti pa smo zvišali na 1950 V. V nasprotju z do sedaj objavljenimi študijami nismo opazili razlike v mišični kontrakciji. Pri "klasični" elektrokemoterapiji z bleomicinom je bilo 50 % (3/6 miši) popolnih odgovorov, pri visoko-frekvenčni z bleomicinom pa 86 % (7/6 miši) popolnih odgovorov. Razlika med skupinama sicer ni signifikantna, pri obeh terapijah se je preživetje živali znatno podaljšalo, v primerjavi z nezdravljeno kontrolno skupino in kontrolno skupino miši, ki smo jim dovedli le elektroporacijske pulze. Živali so dobro prenašale obe vrsti elektrokemoterapije, po zdravljenju se njihova telesna teža ni spremenila za več kot 5 %, prav tako nismo opazili smrtnosti, povezane z zdravljenjem.



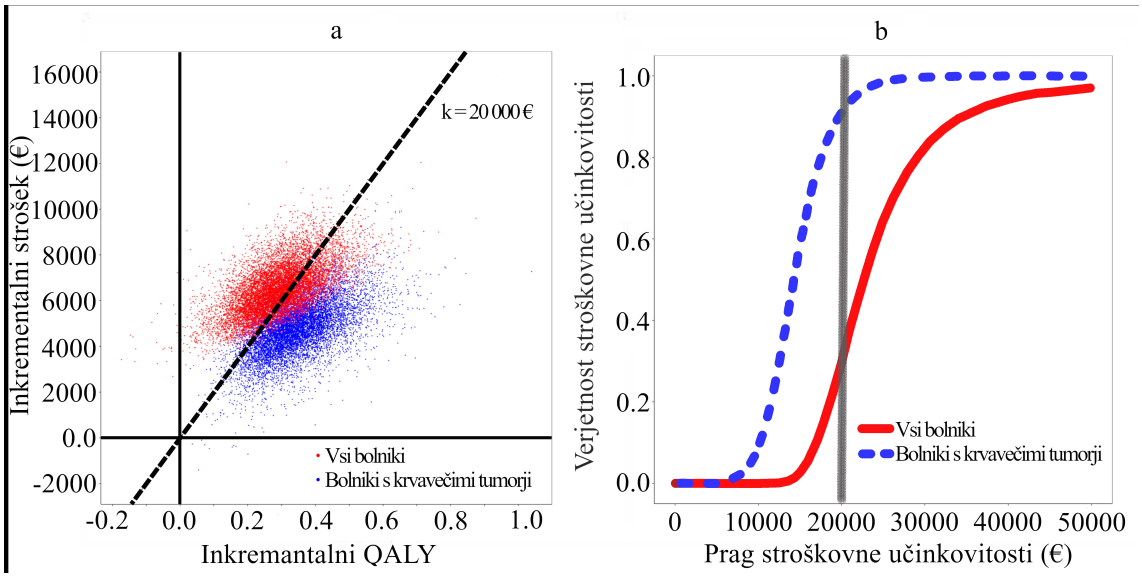
Slika III.7: Osem 200 μs dolgih sekvenc s ponavljalno frekvenco 1 Hz, vsaka sekvenca je bila sestavljena iz 50 bipolarnih pulzov tipa 1-1-1-1 μs (trajanje pozitivne polpreiode-pavza med polperiodama-trajanje negativne polperiode-pavza med bipolarnimi impulzi) amplitude 1950 V, je bilo dovedenih s pomočjo ploščatih elektrod na tumor, ki se je nahajal na boku miške.

III.IV Stroškovna analiza elektrokemoterapije

V sklopu članka 7 smo predstavili osnovne koncepte in postopke vrednotenja tehnologij v zdravstvu *(ang.: Health Technology Assessment)* in analize stroškovne učinkovitosti ter predstavili študijo stroškovne analize *(ang.: Cost-Effectiveness Analysis)* elektrokemoterapije, kot terapije za zdravljenje bazalnoceličnega karcinoma in kožnega melanoma. Nekaj stroškovnih analiz elektrokemoterapije je že bilo narejenih, vendar zaradi pomanjkanja podatkov o učinkovitosti zdravljenja, v smislu povečanja kakovosti življenja bolnikov *(ang.: Quality of Life)*, zaključki ne morejo biti točni. Da bi izdelali čim bolj relevantno analizo v sedmem članku pred-

stavljamo dva splošna Markova modela in njuni poenostavljeni različici. Modela sta zasnovana posebej za analizo elektrokemoterapije bazalnoceličnega karcinoma in kožnega melanoma. Dodatno so opredeljeni tudi potrebni podatki za uspešne izračune. Manjkajoče podatke smo skušali zbrati v okviru različnih študij, ki še vedno tečejo, vključno z randomiziranimi kliničnimi študijami. Predstavljeno je tudi priporočilo za poročanje, ki bi olajšalo zbiranje podatkov. S tem prispevkom smo predvsem želeli dvigniti splošno ozaveščenost o pomembnosti številčnega poročanja o kakovosti življenja in uporabnosti oziroma pomenu vprašalnikov EQ-5D, ki na prvi pogled morda nista samoumevna, vendar sta bistvenega pomena za analizo stroškovne učinkovitosti.

V sklopu članka 8 predstavljamo stroškovno učinkovitost zdravljenja kožnega melanoma IV in IIIc stadija v Sloveniji z napravo Cliniporator (IGEA, Italija). Pacientom, vključenim v študijo, je elektrokemoterapija izboljšala kakovost življenja za 0,29 QALY v celotni življenjski dobi, pri zvišanju stroškov za 6.568 €. Pričakovana dodatna neto korist za zdravje *(ang.: incremental net health benefit)* elektrokemoterpije je v primerjavi s paliativno terapijo -0,037 QALY, kar pomeni, da je pri uporabljenem pragu stroškovne učinkovitosti 20.000 €(prag za Slovenijo, na podlagi GDP) elektrokemoterapija nekoliko manj učinkovita kot paliativna terapija, čeprav je negotovost stroškovne učinkovitosti precej velika. Za podskupino bolnikov s krvavečimi tumorji elektrokemoterapija zviša kakovost življenja za 0,34 QALY s stroški višjimi za 4.863 €. Izračun torej kaže, da je elektrokemoterapija stroškovno učinkovitejša samo za bolnike s krvavečimi tumorji, s pričakovano dodatno neto koristjo za zdravje 0,10. Rezultati verjetnostne analize občutljivosti *(ang.: probabilistic sensitivity analysis)* kažejo veliko negotovost stroškov in QALY elektrokemoterapije za obe skupini bolnikov, vendar kljub veliki razpršenosti simulacija ostaja v drugem kvadrantu ravnine stroškovne učinkovitosti, kar pomeni, da je velika verjetnost, da je elektrokemoterapija bolj učinkovita in dražja v primerjavi s paliativno terapijo (Slika III.8 a). Ob predpostvki, da je prag stroškovne učinkovitosti za Slovenijo (izračunano na podlagi GDP) 20.000 €, je verjetnost, da bo elektrokemoterapija stroškovno učinkovita 0,3 za celoten vzorec in 0,91 za bolnike s krvavečimi tumorji (Slika III.8 b).



Slika III.8: : (a) Ravnina stroškovne učinkovitosti. (b) Verjetnost, da je elektrokemoterapija stroškovno učinkovita za vse bolnike in bolnike s krvavečimi tumorji.

IV Zaključek

Elektroporacija je vsestranska tehnologija, ki je že uveljavljena na številnih področjih. Merjenje napetosti in toka dovedenih elektroporacijskih pulzov je ključno za doseganje učinkovite elektroporacije, saj le tako lahko zagotovimo kakovost aplikacije. Najbolj zahtevno je natančno merjenje visokonapetostnih kratkih nanosekundnih pulzov. Poudariti je potrebno, da se pasovna širina sond konča pri -3 dB, kar pomeni, da ima sonda na koncu pasovne širine napako -29 %. Napaka -29 %, je sprejemljiva za elektroniko, vendar pa ima lahko amplituda, ki odstopa za 29 % drugačen biološki učinek [103, 104]. Ker sta permeabilizacija in preživetje celic sigmoidni funkciji napetosti, lahko ima že več kot 10 % razlika v napetosti bistveno drugačen biološki učinek [103, 104]. Pri uporabi merilnih sond se moramo vedno zavedati napake. Pri komercialno dostopnih sondah je merilna napaka navedena v podatkovnem listu, vendar je včasih kljub temu spregledana, prototipne sonde pa je pred uporabo potrebno pravilno umeriti in ovrednotiti merilno napako.

Na področju elektroporacije se v zadnjem času pojavlja vse več neuspešnih ponovitev že objavljenih raziskav [30]. Mislimo, da je poglavitni razlog za nastalo situacijo neustrezno oziroma površno poročanje o metodah objavljenih raziskav, ki opisujejo/uporabljajo elektroporacijo. Prav tako raziskovalci pogosto v poročila ne vključujejo meritev dovedenih pulzov [18, 31], kar pomeni, da je nepristranskost nekaterih raziskav vprašljiva. Raziskovalce na področju elektroporacije je potrebno vzpodbuditi k večji ponovljivosti raziskav, kar pa je mogoče doseči le z ustreznim merjenjem, standardiziranimi poročili in pravilno uporabo elektroporatorjev ter elektrod. Če navedemo primer: elektroporacija kože je obetavna metoda zdravljenja, pri kateri s transdermalnim vnosom zdravil, gensko elektrotransfekcijo, elektrokemoterapijo ali ireverzibilno elektroporacijo zdravimo različna kožna obolenja. Objavljenih je bilo že ogromno *in vitro* in *in vivo* študij, pri katerih so raziskovalci uporabljali različne geometrije elektrod, karakteristike pulzov in elektroporatorje. Rezultate študij, pri katerih so uporabili električne pulze različnih karakteristik, je včasih težko (če ne celo nemogoče) primerjati. Razlogi za počasno umestitev elektroporacije kože za transdermalni vnos v klinično prakso so torej nestandardni parametri pulzov, nestandardne geometrije elektrod, elektroporatorji, ki se ne ujemajo s svojimi tehničnimi specifikacijami ali tehničnih specifikacij celo

nimajo, pomanjkljivo poročanje o karakteristikah uporabljenih električnih pulzov in porazdelitvi električnega polja, neustrezno merjenje napetosti in toka ter bistveno drugačna struktura kože živali in ljudi. Kljub temu je bilo večkrat pokazano, da lahko elektroporacija zniža stroške in olajša postopke zdravljenja. Iz objavljenih študij lahko sklepamo, da so terapije, ki temeljijo na elektroporaciji, varne in imajo malo ali pa so povsem brez stranskih učinkov. Klinične študije, ki temeljijo na elektroporaciji, so zelo vzpodbudne, zato verjamemo, da bo elektroporacija v prihodnosti nepogrešljiva pri genskem zdravljenju, zdravljenju raka, zdravljenju okužb, intrakardialni ablaciji in cepljenju. Vendar pa so nadaljnji razvoj standardnih protokolov za zdravljenja, ki temeljijo na elektroporaciji, regulacija razvoja elektroporatorjev in usposabljanje uporabnikov bistveni za uspešno vključitev v klinično prakso. Prav tako imajo terapije, ki temeljijo na elektroporaciji, velik potencial za uporabo v klinični praksi tudi pri socialnoekonomsko prikrajšanih populacijah. Trenutno je cena elektroporacije relativno visoka, predvsem zaradi dragih elektroporatorjev in elektrod, vendar bi lahko razvili nizkocenovne različice elektroporatorjev, saj tehnološko ni težko razviti elektroporatorja, bolj zahtevno je zagotavljanje kakovosti in varnosti, ker se karakteristike bioloških bremen precej razlikujejo že od vzorca do vzorca in še bolj pa od tkiva do tkiva. Zaradi velikih razlik v karakteristikah bioloških bremen lahko dovedeni pulzi odstopajo od prednastavljenih. Nizka impedanca bremena (tkiva ali celične suspenzije) zahteva velike moči/tokove, kar pogosto vodi do občutnega padca napetosti na bremenu. Protokoli, pri katerih se dovede večje število pulzov (ali daljše pulze), lahko privedejo do neželenega padca napetosti na bremenu. Z umestitvijo specifičnega standarda za posamezno aplikacijo bodo elektroporacijske naprave varnejše, zdravljenje bolj učinkovito in bolj ponovljivo, kar bo omogočilo hitrejši in učinkovitejši napredek na področju elektroporacije, poleg tega tudi pri zdravljenju in terapijah, ki temeljijo na elektroporaciji. Z uvedbo posebnega standarda za klinične elektroporatorje se bo izboljšala kakovost medicinskih elektroporatorjev, kar bo doprineslo k boljšemu in bolj učinkovitemu zdravljenju raka. Da lahko začnemo razmišljati o predlogu standarda za elektroporacijo, je potrebno najprej določiti tehnične specifikacije in tolerance, nato pa še protokole testiranja. V okviru člankov 2 in 3 smo predlagali minimalne tehnične zahteve in tolerance, v sklopu članka 4 pa smo razvili elektronski emulator biološkega bremena, ki omogoča vzpostavitev standardiziranih postopkov testiranja. Ker so biološka bremena nelinearna in uporno kapacitivna, ad hoc biološka bremena (npr. gomoljnice) in upori niso primerni za testiranje elektroporacijskih naprav, ker niso primeren približek terapevtskega biološkega bremena. Zato smo razvili elektronski emulator biološkega bremena, ki omogoča

trajno testiranje elektroporacijskih naprav in objektivno primerjavo delovanja različnih elektroporatorjev ali delovanje elektroporatorja skozi življenjsko dobo. Razvoj emulatorja je prvi korak k standardizaciji in uveljavitvi standardiziranega protokola za testiranje ali oceno elektroporatorjev, ki omogoča splošen napredek na področju elektroporacije. Določitev karakteristik biološkega bremena je pomemben korak pri razvoju elektroporatorjev, ki pa je prepogosto zanemarjen. Neustrezna karakterizacija biološkega bremena lahko privede do neoptimalnih rešitev in zviša zahtevnost razvoja. Na podlagi objavljenega članka 4 lahko s kvalitetnimi meritvami natančno določimo karakteristiko biološkega bremena in razvijemo emulator, ki olajša in zviša kakovost razvojnega procesa elektroporatorja.

Elektroporacijska naprava, razvita v okviru te doktorske disertacije, deluje v skladu s pričakovanji, maksimalna izhodna napetost je 4 kV, teoretični maksimalni tok 131 A, minimalna dolžina pulza 200 ns in maksimalna ponavljalna frekvenca 2 MHz. Naprava generira asimetrične bipolarne pulze, kar pomeni, da lahko uporabnik hkrati nastavi asimetrijo trajanja pulzov in napetostno amplitudno asimetrijo. Tako omogoča raziskovanje še neznanih učinkov, nadaljno raziskovanje oziroma nadgradnjo raziskav učinka občutljivosti in preklica ter *in vivo* raziskave visokofrekvenčne elektroporacije. Naprava je bila testirana *in vivo* pri prvi visokofrekvenčni elektrokemoterapiji na miših. Spremljali smo učinkovitost terapije in krčenje mišic med dovajanjem električnih pulzov. Rezultati kažejo, da je učinkovitost HF-ECT z bleomicinom in cisplatinom primerljiva s "klasično" ECT z bleomicinom in cisplatinom. V nasprotju z do sedaj objavljenimi študijami krčenje mišic ostaja signifikantno tudi v primeru visokofrekvenčne elektroporacije, vendar smo bili prvi, ki smo električne pulze dovajali prek kože s ploščatimi elektrodami.

Razvili smo dva splošna Markova modela za CEA, - enega za kožni melanom in drugega za bazalno celični karcinom (BCC) - in njuni reducirani verziji, ki sta sestavljeni samo iz stanj, ki so pomembna za uporabo nove tehnologije, kar je v našem primeru elektrokemoterapija. Ker je elektrokemoterapija nova tehnologija, še vedno ni v uporabi pri vseh pacientih, a se z višanjem števila študij povečuje njena verodostojnost in se zato njena uporaba postopoma dviga po lestvici stopnje rakavega obolenja. Realna analiza stroškovne učinkovitosti za določeno vrsto raka olajša nakup opreme in napredovanje kliničnih študij. Poleg tega lahko napoved stroškovne učinkovitosti vpliva tudi na naslednji krog zbiranja sredstev. Vendar je za vsako obolenje potrebno razviti specifični model, zato da vključimo vse specifične pojave pri določeni vrsti raka. Elektrokemoterapijo je potrebno primerjati z uveljavljenimi

terapijami, ki pa so odvisne od vrste rakavega obolenja, lokacije in velikosti tumorja.

V sklopu doktorske disertacije smo za elektrokemoterapijo kožnega melanoma IV in IIIc stadija ocenili stroškovno učinkovitost. Rezultati kažejo, da elektrokemoterapija sicer zviša kakovost življenja pacientov po posegu, vendar je verjetnost, da je terapija stroškovno učinkovita, dokaj nizka (približno 30 %), kar pomeni, da bi bilo potrebno znižati, ceno za uspešno implementacijo v klinično prakso. Razen v primeru, ko obravnavamo paciente, ki imajo krvaveče tumorje, se izkaže, da je terapija stroškovno učinkovita (verjetnost naraste na 0,91). Stroškovno učinkovitost bi najlažje zagotovili z znižanjem cene elektrode, ki predstavlja skoraj polovico celotnega stroška posega.

V Izvirni prispevki k znanosti

Ocene elektroporatorjev in predlog standardizacije elektroporatorjev kot samostojnih medicinskih naprav

Proizvajalci elektroporatorjev s prikrivanjem parametrov generiranih pulzov ovirajo razvoj elektroporacijskega področja. Nekateri namerno načrtujejo naprave tako, da merjenje dovedenih pulzov ni mogoče, tehnične specifikacije izhodnih pulzov ne sovpadajo z zmogljivostmi naprave in uporabnika ne obvestijo o napakah pri dovajanju pulzov. Posledično poročila študij ne vsebujejo potrebnih podatkov za reprodukcijo poskusov. Zato je za boljši vpogled v trenutno stanje in trende razvoja na elektroporacijskem področju narejen nov pregled komercialno dostopnih elektroporatorjev, njihovih značilnosti, omejitev in slabosti. Prav tako je podano strokovno mnenje o napravah za elektroporacijo kože. Pri kakovosti merjenja in dovajanju pulzov smo se osredotočili na področje nanosekundne elektroporacije, kjer je merjenje najbolj zahtevno. S popisom in analizo smo poudarili in dvignili ozaveščenje o pomembnosti merjenja, saj lahko samo z ustreznim merjenjem zagotavljamo kakovost ter spodbujamo proizvajalce, naj ne prikrivajo parametrov generiranih pulzov. Tako smo pripravili priporočila za standardizacijo, ki se osredotočajo predvsem na oceno pravilnega ali nepravilnega delovanja elektroporatorjev, in razvili elektronski emulator biološkega bremena med elektroporacijo, ki omogoča trajno testiranje in nepristransko primerjavo delovanja različnih elektroporatorjev.

Razvoj novega bipolarnega visokofrekvenčnega visokonapetostnega pulznega generatorja - elektroporatorja

Nedavne raziskave na področju preklica, občutljivosti in vzbujalnih učinkov elektroporacijskih pulzov so nas motivirale, da razvijemo visokofrekvenčni, visokonapetostni, bipolarni generator pravokotnih pulzov. Generator razvit v sklopu te doktorske disertacije temelji na nadgradnji že obstoječih rešitev pulznih generatorjev. Naprava generira visokonapetostne pulze z maksimalno napetostjo 4 kV in minimalnim časom preklopa med pozitivno in negativno polperiodo. Minimalna dolžina pulza je 200 ns maksimalna ponavljalna frekvenca pa 2 MHz. Nova naprava omogoča raziskave vnosa snovi in vzbujanje celic v *in vivo* pogojih, možnost generiranja homogenega električnega polja znotraj heterogene strukture tkiva, zmanjšanje živčnega in mišičnega vzbujanja ter občutka bolečine z vi-

sokofrekvenčnimi bipolarnimi pulzi. Od že obstoječih naprav se razlikuje predvsem v tem, da generira tudi asimetrične bipolarne pulze, ki jih potrebujemo za raziskave efekta občutljivosti in preklica. Razvit elektroporator smo preizkusili v prvi *in vivo* visokofrekvenčni elektrokemoterapiji (HF-ECT), pri kateri se je izkazalo, da je HF-ECT z bleomicinom in cisplatinom enako učinkovita kot uveljavljena "klasična" ECT z bleomicinom in cisplatinom.

Ocena cenovne učinkovitosti elektrokemoterapije v terapiji bazalnoceličnega karcinoma in kožnih metastaz melanoma

Nekaj stroškovnih analiz terapij, ki uporabljajo elektroporacijo, je že bilo narejenih, vendar nobena ne vključuje kakovosti življenja (QoL), ki pa je ključnega pomena pri vrednotenju novih tehnologij v medicini *(ang.: Health Technology Assessment (HTA))*. Glavni razlog je najverjetneje neustrezno zbiranje podatkov o kakovosti življenja pacientov. Sklepamo, da v sklopu kliničnih študij zbiranje podatkov ni bilo sistematično izvedeno. V naši študiji stroškovne analize elektroporacije smo se osredotočili na elektrokemoterapijo kožnega melanoma in bazalno celičnega karcinoma (BCC). Za omenjena tipa rakavega obolenja smo se odločili, ker se terapiji na teh dveh vrstah raka izvajata najdlje in imata dobre rezultate. Razvili smo dva Markova modela, za vsako vrsto obolenja posebej, nato pa definirali potrebne podatke in jih zbrali za primer kožnega melanoma IV in IIIc stadija. Izračunali smo stroškovno učinkovitost elektrokemoterapije kožnega melanoma IV in IIIc stadija za paciente zdravljene na Onkološkem inštitutu v Ljubljani z napravo Cliniporator in pripadajočimi elektrodami ter podali predloge, kako izboljšati stroškovno učinkovitost. Realna stroškovna analiza dviga ozaveščenje o novi terapiji in pripomore k bolj racionalni porabi sredstev ter podaja informacijo o predvidenem stroškovnem okviru za uspešno implementacijo v klinično prakso.

High voltage pulse generation for electroporation based technologies and therapies

1 Introduction

1.1 Electroporation - The phenomenon

The first observations of effects of pulsed electric fields on biological material dates more than 250 years back. But in the past two decades applications of electroporation emerged in medicine, food processing and biotechnology [1]. The phenomenon of electroporation leads to increased cell membrane permeability. Due to exposure to high voltage electric pulses, cell membrane becomes permeable to molecules, which otherwise can not cross the membrane [14]. Several theoretical descriptions of the electroporation phenomenon have been proposed. The most established and likely is that electroporation is based on the aqueous pores, which form in the lipid bilayer [109, 110, 7]. When cells are exposed to pulsed electric field, voltage is induced across their membranes. If the induced voltage is higher than the electroporation threshold voltage, the rearrangement of membrane components is triggered, which leads to the formation of hydrophilic pores in the bilayer, the presence of which increases the ionic and molecular transport to otherwise impermeable membranes [111, 7]. Electroporation is reversible, if the cell afterwards fully recovers. However, when the damage of electroporation is too excessive, the electroporation is irreversible [6] and the cell dies presumably due to a loss of cell homeostasis [11]. Experimental observation of the pore formation was not successful with known techniques, but molecular dynamic (MD) simulation provides convincing corroboration [112, 113].

1.2 Electroporation applications in medicine

Electroporation is a platform technology, which is already well established in medicine, food processing and biotechnology [3, 2]. The technology holds great promise also in other fields, such as biomass production [114] and transdermal transport [115, 116]. However, hereinafter only those which relate to PhD topic are presented, i.e. ElectroChemoTherapy (ECT) and IrReversible Electroporation (IRE).

1.2.1 Electrochemotherapy

ECT is an antitumor therapy [49, 50]. Locally applied high voltage electric pulses lead to a transient permeabilization of cells in tissue, which in case of a proper application, covers all tumor cells [51]. Diffusion of chemotherapeutic drug (bleomycin or cisplatin) is thou enabled and higher cytotoxicity is reached [117]. For a successful application, a sufficiently high drug concentration and appropriate electric field distribution within the tumor should be delivered to the targeted area [35]. Electrochemotherapy is a highly efficient treatment, with complete response rates, based on a single treatment between 60 to 70 % and objective response rates up to 80 % [52]. Additionally it is comparable if not even more efficient than other similar skin targeted therapies [53]. At the beginning ECT was mainly used in treatment of cutaneous tumors and skin metastases, following Standard Operating Procedures (SOP) [54], later it was demonstrated that ECT can be successfully used also for treatment of deep-seated tumours. Recently new strategies were developed which enable treatment of deep-seated tumours, including bone metastases, liver malignancies, and pancreatic, prostate and gastrointestinal tumors [9].

1.2.2 Irreversible Electroporation

IRE is used as "non-thermal" ablation of normal and tumor tissue, as cells primarily die due to the membrane permeabilization and not due to increase of tissue's temperature [61]. It was shown that IRE does not cause the denaturation of proteins and is not affected by decreased blood flow, additionally rapid activation of the immune system, no scarring and the potential ability to treat tumors near large blood vessels was observed [11]. However a local temperature increases around the electrodes, can be significant at higher amplitudes, due to high number of pulses delivered to limited volume of tissue [62, 63]. Also muscle contractions associated with the high voltage pulses are present during treatment [67]. But recently in a series of studies, authors showed that by applying bursts of high-frequency, bipolar pulses also termed as H-FIRE (High-Frequency IrReversible Electroporation) pulses, muscle contractions can be avoided during IRE without compromising the non-thermal mechanism of cell death [67] and additionally the electric field distribution is more homogeneous. Even the transmembrane transfer of molecules may be achieved with bursts of short microsecond long pulses, however H-FIRE pulses need considerably larger voltage amplitudes for cell disruption in comparison to longer monopolar pulses. [118]. Even more, the most resent research claims it is possible to use H-FIRE in electrochemotherapy, but again, at the expense of higher electric

fields than in classical ECT [102].

1.3 Dosimetry in electroporation

A pulsed power generator termed as electroporator and electrodes are used to expose the cell to pulsed electric field [23]. Electroporator generates electrical pulses with specific shape, amplitude, duration, number, repetition rate and sequence (bursts of pulses) [24, 119]. By applying electrical pulses to the electrodes, which are in direct contact with the tissue, an electric field is generated in tissue with specific distribution and intensity [43].

1.3.1 Electric pulse generators

Different applications require particular pulse shapes, voltage amplitudes, pulse width, pulse repetition rates and number of pulses or bursts of pulses [23, 24]. For the introduction of small molecules, pulses which generate the electric fields strengths of approximately 1 kV/cm, with pulse widths from μs to ms range are used. For the introduction of large molecules different protocols have been established, higher electric field strengths of few kV/cm, with pulse widths in rage from few to hundred μs, or low voltage pulses with few hundred V/cm and ms pulse widths, or even the combination of both. For electroporation of the cell organelle membranes, pulses with widths in ns range and amplitudes of tens of kV/cm or more are used [111]. Therefore specific pulse generators, i.e. electroporators have to be designed and developed for each application. When designing an electroporation device one should always keep in mind that, biological sample as a load has resistive–capacitive nature and can vary from sample to sample and in addition the impedance of a biological sample decreases during the pulse delivery [105, 120].

Micro- and millisecond square wave pulses are usually generated by a capacitor discharge circuit, with fast power MOSFET or IGBT used as switches. To minimise a voltage drop on the load, all the required energy must be generated and stored in the capacitor before the pulse delivery. Meaning large capacitor or capacitor banks are needed, but this is resulting in difficult voltage modification. Additionally, low impedance of a load (tissue or cell suspension) require large power/currents, which quite often leads to significant voltage drop on the load. Also when using protocols in which many pulses are delivered to the load, with higher pulse repetition rates, significant amplitude voltage drop, with each successive pulse can be observed, if the energy storage is not sufficient. To achieve efficient electric field

that enables electroporation, high voltages and currents are required, therefore generator construction is challenging. The most complicated circuit designs are required, for generation of high power and short duration pulses, with fast switches. To generate high voltage square wave pulses three different circuit concepts are used, i.e. Marx generator, transistors in series or modular square wave generator [121, 122, 123, 124]. For the generation of high frequency and high voltage square wave pulses Radio Frequency MOSFET (RF-MOSFET) or Silicon Carbide MOSFET (SiC MOSFET) are mainly used. For the bipolar pulse generation a H-bridge solution is the most established. Short rise time of high voltage pulses is provided by modular generator and short fall times can be achieved by short circuiting of the load [125]. Modular generator is capable of delivering currents of several amperes and has a capability to generate various pulse duration [122], amplitudes and pulse repetition rates. The topology is simple as it consists of n individually controlled voltage sources, which are at any time available for the participation in the output pulse formation. On the output we have a series of n transistors that connect sources in series, sources that do not participate in output pulse formation, have no current, since the load current is pushed through diode. The output pulse rise and fall time depend only on the speed of transistors, there are no additional pulse repetition rate limits. For the generation of bipolar pulses, a transistor bridge that enable polarity change is added at the output. Diagonal bridge transistors in case of proper control change the polarity of the output voltage [125].

1.3.2 Electric pulse delivery and standardization

We distinguish between prototype and commercially available electroporators. A considerable number of electroporation devices can be found on the market, some designed for specific applications and some for multifunctional laboratory use. Several reviews have been published in which commercially available electroporators were described but not really compared [94, 93, 8]. Even more, as the electroporation market grows more and more manufacturers conceal the output pulse parameters of their devices, claiming that it is their intellectual property. We have already reached a critical point on the field of gene transfection, where pre-programmed electroporation procedures are most commonly used by researchers, without even knowing basic pulse parameters such as amplitude. Pre-programmed and concealed operation of electroporation devices limits researchers, restricts and

hinders sharing, comparing and reproduction of results, consolidation of knowledge and the development of new knowledge. Failed efforts to confirm other group's published papers work are unfortunately increasing in electroporation as in biomedical research in general [30]. In the field of electroporation, this may be because in some studies the determination and description of the electric fields to which the cells were exposed during experimental work were not adequately reported, or at least were not reported in sufficient detail. Measurement of electroporation pulses is crucial to adequately determine and control the quality and the delivery of pulses. Very short (from 1 to 20 ns) electroporation pulses are the one most challenging to be measured, because of finite pulse propagation speed (20 cm/ns in transmission line) and associated pulse reflections [18].

While all of the currently present problems could be avoided, or could be at least reduced with the implementation of a particular standard for electroporators. A clinical electroporator is a medical device, therefore it must comply with medical device standards. In Europe, it is regulated by a Medical Device Regulation - MDR (EU 2017/745) and in the USA by Code of Federal Regulation - CFR (Title 21). The usability, safety, and risk management are strictly defined, however, none of the existing regulative directly addresses the operation or electroporators performance requirements [126, 127, 128]. Even more, a laboratory electroporator is not classified as a medical device and therefore the regulation is even less strict.

1.4 Health Technology Assessment

Screening and evaluation of medical technologies is becoming crucial, as in the most developed countries health care expenditures are constantly increasing, while at the same time budgets are decreasing [129]. In Slovenia, health care spending is around 8.6 % of GDP which is under the European average, that was in year 2012 evaluated to 10.2 % of GDP. Moreover, projections show that Slovenian population is aging and that in 2050 29.8 % of the Slovenian population will be older than 65 years, what will most likely result in a significant rise of health care expenses. Simulations show that the increase of health care expenses pre year will rise from 0.6 %/year in 2016 to 2.8 %/year by the year 2060 [130]. Independently from the relative or absolute expenditure per year, the health care national budget is in any case limited. Therefore, decision makers have to select carefully how to invest public money, as the introduction of a new technology will result in the exclusion of an old one. Health Technology Assessment (HTA) is a systematic evaluation

of the properties and effects of a health technology, addressing mainly the clinical effectiveness, safety and cost-effectiveness. HTA helps health technology decision makers allocate resources, thus allowing better management of health technologies and innovations, and provides guidelines for competitors, developers and researchers. Health technology is evaluated by systematic tools and modelling for instance cost-effectiveness can be evaluated by Cost-Effectiveness Analysis (CEA) [131, 82].

1.4.1 Cost-Effectiveness Analysis

CEA compares health care expenses with treatment outcomes in term of their utility to patient measured in Quality Adjusted Life Years (QALY) [132]. QALY is a generic measure of disease burden that includes both the Quality of Life (QoL) and the quantity of life lived. In HTA it is assumed that people in their lives move through different health states and each of them has a specific value attached. QALY in CEA represents years of life subsequent to a health care intervention that are weighted or adjusted for the QoL experienced by the patient during those years [83, 133]. QoL is normalized to a standardized scale with range from 0.0 (death) to 1.0 (perfect health) and linear distribution in between. Widely used standardized instrument for measuring QoL is an EuroQol five dimensions questionnaire (EQ-5D) [85]. For the CEA analysis, data collection methods such as: systematic literature review; meta-analysis; modelling; group judgment; unstructured literature review and expert's opinions can be used [54]. However, it can happen that for a specific new technique or an innovation, there is not even a single primary study (empirical research study) available. In this case a potential source of bias must always be considered and documented [133]. In order to incorporate conditionality and uncertainty of collected data, regardless of the reliability of the data, decision models are used to simulate adjustments of projections of existing primary data. With modeling patients conditions, treatment efficacy, treatment and maintaining costs and incidence of the disease projections to a future costs and outcomes of the treatments or innovations, can be made. Decision making trees, fuzzy logic and state transition modeling, such as Markov model and Monte Carlo simulation are most often used. Decision trees are not appropriate for modeling of diseases with a recurrent health states. In such cases a state transition models should be used, where the probability of transition between all the states and remaining in each state is incorporated. In the Markov model for HTA each state represents a specific state of health, or stage of disease, between which patients migrates. There is always one state that represents death, which is considered an absorbing state, as it does not have a feedback

connection. Other health states are defined accordingly to the disease studied. The model's structure should be as simple as possible, as it is not necessary to model the full complexity of the disease. Model complexity (i.e. number of parameters and model order) should every time be limited by the quantity and quality of available data [134]. More aggregated structure that however still includes fundamental disease process and interventions is often the best solution. The innovation cannot be cost-effective by itself; therefore, CEA always includes a comparison of a new to a benchmark technology.

2 Aim

The aim of this thesis is quite versatile, it combines three different topics, with a common main topic called electroporation.
The main topic of the first aim was to review currently available commercial electroporation devices, their operation, and usage. Thought testing and evaluation of the devices, a need for an electronic emulator of the biological load becomes apparent. Therefore the aim extended to the development of an electronic circuit that behaves in the same way as biological load, during electroporation. Additionally, due to the review, the focus moved also on the measurement of delivered pulses during electroporation, which enables quality assurance. Dosimetry in electroporation and measurement of nanosecond pulses, which is the most challenging, becomes an area of interest. This topic concluded with the idea that standardization, in the field of electroporation, is necessary. Electroporation is a platform technology and a specific standard for each application or field should be established. Nevertheless, some basic technical specifications and tolerances can be applied to all electroporators. The aim was to write the recommendations for the standardization of electroporation devices.

The second aim was to develop a new versatile high voltage electroporator. Recent insights on H-FIRE raised interest in the exploration of the sensitization and cancelation effects described below. The sensitization effect is defined as a total molecular increase or decrease of uptake in comparison to applying a single dose to a split dose. For pulses of short duration (in a scale of a few hundred nanoseconds), a cancellation of biological effects have been detected [19, 20, 21, 22]. Cancellation effect is defined as a decrease of uptake when one monopolar pulse closely followed by a second monopolar pulse of the opposite polarity (actually one bipolar pulse) is used instead of only one monopolar pulse [91]. However, uniform electroporation of the heterogeneous structure of tissues is a technological challenge that could be overcome by electroporation with high-frequency, bipolar pulses [92]. To allow us to study all the above-described effects *in vivo*, the output voltage of the new generator should reach 4 kV, and the shortest pulse duration and pause between the bipolar pulses should be 1 μs. The simplest way to generate high-frequency square wave pulses is to connect and disconnect the charged capacitor to the load by the silicon transistor. According to the electroporators review [8, 93, 94] and economic

evaluations [95, 96] that have been done by now, we came to the conclusion, we have identified the need to develop. The development of a bipolar pulse generator with a maximum voltage of 4 kV and a minimum duration time lower than 1 μs, would enable an evaluation of biological effects of very high and at the same time short electric pulses. Both *in vitro* and *in vivo*, we believe advantage of those specific pulse characteristics might be in the reduction of an electric field induced muscle contraction and pain sensation [97, 67, 98]. In order to reduce movement, muscle relaxants are currently administered prior to treatment and local or general anaesthesia is used, but the dosage of those relaxants, need to be monitored throughout the procedure [99, 100]. It was shown that the electric field threshold for muscle contraction is two times lower than the threshold for electroporation [98]. It was already reported that bipolar pulses [101] and H-FIRE pulses [67] reduce muscle contraction. It was also shown that IRE can be performed with a phase duration of 1 μs by increasing the number of pulses and electric field strength [67]. An additional aim becomes apparent, which included *in vivo* experiments with the developed device. The feasibility of High-Frequency electrochemotherapy (HF-ECT) and evaluation of muscle contractions present during the pulse delivery were researched.

In order to define the budget for the development of a new clinical electroporation device, the focus, in the scope of the third aim, is on to Heath Technology assessment, in more detail to the Cost-Effectiveness Analysis (CEA). A few CEA have already been done for electroporation [9, 96], but due to the lack of information obtained, especially about QoL increase, results are inadequate and not accurate. Electrochemotherapy of skin melanoma and basal cell carcinoma (BCC), as these two therapies are used for the longest time and are also very successful are economically evaluated.

3 Results and Discussion

Three original scientific contributions were made in this thesis:

1. Evaluation of electroporators and recommendations for standardization of electroporator as a standalone medical device;
2. Development of a novel bipolar high-frequency high-voltage pulse generator - electroporator;
3. Cost-effectiveness analysis of electrochemotherapy for treatment of basal cell carcinoma and skin melanoma;

The results and discussion section consists of eight papers and a book chapter, which presents the work done in the scope of this thesis.

The first scientific contribution is addressed in a book chapter, which was publishes in 2017 and five scientific papers. Paper 1 and 4 were submitted to the SCIE ranking journals and are under the review process. Paper 2 and 3 were accepted for publication and paper 5 is already published.

Book chapter (Pirc, Reberšek and Miklavčič) published in Dosimetry in bioelectromagnetics, titled *Dosimetry in electroporation-based technologies and treatments* provides a brief introduction in electroporation field. The physical dosimetry of pulse delivery is described in detail, with the focus on measuring. We discuss why measuring is necessary and provide basic guidelines on how to measure and report. A review of commercially available electroporators for ECT IRE and GET and different electrodes for electroporation, at the time of writing, is presented in two tables. We contacted 25 manufacturers, but only 13 were pleased to cooperate, others did not want to disclose the output pulse characteristics of their devices. As the electroporation market grows more and more manufacturers conceal the output pulse parameters of their devices, claiming that it is their intellectual property. We concluded the field of electroporation is in need of promoting reproducible research, which can only be achieved by adequate measurements, standardized reports, and proper use of electroporators and electrodes.

Paper 1 (Dermol-Černe, Pirc, and Miklavčič), titled *Mechanistic view of skin electroporation – models and dosimetry for successful applications: an expert review* contains a more recent and focused review of electrode configurations and pulse generators, prototypes as well as commercially available models, used in skin electroporation. An expert opinion about each of the reviewed devices is given and the conclusion that electrode configuration and electroporators are not adequately reported and therefore a comparison of studies is difficult, if not impossible, is made. For each skin electroporation treatment, systematic studies determining the optimal parameters should be performed and treatment parameters standardized.

Paper 2 (Pirc, Reberšek and Miklavčič), titled *Functional requirements and quality assurance necessary for the successful incorporation of electroporation based therapies into clinical practice* provides recommendations for the development of clinical electroporators. Electroporation based therapies have a huge potential for implementation into clinical practice in socioeconomically disadvantaged populations. But currently, the price of electroporators and electrodes is relatively high, however, custom low budget devices can be developed. Functional requirements for a medical electroporator and safety guidelines, with the focus on medical device standard and FDA requirements are described. Quality assurance, the importance of measurement during the pulse delivery and additionally, recommendations for standardization are also addressed in the paper.

Paper 3 (Cvetkoska, Pirc, Reberšek, Magjarević, and Miklavčič), titled *Towards standardization of electroporation devices and protocols* proposes guidelines for the design of clinical electroporators and define minimal requirements for their safe and efficient use which can be incorporated within the particular standard in the future. In the paper, we describe the electroporator's output parameters and define tolerances on the base of the standard operating procedure for electrochemotherapy.

Paper 4 (Pirc, Balosetti, Miklavčič and Reberšek), titled *Electronic emulator of biological load during electroporation*, in detail describes an innovative method for the evaluation of clinical, laboratory and prototype electroporation devices during the development process, or to evaluate their final performance considering at least from the perspective of output pulse parameters. We developed an analog electrical circuit that has equivalent impedance as the beef liver tissue during high voltage pulse delivery and or electroporation. An equivalent circuit was simulated, build and validated. Proposed emulator facilitates standard implementation, it

enables the development of standardized testing protocols, which are fundamental for quality assurance.

Paper 5 (Pirc, Miklavčič and Reberšek), titled *Nanosecond Pulse Electroporator with Silicon Carbide MOSFETs, Development and Evaluation*, is focused on nanosecond electroporation, which is studied for more than a decade, but it is still not entirely understood, we believe one of a contributing reason for this situation, is also a unique, group-specific, prototype hardware equipment and challenging measuring methods. In the scope of this paper, we improve the performance of the high-voltage nanosecond pulse generator by introducing Silicon carbide (SiC) MOSFETs. The developed generator can deliver from 500 V to more than 6 kV, approximately 8 ns pulses to a 50 Ω load. The measurement and evaluation process are described in detail. Additionally, we emphasize on the size of an error which occurs during measurements.

Second scientific contribution is addressed in a one scientific paper, which is ready for submission to the SCIE ranking journal.

Paper 6 (Pirc, Miklavčič, Uršič, Serša and Reberšek), titled *High frequency and high voltage asymmetric bipolar pulse generator for electroporation based technologies and therapies*, describes the development and evaluation process of an electroporator that generates as well, symmetrical as asymmetrical (duration and amplitude) bipolar pulses. The voltage amplitude can reach up to 4 kV, while the maximal theoretical load current is 131 A, the minimal pulse duration is 200 ns. The device was also evaluated *in vivo*, the first *in vivo* use of HF-EP (high frequency) in electrochemotherapy was performed and the desired performance was reached. Additionally, the acceleration of mice leg during the pulse delivery was measured in order to evaluate muscle contractions. The design proposed in this paper enables faster development and spread of the technology and *in vivo* experiments, due to the high maximal current of developed device.

Third scientific contribution is addressed in two scientific paper, paper 7 was published in 2018 in slovenian medical journal, while paper 8 is ready for submission to SCIE ranking journal.

Paper 7 (Pirc, Pecchia, Reberšek, Serša, Snoj, Grošelj, Miklavčič), titled *Study*

design of a medical device pre-market assessment: a case study on electrochemotherapy, consists of two parts: the first part presents basic principles and concepts of health technology assessment and cost-effectiveness analysis, and the second part reports study design of an early cost-effectiveness analysis of electrochemotherapy for the treatment of basal-cell carcinoma and skin melanoma. Two general Markov models and their reduced versions, designed specifically to assess electrochemotherapy of basal-cell carcinoma and skin melanoma, were developed in the scope of this paper. The data required for successful calculations have been identified, some of which were missing at the time of writing, therefore recommendations for data collection process and follow-up reporting were made. With this paper, we tried to raise awareness about the importance of numeric quality of life reporting and usefulness/meaning of EQ-5D questionnaire that might not be self-evident at first sight but are crucial for cost-effectiveness analysis.

Paper 8 (Pirc, Federici, Bošnjak, Perić, Reberšek, Pecchia, Glumac, Čemažar, Snoj, Serša, and Miklavčič), titled *Early cost-effectiveness analysis of electrochemotherapy as a prospect treatment modality for skin melanoma*, describes a study of an early cost-effectiveness analysis of electrochemotherapy with Cliniporator in patients with stage IV and IIIc skin melanoma in Slovenia. In the whole patient population, ECT of skin melanoma stage IV and IIIc is expected to increase the quality of life by 0.29 QALYs, at the higher cost of 6,568 €. At the cost-effectiveness threshold of 20,000 €, the probability of ECT being cost-effective compared to standard of care is estimated to be 0.3 and 0.91 for the whole patient sample and the patients with bleeding lesions respectively. For the whole sample population, a reduction in the price of the electrodes by half is expected to increase the probability of being cost-effectiveness from 0.3 to approximately 0.64.

3.1 Book chapter

Title: **Dosimetry in electroporation-based technologies and treatments**

Authors: Eva Pirc, Matej Reberšek and Damijan Miklavčič

Publication: Dosimetry in bioelectromagnetics

Publisher: Boca Raton: CRC Press

Year: 2017

DOI: 10.1201/9781315154572-13

eBook ISBN: 9781315154572

12

Dosimetry in Electroporation-Based Technologies and Treatments

Eva Pirc, Matej Reberšek, and Damijan Miklavčič
University of Ljubljana

To measure is to know.
If you cannot measure it, you cannot improve it.

Lord Kelvin

12.1 Introduction

Electroporation is a platform technology that is already established in medicine and food processing (Haberl et al., 2013a). It is based on increased cell membrane permeability due to exposure to electric pulses (Weaver, 1993; Kotnik et al., 2012). If the cell is able to fully recover afterwards, we call it reversible electroporation; when the damage is too great and the cell dies, we call it irreversible electroporation (IRE). Electrochemotherapy (ECT) is an antitumor therapy in which locally applied high-voltage (HV) pulses trigger a transient permeabilization of tumor cells. Diffusion of a chemotherapeutic drug (bleomycin or cisplatin) is enabled, resulting in higher cytotoxicity. Effectiveness is accomplished if sufficient drug concentration and electric field in the tumor are achieved. ECT is a highly efficient treatment, with complete response rates of between 60% and 70% on a single treatment and with objective response rates up to 80% (Mali et al., 2013),

and is comparable to, if not more efficient than, other similar skin-directed technologies (Spratt et al., 2014). It is used in the treatment of cutaneous and subcutaneous tumors, following standard operating procedures (SOPs; Mir et al., 2006).

If the exposure of the cell to electric field is too excessive, it dies, presumably due to a loss of homeostasis. IRE is used as an ablation method for normal and tumor tissues. It is called "nonthermal" ablation, because cells primarily die due to membrane permeabilization and not due to the increase in the temperature of the tissues. However, we should not overlook local temperature increases around the electrodes, which can be significant at higher amplitudes, increased duration, or number of pulses (Garcia et al., 2014; Kos et al., 2015). Considerable research has also been undertaken in the area of gene transfection and biopharmaceutical drugs stimulating an immune response. Gene electrotransfer (GET) is a nonviral method for delivering DNA molecules into cells. DNA vaccination using electric pulses and clinical trials of GET of DNA with interleukin-12 in patients with metastatic melanoma also has shown great promise in clinical practice (Heller et al., 2001; Haberl et al., 2013a; Lambricht et al., 2016).

Many of its biotechnological applications such as inactivation of microorganisms and extraction of biomolecules have only recently started to emerge, while nonthermal food pasteurization is already being used in the industry (Toepfl et al., 2006; Kotnik et al., 2015). Electroporation is more commonly termed as pulsed electric field (PEF) treatment in food technology. Food preserved by PEF maintains color and flavor, and the antioxidant levels also stay unaffected (Haberl et al., 2013). It is efficient for increasing the shelf life of liquid food (Toepfl et al., 2006). A combination of mild preheating to 60°C and subsequent electroporation reduces the energy needed for efficient disinfection to 40 kJ/L (Gusbeth and Frey, 2009).

Microalgae are currently the most intensely investigated feedstock for biomass production with electroporation; they are getting implemented in biofuel applications (Golberg et al., 2016; Postma et al., 2016). A combination of grape fermentation and electroporation led to an increased content of polyphenolic compounds and less acidity, thereby resulting in a slightly smoother taste and color intensity in wine (Mahnič-Kalamiza et al., 2014). Overall, it is a fast-growing field with great potential.

12.1.1 Electroporation—The Phenomenon

Electroporation is a phenomenon in which cells that are exposed to a high enough electric field increase permeability and conductivity of their membranes. Each biological cell is surrounded by a membrane that mainly consists of phospholipids. Lipids in aqueous conditions spontaneously form a two-molecule thick layer as a result of their dielectric properties. Water and water-soluble molecules cannot pass the entirely intact barrier only by diffusion (Deamer and Bramhall, 1986). In addition, biological membrane also contains glycolipids, cholesterol, and various proteins, which enable selective transport of some molecules from intracellular space to the cell interior and vice versa (Kotnik et al., 2012).

Several theoretical descriptions of the electroporation phenomenon have been proposed. The most established and likely one is that electroporation is based on the aqueous pores formation in the lipid bilayer (Freeman et al., 1994; Weaver and Chizmadzhev, 1996; Kotnik et al., 2012). When cells are exposed to a high pulsed electric field, voltage

is induced across their membranes. This results in the rearrangement of their membrane components, leading to the formation of hydrophilic pores in the bilayer, the presence of which increases the ionic and molecular transport to otherwise impermeable membranes (Pucihar et al., 2008; Kotnik et al., 2012). Experimental observation of the pore formation was not successful with known techniques, but molecular dynamic (MD) simulation provides convincing corroboration. From the electrical point of view, a cell can be modelled as an electrolyte (conductive media), surrounded by an electrically insulated/dielectric shell. Each cell under physiological conditions has a resting transmembrane voltage in the range of −90 to −40 mV (Kotnik et al., 2010). This is a result of ion imbalance in the cytoplasm, controlled by Na^+–K^+ pumps and K^+ leak channels. Na^+–K^+ pumps export Na^+ ions out of the cell and simultaneously import K^+ ions; meanwhile, K^+ ions can freely cross the membrane through K^+ leak channels, to achieve electrical and concentration equilibrium. Applied electric pulses cause local field distortion in the cell and their surroundings. Due to low-membrane conductivity in the vicinity of the cell, the electrical field concentrates mainly in the cell membrane, resulting in electrical potential difference across the membrane. The induced transmembrane voltage superimposes to the resting potential. It can affect transport through the membrane, stimulate cells, and if high enough, lead to the electroporation of cell membrane. Increased cell permeabilization is observed with electric field increase; induced transmembrane voltage is dependent on position, cell shape, and orientation. Delay between external and inducted voltage is in the microsecond range and is determined by the membrane time constant τ_m (Isokawa, 1997). If cells are exposed to electric field in low conductivity medium, delay significantly increases (Kotnik et al., 2010). When short, intense electric pulses (nanosecond pulses, tens of kV/cm, with a period similar to τ_m or shorter) are applied, the outer membrane acts as a short circuit because of cell frequency response, and the applied voltage also appears across the interior of the cell (Kotnik and Miklavčič, 2006). Nanosecond pulses can induce a high enough voltage to cause electroporation of internal organelles (Batista Napotnik et al., 2016). Because organelle interior is electrically more conductive then cytosol, and organelle membrane dielectric permittivity is lower than a cell membrane permittivity, a voltage induced on organelle membrane can exceed the one induced on the cell membrane, resulting in increase of induced voltage amplitude (Kotnik et al., 2010; Retelj et al., 2013). But at the same time, pulses also cause plasma membrane permeabilization (Kotnik et al., 2006; Batista Napotnik et al., 2010).

12.1.2 Physical Dosimetry in Electroporation

The local electric field, i.e., the one "felt" by the cell is the one that leads to membrane electroporation. Applicator/electrode characteristic and applied pulse characteristics define the electric field distribution and intensity. For various applications, different pulse shapes, voltages, duration, repetition frequencies, and sequences are needed. Therefore, special pulse generators have been designed called electroporators. Because biological load characteristics vary considerably, and in addition their conductivity changes due to electroporation during the pulse delivery, development of these devices is challenging.

12.1.2.1 Dosimetry of Pulse Delivery

The electroporation signal is, as mentioned before, characterized by pulse amplitude, shape and duration, number of pulses, pulse repetition frequency, and pulse orientation sequence. Most common pulse shapes that are used in electroporation are square wave (also bipolar), exponential decay, and bell-shaped pulses. When designing an electroporation device, one should always keep in mind that a biological sample as a load has a resistive–capacitive nature and can vary from sample to sample, and in addition the impedance of a biological sample decreases during pulse delivery (Pliquett et al., 2000; Pavlin et al., 2005). The most simple and inexpensive way to generate pulses is by a capacitor discharge circuit (Figure 12.1). When we are dealing with higher voltages, it is more efficient and easier to use smaller capacitors and connect them in to a Marx generator (Figure 12.1c). The main problem here becomes simultaneous switching; the switching element must be chosen with respect to their maximum operating voltage and response rate. The generated pulses have typical capacitor discharge-exponentially decay shape (Reberšek and Miklavčič, 2011; Reberšek et al., 2014). Micro- and millisecond square pulses are usually generated by an HV power supply switching circuit (Figure 12.2a), with fast-power MOSFET (Metal-Oxide-Semiconductor Field-Effect Transistor) or IGBT (Isolated-Gate Bipolar Transistor) used as the switch. All the required energy must be generated and stored in the capacitor before delivery. To minimize a voltage drop, a very large capacitor is needed, resulting in difficult voltage modification. We also have

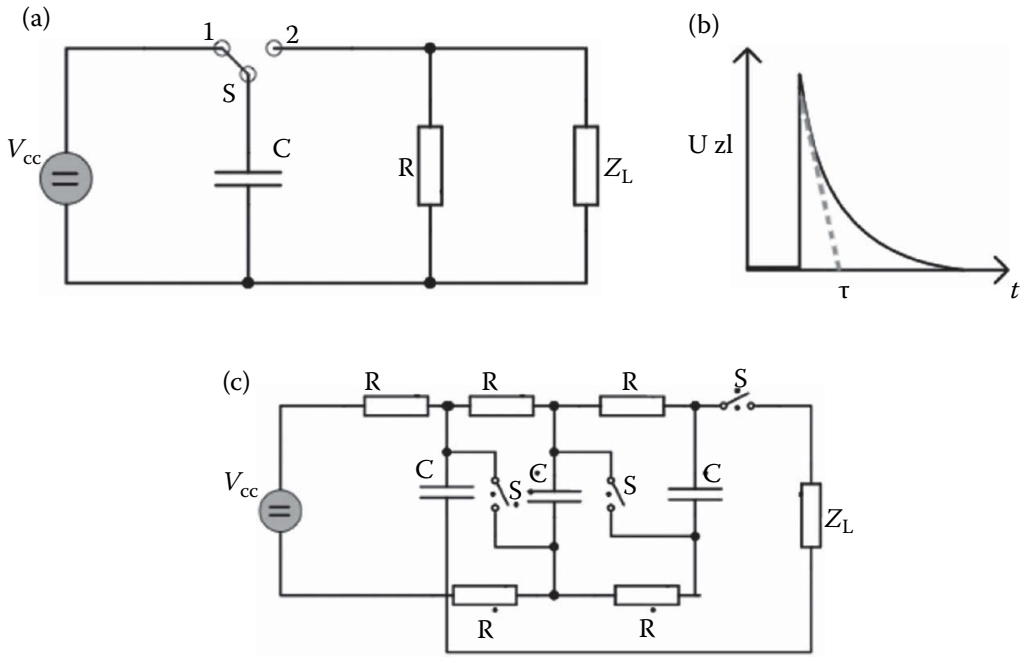


FIGURE 12.1 Panel (a) is capacitor discharge circuit, a built-in resistance R is added to limit the decrease of time constant $\tau = C^* |Z_L| \cdot I_F^* |Z_L| \geq 10R, \tau \approx RC$, resulting in 90% energy dissipation through R. Panel (c) is a proposed Marx bank circuit. Capacitors C are charged in parallel through resistor R and then switched to series building up the voltage to $n^* U$ and discharged through the load Z_L, by switching all switches simultaneously. The maximum applied voltage is equal to the load power supply voltage multiplied by the number of capacitors and time constant. In panel (b), a generated pulse shape is presented, the discharge time is time constant dependent.

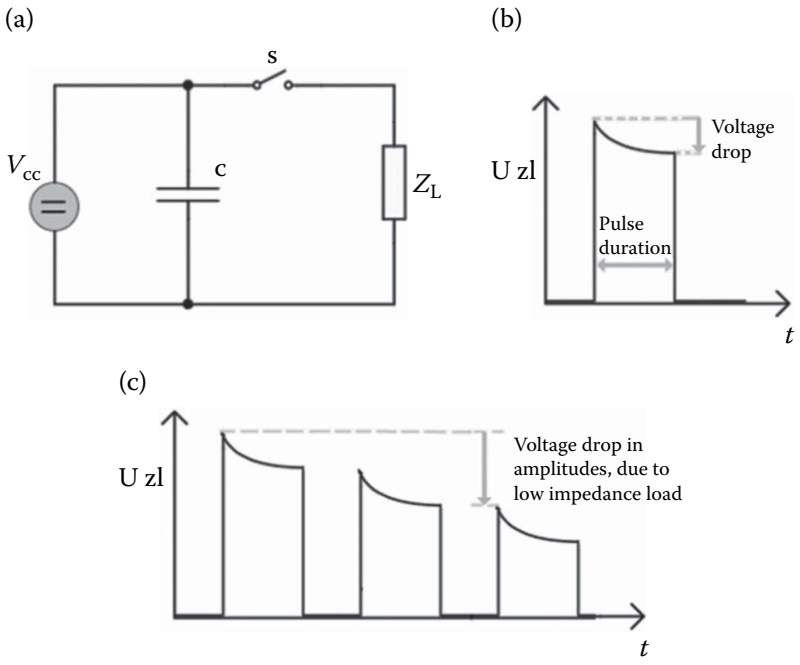


FIGURE 12.2 High-voltage power supply switching circuit (a). The variable power supply V_{CC} defines the amplitude of the output pulse. Switches control pulse duration and pulse repetition frequency. The voltage drop that occurs during pulse is proportional to load impedance $\left(\text{voltage drop} = \text{pulse duration} / \left(C^{*} \left| Z_{L} \right|\right)\right)$ (b). In (c) C an example of reduced amplitude is shown, that can occur in a case of low impedance load that requires too high current.

some time limitations; pulses can be generated after the capacitor is recharged to the preset voltage. Low impedance of a load (tissue or cell suspension) requires large power/currents, which quite often leads to a significant voltage drop. Protocols in which a larger number of pulses are delivered can result in reduced amplitude of pulses (Figure 12.2). This is one of the main reasons why we need to measure when using devices for electroporation. Furthermore, we focus on measuring and quality control.

To achieve an efficient electric field that enables electroporation, we are dealing with HV and currents; therefore, generator construction can be challenging. The shorter the pulses, the more complicated the circuit designs that are required; it is really challenging to generate high-power and short-duration pulses. HV switches with short rise times are needed; spark gaps, UV lasers, SiC MOSFETs, or IGBTs can be used, depending on the application. With nano- and picosecond pulses, pulse-forming networks are a common solution, e.g., a transmission line (Figure 123a). Transmission lines operate in both charging and discharging phases. Generated energy should be stored in a large capacitor and then discharged to the load. After pulse generation, a new pulse can be delivered when the capacitor is recharged, resulting in repetition frequency limitation (Bertacchini et al., 2007; Syamsiana and Putri, 2011; Reberšek et al., 2014).

Electrodes, together with the biological sample, present the load for the pulse generator. The main problem that we encounter here is that electrodes get polarized where they get in touch with the sample due to water molecules and hydrated ions that are present in the surrounding area. It is a frequency-dependent phenomenon, which can be modeled as a capacitor in series with a resistor (Chafai et al., 2015). In a cell suspension, a counterion

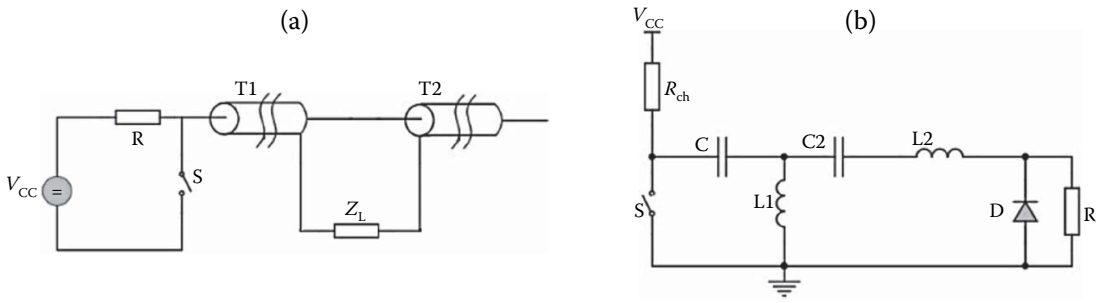


FIGURE 12.3 Concept of Blumlein transmission line (a) and diode opening switch (DOS) circuit (b), commonly used in nanosecond pulse generator designs. The Blumlein transmission line generators have a variable high-voltage power supply *V*, a charging resistor *R*, and two transmission lines. T1 and T2 are charged when the switch is turned off and then discharged through the load when switched. The pulse duration cannot be modified as it equals twice the electrical length of the transmission line, if the impedance of the load is twice the impedance of the transmission line. The DOS generators can be composed of more accessible electrical components than Blumlein generators. Pulse is formed by a diode that must be forward and reverse pumped with adequate sinusoidal current. Diode should stop conducting when the majority of the total energy is stored in L2. That means a current must be maximized at the time of switching. High voltage is switched by diodes, which means MOSFET-s does not need to withstand the whole output amplitude, and does not need to be faster than the output pulse. Pulse duration is determent with diode reverse recovery time. But finding the appropriate matching of capacitors and inductors values in LC oscillator for optimal switching can be challenging. (From Reberšek, M., et al., *IEEE Electr. Insul. Mag.,* 30(3), 8–18, 2014; Sanders, J. M., et al., *IEEE Trans. Dielectr. Electr. Insul.,* 16(4), 1048–1054, 2009.)

layer is formed at each electrode and electric field driving charge transport is reduced, resulting in lower suspension conductivity. At the contacts with tissue, electrodes stimulate the release of electrolytes, resulting in the development of a poorly conductive region where wounds can occur. Luckily, polarization decreases with increasing frequency.

Electrodes must be user friendly; the wires connecting them should be long enough to enable easy handling and smooth application. But each additional wire/connection has some parasitic properties resulting in route losses—the higher the frequencies, the more the parasitic characteristics are manifested. In worst cases, when dealing with nanosecond pulses, the generated pulses at the end of electrodes can completely differ from the ones at the output stage of the electroporator. At high frequencies, reflection on the lines must also be taken into account. The thickness of the wire must be compatible with the output current (Kolb et al., 2006; Batista Napotnik et al., 2016).

Different electrodes are available on the market, and they need to be chosen considering the targeted load and pulse generator restrictions. Electrode geometry and position also determine electric field distribution. *In vitro*, four main groups of electrodes are present: single-cell chambers, macroelectrodes (two-plate electrodes separated for at least 1mm), microelectrodes (glued onto cover glass, with separation of 100 μm), and flow-through chambers (polyethylene or polypropylene used as insulating materials, combined with stainless steel electrodes) (Reberšek et al., 2014). When using nano- or picosecond pulses, impedance matching must be ensured. For *in vivo* use macroplate and needle electrodes are commonly used. Electrochemotherapy standard operating

procedure (ESOP) (Mir et al., 2006) describes three different types of electrodes that were developed within the Cliniporator project and are compatible with the Cliniporator generator (Table 12.1). According to SOP, plate electrodes are recommended for treatment of small and superficial tumor nodules. For treatment of thicker and deeper-seated tumor nodes, needle electrodes are more suitable. During the development of Cliniporator, voltage characteristics—sequences—were determined and acquired for better efficacy (Ongaro et al., 2016). Sometimes, large volumes need to be treated; lately, a new type of electrode that covers larger surfaces has become a subject of study (Ongaro et al., 2016).

12.1.2.2 Measuring

When dealing with electroporation, measuring is crucial for achieving effective electroporation. Quality assurance can only be provided by appropriate measurements. Unfortunately, failed efforts to confirm other group's published work are increasing (Kaiser, 2016). The description of the used equipment and the process are flawed. In many papers describing/using electroporation, insufficient detail is reported, and quite often measurements are not reported (Batista Napotnik et al., 2016; Campana et al., 2016). The electroporation field needs to promote research reproducibility, and to improve this, we further need to try and answer the following questions: "Why measuring is necessary?", "How to measure?", "Which data are significant for researches?", and "Which electroporator to choose?"

For electroporation, you need an electroporator. A considerable number of electroporation devices can be found in the market, some are designed for specific applications and some are multifunctional. Most often, they are compatible with different electrodes. It seems however that as the market grows, manufacturers tell us less about their devices. We have already reached a critical point in the field of gene transfection, where preprogramed electroporation procedures are most commonly used so that quite often the researcher does not even know basic pulse parameters, such as pulse shape, repetition rate, and even less about applied voltage amplitude/electric field strength (see Tables 12.1 and 12.2). The researchers are only aware of the program number they used on their device. This limited information availability restricts and hampers the development of new knowledge. In this case, preengineering of electroporation devices limits researchers and hampers the sharing and comparing of results or protocols and even the further development of gene transfection field.

The most complicated of all are nanosecond electroporation systems. They usually consist of nanosecond pulse generator, transmission line or delivery system, and electroporation chamber/electrodes (Pakhomov et al., 2009; Ibey et al., 2010; Batista Napotnik et al., 2016). When using nanosecond pulses, it should always be taken into account that pulses reflect on impedance change and lose power in the transmission line. If the impedance matching is not guaranteed, reflections are present and load dependent. When load impedance is higher, reflections are positive and add to amplitude that would be present on a matched load. In case of lower load impedance, amplitude on the load will be lower. Pulses can become bipolar and cancelation effect can occur. Nanosecond pulses travel approximately 20 cm/ns in coaxial cable (Batista Napotnik et al., 2016).

12.1.2.3 Why Is Measuring Necessary?

For quality assurance! To be sure that the pulses are delivered and the device operates according to its specifications.

The first and most logical answer is because we want to know if our pulses were successfully delivered and we need to know what was delivered. If the current flows through a load, a delivery was more or less successful. But we still do not know anything about the pulse shape and voltage amplitude, or how many pulses were actually delivered. Due to the nature of electric discharge circuits that are commonly used in electroporation devices, amplitude of successive pulses can be lower with each successive pulse delivered (Figure 12.4), if the pulse repetition rate is in the higher half of device operation range. Low-conductivity media in cuvettes is used for two reasons—to reduce heating of the sample due to the current flow and to facilitate pulse generation. With lower resistant loads, problems can occur because the pulse generators cannot deliver "what they promise," i.e., high currents. A typical cuvette resistance with a low conductivity media is somewhere between 100 and 50 Ω. But high conductive media can have a resistance between 10 and 15 Ω, and even lower, requiring even up to 10 × higher currents. This large variation of load characteristics represents a great challenge in electroporator design resulting in different solutions. Because of software or hardware errors, devices can have an unexpected delay during generation, one or more pulses may be omitted, a voltage amplitude may deviate from the expected value, etc. In the case of clinical medical devices with CE certification, these errors should not occur, or if they do, an alarm must be triggered. But when we are dealing with self-developed or commercial devices not classified as medical devices, monitoring is necessary. Also, electronic components and, consequently, devices are aging, and their characteristics may change with time. Built-in measurement systems are usually comfortable solutions, but some manufacturers are taking shortcuts. If the measurement system is a part of the device, it needs periodic calibration; so if this is not part of the unit maintenance, the measurement system is questionable.

The second reason why measuring is necessary is the reproducibility of research results. For research reproducibility, at least similar, if not exactly the same, pulses are needed. Different research groups have different electroporation devices whose output pulses may derogate from specified shapes/parameters. If we know exactly what kind of pulse is needed, a custom setting might lead us towards better matching. Not reporting pulse parameters hinders the comparison of results and hinders progress of research.

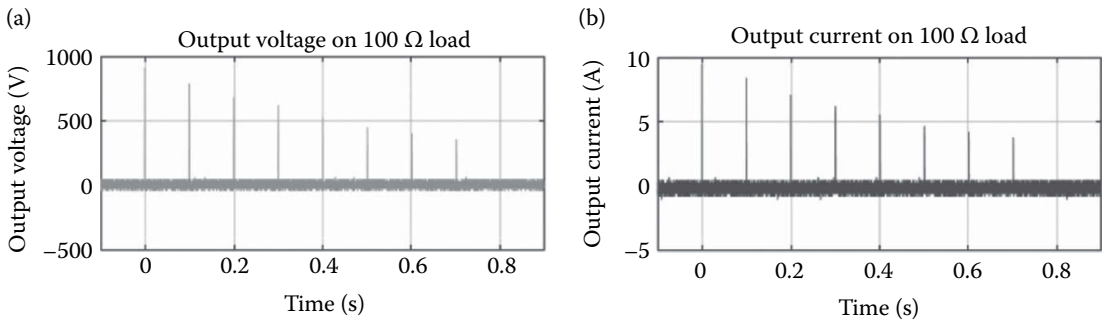


FIGURE 12.4 An example of output pulse measurements, we used a SENNEX electroporator with surface pin electrodes. The biological load was simulated with a 100 Ω resistor. Amplitude of each successive pulse is lower, due to improper operation. The last pulse does not even reach 50% of the preset amplitude. Panel (a) shows output voltage measured on 100 Ω load and (b) output current.

12.1.2.4 How and What to Measure and How to Report

Only correct measurement will upgrade our research, reduce resources used, and help advance the field by contributing to enhancing/improving reproducibility. The easiest way is to measure applied voltage and current. We need an adequate measurement from our oscilloscope and probes. What is appropriate depends on what we want to measure. First, we need to know what pulses are expected—at least amplitude, repetition frequency, and pulse duration. All oscilloscopes have limitations; a bandwidth tells us how fast it follows the signal changes, or more theoretically said, the maximum frequency range that it can measure (Figure 12.8). Closely related to frequency bandwidth is rise time specification. The specified rise time of an oscilloscope defines the fastest rising pulse it can measure. If not specified, it can be calculated as Rise time = 0.35/Bandwidth. For most applications, micro- or millisecond pulses are used; oscilloscopes and probes with a few MHz bandwidth are thus suitable. Measurement gets complicated when we reach the nanosecond HV pulses, where GHz range bandwidths and high rise times are needed. To minimize stray inductance and capacitances and reflections on lines, probes should be located as close to the electrodes as possible, with no additional connecting wires.

For the adequate presentation of pulses delivered, we propose to attach at least two measurements to your publications. One of a single pulse zoomed and another with reduced time scale, where all delivered pulses are displayed (if the number of pulses is low enough to keep a measurement meaningful) (Figure 12.5). If attaching the measurement does not appear to be suitable, an adequate description of a pulse, common notations, and pulse parameters are required. But in some cases when pulses strongly deviate from classical forms, an image tells us a lot more. An example of pulse characteristics determination is given in Figure 12.7.

Exponential decay pulses (Figure 12.6a) are best described by their maximum value, A_{MAX}, and time constant, τ. The value of time constant depends on circuit output stage characteristics. It is defined as the time maximum amplitude A_{MAX}, which drops to 37% of A_{MAX}. Square wave pulses are described with amplitude at high stage (that is choose to best fit the high level) and time t_{FWHM} (Full Width at Half Maximum-FWHM). t_{FWHM} is best described as the time passed between when the pulse reaches 50% of maximal amplitude at the rising and falling phase. Other pulse shapes are best described if we

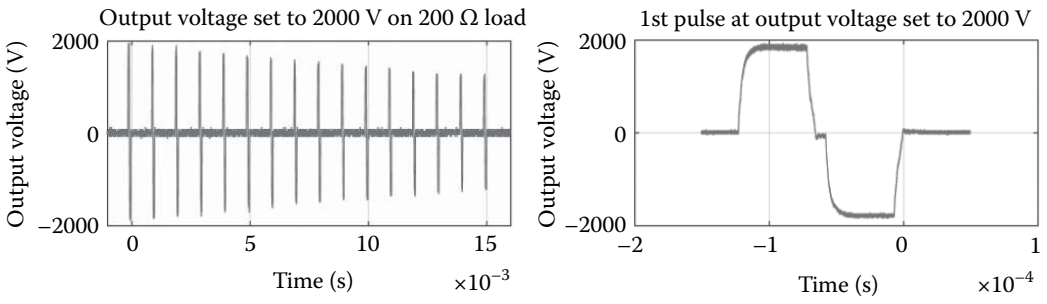


FIGURE 12.5 An example of proposed measurements to accompany the report. For the adequate presentation of pulses delivered, we propose that you attach at least two measurements to your publications. One of a single pulse zoomed and another with reduced time scale, where all delivered pulses are displayed (if the number of pulses is low enough to keep a measurement meaningful) The example measurements were made with the help of a CHEMIPULSE IV electroporator.

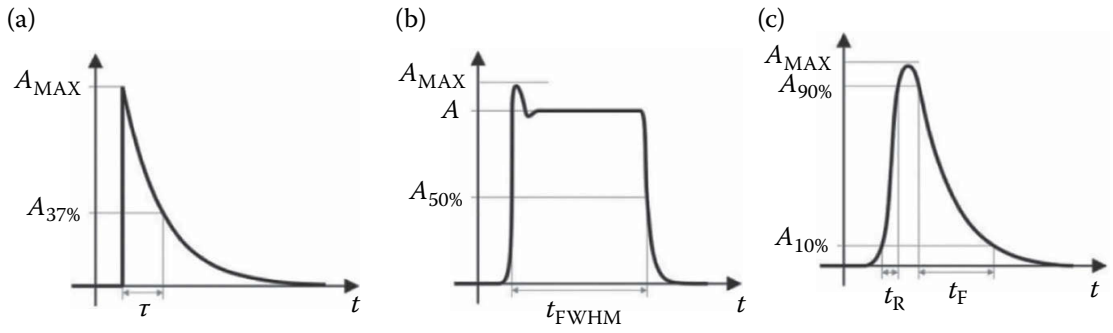


FIGURE 12.6 (a) Exponential decay pulse, (b) square wave pulse, and (c) Gaussian or bell-shaped pulse.

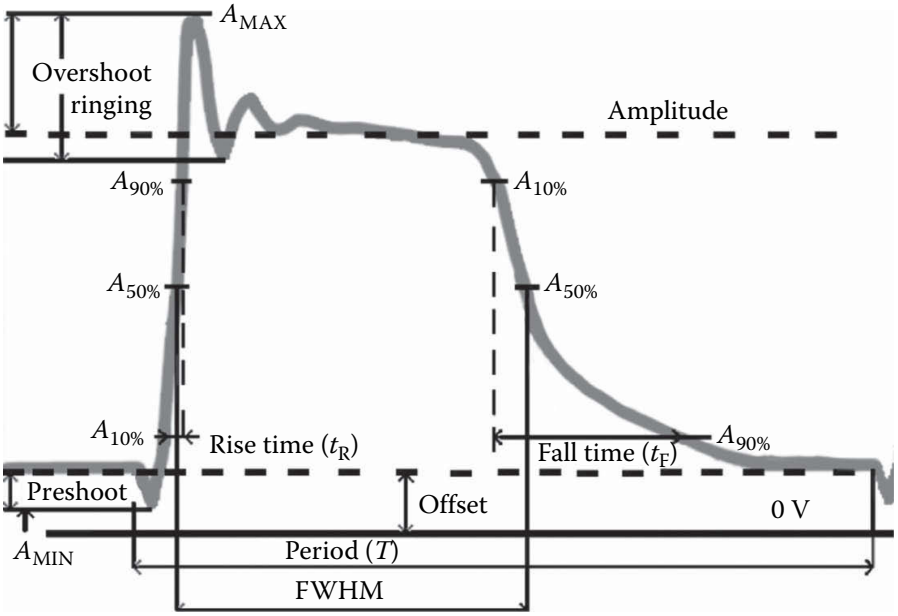


FIGURE 12.7 Pulse characteristics: a presentation of useful terms for description.

define their rise (t_R) and fall times (t_F) and maximum amplitude A_{MAX}. Rise time is time required for a pulse to rise from 10% to 90% of its steady value. Similarly, fall time is the time taken for the amplitude of a pulse to decrease from a specified value (usually 90% of the peak value exclusive of overshoot or undershoot) to another specified value (usually 10% of the maximum value exclusive of overshoot or undershoot) (Reberšek et al., 2014).

12.1.3 Biophysical Dosimetry in Electroporation

Biological cells can be electroporated in suspension, attached, or in tissue. We distinguish *in vitro* and *in vivo* electroporation; the connection between them is not taken for granted. New applications of medical electroporation are first demonstrated *in vitro*, if their efficacy is shown also *in vivo* in an appropriate animal model, human clinical studies can be done (Hofmann., 2000). Electric pulse parameters for effective *in vivo* application can be determined from *in vitro* experiments considering application specifications (Maček-Lebar et al., 2002).

12.1.3.1 Electroporation *In Vitro*

Cell membrane electroporation and consequently increased membrane permeability is controlled by the electric field strength. Because cells are in suspension and we usually work with low cell volume fractions, we can assume the surrounding field is homogenous and uniform throughout all the conducting media (Susil et al., 1998; Kotnik et al., 2010). But induced transmembrane voltage is not uniform on the cell surface, it is dependent on cell size, membrane characteristics and orientation to the field, frequency, and time and space (Teissié and Rols, 1993). *In vitro,* we are dealing with dilute cell suspensions, where the local field outside cells does not affect other cells. If volume fractions are higher than 10%, the induced transmembrane voltage cannot be easily estimated by Schwan's equation; local cell fields influence each other, and therefore approximate analytical or numerical calculations are needed (Pavlin et al., 2005). Even if we increase cell volume fraction of cells, there is still a big difference between tissue and a dense suspension. Plated cells are permeabilized with lower electroporation parameters than when in suspension (Towhidi et al., 2008). In tissues, cells form specific structures and are in contact with each other (Kotnik et al., 2010). *In vitro* experiments can be performed in electroporation chambers, especially with short pulse durations (nano- and picosecond pulses); chamber characteristics such as frequency responses can have a great impact on the results. Results are different when using different cuvettes; pulses are usually applied through two electrodes; the field delivered in is consequently different. From *in vitro* to *in vivo*, one needs to keep in mind that electric pulses are much larger compared to diameters of the cells (Maček-Lebar et al., 2002).

12.1.3.2 Electroporation *In Vivo*

In case of *ex vivo* electroporation of tissues, or *in vivo* electroporation, the electric field can no longer be considered homogenous because tissue is a highly inhomogeneous conductor. Some biological materials are also anisotropic, and therefore electric field orientation must also be considered. Tissue inhomogeneity is frequency dependent, it varies from tissue to tissue and is smaller at higher frequencies. Tumors mostly have a higher water content as a result of cellular necrosis (Miklavčič et al., 2006). In preclinical and clinical studies a few years ago, authors often considered the treated tissues as being linear electric conductors (i.e., with constant tissue conductivities) (Čorović et al., 2013).

Cell membranes have low electric conductivity in comparison to cytoplasm and extracellular medium. Electroporation changes the conductivity of cells, and thereby the field distribution is changed (Sel et al., 2005). To analyze tissue electroporation, we need to know the characteristics of the treated tissue. A macroscopic description is the most common and is described by specific conductivity and relative permittivity. Applied voltage rests among the most resistive tissue, which in the case of external electroporation is the skin. Skin conductivity is 10–100 times lower than the tissue underneath. Restive heating occurs and should be considered so as to avoid damaging healthy cells (Lacković et al., 2009; Kos et al., 2012). Numerical methods are used to define the local electric field distribution within the tumors (Miklavčič et al., 2010; Edhemovic et al., 2011). For deep-seated tumors and tumors in internal organs, which are surrounded by tissues with different electric properties, individualized

patient-specific treatment planning is required (Miklavčič and Davalos, 2015). Tumors vary in shape, size, and location. The shape and position of the used long-needle electrodes and even the applied voltage (Hjouj and Rubinsky, 2010; Edhemovic et al., 2011; Pavliha et al., 2012) are analyzed and optimized for each tumor; coverage of the whole tumor with a sufficiently high electric field (which is one of the two prerequisites for successful treatment) (Miklavčič et al., 1998, 2006) can currently only be assured by means of numerical modeling of electric field distribution (Pavliha and Kos, 2013). Electric field calculations based on real input data are performed. Image-guided

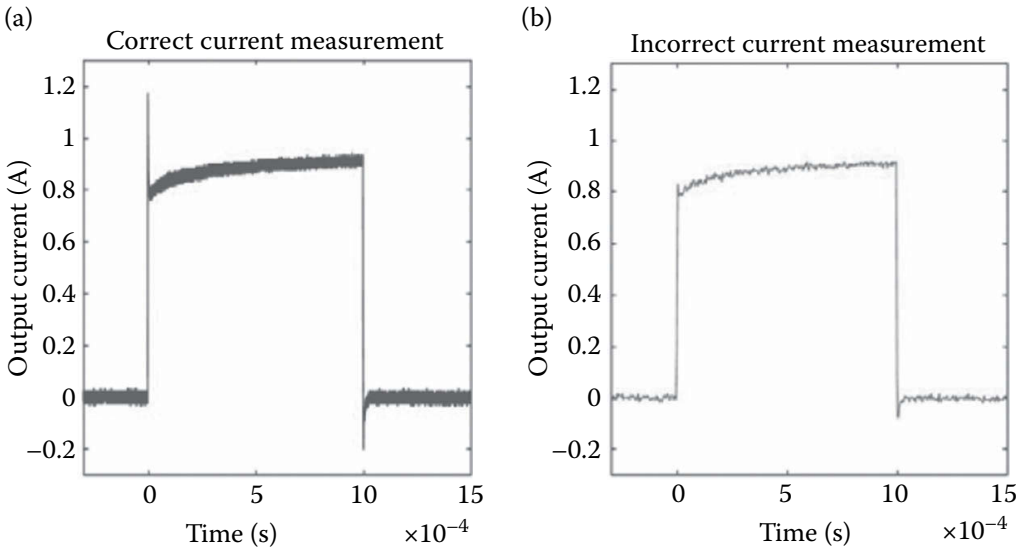


FIGURE 12.8 Current measurement examples, in (b) an oscilloscope with a too low bandwidth was used, consequently current spike was not detected. The spike is clearly visible in (a), that was captured by a faster oscilloscope.

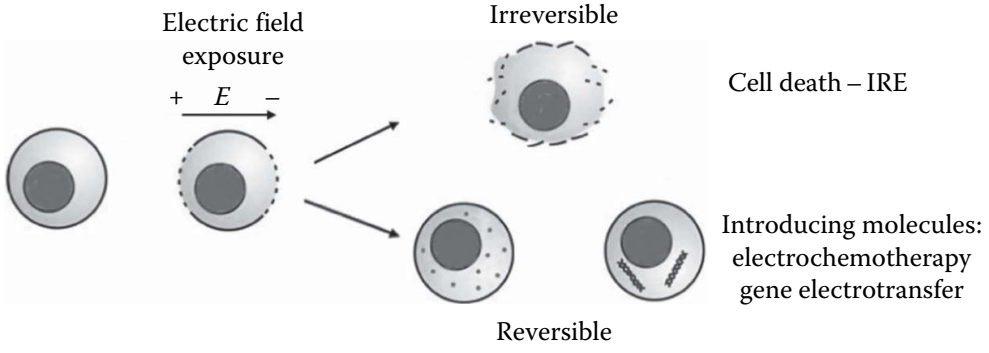


FIGURE 12.9 Symbolic representation of different electroporation applications. When externally applied electric field reaches the cell membrane threshold value, the cell gets permeabilized. We distinguish reversible and irreversible electroporation. The result of irreversible electroporation is cell death, which we exploit for nonthermal ablation, so-called IRE. In case of reversible electroporation, cell membrane can fully recover after the electroporation process. During the electroporation process, molecules are introduced into the cell at electrochemotherapy and gene electrotransfer.

insertion of electrodes is used (Kos et al., 2010; Miklavčič et al., 2010; Grošelj et al., 2015). Nonthermal IRE is also an electroporation-based application that is used for ablation of pathological tissue, it similarly requires a specific treatment planning. But in that case, calculations of temperature increase should also be considered (Županič and Miklavčič, 2011) as the conductivity increases with temperature. It is also necessary to measure *in vivo,* the voltage and current measurement of applied pulses and electrical impedance tomography (EIT) are common, but they do not tell us, how the electrical conductivity of tissue affects the electric field distribution. MR-EIT (Magnetic Resonance-Electrical Impedance Tomography) enables reconstruction of electric field distribution by measuring the electric current density distribution and electric conductivity during electroporation by using MR imaging and numeric algorithms (Kranjc et al., 2015).

12.2 Applications

Further on We will focus on three main applications (Figure 12.9). The most established ECT, IRE, is used for tissue ablation and gene electrotransfection. In each section, we review pulses used, their characteristics, and main principles. For each application, we tried to discover if researches report adequate data. At the end of the chapter, a review of commercially available electroporation devices can be found, including their characteristics. We have summarized all the important parameters, so as to help researchers select the appropriate device for their application. Table 12.2 describes their specifications and limitations. We focused on devices available for ECT, IRE, and gene electrotransfer. After a detailed review of the manufacturer's Internet pages and literature, we wrote to all the listed producers and asked them to kindly review the data we found in literature, device specifications, and on the Internet and update if necessary. We wrote to 25 producers: 13 were pleased to cooperate, the data they provided can be found in Tables 12.1 and 12.2. Three producers (LONZA, MaxCyte, and Ichor Medical Systems) replied to our email and informed us that the requested information was confidential. The data collection lasted for 1 month, with one reminder email. We contacted producers through their official emails published on their homepages. In addition, we also wrote directly to employees, whose contact information we have in our database. There have been no responses from BIO-RAD, Eppendorf, NEPA GENE, Oncosec, Scientz, Sigma-Aldrich, Thermo Fischer, and Tritech Research. Overall, the ones that do not specify their pulse parameters did not change their mind, only Inovio is excluded. We could not find any technical specifications or generated pulse descriptions of their devices, but in the end they provided all the missing data. Most of the producers who did not cooperate sell devices that are mainly used in the biopharmaceutical drug industry. When we are working with a device that has a CE mark, a small derogation from the specification is allowed. But due to aging and the huge diversity in biological load characteristics, control with an external measuring system is required for quality assurance. Noncertified devices can generate pulses that highly deviate from the preset values, so the use of an external measuring system is necessary.

12.2.1 Electrochemotherapy

One of the leading applications on the electroporation field is ECT. It is highly effective, with complete response rates between 60% and 70% and objective response rates of about 80% (Mali et al., 2013). It is suitable for treatment of cutaneous and subcutaneous tumors of different histotypes, both skin and nonskin cancers, as well as metastases. European Standard Operating Procedures of Electrochemotherapy (ESOPE) have been established in 2006; it increased reproducibility and improved clinical practice results (Campana et al., 2016). SOP however only defines ECT for smaller skin tumors (<3 cm in diameter); we still do not have any guidelines for internal or larger tumor ECT. National Institute for Health and Care Excellence (NICE) has recognized ECT as an integral part of the multidisciplinary treatment for patients with skin metastases of nonskin origin and melanoma (NICE interventional procedure guidance IPG 446, http://www.nice.org.uk/guidance/ipg446) (Campana et al., 2016). Lately, ECT has been introduced into the treatment of deep-seated tumors, it is really suitable for treatment of liver metastases, especially when they are located close to major blood vessels and consequently not manageable with surgery (Edhemovic et al., 2014). Recently, recommendations for improving the quality of reporting clinical ECT studies has been released (Campana et al., 2016), on initiative of the Steering Committee of the COST TD 1104 Action. Really good guidelines could raise the level of research even higher. That is a good example for other, high-quality applications. Standardized reporting enables faster and greater progress (Miklavčič et al., 2014).

But the main challenge is still the successful use of the application, the presence of a cytotoxic agent within tumor tissue, and adequate coverage of the tumor with electric pulses above the threshold of reversible membrane electroporation are crucial. Some studies have been conducted that have introduced the method for the determination of effective electrical parameters for ECT from a systematic *in vitro* study performed on cells in culture (Maček-Lebar et al., 2002; Larkin et al., 2007). It has been proven that ECT parameters optimized *in vitro* are applicable *in vivo*. Currently, eight or two groups of four 100 µs square wave pulses with a repetition frequency of 1 Hz or 5 kHz are most commonly used. In the ESOPE clinical study, a 5-kHz electric pulse repetition frequency was used based on preliminary data assuming that higher electric pulse frequency has a comparable effect as lower pulse repetition frequency in ECT (Marty et al., 2006). The advantages of a higher frequency are shorter duration of electroporation, the sensation of only one application of electric pulses and also muscle contraction is obtained only right after the electric pulses, delivery, therefore an electrode displacement due to muscle contraction during pulse' delivery is avoided. Patients report less pain is associated with 5 kHz than with 1-Hz repletion frequency electroporation (Županič et al., 2007; Serša et al., 2010). Pulse voltage amplitude is most commonly somewhere between 200 and 1000 V. It is electrode and target tissue dependent, which means it should be set to the value that ensures the electric field between the electrodes is higher than 400 V/cm. From Table 12.1 various implementations of electrodes and associated voltage amplitudes can be observed. Within a Cliniporator project, a clinical electroporator was designed, which is classified as a medical device and it is for now the only one with a medical device CE mark. SOP bases on the use of Cliniporator with associated electrodes, but

new electric pulse generators are coming to the market, with new electrodes that might have a completely different design, we always need to keep in mind that the voltage amplitude must be optimized specifically for each electrode configuration.

As with all treatments ECT also has some side effects. Transient lesions and some localized pain can appear in areas that are in direct contact with the electrodes (Mir and Orlowski, 1999). A problem can also occur if electroporation pulses interfere with heart muscle rhythm. There is very little chance for this phenomena when the application is used for skin treatment (Mali et al., 2008). But deep-seated tumors, which can be located close to the heart, are also treated with ECT, and even in an open surgery the probability of electroporation pulses interfering with the heart is increasing. The most dangerous possible interference is induction of ventricular fibrillation (Wiggers and Wegria, 1940; Han, 1973; Reilly, 1998). Fibrillation can occur if the amplitude of the applied electric pulses is greater than the threshold level for fibrillation, and if electrical pulses are delivered during late atrial or ventricular systole. The vulnerable period for ventricles is near the peak of the T wave, and for atria in the S wave (Mali et al., 2005). The delivery of electric pulses must be synchronized with the ECG so as to reduce the risk (Mali et al., 2015).

12.2.2 Irreversible Electroporation

IRE as a nonthermal tissue ablation is a promising application for ablation of tumors tissue that is located near bile ducts or blood vessels (Scheffer et al., 2014). IRE causes cell death due to cell membrane electroporation and not due to tissue's temperature increase; however, a local temperature increase occurs around the electrodes, when a greater number of pulses are administered. IRE has almost the same main challenges as ECT, the tumor tissue should be covered with an adequate electric field, but in case of IRE, the electric field should be above the IRE threshold (Rubinsky et al., 2007). In addition, the magnitude should be selected to minimize the electroporation of healthy tissue, so as to avoid significant thermal damage (Shafiee et al., 2009; Županič and Miklavčič, 2011). It is mainly used for the treatment of deep-seated tumors either during open surgery or percutaneously in liver, pancreas, kidney, lung, and other organs.

IRE does not have an SOP, treatment protocols vary with research groups, tumor types, and stages of development. An individual treatment plan required for each specific tumor and is crucial for successful outcome. IRE can be minimally invasive in combination with ultrasound, computed tomography guidance, or magnetic resonance imaging (Jourabchi et al., 2014). In comparison to ECT, safety is even more important, because with IRE we are ablating about 50 cm^3 of tissue and the number of applied pulses is at least 90 (Bertacchini et al., 2007). To achieve IRE threshold, applied electric fields should be higher, i.e., delivered pulses should have amplitudes up to 3000 V and currents up to 50 A (Bertacchini et al., 2007). A Cliniporator VITAE or NanoKnife electroporator is used (their specifications can be found in Table 12.2). Higher electric fields, open surgery, and proximity of the heart raises the risk, delivered pulses might interfere with cardiac activity if delivered at inappropriate heart rhythm phase (Thomson et al., 2011). Pulses should be synchronized with the refractory period of the cardiac rhythm. The overall time for the procedure is extremely short in comparison to benchmark

treatments. It lasts only a few minutes, actual time can be calculated from the number of delivered pulses and average heart rate, because pulse delivery is coupled to the heart rate (Davalos et al., 2005; Bertacchini et al., 2007; Rubinsky et al., 2008).

Traditional IRE is based on the use of a series of unipolar electric pulses, normally accompanied by a significant muscle contraction; therefore, general anesthesia and neuromuscular blocking agents are necessary to prevent muscle contraction (Rubinsky et al., 2008). Researchers are investigating different techniques to minimize the contractions. According to the Golberg and Rubinsky approach, surrounding a central energized electrode with a series of grounded electrodes reduces the volume of tissue exposed to electric fields with the potential to induce contraction (Golberg and Rubinsky, 2012). This procedure requires that at least 16 grounded electrodes be surrounded by one superficially inserted, energized electrode. Arena (Arena et al., 2011) uses high-frequency IRE named H-FIRE. H-FIRE utilizes high frequency, bipolar bursts to eliminate muscle contraction, without sacrificing the efficiency of cell death due to nonthermal electroporation. He showed that H-FIRE at 250 or 500 kHz has the same ablation and precision outcomes as traditional IRE (Arena et al., 2011)

Treatment plans have been developed that can help clinicians. Electrode configuration and pulse parameters are proposed, but a proper electrode placement can be in some cases really challenging (Edd and Davalos, 2007; Kos et al., 2015). Clinical studies are going on all around the world trying to specify the optimal parameters for specific cancer types. Each set of pulse specifications, number of pulses, voltage amplitude, and pulse duration have an effect on IRE outcomes. Pulse length is responsible for thermal effects in tissue. The maximal duration can be calculated for each electric field that would not induce thermal effects or at least minimize them. Typical IRE pulses consist of a series of 100-μs pulses separated by at least 100 μs. The pause between pulses enables a cooldown. Davalos (Davalos et al., 2005) showed that the threshold for IRE in most cell types is at least 800 V/cm. Rubinsky (Rubinsky et al., 2008) proposed that for prostate cancer cells a field of 250 V/cm is sufficient with use of 90 pulses to ensure complete ablation of that region. Raffa demonstrated that performing IRE in the presence of fibril boron nitride nanotubes lowers the necessary voltage threshold required to cause tumor cell death. IRE at 800 V/cm was 2.2 times more effective at causing cell destruction when performed in the presence of fibril boron nitride nanotubes (Raffa et al., 2012). Contradictory to recent guidelines for ECT, there are no specific guidelines on how to report clinical cases and studies.

12.2.3 Gene Transfection

Gene electrotransfer is a promising non-viral gene delivery method (Kandušer et al., 2009). It is used for treatment of cancer and other diseases (Shibata et al., 2006; Daud et al., 2008), for DNA vaccination (Chiarella et al., 2010; Sardesai and Weiner, 2011), and genetic modification of organisms (Golden et al., 2007; Grewal et al., 2009). "Cancer immunoediting" is a process combining the immune system and tumors. The immune system can protect the host against tumor growth, or promote cancer development by selection of tumor variants with reduced immunogenicity (Zou et al., 2005). Immunotherapy can include cancer vaccines based on plasmid DNA (pDNA) vectors (Serša et al., 2015).

Electroporation is used to promote antigen, oligonucleotides, and immunomodulatory molecule delivery in to tumor tissue. They can stimulate the immune system or act on immunosuppressor genes (Serša et al., 2015). *In vitro* electric pulses are frequently used for the transfection of bacterial and eukaryotic cells. *In vivo* the technique is termed DNA electrotransfer, electrogenetherapy, or also gene electrotherapy. It has been successfully used since 1998. However, exact molecular mechanisms of DNA transport are unknown (Kandušer et al., 2009; Serša et al., 2015). DNA transfer can only be achieved by reversible electroporation, because dead cells are not able to express transferred genes (Andre et al., 2010). The DNA must be injected before electroporation; the application requires sufficiently intense electric fields, which means sufficiently long pulses should be applied, but we also need to ensure reversible electroporation. Permeabalized cell membrane should interact with the plasmid; thus a DNA–membrane complex is formed. DNA, then, with an as yet unknown process, is transferred into the cytoplasm and transported to the nucleus. In cases where the application is successful, the process is followed by gene expression (Golzio et al., 2002; Faurie et al., 2010).

Initially, we thought one of the most important mechanisms for efficient gene electrotransfer was electrophoretic movement of DNA during the pulse. A long millisecond, square, or exponential decaying pulses were used with 400–600 V/cm and up to 20 ms long (Bettan et al., 2000). Some studies showed that DNA transfection is enhanced in a combination of short HV and long low-voltage (LV) pulses. It was suggested that HV pulses are crucial for permeabilization of the cell membrane and pore formation, while LV pulses electrophoretically drag negatively charged DNA into the cell. Eight HV pulses 100 µs with amplitude 1300 V/cm followed by one longer 100 ms LV pulse 100 V/cm (Šatkauskas et al., 2002) were proposed. Further, Miklavčič's group showed that short HV pulses are not only crucial but also sufficient for successful DNA delivery, at optimal plasmid concentrations. They suggested that electrophoretic force of LV pulses is crucial in *in vivo* conditions where suboptimal plasmid concentration is the limiting factor for efficient transfection (Kandušer et al., 2009). As induced electric field is tissue dependent, it is important to define targeted tissue. In comparison to ECT, where targeted tissue is always tumor, a definition in the case of gene electrotransfer is more complicated. Electroporation parameters depend on the type of tumor antigen and target tissues, and the target cells in specific tissue are different (Serša et al., 2015). Electric pulse parameters have to be experimentally or numerically optimized for given electrodes' positions and geometry (Županič et al., 2010). Gene electrotransfer efficiency is electroporation media dependent, divalent cations such as Ca^{2+} and Mg^{2+} are necessary for the formation of DNA–membrane complex during the pulses. They act as a bridge between negatively charged DNA and the negatively charged cell plasma membrane, and thus improve DNA–membrane binding (Haberl et al., 2013a).

Classical gene electrotransfection parameters are hard to define; for example, 8×5 ms, 700 V/cm, 1Hz are efficient *in vitro*. Studies show that more than 30% of cells can express the gene coded by plasmid DNA, while preserving cell viability to a large extent (Golzio et al., 2002; Chopinet et al., 2012). In the case of skin tumors, rate significantly decreases *in vivo* (Rols and Teissié, 1998; Čemazar et al., 2009). A lot of studies have been performed and parameters described to enable better gene transfer. In other studies, electric field direction and orientation changes during the pulse delivery have

been shown to increase the area, making DNA entry into the cell more competent. The introduction of DNA only occurs in the part of the membrane facing the cathode. It was shown that the percentage of cells expressing genes increases when electric field direction and orientation change (Pavlin et al., 2011). Also, a new prospect was presented, involving nanosecond electric pulses (pulse duration: 4–600 ns). Very short HV pulses (several tens of kV/cm) are able to disturb membranes of internal organelles, due to cell membranes charging time. We can conclude there is an option short nanosecond pulses can effect the nuclear envelope. Combination of medium or long electrical pulses with short HV nanosecond pulses enhance gene expression by increasing the number of plasmids entering the nucleus (Beebe et al., 2003; Chopinet et al., 2013; Guo et al., 2014).

Overall, we can say that the field of gene electrotransfer is complex and many known and as yet unknown factors mutually affect the process.

Longer electric pulses are optimal for higher transfection efficiency, but they reduce viability. Shorter pulses enable lower transfection efficiency and preserve viability. The number of studies is increasing, fields and applications are spreading, with insufficient and incomplete data and pulse parameters. There are no standard procedures or reporting guidelines defined that would in the future enable a proof of concept. In comparison to ECT, a greater variety of pulses are being used, for each tissue specific pulse parameters are optimal, for each application a specific procedure seems to be required. One of the problems is that electroporation device manufacturers produce devices with preprogramed procedures. Users only select the "appropriate program" and the device ensures optimal transfection. Pulse parameters and characteristics stay unknown due to the device patent. The field is getting more and more chaotic and is in need of a more consistent, explicit, and well-defined research with guidelines on reporting; including electric pulse protocols.

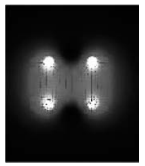
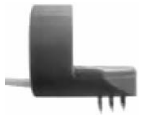
12.3 Conclusion

Electroporation is a platform technology, which is already established in medicine and food processing. When we are dealing with electroporation, measuring is crucial for achieving effective electroporation. Quality assurance can only be provided by appropriate measurements, i.e., measuring the voltage and current using an oscilloscope.

Due to the huge variation in biological load characteristics, delivered pulses may significantly deviate from the pre-set. Low impedance of a load (tissue or cell suspension) requires large power/currents, which quite often leads to significant voltage drop. Protocols in which a larger number of pulses (or long pulses) are delivered can result in reduced amplitude of pulses. The delivered pulse shape repetition frequency, pulse duration, and amplitude must be always monitored. The measurement probes should be located as close to the load as possible, the oscilloscope bandwidth should be high enough, and in the case of nanosecond application, the reflections and losses must be considered.

One of the leading applications in the electroporation field is ECT that already has well-established protocols, reporting guidelines, and good research reproducibility. Unfortunately, failed efforts to confirm other published paperwork are increasing (Kaiser, 2016). We believe the main reason for this situation is flawed descriptions of the

TABLE 12.1 Various Implementations of Electrodes, with Associated Voltage Amplitudes, Provided by the Manufacturers

Producer	Device	Type of electrodes	Number of output channels	Number of electrodes	Electrodes' geometric description		Pulse number	Pulses amplitude
Angiodynamics								
	NanoKnife	Needle	/	1–6 Probe outputs	Probes spaced 1.5 cm apart with the active electrode length set at 2 cm		90 (Pulses for each pair of electrodes)	(100–3000) V
Bionmed Technologies								
	SENNEX	Needle/pin surface	/	4	Linear layout for small tumors and pentagon layout for larger, more extensive tumors. Pin electrodes are 3 mm thick at the top and needle 0.3 mm.		8	1000 V
IGEA								
	Cliniporator EPS02	Needle	7	7	Hexagonal configuration (diameter: 0.7 mm/length: 10mm/20 mm/30 mm)		HV: 4	HV: 730 V
		Needle	2	8 (2 × 4)	Linear configuration (diameter: 0.7 mm/length: 10mm/20 mm/30 mm)		HV: 8	HV: 400 V
		Plate	2	2	Linear configuration (10 mm × 30 mm × 0.8 mm)		HV: 8	HV: 960 V
		Needle	2	6 (2 × 3)	Finger configuration with orthogonal linear needles (diameter: 0.7 mm/ length: 5 mm/10 mm)		HV: 8	HV: 400 V

(*Continued*)

TABLE 12.1 (*Continued*) Various Implementations of Electrodes, with Associated Voltage Amplitudes, Provided by the Manufacturers

Producer	Device	Type of electrodes	Number of output channels	Number of electrodes	Electrodes geometric description		Pulse number	Pulses amplitude
		Needle	2	6 (2 × 3)	Finger configuration with longitudinal linear needles (diameter: 0.7 mm/length: 5 mm/10 mm)		HV: 8	HV: 400 V
		Partially isolated needles	7	7	Hexagonal configuration (diameter: 0.7 mm/length: 40 mm)		HV: 4	HV: 730 V
	Cliniporator VITAE	Needle	2–6	2–6	Single long needle/diameter: 1.2 mm/active part: 1–4 cm; soft tissues custom geometry ECT		HV: 4 + 4 (polarity exchange)	HV: (500–3000) V
		Needle	2–6	2–6	Single long needle/diameter: 1.8 mm/active part 1–4 cm; bones custom geometry ECT		HV: 4 + 4 (polarity exchange)	HV: (500–3000) V
Intracel								
	TSS20 Ovodyne	Silver, tungsten, and platinum electrodes	/	2	Electrodes. Silver electrodes are available made from 0.8 mm diameter silicon rubber insulated silver wire, the exposed pole being flattened into a "paddle" shape approximately 2 × 1 mm. Silver wire length extending beyond the electrode holder is 40 mm. Tungsten electrodes are produced from 0.5 mm o.d. tungsten rod		/	/

(*Continued*)

TABLE 12.1 (*Continued*) Various Implementations of Electrodes, with Associated Voltage Amplitudes, Provided by the Manufacturers

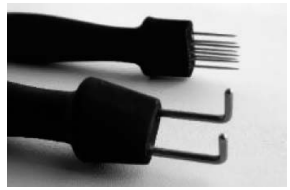
Producer	Device	Type of electrodes	Number of output channels	Number of electrodes	Electrodes geometric description	Pulse number	Pulses amplitude
Inovio							
	CELLECTRA 5PSP	Needle electrodes, intramuscular	/	5	An array consisting of 5 needle electrodes, adjustable from 13, 19, and 25 mm in length depending on BMI, forming a pentagon on a 1-cm circle	3	Max 200 V
	CELLECTRA 2000–5P	Needle electrodes, intramuscular	/	5	An array consisting of 5 needle electrodes, adjustable from 13, 19, and 25 mm in length depending on BMI, forming a pentagon on a 1-cm circle	3	Max 200 V
	CELLECTRA 2000–3P	Needle electrodes, intradermal	/	5	An array consisting of 3 needle electrodes, 3 mm in length, forming an isosceles triangle, 3-mm spacing (short side) and 5-mm spacing (long sides)	2 Sets of 2 pulses	Max 200 V
Leroy BIOTECH							
	ELECTROvet S13		/		All are compatible with plate and needle electrodes		
	ELECTROvet EZ ELECTRO cell B10		/	8 (2 × 4)	Needle electrodes are specially designed and manufactured for the treatment of subcutaneous tumors. 8 mm between the two rows of four needles. Each needle spaced 2 mm apart		
	ELECTRO cell S20 MILLIPULSES		/	2	10-mm length/10-mm spacing between electrodes centers/3 mm		

TABLE 12.2 A Review of Commercially Available Electroporation Devices, Including Their Capabilities and Characteristics

Producer	Device	Pulse description	Pulse number	Pulses amplitude	Pulses duration	Pulses repetition frequency	Pulse sequences	Maximum voltage	Maximum current
Angiodynamics									
	NanoKnife	Square wave	90 (Pulses for each pair of electrodes)	(100–3000) V	(20–100) μs	ECG synchronized, 90, 120, 240 ppm	/	3000 V	50 A
BEX Co., Ltd.									
	CUY21EDIT	Square wave	/	(1–500) V	$T = 0.1–999.9$ ms	/	/	/	$I < 5.0$ A (1–125 V) $I < 2.2$ A (126–250 V) $I < 1.0$ A (251–500 V)
	CUY21EDIT II	Square wave exponential	/	(1–200) V (Square) (1–400) V (Exponential; PP) (1–350) V (Exponential; DP)	$T = 0.05–1000$ ms (Square) $T = 0.01–99.9$ ms (Exponential)	/	/	/	$I < 1$ A (Square) $I < 10$ A (Exponential)
	Genome editor	Square wave	/	(1–200) V	$T = 0.10–1000$ ms	/	/	/	
	CUY21Vitro-EX	Exponential	/	1–900 V (PP) 1–500 V (DP)	$T = 0.01–99.9$ ms	/	/	/	$I < 50$ A
	LF301	Square wave sinus (AC)	/	0–1200 (Square wave) 0–75 V_{rms} (AC)	$T = 0–100$ μs (Square) fAC = 1 MHz; $T = 0–100$ s (Prefusion)/0–10 s (Post-fusion)	/	/	/	$R > 50$ Ω

(*Continued*)

TABLE 12.2 (*Continued*) A Review of Commercially Available Electroporation Devices, Including Their Capabilities and Characteristics

Producer	Device	Pulse description	Pulse number	Pulses amplitude	Pulses duration	Pulses repetition frequency	Pulse sequences	Maximum voltage	Maximum current
Bionmed Technologies									
	SENNEX	Square wave	8	1000 V	100 µs	100 ms	10Imp/s	1000 V	/
BTX-Harvard Apparatus									
	AgilePulse *in vivo* system	/	3 Groups of pulses: from 1 to 10 pulses in each group	(50–1000) V	(0.050–10) ms	(0.200–1000) ms	/	1000 V	At max voltage and minimum resistance: 1000 V/10 Ω = 100 A
	AgilePulse MAX system	/	3 Groups of pulses: from 1 to 10 pulses in each group	(50–1200) V	HV: (0.050–10) ms	(0.200–1000) ms	(1–5000) Hz	1200 V	At max voltage and minimum resistance: 1200 V/10 Ω = 120 A
	ECM 2001	Square wave	1–9	HV: (10–3000) V LV: (10–500) V	HV: (1–99) µs LV: (0.01–0.99) ms	(0.01–0.99) ms (1–99) ms		3000 V	/
		AC		(0–150) V (Vpp)	Duration: (0–99) s	Post fusion—ramp: 1–9 s	1mHz		
	ECM 830	Square wave	1–99	HV: (505–3000) V LV: (5–500) V	HV: (10–600) µs LV: (10–999) µs; (1–999) ms; (1–10) s	100 ms–10 s	/	3000 V	500 A limit at 100 µs
	ECM 630	Exponential decay wave	1–99	HV: (50–2500) V LV: (10–500) V	10 µs–10 s	/	/	2500 V	6000 A in LV mode
	Gemini SC2	Square waves and exponential decay waves	LV: 1–10 HV: 1–2 Exponential decay: 1	(10–3000) V	50 µs–100 ms	100 ms–30 s	/	3000 V	/

(*Continued*)

TABLE 12.2 (*Continued*) A Review of Commercially Available Electroporation Devices, Including Their Capabilities and Characteristics

Producer	Device	Pulse description	Pulse number	Pulses amplitude	Pulses duration	Pulses repetition frequency	Pulse sequences	Maximum voltage	Maximum current
	Gemini X2	Square waves and exponential decay waves	Square wave: LV mode-1–120 (10 per sample) HV mode-1–36 (3 per sample); Exponential decay: 1–12 (R internal <100 Ω) and 1–24 (R internal > 100 Ω)	(5–3000) V	10μs–1s	100 ms–30 s	/	3000 V	/
	ECM 399	Exponential decay waves	1	(2–2500) V	Max. at 500 V: 125 ms; Max. at 2500 V: 5 ms	100 ms–10 s	/	2500 V	/
Cyto Pulse Science, Inc.									
	OncoVet	/	/	(50–1000) V	(0.05–10) ms	(0.2–1000) ms	(1–5000) Hz	/	/
IGEA									
	Cliniporator EPS02	Square wave	LV: 1–10 HV: 1–10	LV: (20–200) V HV: (100–1000) V	LV: (1–200) ms HV: (50–1000) μs	LV: (0.45–500) Hz HV: (1–5000) Hz	24 Configurations	1000 V	LV: 5 A HV: 20 A
	Cliniporator VITAE	Square wave	HV: 4 + 4 (polarity exchange); 4–8	HV: (500–3000) V	100 μs	HV: (1–5000) Hz	Costum	3000 V	50 A
Inovio									
	CELLECTRA 5PSP	Square wave	3	Max 200 V	52 ms	1Hz	/	Max 200 V	0.5 A Constant current

(*Continued*)

TABLE 12.2 (*Continued*) A Review of Commercially Available Electroporation Devices, Including Their Capabilities and Characteristics

Producer	Device	Pulse description	Pulse number	Pulses amplitude	Pulses duration	Pulses repetition frequency	Pulse sequences	Maximum voltage	Maximum current
Intracel	CELLECTRA 2000–5P	Square wave	3	Max 200 V	52 ms	1Hz	/	Max 200 V	0.5 A Constant current
	CELLECTRA 2000–3P	Square wave	2 Sets of 2 pulses	Max 200 V	52 ms	3 Hz	2 Sets seperated by 3 s	Max 200 V	0.2 A Constant current
	TSS20 Ovodyne	Square wave	1–999	(0.1–99.9) V	(1–999) ms	(10–9990) ms	1–999	99.9 V	100 mA TSS20 1000 mA EP21
Leroy Biotech	ELECTROvet S13	Square wave pulse generator	1–10,000 or infinite	0–1350 V	5–5000 µs	0.1–10,000 ms	0.1–10,000 Hz	1300 V	10 A
	ELECTROvet EZ	Square wave pulse generator	1–10,000 or infinite	0–1500 V	5–5000 µs	0.1–10,000 ms	0.1–10,000 Hz	1500 V	25 A
	ELECTRO cell B10	Square wave bipolar pulse generator—high voltage low voltage	1–10,000 or infinite	0–1000 V	5–5000 µs	0.1–10,000 ms	0.1–10,000 Hz	1000 V	10 A
	ELECTRO cell S20	Square wave pulse generator	1–10,000 or infinite	0–2000 V	5–5000 µs	0.1–10,000 ms	0.1–10,000 Hz	2000 V	25 A

(*Continued*)

TABLE 12.2 (*Continued*) A Review of Commercially Available Electroporation Devices, Including Their Capabilities and Characteristics

Producer	Device	Pulse description	Pulse number	Pulses amplitude	Pulses duration	Pulses repetition frequency	Pulse sequences	Maximum voltage	Maximum current
Molecular Devices									
	Axoporator 800 A	Square and bi-level pulses with positive and negative polarity, as well as bipolar	Train duration: 10ms–100 s	±(1–100) V	MONOPOLAR: 200 µs–1s BI-POLAR: 400 µs–1s BI-LEVEL: 10ms–20 s	MONOPOLAR ftrain = (1–2000) Hz BI-POLAR ftrain = (1–2000) Hz BI-LEVEL ftrain = (0.024–50) Hz	Rectangular pulse Bipolar pulse Postive bi-level pulse	5 V peak to peak	±10.0 µA
NPI									
	ELP-01D	Square wave	/	(0–1 D) V	(0–9999.9) ms	(0–9999.9) ms	/	/	/
OnkoDisruptor									
	Onkodisruptor Electroporator	/	/	/	/	50 + 50 µs Pause: 10 µs s	8 Biphasic	1500 V	5 A
Supertech Instruments									
	SP-4a	With RC time constant of the exponential decay of wave	0–99	HV: (200–400) V LV: (0–200) V	(1–250) ms	(10–60,000) ms	Single pulse mode and burst mode (up to 99 pulses)	400 V	119 A

Sources: Angiodynamics, http://www.angiodynamics.com/; BEX Co., Ltd., http://www.bexnet.co.jp/; Bionmed Technologies, http://bionmed.de/; BTX-Harvard Apparatus, http://www.btxonline.com; Cyto Pulse Science, Inc., http://www.cytopulse.com/; IGEA, http://www.igea.it/; Inovio, http://www.inovio.com/; Intracel, http://intracel.co.uk/; Leroy Biotech, https://www.leroybiotech.com; Molecular Devices, http://www.moleculardevices.com/; NPI http://www.npielectronic.de/; OnkoDisruptor, https://www.onkodisruptor.com; Supertech Instruments, http://www.superte.ch/Electroporator.html

Note: We wrote to all listed producers and kindly asked them to review the data we found in literature and on the Internet and update if necessary. While most of the manufacturers were pleased to cooperate, we did not get information about pulse characteristics from others. We are still missing all the information about pulse characteristics from, Lonza, TriTech, Ichor Medical Systems, Thermo Fischer, Eppendorf, Maxcyte, and Oncosec.

equipment used and the process. Many papers describing/using electroporation have reported insufficient details, and quite often measurements are not reported (Batista Napotnik et al., 2016; Campana et al., 2016). The field of electroporation is in need of promoting reproducible research that can only be achieved by adequate measurements, standardized reports, and proper use of electroporators and electrodes.

Acknowledgments

This study was supported by the Slovenian Research Agency (ARRS) and conducted within the scope of the European Associated Laboratory for Electroporation in Biology and Medicine (LEA-EBAM). We also thank all the manufacturers who kindly provided data about their devices.

References

Andre, F. M. and L. M. Mir. 2010. Nucleic acids electrotransfer in vivo: Mechanisms and practical aspects. *Current Gene Therapy* 10, no. 4 (August 1, 2010): 267–80. doi:10.2174/156652310791823380.

Arena, C. B., M. B. Sano, J. H. Rossmeisl, J. L. Caldwell, P. A. Garcia, M. N. Rylander, and R. V. Davalos. 2011. High-frequency irreversible electroporation (H-FIRE) for non-thermal ablation without muscle contraction. *Biomedical Engineering Online* 10 (2011): 102. doi:10.1186/1475-925X-10–102.

Batista Napotnik, T., M. Reberšek, T. Kotnik, E. Lebrasseur, G. Cabodevila, and D. Miklavčič. 2010. Electropermeabilization of endocytotic vesicles in B16 F1 mouse melanoma cells. *Medical & Biological Engineering & Computing* 48, no. 5 (May 2010): 407–13. doi:10.1007/s11517-010-0599-9.

Batista Napotnik, T., M. Reberšek, P. T. Vernier, B. Mali, and D. Miklavčič. 2016. Effects of high voltage nanosecond electric pulses on eukaryotic cells (in vitro): A systematic review. *Bioelectrochemistry* (Amsterdam, Netherlands) 110 (February 27, 2016): 1–12. doi:10.1016/j.bioelechem.2016.02.011.

Beebe, S. J., J. White, P. F. Blackmore, Y. Deng, K. Somers, and K. H. Schoenbach. 2003. Diverse effects of nanosecond pulsed electric fields on cells and tissues. *DNA and Cell Biology* 22, no. 12 (December 2003): 785–96. doi:10.1089/104454903322624993.

Bertacchini, C., P. M. Margotti, E. Bergamini, A. Lodi, M. Ronchetti, and R. Cadossi. 2007. Design of an irreversible electroporation system for clinical use. *Technology in Cancer Research & Treatment* 6, no. 4 (August 2007): 313–20.

Bettan, M., M. A. Ivanov, L. M. Mir, F. Boissière, P. Delaere, and D. Scherman. 2000. Efficient DNA electrotransfer into tumors. *Bioelectrochemistry (Amsterdam, Netherlands)* 52, no. 1 (September 2000): 83–90.

Campana, L. G., A. J. P. Clover, S. Valpione, P. Quaglino, J. Gehl, C. Kunte, M. Snoj, M. Čemažar, C. R. Rossi, D. Miklavčič, and G. Serša. 2016. Recommendations for improving the quality of reporting clinical electrochemotherapy studies based on qualitative systematic review. *Radiology and Oncology* 50, no. 1 (March 1, 2016): 1–13. doi:10.1515/raon-2016-0006.

Čemazar, M., M. Golzio, G. Serša, P. Hojman, S. Kranjc, S. Mesojednik, M.-P. Rols, and J. Teissié. 2009. Control by pulse parameters of DNA electrotransfer into solid tumors in mice. *Gene Therapy* 16, no. 5 (May 2009): 635–44. doi:10.1038/gt.2009.10.

Chafai, D. E., A. Mehle, A. Tilmatine, B. Maouche, and D. Miklavčič. 2015. Assessment of the electrochemical effects of pulsed electric fields in a biological cell suspension. *Bioelectrochemistry* 106, Part B (December 2015): 249–57. doi:10.1016/j.bioelechem.2015.08.002.

Chiarella, P., V. M. Fazio, and E. Signori. 2010. Application of electroporation in DNA vaccination protocols. *Current Gene Therapy* 10, no. 4 (August 2010): 281–86.

Chopinet, L., L. Wasungu, and M.-P. Rols. 2012. First explanations for differences in electrotransfection efficiency *in vitro* and *in vivo* using spheroid model. *International Journal of Pharmaceutics*, Special Issue : Drug Delivery and Imaging in Cancer, 423, no. 1 (February 14, 2012): 7–15. doi:10.1016/j.ijpharm.2011.04.054.

Chopinet, L., T. Batista Napotnik, A. Montigny, M. Reberšek, J. Teissié, M.-P. Rols, and D. Miklavčič. 2013. Nanosecond electric pulse effects on gene expression. *The Journal of Membrane Biology* 246, no. 11 (November 2013): 851–59. doi:10.1007/s00232-013-9579-y.

Čorović, S., I. Lacković, P. Sustaric, T. Sustar, T. Rodic, and D. Miklavčič. 2013. Modeling of electric field distribution in tissues during electroporation. *Biomedical Engineering Online* 12 (2013): 16. doi:10.1186/1475-925X-12-16.

Daud, A. I., R. C. DeConti, S. Andrews, P. Urbas, A. I. Riker, V. K. Sondak, P. N. Munster, D. M. Sullivan, K. E. Ugen, J. L. Messina, and R. Heller. 2008. Phase I trial of interleukin-12 plasmid electroporation in patients with metastatic melanoma. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology* 26, no. 36 (December 20, 2008): 5896–903. doi:10.1200/JCO.2007.15.6794.

Davalos, R. V., L. M. Mir, and B. Rubinsky. 2005. Tissue ablation with irreversible electroporation. *Annals of Biomedical Engineering* 33, no. 2 (February 2005): 223–31. doi:10.1007/s10439-005-8981-8.

Deamer, D. W. and J. Bramhall. 1986. Special issue: Liposomes permeability of lipid bilayers to water and ionic solutes. *Chemistry and Physics of Lipids* 40, no. 2 (June 1, 1986): 167–88. doi:10.1016/00093084(86)90069-1.

Edd, J. F. and R. V. Davalos. 2007. Mathematical modeling of irreversible electroporation for treatment planning. *Technology in Cancer Research & Treatment* 6, no. 4 (August 1, 2007): 275–86. doi:10.1177/153303460700600403.

Edhemovic, I., E. M. Gadzijev, E. Brecelj, D. Miklavčič, B. Kos, A. Županič, B. Mali, T. Jarm, D. Pavliha, M. Marčan, G. Gasljevic, V. Gorjup, M. Music, T. P. Vavpotic, M. Čemažar, M. Snoj, and G. Serša. 2011. Electrochemotherapy: A new technological approach in treatment of metastases in the liver. *Technology in Cancer Research & Treatment* 10, no. 5 (October 2011): 475–85.

Edhemovic, I., E. Brecelj, G. Gasljevic, M. M. Music, V. Gorjup, B. Mali, T. Jarm, B. Kos, D. Pavliha, B. Grcar Kuzmanov, M. Čemažar, M. Snoj, D. Miklavčič, E. M. Gadzijev, and G. Serša. 2014. Intraoperative electrochemotherapy of colorectal liver metastases. *Journal of Surgical Oncology* 110, no. 3 (September 2014): 320–27. doi:10.1002/jso.23625.

Faurie, C., M. Reberšek, M. Golzio, M. Kandušer, J.-M. Escoffre, M. Pavlin, J. Teissié, D. Miklavčič, and M. P. Rols. 2010. Electro-mediated gene transfer and expression are controlled by the life-time of DNA/membrane complex formation. *The Journal of Gene Medicine* 12, no. 1 (January 2010): 117–25. doi:10.1002/jgm.1414.

Freeman, S. A., M. A. Wang, and J. C. Weaver. 1994. Theory of electroporation of planar bilayer membranes: Predictions of the aqueous area, change in capacitance, and pore-pore separation. *Biophysical Journal* 67, no. 1 (July 1994): 42–56.

Garcia, P. A., R. V. Davalos, and D. Miklavčič. 2014. A numerical investigation of the electric and thermal cell kill distributions in electroporation-based therapies in tissue. *PloS One* 9, no. 8 (2014): e103083. doi:10.1371/journal.pone.0103083.

Golberg, A. and B. Rubinsky. 2012. Towards electroporation based treatment planning considering electric field induced muscle contractions. *Technology in Cancer Research & Treatment* 11, no. 2 (April 1, 2012): 189–201. doi:10.7785/tcrt.2012.500249.

Golberg, A., M. Sack, J. Teissié, G. Pataro, U. Pliquett, G. Saulis, T. Stefan, D. Miklavčič, E. Vorobiev, and W. Frey. 2016. Energy-efficient biomass processing with pulsed electric fields for bioeconomy and sustainable development. *Biotechnology for Biofuels* 9, no. 1 (April 27, 2016): 1–22. doi:10.1186/s13068-016-0508-z.

Golden, K., V. Sagi, N. Markwarth, B. Chen, and A. Monteiro. 2007. In vivo electroporation of DNA into the wing epidermis of the butterfly, *Bicyclus anynana*. *Journal of Insect Science* 7, no. 53 (October 1, 2007): 1–8. doi:10.1673/031.007.5301.

Golzio, M., J. Teissié, and M.-P. Rols. 2002. Direct visualization at the single-cell level of electrically mediated gene delivery. *Proceedings of the National Academy of Sciences* 99, no. 3 (February 5, 2002): 1292–97. doi:10.1073/pnas.022646499.

Grewal, R. K., M. Lulsdorf, J. Croser, S. Ochatt, A. Vandenberg, and T. D. Warkentin. 2009. Doubled-haploid production in chickpea (*Cicer arietinum* l.): Role of stress treatments. *Plant Cell Reports* 28, no. 8 (June 19, 2009): 1289–99. doi:10.1007/s00299-009-0731-1.

Grošelj, A., B. Kos, M. Čemažar, J. Urbancic, G. Kragelj, M. Bosnjak, B. Veberic, P. Strojan, D. Miklavčič, and G. Serša. 2015. Coupling treatment planning with navigation system: A new technological approach in treatment of head and neck tumors by electrochemotherapy. *Biomedical Engineering Online* 14, no. Suppl 3 (2015): S2. doi:10.1186/1475-925X14-S3-S2.

Guo, S., D. L. Jackson, N. I. Burcus, Y.-J. Chen, S. Xiao, and R. Heller. 2014. Gene electrotransfer enhanced by nanosecond pulsed electric fields. *Molecular Therapy—Methods & Clinical Development* 1 (September 17, 2014): 14043. doi:10.1038/mtm.2014.43.

Gusbeth, C. A. and W. Frey. 2009. Critical comparison between the pulsed electric field and thermal decontamination methods of hospital wastewater. *Acta Physica Polonica A* 115, No. 6: 1092-1093 (2009).

Haberl, S., D. Miklavčič, G. Serša, W. Frey, and B. Rubinsky. 2013a. Cell membrane electroporation—Part 2: The applications. *IEEE Electrical Insulation Magazine* 29, no. 1 (January 2013): 29–37. doi:10.1109/MEI.2013.6410537.

Haberl, S., M. Kandušer, K. Flisar, D. Hodžić, V. B. Bregar, D. Miklavčič, J.-M. Escoffre, M. Rols, and M. Pavlin. 2013b. Effect of different parameters used for *in vitro* gene electrotransfer on gene expression efficiency, cell viability and visualization of plasmid DNA at the membrane level. *The Journal of Gene Medicine* 15, no. 5 (May 2013): 169–81. doi:10.1002/jgm.2706.

Han, J. 1973. Ventricular vulnerability to fibrillation. In Dreifus, L. S., Likoff, W. (eds): *Cardiac Arrhythmias*. New York: Grune and Stratton, Inc., pp. 87–95.

Heller, R., J. Schultz, M. L. Lucas, M. J. Jaroszeski, L. C. Heller, R. A. Gilbert, K. Moelling, and C. Nicolau. 2001. Intradermal delivery of interleukin-12 plasmid DNA by *in vivo* electroporation. *DNA and Cell Biology* 20, no. 1 (January 2001): 21–26. doi:10.1089/10445490150504666.

Hjouj, M. and B. Rubinsky. 2010. Magnetic resonance imaging characteristics of non-thermal irreversible electroporation in vegetable tissue. *The Journal of Membrane Biology* 236, no. 1 (July 15, 2010): 137–46. doi:10.1007/s00232-010-9281-2.

Hofmann, G. A. 2000. Instrumentation and electrodes for in vivo electroporation. *Methods in Molecular Medicine* 37 (2000): 37–61. doi:10.1385/1-59259-080-2:37.

Ibey, B. L., A. G. Pakhomov, B. W. Gregory, V. A. Khorokhorina, C. C. Roth, M. A. Rassokhin, J. A. Bernhard, G. J. Wilmink, and O. N. Pakhomova. 2010. Selective cytotoxicity of intense nanosecond-duration electric pulses in mammalian cells. *Biochimica Et Biophysica Acta* 1800, no. 11 (November 2010): 1210–19. doi:10.1016/j.bbagen.2010.07.008.

Isokawa, M. 1997. Membrane time constant as a tool to assess cell degeneration. *Brain Research Protocols* 1, no. 2 (May 1997): 114–16.

Jourabchi, N., K. Beroukhim, B. A. Tafti, S. T. Kee, and E. W. Lee. 2014. Irreversible electroporation (nanoknife) in cancer treatment. *Gastrointestinal Intervention* 3, no. 1 (June 2014): 8–18. doi:10.1016/j.gii.2014.02.002.

Kaiser, J. 2016**.** Calling all failed replication experiments. *Science* 351, no. 6273 (February 5, 2016): 548–548. doi:10.1126/science.351.6273.548.

Kandušer, M., D. Miklavčič, and M. Pavlin. 2009. Mechanisms involved in gene electrotransfer using high- and low-voltage pulses—An in vitro study. *Bioelectrochemistry (Amsterdam, Netherlands)* 74, no. 2 (February 2009): 265–71. doi:10.1016/j.bioelechem.2008.09.002.

Kolb, J. F., S. Kono, and K. H. Schoenbach. 2006. Nanosecond pulsed electric field generators for the study of subcellular effects. *Bioelectromagnetics* 27, no. 3 (April 2006): 172–87. doi:10.1002/bem.20185.

Kos, B., A. Županič, T. Kotnik, M. Snoj, G. Serša, and D. Miklavčič. 2010. Robustness of treatment planning for electrochemotherapy of deep-seated tumors. *The Journal of Membrane Biology* 236, no. 1 (July 2010): 147–53. doi:10.1007/s00232-010-9274-1.

Kos, B., B. Valič, T. Kotnik, and P. Gajšek. 2012. Occupational exposure assessment of magnetic fields generated by induction heating equipment—The role of spatial averaging. *Physics in Medicine and Biology* 57, no. 19 (October 7, 2012): 5943–53. doi:10.1088/0031-9155/57/19/5943.

Kos, B., P. Voigt, D. Miklavčič, and M. Moche. 2015. Careful treatment planning enables safe ablation of liver tumors adjacent to major blood vessels by percutaneous irreversible electroporation (IRE). *Radiology and Oncology* 49, no. 3 (September 2015): 234–41. doi:10.1515/raon-2015-0031.

Kotnik, T. and D. Miklavčič. 2006. Theoretical evaluation of voltage inducement on internal membranes of biological cells exposed to electric fields. *Biophysical Journal* 90, no. 2 (January 15, 2006): 480–91. doi:10.1529/biophysj.105.070771.

Kotnik, T., G. Pucihar, and D. Miklavčič. 2010. Induced transmembrane voltage and its correlation with electroporation-mediated molecular transport. *The Journal of Membrane Biology* 236, no. 1 (July 2010): 3–13. doi:10.1007/s00232-010-9279-9.

Kotnik, T., P. Kramar, G. Pucihar, D. Miklavčič, and M. Tarek. 2012. Cell membrane electroporation—part 1: The phenomenon. *IEEE Electrical Insulation Magazine* 28, no. 5 (September 2012): 14–23. doi:10.1109/MEI.2012.6268438.

Kotnik, T., W. Frey, M. Sack, S. H. Meglič, M. Peterka, and D. Miklavčič. 2015. Electroporation-based applications in biotechnology. *Trends in Biotechnology* 33, no. 8 (August 2015): 480–88. doi:10.1016/j.tibtech.2015.06.002.

Kranjc, M., B. Markelc, F. Bajd, M. Čemažar, I. Serša, T. Blagus, and D. Miklavčič. 2015. In situ monitoring of electric field distribution in mouse tumor during electroporation. *Radiology* 274, no. 1 (August 19, 2014): 115–23. doi:10.1148/radiol.14140311.

Lacković, I., R. Magjarević, and D. Miklavčič. 2009. Three-dimensional finite-element analysis of joule heating in electrochemotherapy and in vivo gene electrotransfer. *IEEE Transactions on Dielectrics and Electrical Insulation* 16, no. 5 (October 2009): 1338–47. doi:10.1109/TDEI.2009.5293947.

Lambricht, L., A. Lopes, S. Kos, G. Serša, V. Préat, and G. Vandermeulen. 2016. Clinical potential of electroporation for gene therapy and DNA vaccine delivery. *Expert Opinion on Drug Delivery* 13, no. 2 (February 2016): 295–310. doi:10.1517/17425247.2016.1121990.

Larkin, J. O., C. G. Collins, S. Aarons, M. Tangney, M. Whelan, S. O'Reily, O. Breathnach, D. M. Soden, and G. O'Sullivan. 2007. Electrochemotherapy: Aspects of preclinical development and early clinical experience. *Annals of Surgery* 245, no. 3 (March 2007): 469–79. doi:10.1097/01.sla.0000250419.36053.33.

Maček-Lebar, A., G. Serša, S. Kranjc, A. Groselj, and D. Miklavčič. 2002. Optimisation of pulse parameters *in vitro* for *in vivo* electrochemotherapy. *Anticancer Research* 22, no. 3 (June 2002): 1731–36.

Mahnič-Kalamiza, S., E. Vorobiev, and D. Miklavčič. 2014. Electroporation in food processing and biorefinery. *The Journal of Membrane Biology* 247, no. 12 (December 2014): 1279–1304. doi:10.1007/s00232-014-9737-x.

Mali, B., T. Jarm, F. Jager, and D. Miklavčič. 2005. An algorithm for synchronization of *in vivo* electroporation with ECG. *Journal of Medical Engineering & Technology* 29, no. 6 (December 2005): 288–96. doi:10.1080/03091900512331332591.

Mali, B., T. Jarm, S. Čorovič, M. S. Paulin-Kosir, M. Čemažar, G. Serša, and D. Miklavčič. 2008. The effect of electroporation pulses on functioning of the heart. *Medical & Biological Engineering & Computing* 46, no. 8 (August 2008): 745–57. doi:10.1007/s11517-008-03467.

Mali, B., T. Jarm, M. Snoj, G. Serša, and D. Miklavčič. 2013. Antitumor effectiveness of electrochemotherapy: A systematic review and meta-analysis. *European Journal of Surgical Oncology: The Journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology* 39, no. 1 (January 2013): 4–16. doi:10.1016/j.ejso.2012.08.016.

Mali, B., V. Gorjup, I. Edhemovic, E. Brecelj, M. Čemažar, G. Serša, B. Strazisar, D. Miklavčič, and T. Jarm. 2015. Electrochemotherapy of colorectal liver metastases—An observational study of its effects on the electrocardiogram. *Biomedical Engineering Online* 14, no. Suppl 3 (2015): S5. doi:10.1186/1475-925X-14-S3-S5.

Marty, M., G. Serša, J. R. Garbay, J. Gehl, C. G. Collins, M. Snoj, V. Billard, P. F. Geertsen, J. O. Larkin, D. Miklavčič, I. Pavlovic, S. M. Paulin-Kosir, M. Čemažar, N. Morsli, D. M. Soden, Z. Rudolf, C. Robert, G. C. O'Sullivan, and L. M. Mir. 2006. Electrochemotherapy—An easy, highly effective and safe treatment of cutaneous and subcutaneous metastases: Results of ESOPE (European standard operating procedures of electrochemotherapy) study. *European Journal of Cancer Supplements* 4, no. 11 (November 2006): 3–13. doi:10.1016/j.ejcsup.2006.08.002.

Miklavčič, D., K. Beravs, D. Semrov, M. Čemažar, F. Demsar, and G. Serša. 1998. The importance of electric field distribution for effective in vivo electroporation of tissues. *Biophysical Journal* 74, no. 5 (May 1998): 2152–58.

Miklavčič, D., S. Čorovič, G. Pucihar, and N. Pavselj. 2006. Importance of tumour coverage by sufficiently high local electric field for effective electrochemotherapy. *European Journal of Cancer Supplements* 4, no. 11 (November 2006): 45–51. doi:10.1016/j.ejcsup.2006.08.006.

Miklavčič, D., M. Snoj, A. Županič, B. Kos, M. Čemažar, M. Kropivnik, M. Bracko, T. Pecnik, E. Gadzijev, and G. Serša. 2010. Towards treatment planning and treatment of deep-seated solid tumors by electrochemotherapy. *Biomedical Engineering Online* 9 (February 23, 2010): 10. doi:10.1186/1475-925X-9-10.

Miklavčič, D., B. Mali, B. Kos, R. Heller, and G. Serša. 2014. Electrochemotherapy: From the drawing board into medical practice. *Biomedical Engineering Online* 13 (2014): 29. doi:10.1186/1475-925X-13-29.

Miklavčič, D. and R. V. Davalos. 2015. Electrochemotherapy (ECT) and irreversible electroporation (IRE)-advanced techniques for treating deep-seated tumors based on electroporation. *Biomedical Engineering Online* 14, no. Suppl 3 (August 27, 2015): I1. doi:10.1186/1475-925X-14-S3-I1.

Mir, L. M. and S. Orlowski. 1999. Mechanisms of electrochemotherapy. *Advanced Drug Delivery Reviews* 35, no. 1 (January 4, 1999): 107–18.

Mir, L. M., J. Gehl, G. Serša, C. G. Collins, J.-R. Garbay, V. Billard, P. F. Geertsen, Z. Rudolf, G. C. O'Sullivan, and M. Martya. 2006. Standard operating procedures of the electrochemotherapy: Instructions for the use of bleomycin or cisplatin administered either systemically or locally and electric pulses delivered by the cliniporator™ by means of invasive or non-invasive electrodes. *EJC Supplements* 4, no. 11 (November 1, 2006): 14–25. doi:10.1016/j.ejcsup.2006.08.003.

Ongaro, A., L. G. Campana, M. De Mattei, F. Dughiero, M. Forzan, A. Pellati, C. R. Rossi, and E. Sieni. 2016. Evaluation of the electroporation efficiency of a grid electrode for electrochemotherapy from numerical model to in vitro tests. *Technology in Cancer Research & Treatment* 15, no. 2 (April 1, 2016): 296–307. doi:10.1177/1533034615582350.

Pakhomov, A. G., B. L. Ibey, A. M. Bowman, F. M. Andre, and O. N. Pakhomova. 2009. Nanosecond-duration electric pulses open nanometer-size pores in cell plasma membrane. In *World Congress on Medical Physics and Biomedical Engineering, September 7–12, 2009, Munich, Germany*, edited by O. Dössel and W. C. Schlegel. IFMBE Proceedings 25/13. Springer, Berlin-Heidelberg, pp. 17–20. http://link.springer.com/chapter/10.1007/978-3-642-03895-2_6.

Pavliha, D., B. Kos, A. Županič, M. Marčan, G. Serša, and D. Miklavčič. 2012. Patient-specific treatment planning of electrochemotherapy: Procedure design and possible pitfalls. *Bioelectrochemistry* 87 (October 2012): 265–73. doi:10.1016/j.bioelechem.2012.01.007.

Pavliha, D. and B. Kos. 2013. Planning of electroporation-based treatments using web-based treatment-planning software. *The Journal of Membrane Biology* 246, no. 11 (2013): 833–42. doi:10.1007/s00232-0139567-2.

Pavlin, M., M. Kandušer, M. Reberšek, G. Pucihar, F. X. Hart, R. Magjarevićcacute, and D. Miklavčič. 2005. Effect of cell electroporation on the conductivity of a cell suspension. *Biophysical Journal* 88, no. 6 (June 2005): 4378–90. doi:10.1529/biophysj.104.048975.

Pavlin, M., S. Haberl, M. Reberšek, D. Miklavčič, and M. Kandušer. 2011. Changing the direction and orientation of electric field during electric pulses application improves plasmid gene transfer in vitro. *Journal of Visualized Experiments: JoVE*, no. 55 (September 12, 2011): 3309. doi:10.3791/3309.

Pliquett, U., E. Gersing, and F. Pliquett. 2000. Evaluation of fast time-domain based impedance measurements on biological tissue. *Biomedizinische Technik* 45, nos. 1–2 (January 1, 2000): 6–13.

Postma, P. R., G. Pataro, M. Capitoli, M. J. Barbosa, R. H. Wijffels, M. H. M. Eppink, G. Olivieri, and G. Ferrari. 2016. Selective extraction of intracellular components from the microalga *Chlorella vulgaris* by combined pulsed electric field–temperature treatment. *Bioresource Technology* 203 (March 2016): 80–88. doi:10.1016/j.biortech.2015.12.012.

Pucihar, G., T. Kotnik, D. Miklavčič, and J. Teissié. 2008. Kinetics of transmembrane transport of small molecules into electropermeabilized cells. *Biophysical Journal* 95, no. 6 (September 15, 2008): 2837–48. doi:10.1529/biophysj.108.135541.

Raffa, V., C. Riggio, M. W. Smith, K. C. Jordan, W. Cao, and A. Cuschieri. 2012. Bnnt-mediated irreversible electroporation: Its potential on cancer cells. *Technology in Cancer Research & Treatment* 11, no. 5 (October 1, 2012): 459–65. doi:10.7785/tcrt.2012.500258.

Reberšek, M. and D. Miklavčič. 2011. Advantages and disadvantages of different concepts of electroporation pulse generation. *Automatika (Zagreb)* 52: 12–19.

Reberšek, M., D. Miklavčič, C. Bertacchini, and M. Sack. 2014. Cell membrane electroporation—part 3: The equipment. *IEEE Electrical Insulation Magazine* 30, no. 3 (May 2014): 8–18. doi:10.1109/MEI.2014.6804737.

Reilly, J. P. 1998. *Applied Bioelectricity*. New York, NY: Springer New York, 1998. http://link.springer.com/10.1007/978-1-4612-1664-3.

Retelj, L., G. Pucihar, and D. Miklavčič. 2013. Electroporation of intracellular liposomes using nanosecond electric pulses—A theoretical study. *IEEE Transactions on Biomedical Engineering* 60, no. 9 (September 2013): 2624–35. doi:10.1109/TBME.2013.2262177.

Rols, M. P. and J. Teissié. 1998. Electropermeabilization of mammalian cells to macromolecules: Control by pulse duration. *Biophysical Journal* 75, no. 3 (September 1998): 1415–23.

Rubinsky, B., G. Onik, and P. Mikus. 2007. Irreversible electroporation: A new ablation modality—clinical implications. *Technology in Cancer Research & Treatment* 6, no. 1 (February 1, 2007): 37–48. doi:10.1177/153303460700600106.

Rubinsky, J., G. Onik, P. Mikus, and B. Rubinsky. 2008. Optimal parameters for the destruction of prostate cancer using irreversible electroporation. *The Journal of Urology* 180, no. 6 (December 2008): 2668–74. doi:10.1016/j.juro.2008.08.003.

Sanders, J. M., A. Kuthi, Y. H. Wu, P. T. Vernier, and M. A. Gundersen. 2009. A linear, single-stage, nanosecond pulse generator for delivering intense electric fields to biological loads. *IEEE Transactions on Dielectrics and Electrical Insulation* 16, no. 4 (August 2009): 1048–54. doi:10.1109/TDEI.2009.5211853.

Sardesai, N. Y. and D. B. Weiner. 2011. Electroporation delivery of DNA vaccines: Prospects for success. *Current Opinion in Immunology* 23, no. 3 (June 2011): 421–29. doi:10.1016/j.coi.2011.03.008.

Šatkauskas, S., M. F. Bureau, M. Puc, A. Mahfoudi, D. Scherman, D. Miklavčič, and L. M. Mir. 2002. Mechanisms of *in vivo* DNA electrotransfer: Respective contributions of cell electropermeabilization and DNA electrophoresis. *Molecular Therapy: The Journal of the American Society of Gene Therapy* 5, no. 2 (February 2002): 133–40. doi:10.1006/mthe.2002.0526.

Scheffer, H. J., K. Nielsen, M. C. de Jong, A. A. J. M. van Tilborg, J. M. Vieveen, A. (R. A.) Bouwman, S. Meijer, C. van Kuijk, P. M. van den Tol, and M. R. Meijerink. 2014. Irreversible electroporation for nonthermal tumor ablation in the clinical setting: A systematic review of safety and efficacy. *Journal of Vascular and Interventional Radiology*, Special Issue: Interventional Oncology 25, no. 7 (July 2014): 997–1011. doi:10.1016/j.jvir.2014.01.028.

Sel, D., D. Cukjati, D. Batiuskaite, T. Slivnik, L. M. Mir, and D. Miklavčič. 2005. Sequential finite element model of tissue electropermeabilization. *IEEE Transactions on Biomedical Engineering* 52, no. 5 (May 2005): 816–27. doi:10.1109/TBME.2005.845212.

Serša, G., S. Kranjc, J. Scancar, M. Krzan, M. Cemažar. 2010. Electrochemotherapy of mouse sarcoma tumors using electric pulse trains with repetition frequencies of 1Hz and 5kHz. *The Journal of Membrane Biology* 236, no. 1 (July 2010):155–62. doi:10.1007/s00232-010–9268-z.

Serša, G., J. Teissié, M. Čemažar, E. Signori, U. Kamensek, G. Marshall, and D. Miklavčič. 2015. Electrochemotherapy of tumors as in situ vaccination boosted by immunogene electrotransfer. *Cancer Immunology, Immunotherapy* 64 (2015): 1315–27. doi:10.1007/s00262-015-1724-2.

Shafiee, H., P. A. Garcia, and R. V. Davalos. 2009. A preliminary study to delineate irreversible electroporation from thermal damage using the arrhenius equation. *Journal of Biomechanical Engineering* 131, no. 7 (June 12, 2009): 074509. doi:10.1115/1.3143027.

Shibata, M.-A., Y. Ito, J. Morimoto, K. Kusakabe, R. Yoshinaka, and Y. Otsuki. 2006. In vivo electrogene transfer of interleukin-12 inhibits tumor growth and lymph node and lung metastases in mouse mammary carcinomas. *The Journal of Gene Medicine* 8, no. 3 (March 2006): 335–52. doi:10.1002/jgm.854.

Spratt, D. E., E. A. Gordon Spratt, S. Wu, A. DeRosa, N. Y. Lee, M. E. Lacouture, and C. A. Barker. 2014. Efficacy of skin-directed therapy for cutaneous metastases from advanced cancer: A meta-analysis. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology* 32, no. 28 (October 1, 2014): 3144–55. doi:10.1200/JCO.2014.55.4634.

Susil, R., D. Šemrov, and D. Miklavčič. 1998. Electric field-induced transmembrane potential depends on cell density and organization. *Electro- and Magnetobiology* 17, no. 3 (January 1, 1998): 391–99. doi:10.3109/15368379809030739.

Syamsiana, I. N. and R. I. Putri. 2011. High voltage pulse generator design with voltage control for pulse electric field (PEF) pasteurization. In *2011 International Conference on Electrical Engineering and Informatics (ICEEI)*, 1–5, 2011. doi:10.1109/ICEEI.2011.6021712.

Teissié, J. and M. P. Rols. 1993. An experimental evaluation of the critical potential difference inducing cell membrane electropermeabilization. *Biophysical Journal* 65, no. 1 (July 1993): 409–13.

Thomson, K. R., W. Cheung, S. J. Ellis, D. Federman, H. Kavnoudias, D. Loader-Oliver, S. Roberts, P. Evans, C. Ball, and A. Haydon. 2011. Investigation of the safety of irreversible electroporation in humans. *Journal of Vascular and Interventional Radiology* 22, no. 5 (May 2011): 611–21. doi:10.1016/j.jvir.2010.12.014.

Toepfl, S., A. Mathys, V. Heinz, and D. Knorr. 2006. Review: Potential of high hydrostatic pressure and pulsed electric fields for energy efficient and environmentally friendly food processing. *Food Reviews International* 22, no. 4 (December 1, 2006): 405–23. doi:10.1080/87559120600865164.

Towhidi, L., T. Kotnik, G. Pucihar, S. M. P. Firoozabadi, H. Mozdarani, and D. Miklavčič. 2008. Variability of the minimal transmembrane voltage resulting in detectable membrane electroporation. *Electromagnetic Biology and Medicine* 27, no. 4 (2008): 372–85. doi:10.1080/15368370802394644.

Weaver, J. C. 1993. Electroporation: A general phenomenon for manipulating cells and tissues. *Journal of Cellular Biochemistry* 51, no. 4 (April 1, 1993): 426–35. doi:10.1002/jcb.240051040 7.

Weaver, J. C. and Yu. A. Chizmadzhev. 1996. Theory of electroporation: A review. *Bioelectrochemistry and Bioenergetics* 41, no. 2 (December 1996): 135–60. doi:10.1016/S0302-4598(96)05062-3.

Wiggers, C. J. and R. Wegria. 1940. Ventricular fibrillation due to single, localized induction and condenser shocks applied during the vulnerable phase of ventricular systole. *American Heart Journal* 19, no. 3 (March 1, 1940): 372. doi:10.1016/S0002-8703(40)90014-X.

Zou, P., W. Liu, and Y.-H. Chen. 2005. The epitope recognized by a monoclonal antibody in influenza a virus M2 protein is immunogenic and confers immune protection. *International Immunopharmacology* 5, no. 4 (April 2005): 631–35. doi:10.1016/j.intimp.2004.12.005.

Županič, A. and D. Miklavčič. 2011. Tissue heating during tumor ablation with irreversible electroporation. *Elektrotehniški vestnik* 78, no. (1–2): 42–47.

Županič, A., S. Ribaric, and D. Miklavčič. 2007. Increasing the repetition frequency of electric pulse delivery reduces unpleasant sensations that occur in electrochemotherapy. *Neoplasma* 54, no. 3 (2007): 246–50.

Županič, A., S. Čorović, D. Miklavčič, and M. Pavlin. 2010. Numerical optimization of gene electrotransfer into muscle tissue. *Biomedical Engineering Online* 9 (November 4, 2010): 66. doi:10.1186/1475-925X-9-66.

3.2 Paper 1

Title: **Mechanistic view of skin electroporation – models and dosimetry for successful applications: an expert review**

Authors: Janja Dermol-Černe, Eva Pirc and Damijan Miklavčič

Publication: *Expert Opinion on Drug Delivery*

Impact factor: 5.4 (2018)

Quartile: Q1 (pharmacology & pharmacy)

Rank: 20/267 (pharmacology & pharmacy)

Status: under review

Mechanistic view of skin electroporation – models and dosimetry for successful applications: an expert review

Journal:	*Expert Opinion on Drug Delivery*
Manuscript ID	EODD-2019--0253
Manuscript Type:	Review (Invited)
Keywords:	dosimetry, electrodes, gene electrotransfer, skin electroporation, mathematical models, mesotherapy, pulse generator, skin rejuvenation, transdermal drug delivery, wound disinfection

Abstract

Introduction: *Skin electroporation is a promising treatment for transdermal drug delivery, gene electrotransfer, skin rejuvenation, electrochemotherapy, and wound disinfection. Although a considerable amount of in vitro and in vivo studies exists, the translation to clinics is not as fast as one would hope. We believe the reason lies in the non-standardized dosimetry, i.e. electrode configurations, pulse parameters and pulse generators used. Adequate dosimetry could be determined by mathematical modeling and would allow comparison of protocols and translation into clinics.*

Areas Covered: *We introduce the mechanisms and applications of skin electroporation, review existing mathematical models and compare the influence of different model parameters. We review electrode configurations and pulse generators, prototypes as well as commercially available models.*

Expert opinion*: The reasons for slow translation of skin electroporation treatments into clinics lie in uncontrolled and inadequate dosimetry, poor reporting rendering comparisons between studies difficult, and significant differences in animal and human skin morphology, often dismissed by researchers. Mathematical models enable comparison of studies, however, when the parameters of the pulses and electrode configuration are not adequately reported, as is often the case, comparisons are difficult, if not impossible. For each skin electroporation treatment, systematic studies determining the optimal parameters should be performed and treatment parameters standardized.*

Keywords: computer models, dosimetry, electrodes, gene electrotransfer, mathematical models, mesotherapy, pulse generator, pulse generators, skin electroporation, skin rejuvenation, transdermal drug delivery, wound disinfection

Article highlights

1. Mathematical models of skin electroporation facilitate our understanding of the phenomena, help to reveal relevant parameters for treatment efficacy decrease the number of *in vitro* and *in vivo* experiments needed and enable predictions about the treatment efficacy.
2. The reasons for slow translation of skin electroporation into clinics are non-standard pulse parameters, non-standard electrode configurations, generators not complying with their technical specifications or having no technical specifications at all, lack of reporting on the delivered waveforms, electric field distribution, not performing the current-voltage measurements and significantly different skin structure of animals and humans,.
3. We ask the researchers to follow the guidelines for reporting the dosimetry. The use of high-quality electroporation equipment, with reliable and traceable operation, together with controlled dosimetry and predictive modeling raises the quality of studies and enables faster development of the field and eventual translation into clinics.
4. We propose that the pulse parameters and electrode configurations for skin electroporation should be determined and standardized with the use of mathematical models, taking into account the electric field distribution, electrical, thermal and chemical damage, drug/plasmid distribution and other parameters, deemed relevant for the treatment.
5. When designing new clinical as well as esthetic (for the use in cosmetics) pulse generators, they should comply with existing standards. In Europe, it has to comply with a Medical Device Regulation MDR 2017/745, and in the USA, the device should be approved by the FDA (Food and Drug Administration). Also, new standards specific to electroporation devices should be developed.

1. Introduction

When biological cells are exposed to short high-voltage pulses, electroporation occurs, i.e. pores are formed in the plasma membrane leading to transient permeability increase, and molecules, for which the cell membrane is usually impermeable, can pass across membrane [1]. Therefore, electroporation occurs due to high electric field imposed across a short distance (plasma membrane) causing above-threshold induced transmembrane voltage. In a similar way, electric field across the *stratum corneum* (SC) causes electroporation of skin, i.e. application of electric pulses to the skin disrupts its barrier function by means of skin electroporation.

The main mechanism behind skin electroporation is thus the disruption of the most resistive and impenetrable layer of the skin, the SC. When short high-voltage pulses are applied, small aqueous pores, i.e., local transport regions (LTRs), are formed within the lipids of the SC [2–4]. LTRs are areas of the increased electric conductivity and permeability. When pulses are longer, in the millisecond range, Joule heating causes melting of the lipids around the edge of LTRs as well as around the pre-existing defects and appendages (sweat glands, hair follicles) in the SC, the size of the defects increases and LTRs grow to a few hundred micrometers in diameter. These newly formed defects in the SC decrease its normally very high impedance and allow the electric field to penetrate lower layers of the skin [5,6], thus causing electroporation of the living cells beneath the SC. Additionally, electric pulses cause electrophoretic transport of charged molecules through the LTRs, assisting their transdermal delivery [7]. Electroporation should be distinguished from iontophoresis, which is the application of continuous direct low electric currents to the skin. The two main transport mechanisms of iontophoresis are electrophoresis (moving of charged molecules through skin) and electroosmosis (movement of neutral molecules by convective flow) [8]. In iontophoresis, no LTRs are formed in the SC, most of the transport is through pre-existing defects and appendages and it is mostly used for transdermal delivery of small charged molecules.

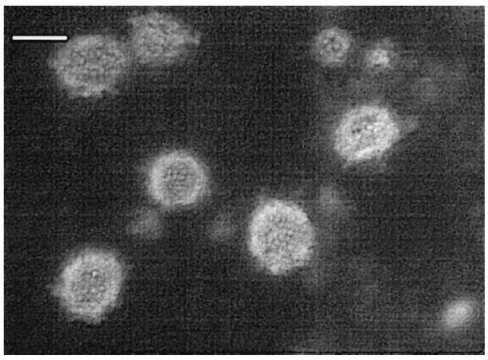


Figure 1: Mechanism of skin electroporation. After high-voltage electric pulses are applied, local transport regions form through which the transport of larger molecules can occur. Here, local transport regions are imaged via calcein transport. Reprinted from Bioelectrochemistry and Bioenergetics, 47 / 1, Pliquett et al., Local transport regions (LTRs) in human stratum corneum due to long and short `high voltage' pulses, 151-161, Copyright (1998), with permission from Elsevier.

2.1 Applications of skin electroporation

Transdermal or intradermal gene electrotransfer [9] is one of the most widely used and promising skin electroporation applications where short-term gene expression is successfully achieved, for example in gene transfer of antigen-presenting cells in immunotherapy such as DNA vaccination [9] or in transfection of skin cells to produce various proteins [10]. In transdermal drug delivery, the aim is a controlled transport of small therapeutic molecules across the skin, for example, to treat pain,

dementia, Parkinson's disease [8,11–13]. In cosmetics, electroporation is used in the so-called 'needless mesotherapy' or 'mesoporation' to rejuvenate the skin [14–16], although our measurements of some devices indicate that instead low-intensity radiofrequency (RF) pulses are delivered. In classical electroporation short, square wave, high voltage electric pulses are delivered to the tissue and the treatment is assumed as "non-thermal", while RF-therapies base on controlled tissue heating with a high frequency alternating current. Pulses of irreversible electroporation are applied to remove the aged cells and to promote the growth of new cells, while extracellular matrix remains undamaged [17], and in superficial wound disinfection to kill the antibiotic-resistant bacteria [18,19]. In electrochemotherapy, cutaneous and subcutaneous tumors are treated with a combination of electric pulses and chemotherapeutic drug [20,21].

Although skin electroporation lends itself as a very promising approach for transdermal drug and gene delivery, the translation into the clinical setting is lagging behind the *in vitro* and *in vivo* studies [13]. In our review, we critically assess the existing applications of skin electroporation, discuss possible reasons for the slow transfer of skin electroporation into clinical use and suggest possible solutions for improving it.

2.2 Models of skin electroporation

As the experimental setups of skin electroporation can vary significantly, direct comparison of results obtained by different electrode configurations and pulse generators are difficult, if not impossible. The models of skin electroporation could be instrumental in comparing results from them, facilitating also the transition from the *in vitro* to the *in vivo* and finally to the clinics as well as decreasing the number of experiments needed. Mathematical models of skin electroporation describe thermal [22,23] or electric [5,6,16,24–28] effects or both coupled together [29–36], primarily for the description of changes in the *stratum corneum* (SC) based on mechanisms of skin electroporation (formation of the defects in the lipid bilayers of the SC, LTR formation and growth [22,30,37], mehcanical deformation of the SC [28]). In the treatment planning of electroporation-based medical treatments, models can be used to calculate the electric field and thermal damage in the cutaneous/subcutaneous tumors and surrounding tissue [27,29,35,38–41], although for standard electrodes and pulses, the standard operating protocols obviate the need to calculate each case separately [20]. Electric and/or thermal models can be coupled to transport models via the diffusion and electrophoresis through the LTRs [30,31,42–45], the dual-porosity model [46], compartmental models [43] and/or regression models [47]. In case of the DNA transport, the electric properties of the injected plasmid DNA were taken into account [48], the efficiency of gene electrotransfer was evaluated according to the predicted plasmid DNA concentration inside the reversibly electroporated tissue [49] as well as taking into account the thermal stress and tissue damage during gene electrotransfer [50]. The extravasation of macromolecules in the size of antibodies or plasmid DNA from blood vessels into the surrounding tissue during skin electroporation was also calculated [51]. Most of the models treat the skin as a bulk tissue of a few layers of different dielectric properties [6,25,27,31,44], or as an equivalent circuit [32,52], however, also multi-scale model, i.e. model which takes into account the microstructure of the skin is available [5]. Recently, a computational study at the level of single lipids in the SC was performed, i.e., a molecular dynamics study, which showed that aqueous pores indeed form in the SC [53]. Some typical examples of the models, progressing from molecular level to elaborated mechanistic macroscale models, are shown in Fig. 2. For example, in a) the molecular dynamics study of pore formation, b) the equivalent circuit, c) electric field distribution in the bulk skin model, d) model of a single LTR or e) several LTRs in the SC, f) single cells from the multi-scale model and g) model of gene electrotransfer.

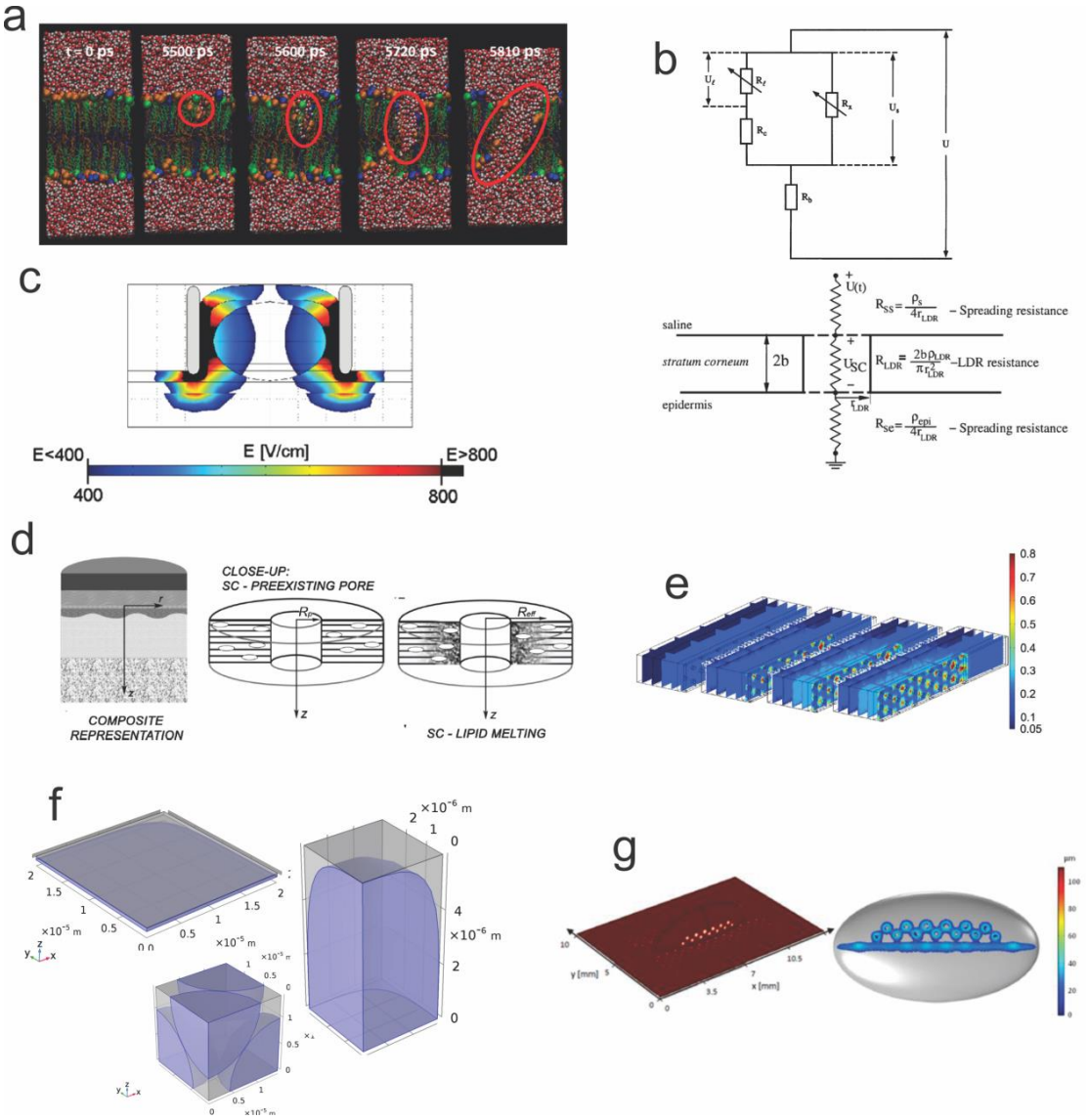


Figure 2: Examples of different models of skin electroporation.
a) Results of the molecular dynamics simulation of the pore formation in the stratum corneum. Reprinted with permission from Gupta R, Rai B. Electroporation of Skin Stratum Corneum Lipid Bilayer and Molecular Mechanism of Drug Transport: A Molecular Dynamics Study. Langmuir. 2018;34:5860–5870. Copyright (2018) American Chemical Society.
b) An equivalent circuit of the skin. Above: the entire SC is modeled with one equivalent circuit. R_b represents the resistance of the bulk solution. The resistance of the skin is represented by two parallel branches with R_x being the pathway through the appendages, R_l through lipid bilayers in the SC and R_c the inner resistance of each compartment of the model. (This model assumes that SC is made of many hydrophilic compartments, separated by boundary bilayers.) Reprinted from Biophysical Journal, 68/3, Chizmadzhev et al., Mechanism of electroinduced ionic species transport through a multilamellar lipid system, 749-765, Copyright (1995), with permission from Elsevier. Below: Only one LDR (local dissipation region) in the SC is modeled with the equivalent circuit. Reprinted from Bioelectrochemistry, 57/1, Martin et al., Theoretical analysis of localized heating in human skin subjected to high voltage pulses, 55-64, Copyright (2002), with permission from Elsevier.
c) A bulk numerical model of the subcutaneous tumor showing electric field distribution when 276 V are applied to parallel plate electrodes. Adapted from [54].
d) The model of a single local transport region formation inside a layered skin model. Left: a layered model of the skin. Middle: The geometry of a pre-existing pore inside the SC. Right: Melting of the lipids around the pre-existing pore and consequent increase in the radius of the LTR. Reprinted from Journal of Biomechanical Engineering, 129/5, Becker SM and Kuznetsov AV, Local Temperature Rises Influence In Vivo Electroporation Pore Development: A Numerical Stratum Corneum Lipid Phase Transition Model, 712-721, Copyright (2007), with permission from Elsevier.

e) A bulk model of the skin with included different dielectric properties of each skin layer and with a model of LTR formation inside the SC. Four three-dimensional slice plots of the conductivity distributions (S/m) represent four stages of the process in chronological order when 400 V is applied. © [2008] IEEE. Reprinted, with permission, from Pavšelj N, Miklavčič D. Numerical Models of Skin Electropermeabilization Taking Into Account Conductivity Changes and the Presence of Local Transport Regions. IEEE Transactions on Plasma Science. 2008;36:1650–1658.
f) The geometry of the cells of the skin (corneocyte, keratinocyte, spheres in the papillary dermis) used in the multi-scale model of skin electroporation. From each cell, equivalent dielectric properties of the respective layer were obtained. © [2018] IEEE. Reprinted, with permission, from Dermol-Černe J, Miklavčič D. From Cell to Tissue Properties—Modeling Skin Electroporation With Pore and Local Transport Region Formation. IEEE Transactions on Biomedical Engineering. 2018;65:458–468.
g) A model of gene electrotransfer with subcutaneously injected plasmid after pulse application. Left: Skin patch with the injected plasmid. SC is shown in red with local conductive pathways in white. Right: Gray ellipsoid is the injected plasmid DNA. Electrophoretic movement of the plasmid DNA through reversibly electroporated volume of the skin tissue is shown as the trajectories of the plasmid DNA movement inside the volume of reversible electroporation due to the non-uniform electric field. © [2019] IEEE. Reprinted, with permission, from Forjanič et al. Electroporation-Induced Stress Response and Its Effect on Gene Electrotransfer Efficacy: In Vivo Imaging and Numerical Modeling. IEEE Trans. Biomed. Eng. 2019;66:2671–2683.

2.3 Pulse generators and dosimetry

In the existing studies, various electrode configurations from invasive to non-invasive are used for pulse application [55]. In Table 1, we summarize the custom prototype electrodes used in different studies and similar commercially available electrodes that researchers could use as well. Pulses of different lengths, shapes, and voltages (schemes are shown in Fig. 3) are applied with different pulse generators (electroporators), listed in Table 2. It needs to be emphasized that a clinical electroporator is a pulse generator, which has to follow the requirements appointed by the local medical regulations and meet the medical device standard IEC 60601 (a series of technical standards for the safety and effectiveness of medical electrical equipment). In Europe, it has to comply with a Medical Device Regulation MDR 2017/745 (which in 2017 replaced Medical Device Directives (93/42/EEC, 98/79/EC and 90/385/EEC) and is in a transition period until May 2020) and in the USA, the device should be approved by the FDA (Food and Drug Administration) for specific indication. In Europe, the area of cosmetic devices has not been well regulated until now but with the new MDR (EU 2017/745), also esthetic devices which present the same characteristics and risk profile as medical devices, are included under the scope of this Regulation.

The design, development and quality assurance of an electroporator is challenging because the electrical characteristics of biological loads vary between tissues, samples from one tissue and parts of the body. SC has a significantly higher impedance than lower skin layers or muscle tissue, meaning that at the same electrode configuration and applied electric pulses the current between the electrodes is higher for invasive than for non-invasive electrodes. Additionally, the invasive and non-invasive electrodes present different risks and are therefore classified into different safety classes of the standards for medical devices. The electric field distribution is also electrode geometry dependent [56]; thus, the comparison of different electrode types and transition from *in vitro* to *in vivo* to the clinical use is only possible through modeling.

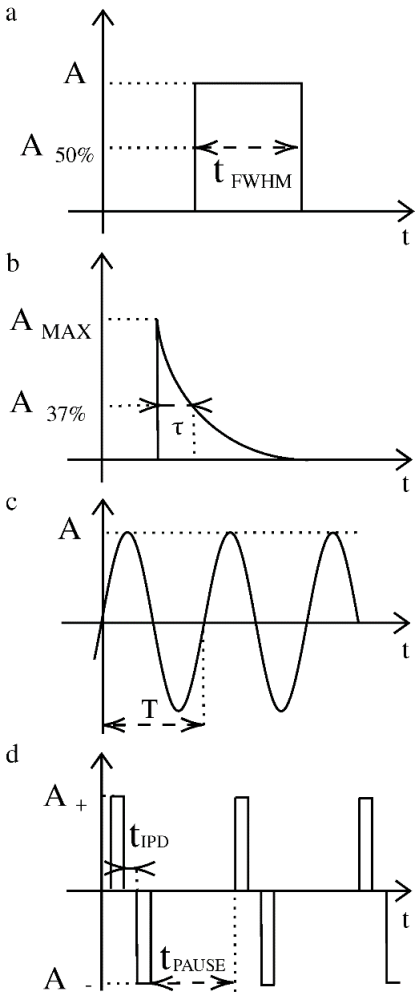


Figure 3: Typical waveforms, applied in skin electroporation treatments. a) Square wave pulses with durations (t_{FWHM} – the time of full width at half maximum) from a few nanoseconds to several hundred milliseconds and pulse amplitudes (A) from a few tens to several hundred volts. b) Exponential pulses with the time constant (τ) from one millisecond to a few hundred milliseconds. c) Sinusoidal waveform, with different periods (T) and amplitudes (A). d) Bipolar pulses with varying pulse durations (t_{FWHM}), delays between half periods (i.e. inter-pulse delays t_{IPD}) and delays between bipolar pulses (t_{PAUSE}).

3. Conclusion

Skin electroporation is a promising modality for treating different conditions with transdermal drug delivery, gene electrotransfer, electrochemotherapy and irreversible electroporation. A considerable amount of *in vitro* and *in vivo* studies exists, which use significantly different electrode configurations, waveforms, and pulse generators. We presented some of the electrode configurations and pulse generators, prototype as well as commercially available, appearing in the literature and pointed out their advantages and drawbacks (see Table 2). Results from studies with different parameters are sometimes difficult (if not impossible) to be compared. Thus, we propose to use mathematical models. They are promising in combining different experimental results and enabling better treatment efficacy

prediction. Alternatively, reporting should follow recommendation and provide detailed description of pulses and electrodes [57–59], that should allow development of models by experts.

4. Expert Opinion

Skin electroporation is a safe, fast, and efficient method of delivering drugs or DNA across the skin and affecting skin structure. Unfortunately, the translation of skin electroporation protocols into clinics is not as fast as one would hope [13]. Already translation from the *in vitro* to the *in vivo* is difficult due to a significantly more complex environment *in vivo*. An interesting link between the *in vitro* and the *in vivo* studies is the *in vitro* 3D reconstructed human skin [60–62] which is more similar to *in vivo* human than rodent skin. Nevertheless, before entering into the clinics, the *in vivo* experiments must be up-scaled to humans, which can result in enormous amounts of drugs/plasmids needed, and scaling up of electrodes and pulse generators.

We identified two main reasons for the hampered transition of skin electroporation into the clinics. 1) *In vivo* studies are usually done on animals (rodents, pigs, rabbits) with significantly different skin structure than humans, which is recognized by the researchers, but they have no way of translating the results. They differ in, among others, the thickness of the layers, their number, the density of the appendages, hydration. The structure of the skin varies significantly also between different people, among different body parts of one person or even of the same part of skin throughout the day. 2) The dosimetry of the delivered pulses is not well controlled. Pulses of various shapes, durations and amplitudes are applied to different electrode configurations with many pulse generators. Some electroporators have vague or non-existent technical specifications, meaning the researchers do not know the exact pulse parameters. The comparison of different devices and reproducibility of experiments are therefore difficult and sometimes impossible. Thus, we suggest making use of mathematical models of skin electroporation, which can be used to calculate and predict the differences among various protocols observed in the literature by taking into account different pulse parameters and skin structure.

4.1 The models of skin electroporation

Models offer an insight into the mechanisms of skin electroporation, and we firmly believe in their significant contribution to the community by elucidating the steps in skin electroporation, the importance of different parameters and by decreasing the number of needed *in vitro* or *in vivo* experiments. In the models, the number and thickness of the layers, their dielectric, thermal and transport properties can easily be changed and various pulse combinations tested without expensive, ethically questionable and time-consuming experimental work.

From models, we can observe that small differences in the skin structure and parameters of applied pulses have a large influence on the treatment outcome. It was theoretically shown that small differences in the thickness of the *stratum corneum* (SC) affected the size of the LTRs and the electrophoretic force, pushing molecules across the skin [30] and thus, the treatment efficacy. Additionally, we conducted a parametric study of a model of electroporation of skin patch as a proof-of-principle of how small changes in the skin or pulse properties can have a large effect on the treatment outcome. We modeled the non-invasive multi-electrode array with 570 V pulses applied between every two pins [10] (Figure 4). The initial parameters of the model (skin geometry and

dielectric properties) were obtained from [5], and electroporation was calculated sequentially as in [54]. The obtained results were our baseline. We modeled 1) the effect of skin hydration and/or age by varying the initial SC conductivity, 2) increase in SC conductivity after electroporation, 3) the body part by varying the thickness of SC, 4) different pulse generator by varying the applied voltage and 5) electric conductivity of all layers (Table 3). We calculated the reversibly and irreversibly electroporated volume and normalized them to the baseline. We observed that only a 2-fold difference in initial or electroporated SC conductivity, which can easily be expected in experiments, increased the electroporated volume up to 30%. Increasing the thickness of the SC to 40 µm instead of 20 µm increased the reversibly electroporated volume but surprisingly, not the irreversibly electroporated. Decreasing the voltage by half did not decrease the electroporated volumes by half as one could expect but to 45% (reversible) or only 15% (irreversible) of the baseline. We can thus see that already small differences in the skin structure, which are more than expected in experiments across different animal species or even in the same subject, could be responsible for the poor reproducibility in applying the *in vivo* results from animal studies to human studies and that modeling of skin electroporation is useful.

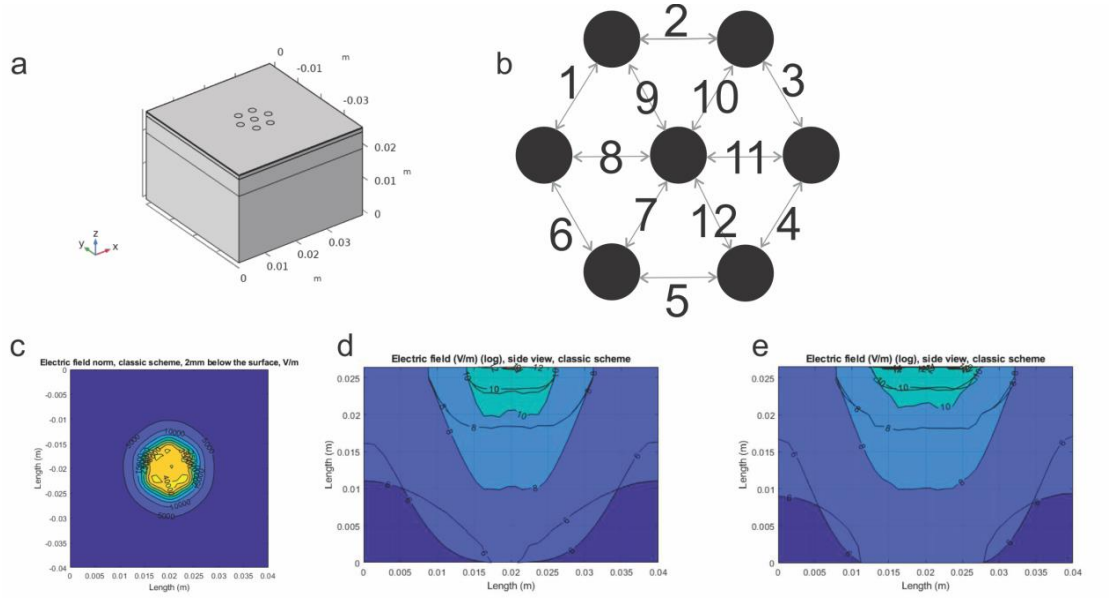


Figure 4: A numerical model of skin electroporation, which we calculated in the scope of this paper. a) The geometry of the layered skin with the circles marking the pins of the multi-electrode array on the surface of the *stratum corneum*. b) Classical pulse application scheme usually used in gene electrotransfer studies. Numbers mark the order of pulses in our simulation. c) The electric field distribution 2 mm below the skin surface (in the hypodermis) when 570 V is applied when following the pulse application scheme on B. d) and e) show the side view of the logarithm of electric field distribution when the thickness of the SC is d) 20 µm or e) 40 µm. We can see that a minor change in the thickness of one layer significantly influences the distribution of electric field even 2 cm below the surface in the muscle layer.

Although the mathematical models of skin electroporation are promising, they do come with their drawbacks. Currently, the largest drawback is the lack of reliable parameter values [5]. The values of the parameters used in the models of human skin, come from porcine skin [23], properties of keratinous fibers [22,23], *ex vivo* human cadavers frozen for different time [24,26,63], geometry of skin cells [5], are deduced from physical constants and/or are based on only a few measurements. Also, the place of the measurement is not always given and the measuring protocol not well described. The values of some parameters are only estimated, especially the dielectric properties of different layers after electroporation. Moreover, the dielectric properties of the skin are anisotropic, which is

only rarely considered in the models. Also, all models should be validated by actual measurement. The models of skin electroporation were mostly constructed for human skin, and some were also validated on it [24,42], however, others were validated on rat [25], porcine [5], mice skin [38], with analytical solutions [22,23,31,34] or were not validated [30]. It was already shown that skin layers differ vastly in their dielectric properties, which significantly affects the results of calculations [23]. However, some models still model skin as one organ of one layer with homogeneous properties [48,64,65], which can lead to erroneous results.

In future, new models should be developed and existing ones improved. More good-quality measurements of properties of skin should be performed and made available. The modeling focus should go in the direction of mechanistic multi-scale modeling and linking the phenomena at different levels – molecular, single-cell, organ, and tissue [66]. The successfully permeabilized/transfected region should be predicted by taking into account the LTR and pore formation, thermal, chemical and electric tissue damage, the amount of the drug/DNA in contact with the cells [49,50], and other parameters, determined to be relevant for skin electroporation. Better models will enable a better treatment outcome prediction and more controlled treatment, which will pave the way to the routine use of skin electroporation in the clinical setting.

4.2 The dosimetry

In the field of skin electroporation, many different pulse generators were used (listed in Table 2). Unfortunately, in most cases, the delivery of electroporation pulses was not properly monitored [67]. Measurement of electroporation pulses is crucial to determine and control their quality and delivery. Current through the electrodes should always be measured to make sure that the pulses were applied to the biological load. Additionally, pulse generators cannot always be trusted due to poor regulation and lack of standardization. Because of large variety of biological loads with significantly different dielectric properties, (also due to different electrode geometries used), pulse generators are not always able to deliver what they promise. In case of loads with low impedance and use of pulses parameters in the higher operation range, the delivered pulses can have a significant voltage drop due to the insufficient energy storage. On the other hand, the voltage amplitude can be limited because of current limitations. Some devices warn the user about the improper operation while others do not. Another problem is that pulse parameters of some devices cannot be changed and/or pre-programmed setups without unknown pulse parameters are used. Consequently, studies often lack the information on pulse specifications (shape, duration, number, voltage, repetition frequency) and thus cannot be reproduced.

Applied pulses are of different shapes, durations, voltages with their spectral energy contained within different parts of the spectrum. It is known that the dielectric properties of tissues are frequency-dependent [68], which influences the electric field distribution across the skin layers and consequently electroporation efficiency [69]. Currently, there are no agreed standard operating procedure pulse parameters for skin electroporation, except for the treatment of cutaneous and subcutaneous tumors [20]. More studies should be performed, determining the most optimal waveform for skin electroporation. Although there is a significant number of pulse generators available on the market, versatile generators should still be developed, for example, for applying bursts of short bipolar pulses, i.e. the high-frequency irreversible electroporation (HF-IRE) pulses [70] to reduce pain and muscle contraction during skin electroporation [13]. The generators, producing the optimal waveforms being applied to optimal electrode configurations should be reliable, simple to use, safe, with available technical specifications, feedback quality measuring system and, when used in the clinics, should comply with the standards for medical devices [71,72].

Various electrode configurations are used in the skin electroporation studies, and the information on electrode geometry is often poor or lacking which additionally renders the dosimetry inaccessible. Moreover, in many studies, only the applied voltage is reported although it was shown that the electric field is the most important parameter influencing the efficiency of electroporation [73] and different electrode configurations cause significantly different electric field distributions [56]. The electrodes are of different materials (stainless steel, platinum, silver, silver chloride, brass, gold) which can cause different chemical reactions and metal release and could affect the treatment outcome [74–76]. Interestingly, it was experimentally shown that gene transfection of skin cells [77], as well as electrochemotherapy of subcutaneous tumors [78], could also be achieved contactless with pulsed magnetic fields (PEMF) which could decrease the chemical contamination and facilitate the use of non-invasive techniques and is worth exploring in the future. Electric field distribution should be calculated and shown for each configuration separately enabling comparison of different electrode configurations. These calculations should be then used to reach an agreement on the most efficient electrode configuration for a chosen skin electroporation treatment.

Various pulse shapes, generators and electrode configurations all contribute to vastly different dosimetry among the published studies, and the dosimetry is not always adequately reported. All this renders comparison of different studies difficult, if not impossible, especially if not all the details are presented. In electrochemotherapy, the pulse parameters and electrode configurations are now standardized to provide safe and efficient treatment for the patients [20]. A similar attempt should also be made in the field of other skin electroporation treatments. We thus ask the researchers to follow the instructions for reporting the dosimetry, as suggested in [57–59,71]. Using good quality pulse generators together with controlled dosimetry, and predictive modeling should increase the efficiency of skin electroporation treatments, enable comparisons between treatments and simplify the translation into clinics.

Acknowledgements

This work was partly supported by the Slovenian Research Agency (ARRS) [research core funding No. P2-0249 and IP-0510] and partly by the European Regional Development Fund (ERDF) and the Slovenian Ministry of Education, Science and Sport within the scope of the Smartgene.si project. The authors would like to thank dr. B. Kos and H. Cindrič for their help with numerical model and dr. M. Reberšek for his help with hardware review.

Annotations to references

* Paper of interest

[5] A paper presenting mechanistic multiscale model of skin electroporation.
[6] A paper presenting a bulk model of skin electroporation, taking into account the presence of separate local transport regions.
[8] A paper reviewing different methods of increasing skin permeability, among them also electroporation.
[31] A paper presenting the model of thermal local transport region expansion via melting of the lipids of the stratum corneum.
[50] A modeling paper on evaluating gene electrotransfer efficiency via electroporation efficiency and electrophoretic transport of plasmid DNA.
[53] A modeling paper showing pore formation in the stratum corneum with molecular dynamics simulations.
[57] A paper giving instructions on correct reporting of experimental factors in food and biotechnological applications of electroporation.

** Paper of particular interest

[1] An excellent review paper on mechanisms and models of electroporation.
[58] A paper giving instructions on correct reporting of experimental factors in biological experiments on electroporation.
[59] A paper giving instructions on correct reporting of experimental factors in clinical experiments with electroporation.
[71] A book chapter on dosimetry in electroporation-based treatments, also giving instructions to researchers on correct reporting of the delivered waveforms.

List of references

[1] Kotnik T, Rems L, Tarek M, et al. Membrane Electroporation and Electropermeabilization: Mechanisms and Models. Annual Review of Biophysics. 2019;48.
[2] Pliquett UF, Vanbever R, Preat V, et al. Local transport regions (LTRs) in human stratum corneum due to long and short `high voltage' pulses. Bioelectrochemistry and Bioenergetics. 1998;47:151–161.
[3] Pliquett UF, Zewert TE, Chen T, et al. Imaging of fluorescent molecule and small ion transport through human stratum corneum during high voltage pulsing: localized transport regions are involved. Biophysical Chemistry. 1996;58:185–204.
[4] Gowrishankar TR, Herndon TO, Vaughan TE, et al. Spatially constrained localized transport regions due to skin electroporation. Journal of Controlled Release. 1999;60:101–110.
[5] Dermol-Černe J, Miklavčič D. From Cell to Tissue Properties—Modeling Skin Electroporation With Pore and Local Transport Region Formation. IEEE Transactions on Biomedical Engineering. 2018;65:458–468.
[6] Pavšelj N, Miklavčič D. Numerical Models of Skin Electropermeabilization Taking Into Account Conductivity Changes and the Presence of Local Transport Regions. IEEE Transactions on Plasma Science. 2008;36:1650–1658.
[7] Denet A-R, Vanbever R, Préat V. Skin electroporation for transdermal and topical delivery. Adv. Drug Deliv. Rev. 2004;56:659–674.
[8] Zorec B, Préat V, Miklavčič D, et al. Active enhancement methods for intra- and transdermal drug delivery: a review. Zdravniški vestnik. 2013;82:339–356.
[9] Lambricht L, Lopes A, Kos Š, et al. Clinical potential of electroporation for gene therapy and DNA vaccine delivery. Expert Opinion on Drug Delivery. 2016;13:295–310.
[10] Kos Š, Blagus T, Čemažar M, et al. Electrotransfer parameters as a tool for controlled and targeted gene expression in skin. Molecular Therapy - Nucleic Acids. 2016;5:e356.

[11] Prausnitz MR, Langer R. Transdermal drug delivery. Nat Biotechnol. 2008;26:1261–1268.
[12] Ita K. Perspectives on Transdermal Electroporation. Pharmaceutics. 2016;8:9.
[13] Schoellhammer CM, Blankschtein D, Langer R. Skin permeabilization for transdermal drug delivery: recent advances and future prospects. Expert Opinion on Drug Delivery. 2014;11:393–407.
[14] Zasada M, Markiewicz A, Drożdż Z, et al. Preliminary randomized controlled trial of antiaging effects of L-ascorbic acid applied in combination with no-needle and microneedle mesotherapy. J Cosmet Dermatol. 2019;18:843–849.
[15] Thappa D, Konda D. Mesotherapy: What is new? Indian J Dermatol Venereol Leprol. 2013;79:127.
[16] Zhang L, Lerner S, Rustrum WV, et al. Electroporation-mediated topical delivery of vitamin C for cosmetic applications. Bioelectrochemistry and Bioenergetics. 1999;48:453–461.
[17] Golberg A, Villiger M, Felix Broelsch G, et al. Skin regeneration with all accessory organs following ablation with irreversible electroporation: Skin regeneration following irreversible electroporation. J Tissue Eng Regen Med. 2018;12:98–113.
[18] Golberg A, Broelsch GF, Vecchio D, et al. Pulsed Electric Fields for Burn Wound Disinfection in a Murine Model: Journal of Burn Care & Research. 2015;36:7–13.
[19] Novickij V, Zinkevičienė A, Perminaitė E, et al. Non-invasive nanosecond electroporation for biocontrol of surface infections: an in vivo study. Sci Rep. 2018;8:14516.
[20] Gehl J, Serša G, Matthiessen LW, et al. Updated standard operating procedures for electrochemotherapy of cutaneous tumours and skin metastases. Acta Oncol. 2018;57:874–882.
[21] Campana LG, Miklavčič D, Bertino G, et al. Electrochemotherapy of superficial tumors – Current status: Seminars in Oncology. 2019;46:173–191.
[22] Becker SM, Kuznetsov AV. Local Temperature Rises Influence In Vivo Electroporation Pore Development: A Numerical Stratum Corneum Lipid Phase Transition Model. Journal of Biomechanical Engineering. 2007;129:712–721.
[23] Becker SM, Kuznetsov AV. Numerical Assessment of Thermal Response Associated With In Vivo Skin Electroporation: The Importance of the Composite Skin model. J. Biomech. Eng. 2007;129:330.
[24] Chizmadzhev YA, Indenbom AV, Kuzmin PI, et al. Electrical properties of skin at moderate voltages: contribution of appendageal macropores. Biophys. J. 1998;74:843–856.
[25] Pavšelj N, Préat V, Miklavčič D. A Numerical Model of Skin Electropermeabilization Based on In Vivo Experiments. Ann Biomed Eng. 2007;35:2138–2144.
[26] Pavšelj N, Miklavčič D. A numerical model of permeabilized skin with local transport regions. IEEE Trans Biomed Eng. 2008;55:1927–1930.
[27] Pavšelj N, Bregar Z, Cukjati D, et al. The Course of Tissue Permeabilization Studied on a Mathematical Model of a Subcutaneous Tumor in Small Animals. IEEE Transactions on Biomedical Engineering. 2005;52:1373–1381.
[28] Pawlowski P, Gallo SA, Johnson PG, et al. Electrorheological Modeling of the Permeabilization of the Stratum Corneum: Theory and Experiment. Biophysical Journal. 1998;75:2721–2731.
[29] Nickfarjam A, Firoozabadi SMP. Parametric study of irreversible electroporation with different needle electrodes: Electrical and thermal analysis. International Journal of Hyperthermia. 2014;30:335–347.
[30] Becker S. Transport modeling of skin electroporation and the thermal behavior of the stratum corneum. International Journal of Thermal Sciences. 2012;54:48–61.
[31] Becker SM, Kuznetsov AV. Thermal in vivo skin electroporation pore development and charged macromolecule transdermal delivery: A numerical study of the influence of chemically enhanced lower lipid phase transition temperatures. International Journal of Heat and Mass Transfer. 2008;51:2060–2074.
[32] Martin GT, Pliquett UF, Weaver JC. Theoretical analysis of localized heating in human skin subjected to high voltage pulses. Bioelectrochemistry. 2002;57:55–64.
[33] Pavšelj N, Miklavčič D. Resistive heating and electropermeabilization of skin tissue during in vivo electroporation: A coupled nonlinear finite element model. International Journal of Heat and Mass Transfer. 2011;54:2294–2302.
[34] Becker SM, Kuznetsov AV. Thermal damage reduction associated with in vivo skin electroporation: A numerical investigation justifying aggressive pre-cooling. International Journal of Heat and Mass Transfer. 2007;50:105–116.
[35] Pliquett U, Nuccitelli R. Measurement and simulation of Joule heating during treatment of B-16 melanoma tumors in mice with nanosecond pulsed electric fields. Bioelectrochemistry. 2014;100:62–68.
[36] Pliquett U, Gusbeth Ch, Nuccitelli R. A propagating heat wave model of skin electroporation. Journal of Theoretical Biology. 2008;251:195–201.
[37] Vanbever R, Pliquett UF, Préat V, et al. Comparison of the effects of short, high-voltage and long, medium-voltage pulses on skin electrical and transport properties. Journal of Controlled Release. 1999;60:35–47.
[38] Daugimont L, Baron N, Vandermeulen G, et al. Hollow Microneedle Arrays for Intradermal Drug Delivery and DNA Electroporation. J Membrane Biol. 2010;236:117–125.
[39] Mi Y, Peng W, Rui S, et al. Multiparametric Finite-Element Simulation and Experiment on Thermal Effects in Skin Tumor Exposed to High-Frequency Nanosecond Pulse Bursts. IEEE Trans. Plasma Sci. 2019;47:924–934.
[40] Čorović S, Županič A, Miklavčič D. Numerical Modeling and Optimization of Electric Field Distribution in Subcutaneous Tumor Treated With Electrochemotherapy Using Needle Electrodes. IEEE Transactions on Plasma Science. 2008;36:1665–1672.
[41] Cornelis FH, Cindrič H, Kos B, et al. Peri-tumoral Metallic Implants Reduce the Efficacy of Irreversible Electroporation for the Ablation of Colorectal Liver Metastases. Cardiovasc Intervent Radiol [Internet]. 2019 [cited 2019 Oct 18]; Available from: http://link.springer.com/10.1007/s00270-019-02300-y.

[42] Becker SM. Skin Electroporation With Passive Transdermal Transport Theory: A Review and a Suggestion for Future Numerical Model Development. J. Heat Transfer. 2011;133:011011.

[43] Chizmadzhev YA, Zarnitsin VG, Weaver JC, et al. Mechanism of electroinduced ionic species transport through a multilamellar lipid system. Biophysical Journal. 1995;68:749–765.

[44] Becker S, Zorec B, Miklavčič D, et al. Transdermal transport pathway creation: Electroporation pulse order. Mathematical Biosciences. 2014;257:60–68.

[45] Edwards DA, Prausnitz MR, Langer R, et al. Analysis of enhanced transdermal transport by skin electroporation. Journal of Controlled Release. 1995;34:211–221.

[46] Mahnič-Kalamiza S, Vorobiev E. Dual-porosity model of liquid extraction by pressing from biological tissue modified by electroporation. Journal of Food Engineering. 2014;137:76–87.

[47] Hirsch AC, Upasani RS, Banga AK. Factorial design approach to evaluate interactions between electrically assisted enhancement and skin stripping for delivery of tacrine. Journal of Controlled Release. 2005;103:113–121.

[48] Gothelf A, Mahmood F, Dagnaes-Hansen F, et al. Efficacy of transgene expression in porcine skin as a function of electrode choice. Bioelectrochemistry. 2011;82:95–102.

[49] Forjanič T, Miklavčič D. Numerical study of gene electrotransfer efficiency based on electroporation volume and electrophoretic movement of plasmid DNA. BioMedical Engineering OnLine [Internet]. 2018 [cited 2018 Jul 13];17. Available from: https://biomedical-engineering-online.biomedcentral.com/articles/10.1186/s12938-018-0515-3.

[50] Forjanič T, Markelc B, Marčan M, et al. Electroporation-Induced Stress Response and Its Effect on Gene Electrotransfer Efficacy: In Vivo Imaging and Numerical Modeling. IEEE Trans. Biomed. Eng. 2019;66:2671–2683.

[51] Čorović S, Markelc B, Dolinar M, et al. Modeling of Microvascular Permeability Changes after Electroporation. Schneditz D, editor. PLOS ONE. 2015;10:e0121370.

[52] Vargas Luna JL, Krenn M, Cortés Ramírez JA, et al. Dynamic impedance model of the skin-electrode interface for transcutaneous electrical stimulation. PLoS ONE. 2015;10:e0125609.

[53] Gupta R, Rai B. Electroporation of Skin Stratum Corneum Lipid Bilayer and Molecular Mechanism of Drug Transport: A Molecular Dynamics Study. Langmuir. 2018;34:5860–5870.

[54] Čorović S, Lacković I, Šuštarič P, et al. Modeling of electric field distribution in tissues during electroporation. BioMedical Engineering OnLine. 2013;12:16.

[55] Gothelf A, Gehl J. Gene Electrotransfer to Skin; Review of Existing Literature and Clinical Perspectives. CGT. 2010;10:287–299.

[56] Čorović S, Pavlin M, Miklavčič D. Analytical and numerical quantification and comparison of the local electric field in the tissue for different electrode configurations. BioMedical Engineering OnLine. 2007;6:37.

[57] Raso J, Frey W, Ferrari G, et al. Recommendations guidelines on the key information to be reported in studies of application of PEF technology in food and biotechnological processes. Innovative Food Science & Emerging Technologies [Internet]. 2016 [cited 2016 Aug 30]; Available from: http://linkinghub.elsevier.com/retrieve/pii/S1466856416301771.

[58] Čemažar M, Serša G, Frey W, et al. Recommendations and requirements for reporting on applications of electric pulse delivery for electroporation of biological samples. Bioelectrochemistry. 2018;122:69–76.

[59] Campana LG, Clover AJP, Valpione S, et al. Recommendations for improving the quality of reporting clinical electrochemotherapy studies based on qualitative systematic review. Radiology and Oncology. 2016;50:1–13.

[60] Madi M, Rols M-P, Gibot L. Efficient In Vitro Electropermeabilization of Reconstructed Human Dermal Tissue. The Journal of Membrane Biology. 2015;248:903–908.

[61] Madi M, Rols M-P, Gibot L. Gene Electrotransfer in 3D Reconstructed Human Dermal Tissue. Curr Gene Ther. 2016;16:75–82.

[62] Bulysheva AA, Burcus N, Lundberg C, et al. Recellularized human dermis for testing gene electrotransfer ex vivo. Biomed. Mater. 2016;11:035002.

[63] Pliquett U, Weaver JC. Electroporation of human skin: simultaneous measurement of changes in the transport of two fluorescent molecules and in the passive electrical properties. Bioelectrochemistry and Bioenergetics. 1996;39:1–12.

[64] Henshaw JW, Zaharoff DA, Mossop BJ, et al. Electric field-mediated transport of plasmid DNA in tumor interstitium in vivo. Bioelectrochemistry. 2007;71:233–242.

[65] Čorović S, Mir LM, Miklavčič D. In Vivo Muscle Electroporation Threshold Determination: Realistic Numerical Models and In Vivo Experiments. The Journal of Membrane Biology. 2012;245:509–520.

[66] Walpole J, Papin JA, Peirce SM. Multiscale Computational Models of Complex Biological Systems. Annu. Rev. Biomed. Eng. 2013;15:137–154.

[67] Reberšek M. Beyond Electroporation Pulse Parameters: From Application to Evaluation. In: Miklavcic D, editor. Handbook of Electroporation [Internet]. Cham: Springer International Publishing; 2017. p. 1–21. Available from: https://doi.org/10.1007/978-3-319-26779-1_222-1.

[68] Gabriel C, Gabriel S, Corthout E. The dielectric properties of biological tissues: I. Literature survey. Phys Med Biol. 1996;41:2231–2249.

[69] Chiapperino MA, Bia P, Caratelli D, et al. Nonlinear Dispersive Model of Electroporation for Irregular Nucleated Cells. Bioelectromagnetics. 2019;bem.22197.

[70] Scuderi M, Reberšek M, Miklavčič D, et al. The use of high-frequency short bipolar pulses in cisplatin electrochemotherapy in vitro. Radiology and Oncology. 2019;53:194–205.

[71] Pirc E, Reberšek M, Miklavčič D. Dosimetry in Electroporation-Based Technologies and Treatments. Dosimetry in Bioelectromagnetic. Boca Raton: Taylor & Francis Group, LLC; 2017. p. 233–268.

[72] Cvetkoska A, Pirc E, Reberšek M, et al. Towards standardization of electroporation devices and protocols. IEEE Instrumentation & Measurement Magazine. 2019;accepted for publication.

[73] Mori K, Hasegawa T, Sato S, et al. Effect of electric field on the enhanced skin permeation of drugs by electroporation. Journal of Controlled Release. 2003;90:171–179.

[74] Kotnik T, Miklavčič D, Mir LM. Cell membrane electropermeabilization by symmetrical bipolar rectangular pulses. Part II. Reduced electrolytic contamination. Bioelectrochemistry. 2001;54:91–95.

[75] Saulis G, Rodaitė-Riševičienė R, Dainauskaitė VS, et al. Electrochemical Processes During High-Voltage Electric Pulses and their Importance in Food Processing Technology. In: Rai V R, editor. Advances in Food Biotechnology [Internet]. Chichester, UK: John Wiley & Sons Ltd; 2015 [cited 2015 Dec 10]. p. 575–592. Available from: http://doi.wiley.com/10.1002/9781118864463.ch35.

[76] Luján E, Marino M, Olaiz N, et al. Towards an optimal dose-response relationship in gene electrotransfer protocols. Electrochimica Acta. 2019;319:1002–1011.

[77] Kardos TJ, Rabussay DP. Contactless magneto-permeabilization for intracellular plasmid DNA delivery in-vivo. Human Vaccines & Immunotherapeutics. 2012;8:1707–1713.

[78] Kranjc S, Kranjc M, Ščančar J, et al. Electrochemotherapy by pulsed electromagnetic field treatment (PEMF) in mouse melanoma B16F10 in vivo. Radiology and Oncology. 2016;50:39–48.

[79] Mossop BJ, Barr RC, Henshaw JW, et al. Electric Fields in Tumors Exposed to External Voltage Sources: Implication for Electric Field-Mediated Drug and Gene Delivery. Ann Biomed Eng. 2006;34:1564–1572.

[80] Caliper Electrodes for Electroporation Applications [Internet]. [cited 2019 Oct 10]. Available from: https://www.btxonline.com/caliper-electrodes.html.

[81] Heller LC, Jaroszeski MJ, Coppola D, et al. Optimization of cutaneous electrically mediated plasmid DNA delivery using novel electrode. Gene Ther. 2007;14:275–280.

[82] Golberg A, Bei M, Sheridan RL, et al. Regeneration and control of human fibroblast cell density by intermittently delivered pulsed electric fields. Biotechnol. Bioeng. 2013;110:1759–1768.

[83] Petri Pulser Electroporation Applicator [Internet]. [cited 2019 Oct 10]. Available from: https://www.btxonline.com/petri-pulser.html.

[84] Accessories ElectroVet [Internet]. Leroy Biotech. [cited 2019 Oct 10]. Available from: https://www.leroybiotech.com/electrovet-ez/accessories/.

[85] Mazères S, Šel D, Golzio M, et al. Non invasive contact electrodes for in vivo localized cutaneous electropulsation and associated drug and nucleic acid delivery. Journal of Controlled Release. 2009;134:125–131.

[86] Guo S, Donate A, Basu G, et al. Electro-gene transfer to skin using a noninvasive multielectrode array. Journal of Controlled Release. 2011;151:256–262.

[87] Heller R, Cruz Y, Heller LC, et al. Electrically Mediated Delivery of Plasmid DNA to the Skin, Using a Multielectrode Array. Human Gene Therapy. 2010;21:357–362.

[88] Donate A, Coppola D, Cruz Y, et al. Evaluation of a Novel Non-Penetrating Electrode for Use in DNA Vaccination. Rubinsky B, editor. PLoS ONE. 2011;6:e19181.

[89] Zorec B, Jelenc J, Miklavčič D, et al. Ultrasound and electric pulses for transdermal drug delivery enhancement: Ex vivo assessment of methods with in vivo oriented experimental protocols. International Journal of Pharmaceutics. 2015;490:65–73.

[90] Pliquett U, Weaver JC. Feasibility of an Electrode-Reservoir Device for Transdermal Drug Delivery by Noninvasive Skin Electroporation. IEEE Trans. Biomed. Eng. 2007;54:536–538.

[91] Huang D, Zhao D, Wang X, et al. Efficient delivery of nucleic acid molecules into skin by combined use of microneedle roller and flexible interdigitated electroporation array. Theranostics. 2018;8:2361–2376.

[92] Maruyama H, Ataka K, Higuchi N, et al. Skin-targeted gene transfer using in vivo electroporation. Gene Ther. 2001;8:1808–1812.

[93] Electrodes and accessories | IGEA [Internet]. [cited 2019 Oct 10]. Available from: https://www.igea.it/en/oncology/information-clinicians/electrodes-and-accessories.

[94] Platinum Needle L-Shaped Electrode for In Vivo Electroporation Applications [Internet]. [cited 2019 Oct 10]. Available from: https://www.btxonline.com/platinum-needle-l-shaped-electrode.html.

[95] Needle Array Electrodes for BTX AgilePulse In Vivo [Internet]. [cited 2019 Nov 5]. Available from: https://www.btxonline.com/needle-array-electrodes-for-agilepulse-in-vivo.html.

[96] In Vivo/In Utero/In Ovo/Ex Vivo Electroporation Electrodes [Internet]. [cited 2019 Oct 10]. Available from: http://www.nepagene.jp/e_products_nepagene_0009.html.

[97] Electrodes for in vivo electroporation | BEX CO., LTD. [Internet]. [cited 2019 Oct 10]. Available from: http://www.bexnet.co.jp/english/product/device/in-vivo/2.html.

[98] Deng Y, Chen J, Zhao Y, et al. Transdermal Delivery of siRNA through Microneedle Array. Scientific Reports. 2016;6:21422.

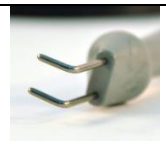
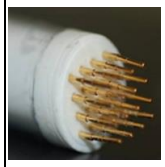
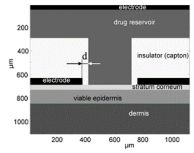
[99] Campana LG, Dughiero F, Forzan M, et al. A prototype of a flexible grid electrode to treat widespread superficial tumors by means of Electrochemotherapy. Radiology and Oncology [Internet]. 2016 [cited 2019 Aug 26];50. Available from: https://content.sciendo.com/view/journals/raon/50/1/article-p49.xml.

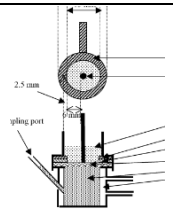
614 [100] MESOFORTE Duo | AMICO GROUP [Internet]. [cited 2019 Oct 29]. Available from:
615 https://www.amicogroup.com/specialization/dermatology/cosmetics/dr-derm/mesotherapy-mesoforte-duo.aspx.
616 [101] El-Kamary SS, Billington M, Deitz S, et al. Safety and Tolerability of the Easy Vax™ Clinical Epidermal Electroporation
617 System in Healthy Adults. Molecular Therapy. 2012;20:214–220.
618 [102] Goldman MP, Hexsel D. Cellulite: Pathophysiology and Treatment. CRC Press; 2010.
619 [103] Lombry C, Dujardin N, Préat V. Transdermal Delivery of Macromolecules Using Skin Electroporation. Pharmaceutical
620 Research. 2000;17:32–37.
621 [104] Orr RM. Technology evaluation: electroporation therapy, Genetronics Inc. Curr. Opin. Mol. Ther. 2000;2:205–210.
622 [105] ACTHYDERM ® - Microlab International Srl ® [Internet]. [cited 2019 Oct 29]. Available from:
623 http://acthyderm.mulab.net/Acthyderm01.html.
624 [106] Skrynska O, Antonova-Rafi J, Khudetskyy I. Treating Skin with Use of Electroporation. 2019;503962 Bytes.
625

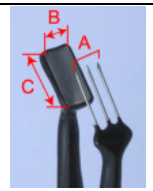
626 **Tables**

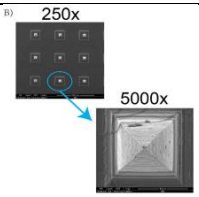
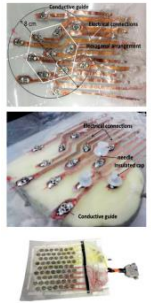
627 Table 1: For skin electroporation, different electrode configurations are used. Here, we show electrode types usually used for skin electroporation in *in vitro*
628 as well as *in vivo* studies. Electrodes are divided into invasive and non-invasive, for each we also state the intended area of use, the manufacturer, the electrode
629 configuration (number of electrodes and their dimensions), material, reference to the study or manufacturer's webpage and an image of the electrodes.

Electrode type	Area of use	Manufacturer	Electrode configuration (number of electrodes and their dimensions)	Material	Reference	Image
Non-invasive electrodes						
PLATE OR CALIPER ELECTRODES	Gene electrotransfer, transdermal drug delivery, electrochemotherapy, skin rejuvenation	BTX - Harvard Apparatus	2 1 x 1 cm, 1.5 x 1.5 cm, 2 x 2 cm	Brass or stainless steel (only 2 x 2 cm)	[79,80]	
		IGEA	2 8 mm gap / 6 mm (older version) 10 mm x 30 mm x 0.8 m	medical grade steel	[48]	
		custom prototype	4 6 mm gap	NA	[81]	
		BTX - Harvard Apparatus	13 spaced 2 mm apart	gold plated	[82,83]	

L-SHAPED ELECTRODES	Transdermal drug delivery Gene electrotransfer Electrochemotherapy	Leroy BIOTECH	2 10 mm gap 10 mm x 3 mm	stainless steel	[84]	
		custom prototype	2 4 mm gap 20 mm x 1 mm	stainless steel	[85]	
PIN SURFACE ELECTRODES	Gene electrotransfer Transdermal drug delivery	custom prototype	16 0.3 mm diameter Grid of 2-mm-apart pins	gold-plated	[86–88]	 Image from [88]
		Iskra Medical	7 spring-loaded pins in honeycomb configuration, spaced 3.5 mm apart	NA	[10,89]	
ELECTRODE-RESERVOIR DEVICE	Transdermal drug delivery	custom prototype	2 should not exceed 200 µm	NA	[90]	

INTERDIGITATED ELECTRODES	Gene electrotransfer	custom prototype	patch-like electroporation array	gold electrodes on pliable parylene substrate	[91]	 Image from [91]
RING AND NEEDLE ELECTRODES	Transdermal drug delivery	custom prototype	ring electrode: (outside diameter 25 mm and inside diameter 15 mm) needle electrode: 3 cm x 3 mm	silver	[73]	
MESO THERAPY ELECTRODES	Transdermal drug delivery	Derm Equipment	a plate electrode 25 mm in diameter	NA	[16]	
Invasive electrodes						
NEEDLE ELECTRODES	Gene electrotransfer Electrochemotherapy	IGEA	7 hexagonal configuration Diameter 0.7 mm, length 10 mm, 20 mm or 30 mm	medical grade steel	[92]	

		IGEA	8 (2 rows of 4) linear configuration Diameter 0.7 mm, length 10 mm or 20 mm or 30 mm	medical grade steel	[93]	
		BTX - Harvard Apparatus	2 3 mm tip length ultra thin diameter	platinum	[94]	
		BTX - Harvard Apparatus	8 or 12 4 or 6 mm gap lengths: 2, 3, 5, 10, 16, 25,	stainless steel	[95]	
		Leroy	8 (two rows of four) Linear configuration 8 mm between two rows, each needle is 2 mm apart 0.88 mm diameter, 15 mm long	stainless steel	[84]	
FORK ELECTRODES	Gene electrotransfer	BEX and NEAPGENE	4 (3 needles + 1 plate) 3 x 2.5 mm needles intervals, length 3 mm or 5 mm or 10 mm, diameter 0.5 mm	stainless steel coated in platinum	[92,96,97]	

MICRONEEDLE ARRAY ELECTRODES	Gene electrotransfer	custom prototype	pyramidal shape, radius of the tip is below 1 µm needles length: 200 µm ± 7 µm (900 needles per array and 121 arrays per wafer. Needle spacing: 90 µm	solid silicon glass titanium ceramic polymer	[38,98]	 Image from [98]
GRID ELECTRODES	Electrochemotherapy	custom prototype	Flexible support and 67 needles, length 5 or 10 mm	stainless steel	[99]	 Image from [99].

NA = not available

Copyrights:

L-shaped prototype electrodes: Reprinted from Journal of Controlled Release, 134 /2, Mazères et al., Non invasive contact electrodes for in vivo localized cutaneous electropulsation and associated drug and nucleic acid delivery, 125-131, Copyright (2009), with permission from Elsevier.

Ring and needle electrodes: Reprinted from Effect of electric field on the enhanced skin permeation of drugs by electroporation, 90/2, Mori et al., Effect of electric field on the enhanced skin permeation of drugs by electroporation, 171-179, Copyright (2003), with permission from Elsevier.

637 Table 2: Pulse generators usually used in studies on skin electroporation. For each manufacturer, we list the existing pulse generators, the intended use, the
638 waveform type, pulse number, amplitude and duration, reference to a study or manufacturer's webpage and our expert opinion on the generator. As we did
639 not have access to all the listed generators, some were not tested or older versions than listed were tested as specified under each expert opinion.

Manufacturer	Pulse generator	Used for	Waveform type	Pulse number	Pulse amplitude	Pulse duration	Reference	Expert opinion
Amico	Mezoforte Duo*	Cosmetics (mesotherapy)	NA	NA	NA	NA	[100]	The device was tested in our laboratory. It delivers standard low voltage radiofrequency (RF) electric pulses to the skin. There are three different applicators available for the device. One for RF and two for mesotherapy - "electroporation". The device allows us to set up the duration of the therapy (minutes), to change the programs (face, body, hand ...) and to change the intensity of the therapy (low, standard, high). It generates a sinusoidal output signal with of 833 kHz. The sinusoidal pulses are generated in bursts (modulated). The duration of the bursts are 4.3 ms for mesotherapy and 30 ms for RF therapy. The delays between the bursts are 8.5 ms for mesotherapy and 17 ms for RF therapy. There are no changes in the output signal characteristics due

								to the change in the selected program. Only the suggested time of treatment varies with programs. The highest voltages achieved measured at the therapeutic surface are 25 Vpp (peak-to-peak) for meso applicator and 32 Vpp for RF applicator.
BTX HARVARD APPARATUS	AgilePulse In Vivo System (formerly the DermaVax device)	Skin rejuvenation Transdermal drug delivery	NA	3 groups of pulses: from 1-10 pulses in each group	(50 - 1000) V	(0.050 - 10) ms	[manufacturer]***	Gemini devices "sense" the load each time before the pulse delivery. We tested Gemini X2 and a single 15 V, 45 ms long pre-pulse is delivered approximately 4 seconds before the pre-set sequence. The maximal current is not defined, but for Gemini X2 we determined it to be close to 11 A. Therefore when working with more conductive loads (< 80 Ω), the device is actually not able to deliver the whole range of pulse amplitudes defined in the specifications. We tested also the ECM 830, This device was also tested also on 50 Ω and 100 Ω resistors. From the measurements, we assume that this electroporator cannot deliver more than 1500 V to 100 Ω load and is almost not to be used for 50 Ω loads and lower.
	ECM 830*		Square wave	1 – 99	HV: (505 - 3000) V LV: (5 - 500) V	HV: (10 - 600) µs LV: (10 - 999) µs; (1 - 999) ms; (1 - 10) s		
	ECM 630		Exponential decay wave	1 – 99	HV: (50 - 2500) V LV: (10 - 500) V	10 µs - 10 s		
	Gemini SC2		Square waves and exponential decay waves	LV: 1 - 10 HV: 1 -2 Exponential decay- 1	(10 - 3000) V	50 µs – 100 ms		

	Gemini X^2*		Square waves and exponential decay waves	Square wave: LV mode-1-120 (10 per sample) HV mode-1-36 (3 per sample); Exponential decay- 1-12 (R internal <100 ohms) and 1-24 (R internal > 100 ohm)	(5 – 3000) V	10 µs – 1 s		Overall a reliable manufacturer, with high quality products. The main drawback are the pre-pulse and missing visualization of delivered pulses.
Cyto Pulse Sciences	Easy Vax	Gene electrotransfer	NA	NA	NA	NA	[9,101]	The device is not commercially available anymore.
DermaWave Company, USA and BTL Industries	DermaWave	Cosmetics (mesotherapy)	NA	NA	NA	NA	[102,chap.12]	Cannot comment, due to the lack of the output pulse specification.
Equibio	Easyject Plus	Transdermal drug delivery	NA	NA	NA	NA	[103]	The data about the device could not be found, on the internet, maybe they were available in the past, it looks like device is not available anymore.

Genetronics Biomedical	MedPulser	Electrochemotherapy	Square wave	NA	~ 200 V/cm	60 ms	[9,104]	The device in not available anymore, Inovio upgraded it to CELLECTRA.
Ichor Medical Systems	TriGrid™Delivery System	Gene electrotransfer	NA	NA	NA	NA	[13]	The manufacturer chose not to provide the pulse characteristics, due to being a subject of their intellectual property.
IGEA	Cliniporator EPS02 *	Electrochemotherapy Gene electrotransfer Transdermal drug delivery	Square wave	LV: 1 - 10 HV: 1 - 10	LV : (20 - 200) V HV : (100 - 1000) V	LV : (1 - 200) ms HV : (50 - 1000) µs	[manufacturer]***	Both devices are clinical electroporators, classified as a medical device with a CE mark. Their operation is reliable and of good quality. We evaluated both and were pleased with their operation. They can however only be used in combination with manufacturers' electrodes, which are quite expensive and for a single-use. The only drawback is too low sampling frequency of the built-in measuring system.
	Cliniporator VITAE*			HV: 4+4 (polarity exchange); 4 -8	HV : (500 - 3000) V	100 µs		
INOVIO	CELLECTRA®: - 5PSP 2000 - 5P 2000 - 3P	Gene electrotransfer	Square wave	3 3	max 200 V	52 ms	[manufacturer]***	The data presented in the table were provided directly from the manufacturer. However, the parameters might have changed over time. The device is built in the shape of an injection gun and it is used *in vivo* intramuscularly. It is not yet FDA approved but clinical trials are in full swing.

				2 sets of 2 pulses				
Jouan	Societe Jouan*		Square wave	one or continues	0 -1500 V	5 µs - 24 ms	[65]	The device is used in our laboratory, pulse shape is not exactly square wave and measuring is necessary because output pulses do not match the preset characteristics. It is not produced anymore and quite rare.
Leroy BIOTECH	ELECTROvet S13	Gene electrotransfer	Square wave	1 - 10 000	0 - 1350 V	5 - 5,000 µs	[manufacturer]***	B10 and S13 have 25 A current limitation and B20 and EZ have only 10 A current limitation. The device evaluates the impedance of the connected load and disables the pulse delivery in case of a too conductive load (the user is properly informed about the fault). Thus, all parameter ranges are not available in all situations. The B10 device was evaluated in our laboratory and some problems were detected at low voltages (under 100 V) and short pulse durations (e.g. 10 µs). Overall a reliable manufacturer, with quality products.
	ELECTROvet EZ			1 - 10 000	0 - 1500 V	5 - 5,000 µs		
	ELECTRO cell B10*		Square wave bipolar	1 - 10 000	0 - 1000 V	5 - 5,000 µs		
	ELECTRO cell S20		Square wave	1 - 10 000	0 - 2000 V	5 - 5,000 µs		
Microlab International	Acthyderm*	Cosmetics (mesotherapy)	NA	NA	NA	NA	[105]	Cannot comment, due to the lack of the output pulse specification. However, the older version was tested in our laboratory and actually delivered radiofrequency waves to the skin.

	Max-E48	Cosmetics (mesotherapy)	NA	NA	NA	NA	[106]	The data about the device could not be found on the internet, maybe they were available in the past, it appears the device is not produced anymore.
OncoSec Medical	IMMUNOPULSE™ IL-12 NeoPulse	Gene electrotransfer	NA	NA	NA	NA	[9,13]	The device is used only in clinical trials; it seems is still under development. The manufacturer declined to provide pulse characteristics, claiming they are a subject of intellectual property.
UltraVolt	Rack-2-500-00230	Power supply unit	Power supply unit	Power supply unit	Power supply unit	Power supply unit	[86]	Not an actual electroporator but a high-voltage power supply. For pulse generation an additional pulse forming circuit is required.

640 * Evaluated in our laboratory; ** Technical specifications approved by the manufacturer; NA = not available; LV = low-voltage pulses, HV = high-voltage
641 pulses

642 Table 3: The volume of reversibly and irreversibly electroporated skin in the layered skin model as
643 calculated in the scope of this paper. First, we calculated the volume of reversibly and irreversibly
644 electroporated volume and then varied the parameters of the model. For each change in parameters,
645 we normalized the results to the results with initial values of the parameters (the baseline)

Change of parameters / Normalized volume	Reversibly electroporated volume	Irreversibly electroporated volume
Baseline	100 %	100 %
Increased SC conductivity (2-times)	124% of the baseline	122% of the baseline
Increased electroporated SC conductivity	123% of the baseline	130% of the baseline
Increased SC thickness	120% of the baseline	98% of the baseline
Decreased voltage (50% of the initial one)	45% of the baseline	17% of the baseline
Changed threshold of electroporation (increased 2-times)	98% of the baseline	91%of the baseline
Increased conductivity of all layers (2-times)	100% of the baseline	124% of the baseline

646 SC = *stratum corneum*

3.3 Paper 2

Title: **Functional requirements and quality assurance necessary for the successful incorporation of electroporation-based therapies into clinical practice**

Authors: Eva Pirc, Matej Reberšek and Damijan Miklavčič

Publication: *Journal of Medical Devices-Transactions of the ASME*, vol. 14, no. 1, pp. 1 - 9

Year: 2019

DOI: 10.1115/1.4045837

Impact factor: 0.539 (2018)

Quartile: Q4 (engineering, biomedical)

Rank: 79/80 (engineering, biomedical)

Journal of Medical Devices

Expert View

Functional Requirements and Quality Assurance Necessary for Successful Incorporation of Electroporation-Based Therapies Into Clinical Practice

Eva Pirc
Laboratory of Biocybernetics,
Faculty of Electrical Engineering,
University of Ljubljana,
Tržaška cesta 25,
Ljubljana 1000, Slovenia
e-mail: eva.pirc@fe.uni-lj.si

Matej Reberšek
Laboratory of Biocybernetics,
Faculty of Electrical Engineering,
University of Ljubljana,
Tržaška cesta 25,
Ljubljana 1000, Slovenia
e-mail: matej.rebersek@fe.uni-lj.si

Damijan Miklavčič[1]
Professor
Laboratory of Biocybernetics,
Faculty of Electrical Engineering,
University of Ljubljana,
Tržaška cesta 25,
Ljubljana 1000, Slovenia
e-mail: damijan.miklavcic@fe.uni-lj.si

Electroporation-based therapies have a huge potential for implementation into clinical practice in socioeconomically disadvantaged populations. Currently, the price of electroporators and electrodes is relatively high, but custom low budget devices can be developed. In the paper, we describe three most established applications in medicine, with the focus on the basic mechanisms, which should be taken into account during the development process of a clinical electroporator. Also, typical pulse parameters used in each of the described applications are defined. In the second part of the paper, we describe technical functional requirements for a clinical electroporator and safety guidelines, with the focus on medical device standard. At the end of the paper, the focus moves to a more general problematic, such as quality assurance and the importance of measurement during the pulse delivery, which we firmly believe is necessary for successful electroporation.
[DOI: 10.1115/1.4045837]

1 Introduction

Electroporation is a phenomenon in which cells that are exposed to an intense pulsed electric field increase permeability and conductivity of their membranes. Each biological cell is surrounded by a cell membrane. The cell membrane consists mainly of phospholipids, which in aqueous conditions, due to its properties, form a bilayer. The bilayer is a stable structure [1] and due to its nonpolar interior, almost impenetrable for polar molecules, dissolved in the aqueous electrolyte. The resting transmembrane potential is in a range of few tens of mV, due to the distribution of ions between the cell exterior and interior [2]. If an external electric field is applied to a biological cell or tissue, the local electric field in the cell and their surroundings is disturbed. The imposed transmembrane voltage superimposes to the cell resting potential. The electrical field "amplifies" in the cell membrane, as the electric potential difference is established across the membrane. The induced transmembrane voltage is conditioned by the orientation of the cell regarding the applied electric field and cell shape. Therefore, due to the pulse application, transport through the cell membrane can be affected, cells can be stimulated, and if the applied electric field is high enough, pulse application can also lead to electroporation of the cell membrane.

According to current understanding, the electroporation is based on the formation of aqueous pores in the lipid bilayer [3–5]. Pores enable the ionic and molecular transport over otherwise impermeable membranes during the pulse. Experimental observation of the pore formation was not successful with known techniques, but molecular dynamic (MD) simulation and statistical thermodynamics provide convincing corroboration of pore formation. Recently, it was also shown that electric pulse exposure causes chemical changes to the lipids, responsible for the transport after the pulse, and modulation of membrane proteins' function [3].

We distinguish between reversible and irreversible electroporation. In the case of reversible electroporation, the cell after the pulse application fully recovers. Thus, through the permeabilized cell membrane, proteins and small or large molecules can be delivered. Additionally, if two cells are close to each other, fusion can occur, due to electroporation. But when the damage of the cell membrane and to the cell is too excessive, the cell dies, presumably due to the loss of cell homeostasis. Electroporation is a platform technology with many applications in different fields [6–9]. It is well established in medicine, where electrochemotherapy (ECT) has been studied for more than two decades [10]. With time, new applications developed and now nonthermal tissue ablation by means of irreversible electroporation (NTIRE) [11], DNA

[1]Corresponding author.
Manuscript received September 3, 2019; final manuscript received December 15, 2019; published online January 31, 2020. Assoc. Editor: Boris Rubinsky.

vaccination [12], and gene therapy [13,14] are studied in detail. In the field of bio- and food technology, electroporation is exploited for inactivation of microorganisms, extraction of biomolecules, fast drying of biomass, and even cooking [8,9].

This paper aims at presenting the electroporation and electroporation-based therapies in medicine, as a prospect solution for socioeconomically disadvantaged people and populations. Because portable, battery-powered, cost-efficient clinical electroporator is not yet available on the market, we also provide guidelines for future developers.

In the first part of the paper, we describe three most established applications in medicine, with the focus on the basic mechanisms, which should be taken into account during the development process of a clinical electroporator. Additionally, for each application, we specify the range of used pulse parameters. In the second part of the paper, functional requirements for each of the previously described applications, safety guidelines, where we focus on compliance with medical device standard and FDA requirements, are defined. In the end, the focus moves to quality assurance and measurements.

2 Electroporation-Based Medical Applications

All electroporation therapies could be used at low cost and in areas with limited resources. Electroporator can be transportable and battery-powered and in case of wide pulse parameter range, could be, in combination with custom made electrodes, used for different medical applications (described below). Currently, electrodes for ECT and IRE are for single-use and quite expensive; however, this cost could and should be reduced with an increase in the use. The electroporator user interface can be developed in a way to facilitate the use; nevertheless, the device operator should be familiar with at least basic mechanisms of electroporation, meaning training is required. Electroporation-based therapies are safe, with little or no side effects, reduce stays in the hospital, can facilitate or replace surgeries and at the same time improve the quality of patient's life's, can be performed in an outpatient basis, and are therefore a promising application for introduction to the clinical practice all around the world. In this section, three applications are described, with the focus on the used electrical pulse characteristics (Fig. 1).

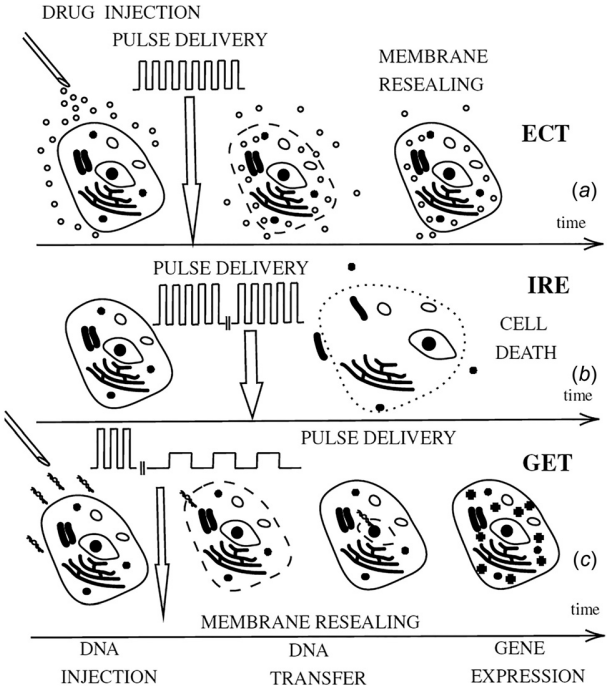


Fig. 1 Three most established electroporation-based medical applications: (*a*) ECT: after chemotherapeutic drug injection, electroporation pulses are delivered to the cell. Electroporation of cell membrane is triggered, which results in increased transport of the drug in the cell interior. (*b*) IRE: after the pulse delivery, the cell dies, due to the loss of homeostasis. (*c*) GET: the injected DNA migrates to the cell membrane, where it is due to electroporation and electrophoretic force, transferred into the cell interior. DNA then migrates toward the nucleus, where it is transported across the nuclear envelope. Afterward, the result of successful GET is gene expression, which can be used for gene therapy and DNA vaccination.

2.1 Electrochemotherapy. The leading electroporation-based therapy is ECT, an antitumor therapy [15–17], at which after injection of the chemotherapeutic drug, locally applied high voltage electric pulses trigger transient permeabilization of cells in a tissue. In the case of an adequate application, all tumor cells [18] get permeabilized, meaning the diffusion, of previously injected chemotherapeutic drug into the cells is thus facilitated. This results in higher cytotoxicity [19] of injected drug. Bleomycin is the most utilized drug for ECT and the intravenous infusion is the most common, while Cisplatin is injected intratumorally [20]. For the success of the application, drug concentration in the treated area should be sufficient and the whole tumor should be exposed to high enough electric field [21]. ECT can be easily repeated, in case of tumor progression or in cases where tumor does not completely respond to ECT after the first treatment. ECT is highly effective, with complete response rates between 60% and 70% and objective response rates about 80%, after a single treatment [10]. It is suitable for the treatment of cutaneous and subcutaneous tumors of different histotypes, both skin and non-skin cancers, as well as metastases. In the last few years, ECT has also been established in deep-seated tumors, including bone metastases, liver malignancies, and pancreatic and prostate cancers, and gastrointestinal tumors. Additionally, lung and brain tumors are currently studied as potential future targets [22]. ECT is a treatment of choice especially when tumors are located close to major blood vessels and consequently not manageable with surgery [23]. European Standard Operating Procedures of Electrochemotherapy (ESOPE) have been established in 2006 [24]; however, SOP only defines ECT for skin tumors smaller than 3 cm in diameter. Therefore, an update of SOP was published in 2018, which provides guidelines for the treatment of primary and metastatic tumors (also >3 cm) of the skin, based on broad experience across treatment centers and medical specialties [20]. National Institute for Health and Care Excellence (NICE), in the United Kingdom, has recognized ECT as an integral part of the multidisciplinary treatment for patients with skin metastases of nonskin origin and melanoma (NICE interventional procedure guidance IPG 446) [25]. For easier understanding of the ECT procedure, we suggest watching the video published in the Journal of Visualized Experiments [26].

A combination of ECT with immunotherapy, GET, radiotherapy, as well as calcium electroporation are promising novelties on the ECT field [22]. Calcium electroporation has been investigated in vitro, in vivo, and in early clinical trials, and it lends itself as safe inexpensive antitumor treatment [27]. A drastic increase of intracellular calcium concentration triggers necrotic cell death, due to acute energy depletion [28]. Calcium affects normal and malignant cells differently (malignant cells are more sensitive to calcium electroporation); it can be administered by other medical professionals (not only oncologists), it is nonmutagenic, and has a long durability [29]. Therefore, calcium ECT seems specifically advantageous for economically and socially disadvantaged countries. However, calcium electroporation is currently performed mainly for tumors localized to the skin and in combination with intratumoral injection, additional attention should be paid to the risk of necrosis. According to first results, calcium electroporation response rates are comparable to bleomycin ECT rates [30] and the same electroporation device can be used for both. In classical

ECT and calcium electroporation, eight square wave 100 μs long pulses with a repetition rate of 1 Hz or 5 kHz are most commonly used (To ensure electroporation, the electric field in tissue should be higher than 400 V/cm [31].). Pulse voltage amplitude is electrode (distance between the electrodes) and target tissue-dependent; in most cases, it is somewhere between 200 and 1000 V in case of skin electroporation and up to 3 kV for deep-seated tumors. Also, a 5 kHz pulse repetition rate is more common due to shorter duration of electroporation; the sensation of only one application of electric pulses (less pain), meaning muscle contraction is present after the pulse application and an electrode displacement due to muscle contraction during pulse delivery is therefore reduced. The SOP [20] in detail defines treatment choices, including pretreatment examinations, anesthesia, drug injection, electrode selection, and characteristic of electric pulses for treatment with a Cliniporator device from IGEA. If a device or electrodes from other manufacturers or custom-made electrodes are used, the electric pulse characteristic should be adjusted to comply with requirements in the SOP.

2.2 Irreversible Electroporation. IRE or NTIRE-nonthermal irreversible electroporation is a promising application for ablation of nontumor and tumor tissue. The cells primarily die due to membrane permeabilization and not due to the increase of tissue's temperature [32,33]. However, a local temperature increases around the electrodes and can be significant at higher amplitudes, also due to the high number of pulses delivered to a limited volume of tissue [34,35]. It was shown that IRE does not cause denaturation of proteins and is not affected by blood flow. Additionally, rapid activation of the immune system, no scarring, and the potential ability to treat tumors near large blood vessels were observed [11]. In case of irreversible electroporation of tumor tissue, the same as in ECT, all tumor tissues should be covered with a high enough electric field; however, for IRE, the electric field should be above the IRE threshold, which is around 700 V/cm [11,36] for approximately 100 pulses. Therefore, to cross the IRE threshold, applied pulse amplitudes go as high as 3000 V, meaning current amplitude values can reach up to 50 A [37] (similar values can also be reached at ECT of deep-seated tumors). The number of applied pulses at IRE is most often above 90 and the pulse duration is around 100 μs. Because the standard operating procedures are not yet defined for the IRE, the pulse parameters of delivered pulses are much more diverse. In addition, an individual treatment plan is required for each specific tumor and is crucial for a successful outcome. IRE is mainly used for the treatment of deep-seated tumors either during open surgery or percutaneously in the liver, pancreas, kidney, lung, and other organs [38]. Because electric fields applied in IRE can cause cardiac arrhythmias, synchronization of pulse delivery with the refractory period of the cardiac rhythm is necessary [39]. The additional issue at IRE is muscle contractions [40], which are associated with the high voltage amplitudes and a large number of pulses delivered at a low repetition rate to avoid excessive tissue heating. Therefore, general anesthesia and neuromuscular blocking agents are necessary to prevent muscle contraction [11]. Recently, in a series of studies, authors showed that by applying bursts of high-frequency bipolar pulses also termed as H-FIRE (High-Frequency IrReversible Electroporation) pulses, muscle contractions can be avoided, without compromising the nonthermal mechanism of cell death [41,42]. Every single monopolar pulse is replaced by a burst of few microsecond long bipolar pulses, with a repetition rate in ranges of few hundred kHz, while the repetition rate of the burst stays the same as pulse repetition rate at IRE. Additionally, in the case of H-FIRE, even the electric field distribution is more homogeneous. Also, the transmembrane transfer of molecules may be achieved with bursts of short few microsecond long pulses; however, H-FIRE pulses need considerably larger voltage amplitudes for cell disruption in comparison to longer monopolar pulses [43]. Recent reports offer the possibility to use H-FIRE pulses in electrochemotherapy, but again, at the expense of higher electric fields than in classical ECT [44].

Tissue ablation with IRE would enable the ablation of previously unresectable tumors. Additional, IRE is in comparison to other treatment modalities that are easy to perform, favorable, because the procedure time is short, and it was also shown, IRE has a low risk of bleeding and perforation [45].

2.3 Gene Therapy and DNA Vaccination. Gene electrotransfection is a promising non-viral gene delivery method [13,46], used for treatment of cancer and other diseases [47,48], regulation of protein levels to enhance or reduce function, or the amelioration of symptoms of iatrogenic or natural disease, DNA vaccination [49,50], and genetic modification of organisms [51,52]. Plasmids or oligonucleotides may also be delivered to explore promoter or gene function [53]. Facilitation of gene expression in vivo by electroporation of plasmid DNA has implications for both vaccine and gene therapy applications [12]. Electroporation promotes antigen, oligonucleotides, and immunomodulatory molecule delivery into tumor tissue, which then stimulate the immune system or act on immunosuppressor genes [54]. Plasmid DNA should be injected before electroporation and should be always delivered to the tissue placed between the electrodes. After or during the electroporation, the cell membrane interacts with injected plasmid and forms a DNA-membrane complex, which is then transported through the cytoplasm to the nucleus. The electroporation has to be reversible because, after the pulse delivery, the cells have to express transferred genes [55]. Great variety of pulses with different pulse parameters are used in this field, both high-voltage short-duration pulses (few hundred μs long pulses and local electric fields around 400 V/cm (HV)) or low-voltage long-duration pulses (from few to hundreds ms long pulses and local electric fields of few tens V/cm (LV)) or even the combination of both. In some studies, it was suggested that HV pulses are crucial for permeabilization of the cell membrane and pore formation, while LV pulses electrophoretically drag negatively charged DNA into the cell [56]. In other studies, the authors proposed the change of electric field direction or orientation of the electrodes during the pulse delivery; thus, the area of DNA entry is enlarged (because DNA transfection occurs only in the part of the membrane facing the cathode) [57]. Additionally, nanosecond pulses with a duration from few to hundreds of nanoseconds and electric field densities up to several tens of kV/cm are currently studied, because it is believed that they affect internal cell membranes and may contribute to increased delivery of plasmid to the nucleus [58]. Overall, it was proven that longer electric pulses give higher transfection efficiency, but they reduce cell viability and shorter pulses give lower transfection efficiency and preserve viability.

In the field of DNA vaccination by electroporation, mainly intramuscular (IM) and intradermal (ID) delivery are studied. The muscle is an attractive tissue for nucleic acid vaccination in a clinical setting due to the accessibility and abundance of the target tissue [59]. Skeletal muscles promote strong humoral and cellular immune responses; however, muscle fibers are quite ineffective in plasmid capture. Therefore, for a successful application, a large volume of plasmid should be injected. The expression of plasmid DNA in the muscles can be prolonged and increased by electroporation. It was reported that by electroporation, the antigen delivery increase up to a 1000 fold over naked DNA delivery alone can be reached [50]. Currently, the development of delivery systems and research in the field of DNA vaccination by electroporation are at full swing [60]. Clinical studies of electroporation in the muscle showed the procedure is well tolerated by patients.

HIV, Human papillomavirus (HPV), Hepatitis C, and cancer DNA vaccination by electroporation are currently investigated [14]. For cancer gene therapy, viral delivery methods such as immune modulators, cell cycle regulators, suicide genes, anti-angiogenic genes, and genes encoding toxins are used and

electroporation mediated delivery is studied. It was shown that the most successful cancer therapeutic genes, delivered by electroporation, which results in significant tumor regression are IL-12 and IFN-α [48,53]. However, studies [61,62] also determined that optimization of electroporation pulse characteristics have a major impact on the success of the application; therefore, further research is necessary. Because vaccination is generally regarded to be one of the most cost-effective interventions in public health, we believe DNA vaccination with electroporation is the most promising application for implementation into economically disadvantaged populations. DNA is more heat stable, easier to produce and store than current vaccines [63]. The transport from the manufacturing to injection into the patient is easier and therefore cheaper, because DNA is more heat stable, and easier to produce and store than current vaccines.

3 Medical Devices for Electroporation

As presented in Sec. 2, different applications require different pulse parameters, such as voltage amplitude, pulse width, pulse repetition rates, and a number of pulses or even bursts of pulses [64,65]. Therefore, specific pulse generators, i.e., electroporators, have to be designed and developed for each application. When designing an electroporation device, one should always keep in mind that biological sample/tissue as a load has resistive–capacitive nature and varies from sample to sample. Additionally, also the impedance of a biological load decreases during the pulse delivery, due to electroporation [66,67].

A considerable number of electroporation devices can be found on the market, some designed for specific applications and some for multifunctional laboratory use. Several reviews have been published, in which pulse characteristics of commercially available electroporators are described [68–70]. But for the incorporation of an electroporator into the clinical practice, an electroporator has to follow the requirements appointed by local medical regulations and meet medical device standards. In Europe, it has to comply with a Medical Device Regulation 2017/745 and in the United States, the device should be approved by the FDA (Food and Drug Administration). The process of incorporation of a medical device in clinical practice in developed countries is thus well established. After the development process and collection of considerable scientific evidence for devices' safety and effectiveness, the first-in-human study is performed, followed by the evaluation of the device in clinical trials, culminating in regulatory approval for use and the adoption of the device [71]. The European CE Mark adoption process requires demonstration of safety, quality, and efficacy and nongovernmental notified bodies regulate the approval and postapproval. While in the United States, demonstration of safety and efficacy is required and regulated by a central governmental agency (FDA) [72]. All clinical trials, studies, or testing have to be authorized by the FDA. And even after a medical device is made available on the market for use, regulations have been established to ensure ongoing postmarket surveillance of device safety and effectiveness [73].

In Europe, currently there are only five certified clinical electroporators, the Cliniporator 2 and Cliniporator VITAE (IGEA S.p.A., Italy), available for ECT and GET, SENNEX (BionMed Technologies, Germany), that is only used for ECT, NanoKnife (AngioDynamics, USA), which may be used for soft-tissue ablation and ePORE (Mirai Medical, Ireland) that is compatible with the EndoVE, which enables a targeted treatment delivered within minutes in an outpatient endoscopy setting. In the United States, the FDA clearance was granted to NanoKnife (AngioDynamics, USA), first for the soft-tissue ablation and recently also for "Direct IRE Cancer Treatment" clinical study (DIRECT), for the treatment of stage III pancreatic cancer. Nevertheless, it has not received clearance for the therapy or treatment of any specific disease or condition. Unfortunately, none of those devices is actually appropriate for implementation in economically disadvantaged populations. The first issue is the cost, for example, a Cliniporator device is sold for 100,000 € (without VAT) and each electrode that is for single-use cost 1200 € and sometimes, more than one set of electrodes per treatment are used. Few cost-effectiveness studies of ECT have been done [74,75], but none of them have taken into account also the increase in the quality of life of patients after the ECT. Therefore, the use of ECT even in developed countries is sometimes hard to justify. Additionally, none of the existing clinical electroporators is battery-powered or easy to transport. Therefore, in the following part of the paper, we provide guidelines for future electroporator manufactures, based on our experiences. No relevant cost-effectiveness or economic studies have been published yet; therefore, it is hard to evaluate its potential for implementation in economically disadvantages population. However, in the case of the development of a low-cost electroporator for all presented applications it can have potential for implementation.

3.1 Functional Requirements. For successful electroporation and development of a quality and reliable electroporator, it is crucial to understand what needs to be achieved when applying electric pulses. Because different applications require different electric pulse characteristics, and because biological loads vary considerably, specific electroporators are designed for each application. However, it is possible to develop a device that could be used for all presented medical applications. Micro- and millisecond square wave pulses are usually generated by an HV power supply switching circuit, with fast power MOSFET or IGBT used as switches [76–79]. The simplest and most cost-efficient solution is a square wave generator concept shown in Fig. 2. The solution is similar to the capacitor discharge circuit; the voltage power supply V constantly charges the capacitor C. Pulse duration, pulse repetition rate, and a number of pulses are defined by the switching sequence, meaning the switch has to be driven by a precise and adequate driving/control system. The output pulse amplitude is defined by the set amplitude of the variable power supply. To minimize a voltage drop on the load, all the required energy must be generated and stored in the capacitor before each pulse delivery. Meaning large capacitor or capacitor banks are needed, especially in the case of IRE, where applied pulse voltage amplitudes are as high as 3 kV and at least 90 pulses are delivered. For the development of a nanosecond electroporator, more complex circuit designs are required such as a transmission line approach (Blumlein line) and diode opening switch and even faster switches are needed, mainly Radio Frequency MOSFET (RF-MOSFET) or Silicon Carbide MOSFET (SiC MOSFET) are used [80]. For the bipolar pulse generation, an H-bridge solution is the most established. The short rise time of high voltage pulses is provided by modular generator and short fall times can be achieved by short-circuiting of the load [81].

For specific treatments (i.e., applications, treatment locations), special application-optimized electrodes are used. SOP [20]

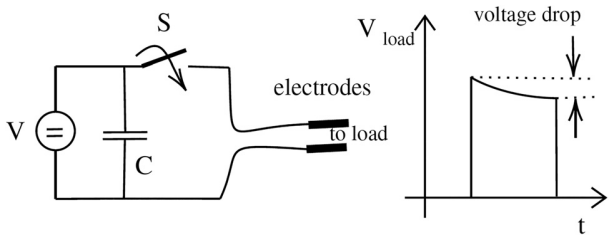


Fig. 2 Square wave generator concept. Switch S discharges capacitor C, through the load. The capacitor C is constantly charged, by a variable voltage source V. The amplitude of the generated pulse is equal to the set voltage of the source. The voltage drop during the pulse is capacitance, load impedance, and pulse duration dependent and should not exceed 10% of the set voltage V.

describes the appropriate use of different electrodes available for ECT in combination with the Cliniporator device. An electroporator should be compatible with different electrode types. However, the device should recognize the electrode type and adjust the output pulse parameters in accordance with the treatment modality. For example, if the distance between the electrodes is larger, then the pulse amplitude should be increased, to ensure the same electric field in the targeted tissue. An important part of the clinical electroporator, from the prospect of the use, is a user interface. Devices for the clinics should be as user-friendly as possible. To prevent misuse and mistakes, the output pulse characteristic should be preprogrammed for specific electrodes and treatment protocols. Meaning the user only selects, i.e., electrode type. However, in the case of deep-seated tumors, where anatomy is more complex, or due to the inability to cover the whole tumor due to its size, with standard electrodes, several single-needle electrodes are used [82]. Special treatment plans are designed for each therapy, to ensure the optimal configuration of the electrodes that adequately cover the tumor with high enough electric field. Meaning, a custom setting of the pulse parameter should be enabled in this case. Due to safety reasons, clinical electroporators are armed by the user, just before the pulse delivery. When capacitors are charged, the device is ready for pulse delivery; however, this active time is limited to a few seconds. If the device is not triggered and pulses are not delivered, it should self-discharge. In the case of IRE and ECT of deep-seated tumors, the pulse delivery should also be synchronized with cardiac rhythm. Both charge and delivery triggers should be made in the form of a pedal or enabling button on the handle, in a way not to obstruct the user. An electroporator should have a built-in measuring system, with an adequate bandwidth. The current and voltage should be constantly monitored to ensure the pulse delivery, with the sampling frequency that is equal or higher to twice the highest frequency contained in the measured signal. The real-time oscilloscope like display of the measurement is desirable but not mandatory. Nevertheless, at least the measured amplitude values of delivered voltage and current should be displayed as feedback and saved for post-treatment analysis if necessary. The evaluation of the delivered pulses is necessary, the device can self-evaluate the delivered pulses, or this can be done by the operator if adequate data are displayed.

3.2 Safety Guidelines. A clinical electroporator has to comply with the general standard for basic safety and essential performance of medical devices, EN/IEC 60601-1 [83]. The manufacturer should ensure that the risk is removed, or if not possible, the risk should be minimized. Therefore, manufacturers have to pay particular attention to choosing adequate voltage insulation, limit leakage currents in accordance with the safety class, limit the output voltage, current and energy and to ensure electromagnetic compatibility by following the standard EN/IEC 60601-1-2 EMC for medical devices.

Safety class of the electroporator is defined by its most risky application. We distinguish between invasive and noninvasive electroporation. If the device is made for more treatment modalities, it should be classified on the basis of the one that represents the highest risk. The same applies to the location of treatment; if the device can be used in surgery for deep-seated tumors, then the location with the highest risk has to be taken into account. Both Cliniporator EPS02 and Cliniporator VITAE have been in accordance with EN 60601-1 classified as Class I regarding the protection against electrical risks and class BF regarding the protection against electric shock. Cliniporator EPS02 in accordance with MDD 93/42 CEE classified as IIa, while Cliniporator VITAE is in class IIb. Additionally, also the following standards have to be considered: ISO 14971 for risk analysis, ISO 13485 for the quality management system, EN/IEC 60601-1-6 and ISO 62366 for usability, ISO 62304 and IEC 80002-1 for medical device software, and IEC 62311 in case of a battery power supply.

3.3 Quality Assurance. Currently, electroporation research and applications are developing fast and growing; therefore, the number of electroporators available on the market is increasing even faster than before. Unfortunately, the quality of some devices is questionable [70]. Some manufacturers intentionally conceal the output pulse parameters of their devices, claiming that it is their intellectual property. The critical point on the field of in vitro gene transfection, where preprogrammed electroporation procedures are most commonly used by researchers, without even knowing basic pulse parameters such as amplitude and pulse duration [70]. The concealed operation of electroporation devices hinders sharing, comparing, and reproduction of results; it limits and restricts researchers and further development of the new knowledge. In the electroporation field as well as in general biomedical research, the increase in failed efforts to confirm other group's published papers work is significant [84]. We believe this is at least in part due to poor quality assurance. Measurement of electroporation pulses is crucial to adequately determine and control the quality and the delivery of pulses. Even the International Medical Device Regulators Forum (IMDRF) gave the harmonization initiative and proposed mutual recognition encouraged by WHO. They engage an external consultant to conduct a more detailed study to examine overseas experience and practices of, and the scope of control on the use of, electroporation medical devices. We can conclude that standardization regarding quality is necessary; however, electroporation is a platform technology, and specific testing protocols for each application or field should be established eventually. Nevertheless, some basic technical specifications and tolerances can be applied to all electroporators. Currently, the manufactures often give ill-defined technical specification, defined pulse parameter ranges cannot be used with all biological loads and/or in all possible combinations. Meaning, for example, in case of low impedance load the number of pulses or pulse duration and the voltage range are limited. Therefore, before actual experiment, the researchers cannot know, if the device will be able to deliver what it promises. Thus, we address the future developers of electroporator medical as well as laboratory to follow the recommended guidelines.

Each device is designed for precisely defined maximal current and some limitation is necessary, for the protection of the device itself. Manufacturers should define the maximal current and the principle of current limitation. Some devices use a pre-pulse to evaluate the load and then adjust the pulse parameter range, in a way to disable the current to go over the limit. If pre-pulse is used, the amplitude and pulse duration should be as low as possible, but the pause between the pre-pulse and preset electroporation pulses should be long (All processes triggered by the pre-pulse must be extinguished before the delivery of actual electroporation pulses.). Pre-pulse parameters should be given in detail in technical specifications (e.g., the amplitude, pulse duration, and exact timing of the pre-pulse regarding the electroporation pulses). Other devices measure the current on the output and then stop the pulse delivery when the current value is too high. Such a measuring system should be fast enough to assure the disconnection within the pulse rise time and in this case, the user should be informed about the interruption of pulse delivery. The third solution is to build in an electroporator an additional impedance, which limits the output current. Meaning that the device will never be able to generate higher current than maximal current and will, therefore, reduce the applied voltage. Again, in this case, the user should be informed about the alternation. Therefore, we propose the following solution. The manufactures should define applications for which the device is made, and then provide the technical specification and tolerances in accordance with the typical load for those applications, e.g., voltage: 200–1000 V (for loads with impedance higher than 50 Ω and for pulse durations up to 1 ms ($N_{MAX} = 10$); 200–2000 V (for loads with impedance higher than 100 Ω and for pulse durations up to 100 μs ($N_{MAX} = 10$);); …, where N_{MAX} is the maximal number of pulses.

On the base of the literature review [66,85–87], our experiences, and current status of technology, we defined the tolerance of the voltage amplitude and pulse duration. Meaning the requirements could be easily fulfilled, with the available electronics and circuit solutions. Published permeabilization and cell survival curves [66,85–87] indicate that more than 15% deviation can result in significantly different electroporation outcome. However, in case of in vivo electroporation, an additional error can be present in electrode placement, treatment planning, muscle contractions (present during the pulse delivery) or other unexpected circumstances. Because the total tolerance is equal to the sum of all tolerances, which may exceed 15%, the developers of electroporation devices should at least follow the state-of-the-art tolerances. Meaning the requirements could be easily fulfilled, with the available electronics and circuit solutions. Therefore, the recommended voltage amplitude tolerance is 10%; thus, the applied voltage amplitude should not be lower than 90% or higher than 110% of preset voltage. Meaning also the voltage of the first to the last pulse should be in this range. The pulse duration should be defined as Full Width at Half Maximum (FWHM) and a deviation from the preset value should not be higher than 8% [85,86], as shown in Fig. 3. The proposed tolerances must be fulfilled, from maximal to minimal settings, which are: maximal pulse amplitude, maximal pulse duration, the maximal number of pulses, and minimal pulse repletion rate when the pulse is applied on a typical load for an application that it is made for. Meaning the list of applications should be defined and a typical load for each of those applications should be developed. Additionally, also electrodes should be standardized and tolerances of the distances between the electrodes, appropriate materials and in case of multiple use, also sterilization and maintenance should be defined. A clinical electroporator should constantly self-test the operation, and periodic evaluation and calibration of the device are mandatory. The tolerances defined in this section address the "classical" electroporation and are not applicable to the nanosecond electroporation, where pulse duration may be the dominant source of deviation.

3.4 Usage Guidelines. Each user as well as developer, of electroporation-based therapies, has to be familiar with basic electroporation mechanisms; therefore, education (e.g., www.ebtt.org) and training (e.g., ESSO course on ECT) are necessary. The application can be successful, only if the applied electric field covers the whole targeted area and if induced voltage is higher than the reversible or irreversible electroporation threshold voltage. It should always be taken into account that the tissue is not homogeneous, meaning the electric field, to which the biological load was exposed during the pulse delivery, cannot be evaluated simply by equation (E = U/d), which defines an electric field as the ratio of applied voltage amplitude and distance between the electrodes. This equation is only valid if the load between the electrodes is homogeneous and plate electrodes are used and the distance between the electrodes is small with respect to electrode dimensions. The operation of an electroporator has to be always monitored; only by adequate measuring, the quality of the pulse delivery can be assured. We need to measure because we need to know, if the pulses were successfully delivered and we need to know, what was delivered. If the current flows through a load, delivery was more or less successful, but we still do not know anything about the pulse characteristics of the delivered pulses. If an electroporator has a built-in measuring system, the user has to evaluate the quality of measurements displayed by the electroporator. Due to the poor regulation of the electroporation field and lack of standard, the quality of built-in measurement systems is sometimes questionable. Because of a large variety in the electrical characteristic of biological loads/tissues, the user has to be familiar with common errors in pulse delivery. When biological loads have low impedance, problems can occur because the pulse generators cannot deliver "what they promise," i.e., high voltage pulses due to too high currents. Or the amplitude of successive pulses can be lower with each successive pulse delivered, if the pulse repetition rate is in the higher half of device operation range, meaning that the electroporator's energy storage is not sufficient. In the worst case, the voltage amplitude can even be lower than electroporation threshold voltage [70]. Additionally, also each user should be familiar with the guidelines for reporting electroporation clinical studies [25].

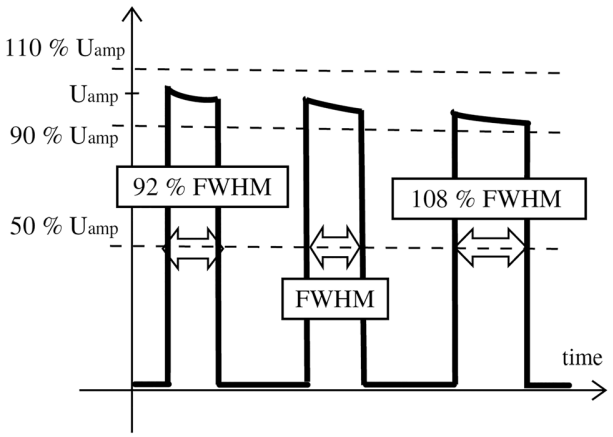


Fig. 3 The applied voltage amplitude should not be lower than 90% or higher than 110% of preset voltage. Meaning also the voltage drop between the first and last pulse at the maximal number of pulses should be in this range. The pulse duration deviation from the preset value should not be higher than 8%. The proposed tolerances must be fulfilled in case of maximal settings, which are: maximal pulse amplitude, maximal pulse duration, the maximal number of pulses, and minimal pulse repletion rate when the pulse is applied on a typical load for an application that it is made for.

4 Discussion

Electroporation-based therapies have a huge potential for implementation into clinical practice in socioeconomically disadvantaged populations. Electrochemotherapy is easy, quick to perform, and inexpensive. Only suitable room for patient preparation and treatment, an electroporator with suitable electrodes, a physician in charge, and a nurse (and an assistant trained in handling the electroporator) are required for treatment of cutaneous tumors and skin metastases. Because the treatment is safe, with mainly no side effects, a patient can wait for a while in the hospital for the observation, but do not need to spend a night. The treatment can be performed on any part of the body, on different tumor types with the same electroporation device, only appropriate electrodes have to be selected [88]. The SOP [20] should always be followed. Calcium electroporation is even cheaper because calcium is commercially available and regularly used in most hospitals. It can administer by other medical professionals (not only oncologists); it is nonmutagenic and has long durability. Additionally, calcium has an excellent safety profile, both for use in patients and for staff, and would not need administration by staff accredited to administer chemotherapy [27]. However, calcium can only be used for tumors localized in the skin and has to be administered locally.

The main advantages of IRE are apoptotic cell death, the sharp boundary between the treated and untreated areas, and the overall time for the procedure is extremely short in comparison to benchmark ablation treatments. The pulse application lasts only a few minutes; actual time can be calculated from the number of delivered pulses and average heart rate [89,90]. IRE also enables the ablation of tumors in areas previously contraindicated for thermal ablation [90].

Gene therapy by electroporation is a highly efficient method, with delivery efficiency better than many non-viral vectors [91]. It

substantially increases DNA delivery and DNA vaccine potency. The preclinical development of electroporation is focused on tissues that are easily accessible and the application technique is simple and takes only a few seconds after DNA injection. Electroporation is a useful strategy to improve DNA-based vaccination protocols; it stimulates both the humoral and cellular immune responses and if necessary, they can contain several antigen epitopes [49]. Additionally, DNA is more heat stable, easier to produce and store than current vaccines [63]. The transport from the manufacturing to injection into the patient is easier and therefore cheaper, meaning it is perfect for warm climates like the African continent. They do not display an environmental hazard since there is no risk of replication and only contain the antigen encoded [63].

Because different applications require different electric pulse characteristics, and because biological loads vary considerably, specific electroporators are designed for each application. However, it is possible to develop a device that could be used for all presented medical applications. But, the price of such a device would rise, the certification process would be more challenging, and the size and weight would increase in comparison to a single application device. A battery-powered electroporator would enable DNA vaccination even in areas without electricity. Currently, the price of electroporation is relatively high, but custom low budget devices can be developed. Even cheap single-use electrodes, with mass production or multiple-use electrodes with a defined sterilization process, would significantly reduce the price of electroporation.

5 Conclusions

Electroporation is a platform technology, with high potential, it can reduce cost and facilitate treatment procedures. According to the ongoing studies the electroporation-based therapies are safe, with little or no side effects [7]. The clinical data published on electroporation based applications are quite encouraging; therefore we believe, that in the future, electroporation will be indispensable in cancer treatment, infection disease treatment, intracardiac ablation [92], and vaccination [91]. Further development of standard electroporation treatment protocols, regulation of electroporator development, and user training, however, remain essential for successful incorporation into clinical practice. Electroporation-based therapies have a huge potential for implementation into clinical practice also in socioeconomically disadvantaged populations. Currently, the price of electroporation is relatively high, mainly due to costly electroporators and electrodes, but custom low budget devices can be developed. It is not technically difficult to develop an electroporator, more challenging is the quality assurance, because biological loads characteristics vary considerably from sample to sample and even more from tissue to tissue. Therefore, measuring of applied electric pulses is crucial for a successful application.

Acknowledgment

This study was supported by the Slovenian Research Agency (ARRS) (MRIC UL IP-0510, P2-0249, and funding for Junior Researchers to Eva Pirc).

Nomenclature

ECT = electrochemotherapy
GET = gene electrotransfer
IRE = Irreversible electroporation
H-FIRE = high frequency-Irreversible electroporation
DNA = deoxyribonucleic acid
FDA = food and drug administration
HV = high voltage
LV = low voltage
WHO = Word Health Organization

References

[1] Deamer, D. W., and Bramhall, J., 1986, "Permeability of Lipid Bilayers to Water and Ionic Solutes," Chem. Phys. Lipids, **40**(2–4), pp. 167–188.
[2] Kotnik, T., Pucihar, G., and Miklavčič, D., 2010, "Induced Transmembrane Voltage and Its Correlation With Electroporation-Mediated Molecular Transport," J. Membr. Biol., **236**(1), pp. 3–13.
[3] Kotnik, T., Rems, L., Tarek, M., and Miklavčič, D., 2019, "Membrane Electroporation and Electropermeabilization: Mechanisms and Models," Annu. Rev. Biophys., **48**(1), pp. 63–91.
[4] Freeman, S. A., Wang, M. A., and Weaver, J. C., 1994, "Theory of Electroporation of Planar Bilayer Membranes: Predictions of the Aqueous Area, Change in Capacitance, and Pore-Pore Separation," Biophys. J., **67**(1), pp. 42–56.
[5] Weaver, J. C., 1993, "Electroporation: A General Phenomenon for Manipulating Cells and Tissues," J. Cell. Biochem., **51**(4), pp. 426–35.
[6] Miklavčič, D., 2012, "Network for Development of Electroporation-Based Technologies and Treatments," J. Membr. Biol., **245**(10), pp. 591–598.
[7] Yarmush, M. L., Golberg, A., Serša, G., Kotnik, T., and Miklavčič, D., 2014, "Electroporation-Based Technologies for Medicine: Principles, Applications, and Challenges," Annu. Rev. Biomed. Eng., **16**(1), pp. 295–320.
[8] Kotnik, T., Frey, W., Sack, M., Haberl Meglič, S., Peterka, M., and Miklavčič, D., 2015, "Electroporation-Based Applications in Biotechnology," Trends Biotechnol., **33**(8), pp. 480–488.
[9] Mahnič-Kalamiza, S., Vorobiev, E., and Miklavčič, D., 2014, "Electroporation in Food Processing and Biorefinery," J. Membr. Biol., **247**(12), pp. 1279–1304.
[10] Mali, B., Jarm, T., Snoj, M., Serša, G., and Miklavčič, D., 2013, "Antitumor Effectiveness of Electrochemotherapy: A Systematic Review and Meta-Analysis," Eur. J. Surg. Oncol., **39**(1), pp. 4–16.
[11] Rubinsky, B., 2007, "Irreversible Electroporation in Medicine," Technol. Cancer Res. Treat., **6**(4), pp. 255–59.
[12] Widera, G., Austin, M., Rabussay, D., Goldbeck, C., Barnett, S. W., Chen, M., Leung, L., Otten, G. R., Thudium, K., Selby, M. J., and Ulmer, J. B., 2000, "Increased DNA Vaccine Delivery and Immunogenicity by Electroporation In Vivo," J. Immunol., **164**(9), pp. 4635–4640.
[13] Mir, L. M., Bureau, M. F., Gehl, J., Rangara, R., Rouy, D., Caillaud, J.-M., Delaere, P., Branellec, D., Schwartz, B., and Scherman, D., 1999, "High-Efficiency Gene Transfer Into Skeletal Muscle Mediated by Electric Pulses," Proc. Natl. Acad. Sci. U. S. A., **96**(8), pp. 4262–4267.
[14] Sokołowska, E., and Błachnio-Zabielska, A. U., 2019, "A Critical Review of Electroporation as a Plasmid Delivery System in Mouse Skeletal Muscle," Int. J. Mol., **20**(11), p. 2776.
[15] Miklavčič, D., Mali, B., Kos, B., Heller, R., and Serša, G., 2014, "Electrochemotherapy: From the Drawing Board Into Medical Practice," BioMed. Eng. OnLine, **13**(1), p. 29.
[16] Stepišnik, T., Jarm, T., Grošelj, A., Edhemović, A., Djokić, M., Ivanecz, A., and Trotovšek, B., 2016, "Elektrokemoterapija—Učinkovita Metoda Zdravljenja Tumorjev S Kombinacijo Kemoterapevtika in Električnega Polja," Zdravniški Vestn., **85**(1), pp. 41–55.
[17] Campana, L. G., Edhemovic, I., Soden, D., Perrone, A. M., Scarpa, M., Campanacci, L., Cemazar, M., Valpione, S., Miklavčič, D., Mocellin, S., Sieni, E., and Sersa, G., 2019, "Electrochemotherapy—Emerging Applications Technical Advances, New Indications, Combined Approaches, and Multi-Institutional Collaboration," Eur. J. Surg. Oncol., **45**(2), pp. 92–102.
[18] Soden, D. M., Larkin, J. O., Collins, C. G., Tangney, M., Aarons, S., Piggott, J., Morrissey, A., Dunne, C., and O'Sullivan, G. C., 2006, "Successful Application of Targeted Electrochemotherapy Using Novel Flexible Electrodes and Low Dose Bleomycin to Solid Tumours," Cancer Lett., **232**(2), pp. 300–310.
[19] Serša, G., Miklavčič, D., Čemažar, M., Rudolf, Z., Pucihar, G., and Snoj, M., 2008, "Electrochemotherapy in Treatment of Tumours," Eur. J. Surg. Oncol., **34**(2), pp. 232–240.
[20] Gehl, J., Serša, G., Matthiessen, L. W., Muir, T., Soden, D., Occhini, A., Quaglino, P., Curatolo, P., Campana, L. G., Kunte, C., Clover, A. J. P., Bertino, G., Farricha, V., Odili, J., Dahlstrom, K., Benazzo, M., and Mir, L. M., 2018, "Updated Standard Operating Procedures for Electrochemotherapy of Cutaneous Tumours and Skin Metastases," Acta Oncol., **57**(7), pp. 874–882.
[21] Miklavčič, D., Čorović, S., Pucihar, G., and Pavšelj, N., 2006, "Importance of Tumour Coverage by Sufficiently High Local Electric Field for Effective Electrochemotherapy," Eur. J. Cancer Suppl., **4**(11), pp. 45–51.
[22] Campana, L. G., Miklavčič, D., Bertino, G., Marconato, R., Valpione, S., Imarisio, I., Dieci, M. V., Granziera, E., Čemaža, M., Alaibac, M., and Serša, G., 2019, "Electrochemotherapy of Superficial Tumors—Current Status:: Basic Principles, Operating Procedures, Shared Indications, and Emerging Applications," Semin. Oncol., **46**(2), pp. 173–191.
[23] Edhemovic, I., Brecelj, E., Gasljevic, G., Marolt Music, M., Gorjup, V., Mali, B., Jarm, T., Kos, B., Pavliha, D., Grcar Kuzmanov, B., Cemazar, M., Snoj, M., Miklavcic, D., Gadzijev, E. M., and Sersa, G., 2014, "Intraoperative Electrochemotherapy of Colorectal Liver Metastases," J. Surg. Oncol., **110**(3), pp. 320–27.
[24] Mir, L. M., Gehl, J., Serša, G., Collins, C. G., Garbay, J.-R., Billard, V., Geertsen, P. F., Rudolf, Z., O'Sullivan, G. C., and Marty, M., 2006, "Standard Operating Procedures of the Electrochemotherapy: Instructions for the use of Bleomycin or Cisplatin Administered Either Systemically or Locally and Electric Pulses Delivered by the CliniporatorTM by Means of Invasive or Non-Invasive Electrodes," EJC Suppl., **4**(11), pp. 14–25.
[25] Campana, L. G., Clover, A. J. P., Valpione, S., Quaglino, P., Gehl, J., Kunte, C., Snoj, M., Čemažar, M., Rossi, C. R., Miklavčič, D., and Serša, G., 2016, "Recommendations for Improving the Quality of Reporting Clinical

Electrochemotherapy Studies Based on Qualitative Systematic Review," Radiol. Oncol., **50**(1), pp. 1–13.
[26] Serša, G., and Miklavčič, D., 2008, "Electrochemotherapy of Tumours," J. Visualized Exp., **22**, p. e1038.
[27] Frandsen, S. K., Gissel, H., Hojman, P., Tramm, T., Eriksen, J., and Gehl, J., 2012, "Direct Therapeutic Applications of Calcium Electroporation to Effectively Induce Tumor Necrosis," Cancer Res., **72**(6), pp. 1336–1341.
[28] Staresinic, B., Jesenko, T., Kamensek, U., Krog Frandsen, S., Sersa, G., Gehl, J., and Cemazar, M., 2018, "Effect of Calcium Electroporation on Tumour Vasculature," Sci. Rep., **8**(1), p. 9412.
[29] Hoejholt, K. L., Mužić, T., Jensen, S. D., Dalgaard, L. T., Bilgin, M., Nylandsted, J., Heimburg, T., Frandsen, S. K., and Gehl, J., 2019, "Calcium Electroporation and Electrochemotherapy for Cancer Treatment: Importance of Cell Membrane Composition Investigated by Lipidomics, Calorimetry and In Vitro Efficacy," Sci. Rep., **9**(1), p. 4758.
[30] Falk, H., Lambaa, S., Johannesen, H. H., Wooler, G., Venzo, A., and Gehl, J., 2017, "Calcium Electroporation for Treatment of Cutaneous Metastases; a Randomized Double-Blinded Phase II Study, Comparing the Effect of Calcium Electroporation With Electrochemotherapy," Acta Oncol., **56**(8), pp. 1126–1131.
[31] Pavšelj, N., and Miklavčič, D., 2008, "A Numerical Model of Permeabilized Skin With Local Transport Regions," IEEE Trans. Biomed. Eng., **55**, pp. 1927–1930.
[32] Edd, J. F., Horowitz, L., Davalos, R. V., Mir, L. M., and Rubinsky, B., 2006, "In Vivo Results of a New Focal Tissue Ablation Technique: Irreversible Electroporation," IEEE Trans. Biomed. Eng., **53**(7), pp. 1409–15.
[33] Rubinsky, B., Onik, G., and Mikus, P., 2007, "Irreversible Electroporation: A New Ablation Modality—Clinical Implications," Technol. Cancer Res. Treat., **6**(1), pp. 37–48.
[34] Kos, B., Voigt, P., Miklavcic, D., and Moche, M., 2015, "Careful Treatment Planning Enables Safe Ablation of Liver Tumors Adjacent to Major Blood Vessels by Percutaneous Irreversible Electroporation (IRE)," Radiol. Oncol., **49**(3), pp. 234–241.
[35] Garcia, P. A., Davalos, R. V., and Miklavčič, D., 2014, "A Numerical Investigation of the Electric and Thermal Cell Kill Distributions in Electroporation-Based Therapies in Tissue," PloS One, **9**(8), p. e103083.
[36] Neal, R. E., Millar, J. L., Kavnoudias, H., Royce, P., Rosenfeldt, F., Pham, A., Smith, R., Davalos, R. V., and Thomson, K. R., 2014, " In Vivo Characterization and Numerical Simulation of Prostate Properties for Non-Thermal Irreversible Electroporation Ablation," Prostate, **74**(5), pp. 458–468.
[37] Bertacchini, C., Margotti, P. M., Bergamini, E., Lodi, A., Ronchetti, M., and Cadossi, R., 2007, "Design of an Irreversible Electroporation System for Clinical Use," Technol. Cancer Res. Treat., **6**(4), pp. 313–320.
[38] Scheffer, H. J., Nielsen, K., de Jong, M. C., van Tilborg, A. A. J. M., Vieveen, J. M., Bouwman, A. R. A., Meijer, S., van Kuijk, C., van den Tol, P. M. P., and Meijerink, M. R., 2014, "Irreversible Electroporation for Nonthermal Tumor Ablation in the Clinical Setting: A Systematic Review of Safety and Efficacy," J. Vasc. Interventional Radiol., **25**(7), pp. 997–1011.
[39] Ball, C., Thomson, K., and Kavnoudias, H., 2010, "Irreversible Electroporation: A New Challenge in "Out of Operating Theater," Anesth. Analg., **110**(5), pp. 1305–1309.
[40] Golberg, A., and Rubinsky, B., 2012, "Towards Electroporation Based Treatment Planning Considering Electric Field Induced Muscle Contractions," Technol. Cancer Res. Treat., **11**(2), pp. 189–201.
[41] Arena, C. B., Sano, M. B., Rossmeisl, J. H., Caldwell, J. L., Garcia, P. A., Rylander, M. N., and Davalos, R. V., 2011, "High-Frequency Irreversible Electroporation (H-FIRE) for Non-Thermal Ablation Without Muscle Contraction," BioMedical Eng. OnLine, **10**(1), p. 102.
[42] Latouche, E. L., Arena, C. B., Ivey, J. W., Garcia, P. A., Pancotto, T. E., Pavlisko, N., Verbridge, S. S., Davalos, R. V., and Rossmeisl, J. H., 2018, "High-Frequency Irreversible Electroporation for Intracranial Meningioma: A Feasibility Study in a Spontaneous Canine Tumor Model," Technol. Cancer Res. Treat., **17**, pp. 1–10.
[43] Sweeney, D. C., Reberšek, M., Dermol, J., Rems, L., Miklavčič, D., and Davalos, R. V., 2016, "Quantification of Cell Membrane Permeability Induced by Monopolar and High-Frequency Bipolar Bursts of Electrical Pulses," Biochim. Biophys. Acta, **1858**(11), pp. 2689–2698.
[44] Scuderi, M., Reberšek, M., Miklavčič, D., and Dermol-Černe, J., 2019, "The Use of High-Frequency Short Bipolar Pulses in Cisplatin Electrochemotherapy In Vitro," Radiol. Oncol., **53**(2), pp. 194–205.
[45] Lee, J. M., Choi, H. S., Kim, E. S., Keum, B., Seo, Y. S., Jeen, Y. T., Lee, H. S., Chun, H. J., Um, S. H., Kim, C. D., and Kim, H., 2019, "Characterization of Irreversible Electroporation on the Stomach: A Feasibility Study in Rats," Sci. Rep., **9**(1), p. 9094.
[46] Rosazza, C., Meglič, S. H., Zumbusch, A., Rols, M.-P., and Miklavčič, D., 2016, "Gene Electrotransfer: A Mechanistic Perspective," Curr. Gene Ther., **16**(2), pp. 98–129.
[47] Shibata, M.-A., Ito, Y., Morimoto, J., Kusakabe, K., Yoshinaka, R., and Otsuki, Y., 2006, "In Vivo Electrogene Transfer of Interleukin-12 Inhibits Tumor Growth and Lymph Node and Lung Metastases in Mouse Mammary Carcinomas," J. Gene Med., **8**(3), pp. 335–352.
[48] Daud, A. I., DeConti, R. C., Andrews, S., Urbas, P., Riker, A. I., Sondak, V. K., Munster, P. N., Sullivan, D. M., Ugen, K. E., Messina, J. L., and Heller, R., 2008, " Phase I Trial of Interleukin-12 Plasmid Electroporation in Patients With Metastatic Melanoma," J. Clin. Oncol., **6**(36), pp. 5896–5903.
[49] Chiarella, P., Fazio, V. M., and Signori, E., 2010, "Application of Electroporation in DNA Vaccination Protocols," Curr. Gene Ther., **10**(4), pp. 281–286.
[50] Sardesai, N. Y., and Weiner, D. B., 2011, "Electroporation Delivery of DNA Vaccines: Prospects for Success," Curr. Opin. Immunol,., **23**(3), pp. 421–429.
[51] Golden, K., Sagi, V., Markwarth, N., Chen, B., and Monteiro, A., 2007, "In Vivo Electroporation of DNA into the Wing Epidermis of the Butterfly, Bicyclus Anynana," J. Insect Sci., **7**(53), pp. 1–8.
[52] Grewal, R. K., Lulsdorf, M., Croser, J., Ochatt, S., Vandenberg, A., and Warkentin, T. D., 2009, "Doubled-Haploid Production in Chickpea (Cicer Arietinum l.): Role of Stress Treatments," Plant Cell Rep., **28**(8), pp. 1289–1299.
[53] Heller, L. C., and Heller, R., 2006, "In Vivo Electroporation for Gene Therapy," Human Gene Ther., **17**(9), pp. 890–897.
[54] Serša, G., Teissié, J., Čemažar, M., Signori, E., Kamensek, U., Marshall, G., and Miklavčič, D., 2015, "Electrochemotherapy of Tumors as in Situ Vaccination Boosted by Immunogene Electrotransfer," Cancer Immunol., Immunother., **64**, pp. 1315–1327.
[55] Andre, F. M., and Mir, L. M., 2010, "Nucleic Acids Electrotransfer In Vivo: Mechanisms and Practical Aspects," Curr. Gene Ther., **10**(4), pp. 267–80.
[56] Kandušer, M., Miklavčič, D., and Pavlin, M., 2009, "Mechanisms Involved in Gene Electrotransfer Using High- and Low-Voltage Pulses—An In Vitro Study," Bioelectrochemistry, **74**(2), pp. 265–271.
[57] Faurie, C., Reberšek, M., Golzio, M., Kanduser, M., Escoffre, J. M., Pavlin, M., Teissie, J., Miklavčič, D., and Rols, M. P., 2010, "Electro-Mediated Gene Transfer and Expression Are Controlled by the Life-Time of DNA/Membrane Complex Formation," J. Gene Med., **12**(1), pp. 117–125.
[58] Chopinet, L., Batista-Napotnik, T., Montigny, A., Rebersek, M., Teissié, J., Rols, M.-P., and Miklavčič, D., 2013, "Nanosecond Electric Pulse Effects on Gene Expression," J. Membr. Biol., **246**(11), pp. 851–859.
[59] Broderick, K. E., and Humeau, L. M., 2017, "Enhanced Delivery of DNA or RNA Vaccines by Electroporation," *Methods and Protocols*, T. Kramps and K. Elbers, eds., Springer, New York, pp. 193–200.
[60] Jiang, J., Ramos, S. J., Bangalore, P., Fisher, P., Germar, K., Lee, B. K., Williamson, D., Kemme, A., Schade, E., McCoy, J., Muthumani, K., Weiner, D. B., Humeau, L. M., and Broderick, K. E., 2019, "Integration of Needle-Free Jet Injection With Advanced Electroporation Delivery Enhances the Magnitude, Kinetics, and Persistence of Engineered DNA Vaccine Induced Immune Responses," Vaccine, **37**(29), pp. 3832–3839.
[61] Heller, L. C., 2016, "Principles of Electroporation for Gene Therapy," *Handbook of Electroporation*, D. Miklavčič, ed., Springer International Publishing, Cham, Switzerland, pp. 1–16.
[62] Marjanovic, I., Haberl, S., Miklavčič, D., Kanduser, M., and Pavlin, M., 2010, "Analysis and Comparison of Electrical Pulse Parameters for Gene Electrotransfer of Two Different Cell Lines," J. Membr. Biol., **236**(1), pp. 97–105.
[63] Gothelf, A., and Gehl, J., 2010, "Gene Electrotransfer to Skin; Review of Existing Literature and Clinical Perspectives," Curr. Gene Ther., **10**(4), pp. 287–299.
[64] Reberšek, M., Miklavčič, D., Bertacchini, C., and Sack, M., 2014, "Cell Membrane Electroporation—Part 3: The Equipment," IEEE Electr. Insul. Mag., **30**(3), pp. 8–18.
[65] Reberšek, M., 2017, "Beyond Electroporation Pulse Parameters: From Application to Evaluation," *Handbook of Electroporation*, in D. Miklavčič, ed., Springer International Publishing, Cham, Switzerland, pp. 977–997.
[66] Pavlin, M., Kandušer, M., Reberšek, M., Pucihar, G., Hart, F. X., Magjarević, R., and Miklavčič, D., 2005, "Effect of Cell Electroporation on the Conductivity of a Cell Suspension," Biophys. J., **88**(6), pp. 4378–4390.
[67] Ivorra, A., Al-Sakere, B., Rubinsky, B., and Mir, L. M., 2009, "In Vivo Electrical Conductivity Measurements During and After Tumor Electroporation: Conductivity Changes Reflect the Treatment Outcome," Phys. Med. Biol., **54**(19), p. 5949.
[68] Puc, M., Čorović, S., Flisar, K., Petkovšek, M., Nastran, J., and Miklavčič, D., 2004, "Techniques of Signal Generation Required for Electropermeabilization. Survey of Electropermeabilization Devices," Bioelectrochemistry, **64**(2), pp. 113–124.
[69] Reberšek, M., and Miklavčič, D., 2010, "Concepts of Electroporation Pulse Generation and Overview of Electric Pulse Generators for Cell and Tissue Electroporation," *Advanced Electroporation Techniques in Biology and Medicine*, in A. G. Pakhomov, D. Miklavčič, and M. S. Markov, eds., CRC Press, Boca Raton, FL, pp. 323–339.
[70] Pirc, E., Reberšek, M., and Miklavčič, D., 2017, "Dosimetry in Electroporation-Based Technologies and Treatments," *Dosimetry in Bioelectromagnetics*, M. S. Markov, ed., CRC Press, Taylor & Francis Group, Boca Raton, FL, pp. 233–268.
[71] Marcus, H. J., Payne, C. J., Hughes-Hallett, A., Marcus, A. P., Yang, G.-Z., Darzi, A., and Nandi, D., 2016, "Regulatory Approval of New Medical Devices: Cross Sectional Study," BMJ, **353**, pp. 1–5.
[72] Kaplan, A. V., Baim, D. S., Smith, J. J., Feigal, D. A., Simons, M., Jefferys, D., Fogarty, T. J., Kuntz, R. E., and Leon, M. B., 2004, "Medical Device Development," Circulation, **109**(25), pp. 3068–3072.
[73] Ross, J. S., Blount, K. L., Ritchie, J. D., Hodshon, B., and Krumholz, H. M., 2015, "Post-Market Clinical Research Conducted by Medical Device Manufacturers: A Cross-Sectional Survey," Med. Devices, **8**, pp. 241–249.
[74] Pirc, E., Pecchia, L., Reberšek, M., Serša, G., Snoj, M., Grošelj, A., and Miklavčič, D., 2018, "Study Design of a Medical Device Pre-Market Assessment: A Case Study on Electrochemotherapy," Zdravniški Vestn., **87**(1–2), pp. 22–40.
[75] Colombo, G. L., Matteo, S. D., and Mir, L. M., 2008, "Cost-Effectiveness Analysis of Electrochemotherapy With the Cliniporator™ versus Other Methods for

the Control and Treatment of Cutaneous and Subcutaneous Tumors," Ther. Clin. Risk Manage., **4**(2), pp. 541–548.
[76] Sack, M., Schultheiss, C., and Bluhm, H., 2005, "Triggered Marx Generators for the Industrial-Scale Electroporation of Sugar Beets," IEEE Trans. Ind. Appl., **41**(3), pp. 707–714.
[77] Elgenedy, M. A., Darwish, A., Ahmed, S., and Williams, B. W., 2017, "A Transition Arm Modular Multilevel Universal Pulse-Waveform Generator for Electroporation Applications," IEEE Trans. Power Electron., **32**(12), pp. 8979–8991.
[78] Elserougi, A., Massoud, A., and Ahmed, S., 2016, "Conceptual Study of a Bipolar Modular High Voltage Pulse Generator With Sequential Charging," IEEE Trans. Dielectrics Electr. Insulations, **23**(6), pp. 3450–3457.
[79] Novickij, V., 2016, "High-Frequency Submicrosecond Electroporator," Biotechnol. Biotechnol. Equip., **30**, pp. 1–7.
[80] Pirc, E., Miklavčič, D., and Reberšek, M., 2019, "Nanosecond Pulse Electroporator With Silicon Carbide MOSFETs, Development and Evaluation," IEEE Trans. Biomed. Eng., **66**(12), pp. 3526–3533.
[81] Grainys, A., Vitalij, N., and Novickij, J., 2016, "High-Power Bipolar Multilevel Pulsed Electroporator," Instrum. Sci. Technol., **44**(1), pp. 65–72.
[82] Groselj, A., Kos, B., Čemažar, M., Urbancic, J., Kragelj, G., Bošnjak, M., Veberic, B., Strojan, P., Miklavčič, D., and Serša, G., 2015, "Coupling Treatment Planning With Navigation System: A New Technological Approach in Treatment of Head and Neck Tumors by Electrochemotherapy," Biomed. Eng. Online, **14**(Suppl 3), p. S2.
[83] Pal, K., Kraatz, H.-B., Khasnobish, A., Bag, S., Banerjee, I., and Kuruganti, U., 2019, *Bioelectronics and Medical Devices: From Materials to Devices—Fabrication, Applications and Reliability*, Woodhead Publishing, Cambridge, UK.
[84] Kaiser, J., 2016, "Calling All Failed Replication Experiments," Science, **351**(6273), p. 548.
[85] Puc, M., Kotnik, T., Mir, L. M., and Miklavčič, D., 2003, "Quantitative Model of Small Molecules Uptake After In Vitro Cell Electropermeabilization," Bioelectrochemistry, **60**(1–2), pp. 1–10.
[86] Rols, M.-P., and Teissié, J., 1998, "Electropermeabilization of Mammalian Cells to Macromolecules: Control by Pulse Duration," Biophys. J., **75**(3), pp. 1415–1423.
[87] Novickij, V., Girkontaitė, I., Grainys, A., Zinkevičienė, A., Lastauskienė, E., Švedienė, J., Paškevičius, A., Markovskaja, S., and Novickij, J., 2016, "Measurement of Transient Permeability of Sp2/0 Myeloma Cells: Flow Cytometric Study," Meas. Sci. Rev., **16**(6), pp. 300–304.
[88] Serša, G., Cemazar, M., and Rudolf, Z., 2003, "Electrochemotherapy: Advantages and Drawbacks in Treatment of Cancer Patients," Cancer Ther., **1**, pp. 133–142.
[89] Kalra, N., Gupta, P., Chawla, Y., and Khandelwal, N., 2015, "Locoregional Treatment for Hepatocellular Carcinoma: The Best is Yet to Come," World J. Radiol., **7**(10), pp. 306–318.
[90] Silk, M., Tahour, D., Srimathveeravalli, G., Solomon, S. B., and Thornton, R. H., 2014, "The State of Irreversible Electroporation in Interventional Oncology," Semin. Interventional Radiol., **31**(2), pp. 111–117.
[91] Bolhassani, A., Khavari, A., and Orafa, Z., 2014, "Electroporation—Advantages and Drawbacks for Delivery of Drug, Gene and Vaccine," *Application of Nanotechnology in Drug Delivery*, A. D. Sezer, ed., IntechOpen, Rijeka, Croatia, Chap. 11.
[92] Stewart, M. T., Haines, D. E., Verma, A., Kirchhof, N., Barka, N., Grassl, E., and Howard, B., 2019, "Intracardiac Pulsed Field Ablation: Proof of Feasibility in a Chronic Porcine Model," Heart Rhythm, **16**(5), pp. 754–764.

3.4 Paper 3

Title: **Towards standardization of electroporation devices and protocols**

Authors: Aleksandra Cvetkoska, Eva Pirc, Matej Reberšek, Ratko Magjarević and Damijan Miklavčič

Publication: *IEEE Instrumentation & Measurement Magazine*

Year: 2020

Impact factor: 1.024 (2018)

Quartile: Q4 (engineering, electrical & electronic; instruments & instrumentation)

Rank: 209/266, 52/61 (engineering, electrical & electronic; instruments & instrumentation)

Status: accepted for publication

Towards standardization of electroporation devices and protocols

Aleksandra Cvetkoska, Eva Pirc, Matej Reberšek, Ratko Magjarević, Damijan Miklavčič

Cell exposure to high-voltage, short-duration electric pulses can lead to temporary formation of hydrophilic pores in the plasma membrane and increase of membrane's permeability which consequently increases the transmembrane transport of molecules that are otherwise unable to cross the membrane. This phenomenon termed as membrane electroporation is currently an applicable technique in different areas such as biomedicine, biotechnology, food technology and environmental applications. Electroporation pulses are generated by pulse power generators known as electroporators and delivered to the cells (in tissue) via electrodes. The objective of this paper is to review and compare characteristics of the electroporation applications and equipment described in literature and/or present on the market. Since there are no specific standards or regulations referring particularly to the safety of medical devices with intended medical use for electroporation, we propose guidelines for the design of clinical electroporators and define minimal requirements for their safe and efficient use which can be incorporated within the particular standard in the future. In order to facilitate the comparison of the data obtained by different research groups and to enable reproduction of results under the same conditions, we want to stress the necessity of defining the electroporator's output parameters and define tolerances of electroporation parameters for electroporation-based therapies.

Introduction

The phenomenon of electroporation

Each biological cell is protected from its surroundings by the plasma membrane which is composed of a two-molecule thick layer of lipids. The plasma membrane would be a mostly impenetrable barrier if there were not various proteins, which enable transport of specific molecules across the membrane. However, cell exposure to high-voltage, short-duration electric pulses can lead to temporary formation of hydrophilic pores in the bilayers and increase of plasma membrane permeability which consequently causes increase in transmembrane transport of molecules that are otherwise unable to cross the membrane [1]. This phenomenon termed as membrane electroporation/permeabilization can be either reversible, when the cell exposure to electric field is short enough for the cells to fully recover; or irreversible, when their exposure leads to cell death.

Electroporation applications

Electroporation has become a relevant technique in different areas such as biomedicine, biotechnology, food technology and environmental applications [2]. Reversible electroporation is already a well-established method in medicine, which combined with administration of otherwise low-permeant chemotherapeutic drugs to tumor cells, results in highly efficient local antitumor therapy called electrochemotherapy (ECT). Locally applied short, high-voltage (HV) pulses in the range of few hundreds of volts to few kilovolts increase the permeability of tumor cells membranes in order to facilitate cellular uptake of chemotherapeutic drugs like bleomycin and cisplatin, thus increasing their cytotoxicity [3]. Electroporation is also used for cell gene transfection, i.e. gene electrotransfer (GET), which is a non-viral gene delivery method that uses reversible electroporation for delivering DNA molecules to cells. Skin and muscle are promising targets for gene delivery, thus GET has been used in many medical applications, including vaccination, wound healing and cancer treatment. On the other hand, the potential of irreversible electroporation in medicine appeared as a nonthermal irreversible electroporation (NTIRE), also termed Pulsed Field Ablation (PFA), which has enabled the ablation of

undesirable (malignant or arrhythmogenic) tissue with minimal damage to blood vessels and nerve conduits in the treated area [4]. Furthermore, electroporation emerged in different applications of biotechnology [5] and it is efficiently utilized for heritable genetic modification of microorganisms (electrotransformation), extraction of biomolecules, inactivation of microorganisms, and improving the mass transport in food processes. Some of the applications of electroporation are presented on Fig. 1.

Generating electrical pulses

To achieve successful electroporation, cells have to be exposed to sufficiently high electric fields (related also to duration of exposure). Electroporation pulses are electrical pulses, which are generated by pulse power generators known as electroporators and delivered to the cells (in tissue) via electrodes. Electrode geometry and tissue dielectric properties define the electric field intensity. Regardless of the application, electroporators have one common task: electrical pulses generation and delivery.

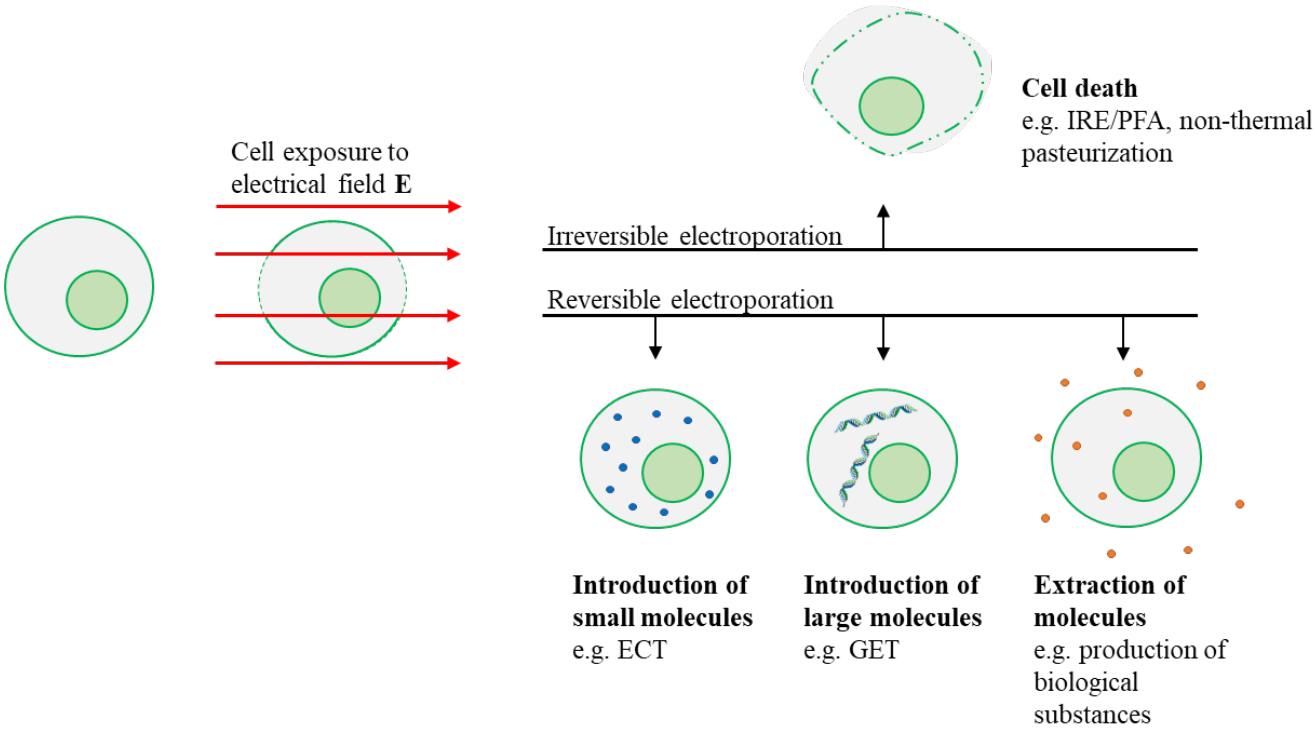


Fig. 1. *Various electroporation applications. When exposure of the cell to sufficiently high electric field reaches the cell membrane threshold value, the cell gets permeabilized. Membrane electroporation/permeabilization can be either reversible, when the cell exposure to electric field is short enough for the cells to fully recover; or irreversible, when their exposure leads to cell death. In case of reversible electroporation, during the electroporation process, molecules can be introduced into the cell (electrochemotherapy (ECT), gene electrotransfer (GET)) or molecules can be extracted from the cell.*

Electroporation success depends on the parameters of the delivered electrical pulses. The parameters of electrical pulses at the output of commercially available electroporators may vary in shape, voltage/current amplitude, pulse duration, number of pulses in a pulse train, and pulse repetition rate [6]. In electroporation, series of pulses is most commonly used for the treatment. If all pulses in a train/series of pulses are similar, the definition of the characteristics of only a single pulse and its repetition rate is sufficient for defining the treatment. The amplitude of the generated pulses may range from a few tens of volts (e.g. for GET) to a few kilovolts (e.g. for IRE) and even tens of kilovolts for microbial inactivation in liquid food pasteurization, with durations that range from nanoseconds to milliseconds for single pulse or train of pulses with

predefined pulse repetition rate. Pulses may be preset for a specific application or precisely defined for a particular patient.

An electroporation pulse generator comprises a HV power supply, a pulse generator, a control unit, a user interface and an output module (Fig. 2). The user interface enables setting of pulse parameters as required for a specific application. It is preferable that the device measures the parameters of delivered pulses and generates warnings in case of malfunction and/or incorrect operation.

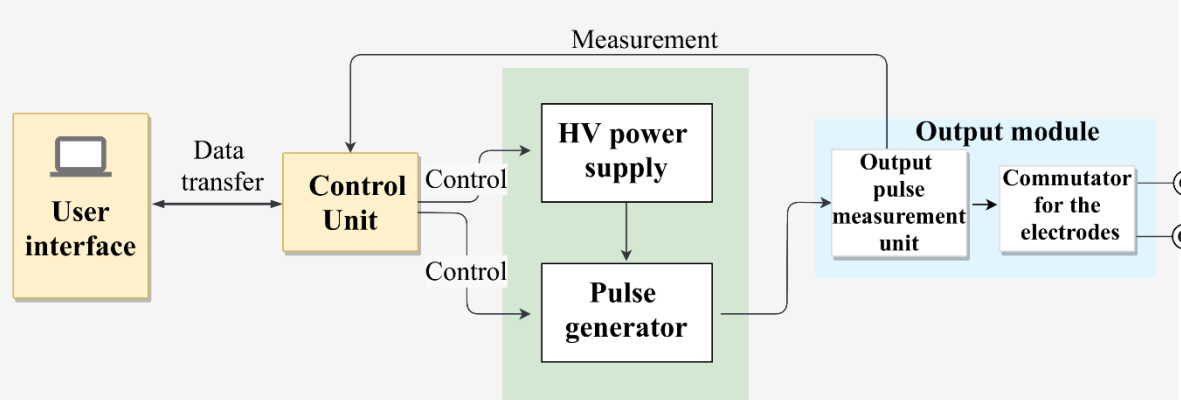


Fig. 2. *Block diagram of an electroporator. An electroporator comprises a user interface, to enable setting of the pulse; a control unit; a high-voltage (HV) power supply and pulse generator for generation and forming of electrical pulses; an output module, and in case of multiple electrodes, a commutator for switching the voltage pulses to different electrodes. Measuring of the delivered pulses is preferable and warnings in case of malfunction and/or incorrect operation should be generated.*

For generation of electrical pulses, HV power supply and pulse generator (for pulse shaping) are needed. The output module consists of an output pulse measurement unit and a commutator for switching high voltage pulses to different electrodes (if multiple electrodes are used).

Electroporators for specific applications

Electroporators are mainly classified as clinical, industrial or laboratory [7]. Electrodes together with the biological sample define the load for the output stage of the electroporator. Thus, classification of the electrodes is according to targeted load, i.e. cells (single-cell chambers, micro-electrodes, macro-electrodes and flow-through chambers) or tissue (plate, parallel needle array, hexagonal needle, finger and adjustable electrodes). Since biological loads as well as the electroporated volume intrinsically differ in different electroporation-based applications, pulses with specifically predefined parameters have to be used. Therefore, electroporators are often designed and developed for specific applications, where quality and efficiency have to be assured [8].

Clinical electroporators

Electroporation-based medical/clinical applications such as ECT, GET, IRE and PFA, have so far been focused predominantly on cancer treatments. Electroporators used for medical treatments in clinics, i.e. clinical electroporators, have been developed to implement antitumor therapy using a limited number of predefined settings of electrical pulses in associations with chemotherapeutic drugs (ECT) or foreign substances such as DNA (GET) within protocols based on reversible electroporation or as stand-alone ablation treatment based on irreversible electroporation (IRE). The target, e.g. tumor tissue should be covered with sufficiently high electric field, which should be above the threshold of reversible or irreversible electroporation 500-600 V/cm local electric field (which often requires pulse amplitudes up to 3000 V and currents up to 50 A), to achieve the desired effect. Therefore, HV pulse generators are needed

which should not present potential risk for hazardous event for the patient, operator and nearby devices.

Clinical electroporators are considered to be medical devices and thus, patient and operator safety must be ensured under both normal and single-fault conditions. Furthermore, they are obligated to meet medical devices' standards and follow the requirements defined by local medical regulations, e.g. Medical Device Regulation 2017/745 in Europe or CFR (Code of Federal Regulations) Title 21 in the United States (US), in order to get approval for selling the device on the market, e.g. certification mark (CE) in Europe or FDA (Food and Drug Administration) approval in the US. In spite of agreements signed between EU and the US, a clinical electroporator approved for use in Europe cannot be automatically used in the US and vice versa, because each country has different regulatory regimes.

Based on our review, there are only few certified clinical electroporators on the market. The most used clinical electroporators are the Cliniporator and the NanoKnife System. In Europe, the Cliniporator (two different models: Cliniporator EPS02 and Cliniporator VITAE), manufactured by IGEA S.p.A. (Carpi MO, Italy), used for both ECT and GET was approved by the Italian notified body and has CE mark. Another certified clinical electroporator in Europe is SENNEX (BionMed Technologies, Germany), which is used only for ECT. Recently, a new CE approved clinical electroporation generator ePORE (Mirai Medical, Galway, Ireland) was developed for simple and reliable delivery of ultra-short electrical pulses up to 250 kHz to enable treatment on an outpatient endoscopy basis. On the other hand, in the US, the NanoKnife System, manufactured by AngioDynamics, Inc. (Queensbury, N.Y, U.S) was approved by the FDA for surgical ablation of soft tissue [9]. Additionally, it is CE marked for Europe and has license approval for Canada for soft tissue ablation. However, it does not have FDA clearance for other treatments and therapies for specific disease or condition.

To help verify the safety of medical devices, electrical safety standards have been established in the US, European countries and other parts of the world. The first medical standard for medical electrical equipment, IEC 60601 was published in 1977 by the International Electrotechnical Commission (IEC) which is a family of technical standards whose scope covers the safety, essential performance and electromagnetic compatibility of medical electrical equipment and systems. As of 2011 it consists of a general standard for medical electrical equipment: 60601-1 (EN 60601-1:2006/A1:2013 in EU or IEC 60601-1:2005/A1:2012 in the US), ten collateral standards and about sixty particular standards. Collateral standards (numbered 60601-1-X) define the general requirements for certain aspects of safety and performance, e.g. Electromagnetic Compatibility (IEC 60601-1-2). Particular standards (numbered 60601-2-X) define particular requirements for specific products or specific measurements built into products and add conditions not mentioned in 60601-1 or explain how to simplify implementation of 60601-1 to a particular device type, e.g. cardiac defibrillators (IEC 60601-2-4). In spite of the sixty particular standards, a particular standard for clinical electroporators currently does not exist. Therefore, it will be necessary to define additional rules for manufacturing and safe and efficient use of clinical electroporators as relatively new medical devices in addition to those defined by ISO and EN/IEC standards.

Considering the general standard for medical devices EN/IEC 60601-1, key safety factors that have to be considered in electroporator's design include: voltage and energy limits, adequate insulation, limitation of leakage currents, electromagnetic compatibility requirements as presented in the standard EN/IEC 60601-1-2 and consideration of fault operations while maintaining quality, efficiency and smooth operation of the device. Other standards to be considered for developing clinical electroporators are ISO 14971 for risk analysis, ISO 13485 for quality management system, EN/IEC 60601-1-6 and ISO 62366 for usability, ISO 62304

and IEC 80002-1 for medical device software and IEC 62311 in case of a battery powered clinical electroporator.

Industrial electroporators

In biotechnology and even more in food processing technology, high-power and high-voltage electroporators are needed due to the requirement to electroporate large volumes of liquid. In food processing, electroporation is more frequently termed as pulsed electric field (PEF) treatment used for food structure modification or liquid food pasteurization. By PEF treatment, permeabilization of biological cells is achieved, mass transfer is improved, and therefore an efficient way for extraction of liquid and valuable substances from cells is enabled. PEF treatment systems are usually composed of a pulse power generator and a treatment chamber.

Depending on the application, a suitable pulse generator has to be chosen with adequate parameters:

- pulse shape;
- peak voltage, which is highly depended on the desired application;
- peak current, which is determined by the object and volume being treated;
- geometry of the treatment chamber;
- average power required, depended on the desired processing capacity (kilograms/h or liters/h).

In terms of power requirements, scale-up from several kW for laboratory to more than 100 kW for continuous-flow industrial-level processing was achieved. Therefore, regular average power of contemporary PEF devices ranges between 30 kW and 400 kW [10]. Commercial PEF treatment applications are mostly set up in potato (tuber) industry, fruit juice preservation, and vegetable processing. For juice processing, electroporation treatment systems with continuous flow have already been established with capacity of 8000 liters/h, whereas for potato processing capacity ranges up to 50-80 tons/h.

The use of new processes applied in food industry always requires appropriate process control options and set up of a systematic preventive approach to food safety – HACCP (Hazard Analysis Critical Control Point). HACCP has seven principles that need to be followed, stated in the international standard ISO 22000 FSMS 2011. In the US, the adoption of different technologies in food processing industry is also subject to the regulation of the FDA and in EU it falls under the Regulation EU 2015/2283 on novel foods. Regarding safety of the device, protection against electric shock in case of insulation failure is important when using the device in wet environments. For this reason, wineries for example, are equipped with RCD (Residual Current Devices) responsive to a leakage current of about 30 mA. Furthermore, electromagnetic compatibility according to standards is recommended. Thus, the pulse circuit has to be shielded with metal housing and mains and leads to the control circuity should be protected against over-voltage [11].

Laboratory electroporators

For conducting experiments in the laboratory, users can choose between several commercially available laboratory electroporators. Choosing the right laboratory electroporator can be crucial for experiments and treatment protocols as some laboratory electroporators have limited range and control over pulse parameters.

Important step to be considered during electroporation is to assure pulse measuring and monitoring because only few electroporators can report and provide accurate measurements. Large variation of load characteristics is another reason to measure. The electrical properties

of the sample between the electrodes might affect the current delivered (conductivity versatility). The resistance of the cuvette, for example, can vary depending on the conductivity of the media which can drastically change the required current.

However, in laboratories where experiments are done, oscilloscopes and current probes are often not readily available. Therefore, built-in measurement systems should be provided in order to be used with laboratory electroporators. The device should be able to perform self-tests to ensure flawless operation and detect single faults. Some use "test" pulses which should be specified and should not affect/change the sample or influence the outcome of the result. Furthermore, the device should be able to interact with the operator in order to ensure safe and efficient treatment and generation of output pulses, which ensure effective experiment. The accuracy of measurements should be specified in advance and measuring and comparison of results during experiments should be reported. Periodic calibrations of the device and equipment need to be made as well as electrode replacement based on predefined intervals. In case of using single-use electrodes or electroporation cuvettes, safe disposal after the experiment should be provided due to the chemical reactions that can change the electrical properties of the electrodes in the next experiment. Recently, nanosecond electroporators were introduces and are now being used in laboratory setups. Here measurement protocols and delivery of the pulses are more challenging. Special attention and more advanced measurement setups are required, as those electroporators mainly do not have built-in measurement system.

All of these problems and requirements could be solved with implementation of a specific standard for electroporators based on the application. As laboratory electroporators are not considered to be medical devices, medical device standards do not apply and therefore, the only solution is gathering all previously mentioned requirements into one standard and agreeing on requirements that will be followed by all manufacturers to enable comparability and reproducibility of research and laboratory results. Additionally, electroporation cuvettes and electrodes should be standardized with defined tolerances and materials used.

Recommendations for further research and standardization

Nowadays, electroporation industry is ascending even faster than before. Taking into consideration the new technologies, treatment protocols, increased research intensity and knowledge, we already have well-established protocols, equipment and promising treatments.

For achieving successful and efficient electroporation, it is necessary to have well defined output pulses and measure them in each treatment or experiment to make sure that the pulses are delivered as requested. Thus, suitable reproduction and comparison of results can be made if necessary. When delivering electrical pulses for electroporation, regardless of the application, it is very important to provide complete reports in order to enable comparability and reproducibility of the results [4], [12], [13], [14]. A description of pulses and how the electrical pulses were measured is necessary. The researchers must provide all specifications of the measuring equipment, the point of measurement and state if the pulses were delivered and measured in each experiment. Additionally, parameters and complete time-domain waveforms of the pulses should be provided with an appropriate description of the electroporator and electrodes used. For commercial equipment, the name of the company and model should be specified. If the pulse generator is a laboratory prototype or specially manufactured unit, suitable description of the components, electrical configuration, measurement and data acquisition systems should be provided. Lastly, the electric field induced by the delivered pulses inside the biological load should be calculated and/or all data describing

the electric field should be listed (i.e. electrode shape and their position with respect to the treated sample/tissue).

Currently, we can say that we have a developing market for clinical electroporators and new electroporators designed for specific applications are coming up. However, the absence of industry, laboratory and medical specific standards may eventually become an obstacle for further development of approved electroporation devices and associated equipment. Papers calling for standardization for other applications of electroporation have already been published, e.g. for standardization of IRE techniques and protocols, in which authors propose a set of technical recommendations for the use of IRE for treatment of locally advanced pancreatic cancer [15]. Having a specific standard for each application will simplify the harmonization of all commercial, certified electroporators and improve the safety, quality and efficiency of these devices. Current problems like voltage drop during pulse delivery, unknown pulse parameters, insufficient electrical field, non-comprehensive reports can be solved by stating limits and recommendations for voltage/current, energy, load, electrodes used, insulation and design.

The standard should define:

- maximum tolerances of generated pulses compared to expected values by considering the plasma membrane permeabilization of the load and technical limitations of electroporation device development;
- how to provide technical specifications of the device, i.e. to provide specifications together with conditions under which they are achieved, e.g. to define maximum amplitude of the pulses together with the pulse duration range and load resistance at which it can be achieved;
- pre-pulses (amplitude, pulse duration and exact timing regarding the preset sequence), if used;
- how to implement safety features like galvanic isolation, current, energy and voltage limitations, warnings if the pulse delivery was stopped or limited, or if any other unforeseeable event or malfunction has occurred;
- by which load the electroporators should be tested to ensure effectiveness at specific applications, or to ensure predictable operation or operation within tolerances (this can be quite challenging in case of delivery of nanosecond pulses as pulse reflections can occur due to the dynamics and variability of the biological load, i.e. conductivity increase due to electroporation);
- which electroporation cuvettes and electrodes should be used;
- maximum tolerances of the distances between the electrodes.

The standard should also consider procedures for different materials used and recommend a way of defining a treatment volume and an electric field distribution between the electrodes.

Recommendation for electrochemotherapy device standardization

ECT is an established cancer treatment used in clinics [16] for safe and convenient treatment of cutaneous and subcutaneous tumors following standard operating procedure (SOP) [17], [18]. The pulses are delivered to target tissue via electrodes, which are considered to be medical accessories, used only in combination with a particular pulse generator (mostly used is the Cliniporator EPS02). If the electrodes are placed on the patient's skin, e.g. plate or non-penetrate electrodes, they are considered to be non-invasive medical accessories, used to treat cutaneous tissues. In case they are intended to be placed inside the patient's body, e.g. needle electrodes, they are considered to be invasive, used to treat deeper tissues.

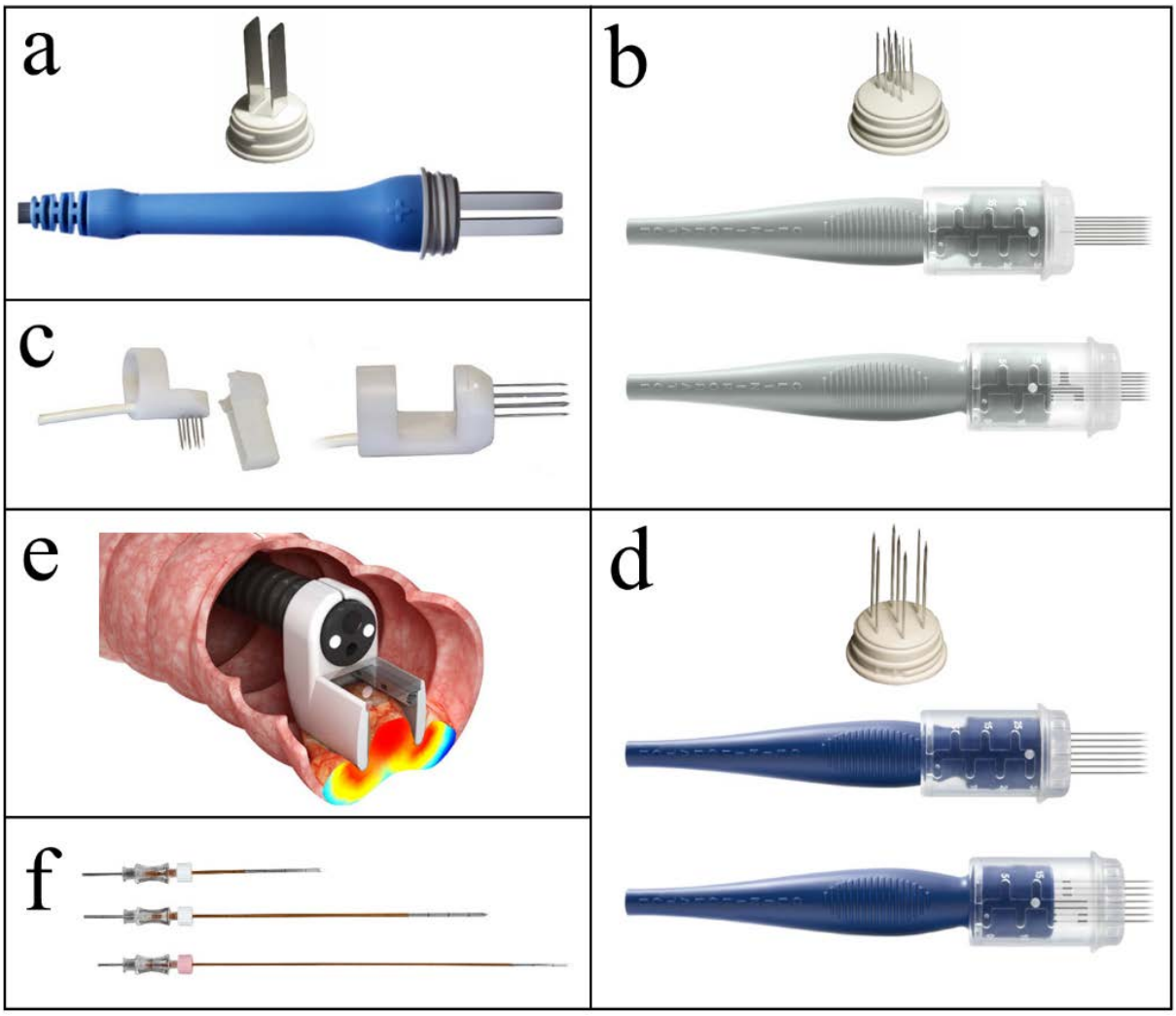


Fig. 3. *Different types of electrodes (by IGEA S.p.A.). a) Plate electrodes. b) Linear (parallel needle) array electrodes (first top image), adjustable linear needle electrodes with needle-length adjustment with 5 mm increment (bottom two images). c) Finger electrodes with orthogonal linear needles (left) and longitudinal linear needles (right). d) Hexagonal needle electrodes (first top image), adjustable hexagonal configuration needle electrodes with needle-length adjustment with 5 mm increment (bottom two images). e) Endoscopic electrode EndoVE (Endoscopic Vacuum Electrode) which is mounted at the head of an endoscope and utilizes a vacuum source to drag the tissue alongside with the electrode. f) Individual (long) needle electrodes for variable electrode-geometry (from 2 to 6 electrodes with 16- to 30 cm long needle and active tip of 3 or 4 cm).*

The updated SOP [18] defines five types of electrodes (made of stainless-steel) that are commercially available (IGEA S.p.A, Carpi MO, Italy) and can be used together with the Cliniporator depending on the treated area:

- **Plate electrodes** with 8 mm gap in-between, used for superficial skin lesions (Fig. 3a).
- **Linear array electrodes** (parallel needle array) that have 2 arrays of 4 needles (with needle length of 10-, 20- or 30 mm), separated by 4 mm distance, used for smaller tumors (recommended to be used for tumors in the facial region) with local anesthesia (Fig. 3b).
- **Hexagonal needle electrodes** with needle length of 10-, 20- or 30 mm, used for treatment of larger areas, e.g. cutaneous metastases (Fig. 3d).
- **Finger electrodes** (longitudinal or orthogonal) with needle length of 5- or 10 mm, used for treatment of mucosal tumors, e.g. in the oral cavity (Fig. 3c).

- **Adjustable electrodes** (linear (Fig. 3b) or hexagonal (Fig. 3d)) allow adjustments in needle length (from 5 mm to 40 mm with 5 mm increment) for better support in treatments of tumors with heterogeneous size.

In addition, endoscopic electroporation system EndoVe (Mirai Medical, Galway, Ireland) was developed to be used with the ePORE electroporation generator, which is also suitable for the Cliniporator (Fig. 3e). Furthermore, long freely-placeable needle electrodes (Fig. 3f) were introduced and new minimally invasive laparoscopic expandable needle electrodes are being developed by IGEA S.p.A [3], [16].

Pulse parameters are defined in the SOP as a result of numerous previously conducted studies. For each pulse delivering, 8 square-wave pulses of 100 µs with pulse amplitude of about 1000 V (1000 V up to 1300 V) across 8 mm distance between plate electrodes should be delivered at repetition rate of either 1 Hz or 5 kHz. ECT with pulse repetition rate of 5 kHz is mandatory for hexagonal needle electrodes because the treatment (delivering 8 x 12 = 96 pulses) with 1 Hz repetition rate would extend over prohibitively long time and high-frequency (5 kHz) pulses reduce the number of contractions. Nevertheless, several applications may be needed to cover the whole tumor volume in a single session.

All electrodes which are commercially available and meant to be used with the Cliniporator are for single use for a particular patient and only for a single session (for one nodule or several similar nodules in the same patient). In case of more nodules of different sizes, more than one electrode type may be needed for a particular patient in the same session.

The galvanic isolation of the output is preferably implemented in the power supply and not in the output module in order to have accurate measurement of the output signal. Output current and voltage are measured at the output of the pulse generator to implement current, energy and voltage limitations. The SOP should define the maximum expected current or minimal expected resistance of the load. The maximum current of the device should be 10 % or 20 % higher than the maximum expected current, which is 20 A for the Cliniporator EPS02. SOP defines the maximum treatment voltage as 1300 V/cm voltage-to-distance ratio times 8 mm which is equal to 1040 V. The maximum voltage is defined by the SOP and tolerances, and is 1000 V for the Cliniporator EPS02. The maximum energy should be equal to the maximum treatment time, times maximum current, times maximum voltage.

Considering the SOP, for square wave pulses (described by the amplitude and the pulse duration t_{FWHM} (FWHM – Full Width at Half Maximum)) we propose the following tolerances:

- the pulse amplitude between 15 % and 85 % of FWHM should not rise over or fall below 110 % or 90 % of SOP amplitude (Fig. 4a);
- the FWHM should not be longer or shorter than ± 8 % of SOP FWHM;
- delivered number of pulses should be exactly the same as in the SOP and variations of this parameter are not allowed;
- pulse repetition rate may deviate from SOP pulse repetition rate (for both options) for maximum ± 5 %.

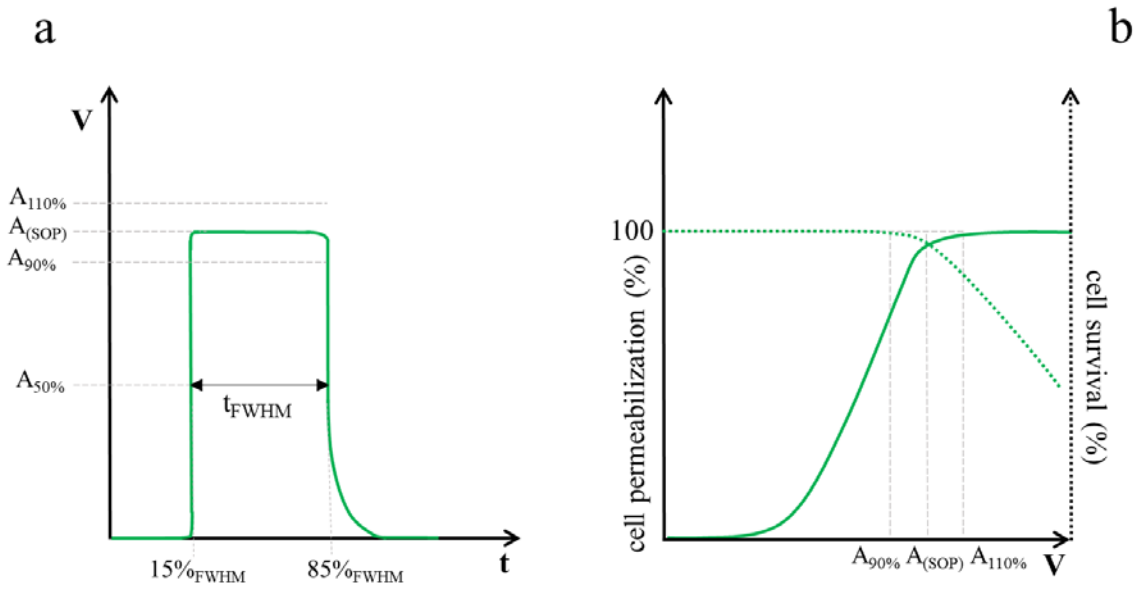


Fig. 4. *a) Electroporation square wave pulse – pulse parameters and tolerances. b) Permeabilization curve (solid line) and cell survival (dashed line) adapted from* [19] *– tolerances. For amplitudes between 90 % and 110 % of the defined SOP amplitude will still be possible to achieve efficient treatment. With amplitudes values higher than 110 %, cell survival will be increasingly lowered and can lead to IRE while with amplitudes values lower than 90 %, the cell permeabilization will decrease and can lead to inefficient tumor treatment.*

For successful ECT it is important to keep within these tolerances as we calculated them to define the maximum deviations where it is still possible to achieve the desired effect. For example, higher (more than 110 % of the amplitude) or lower (less than 90 %) values of the SOP amplitude can lead to IRE or insufficient electric field for ECT, respectively. Based on the permeabilization curves (Fig. 2 from reference [19]), even for pulses with the lowest or highest defined tolerances (for pulse amplitude and pulse durations) will still be possible to stay on the part of the permeabilization curve where the treatment will be efficient (Fig. 4b).

ECT device should work within the tolerances on zero load and on electronic emulator of ECT load. Operation of the limitation should be tested by the device on power up and conformity by using electronic emulator of biological load.

All electrodes should be manufactured utilizing a biocompatible material, usually stainless-steel. However, materials tested by the requirements stated in the ISO 10993 series of standards for biological evaluation of medical devices that come into direct or indirect contact with biological tissues (parts -1, -5 and -10 are the most important) may be allowed.

Following the SOP all electrodes need to be for single use. We propose the option of using multiple-use electrodes. In this case, clear instructions for electrodes' cleaning and maintenance after every treatment should be provided. Moreover, multiple-use electrode replacement on predefined intervals should be stated and provided in the instruction for use.

The allowed tolerances for the diameters of the electrodes, the distances between the electrodes and the length of the needles should also be defined.

Conclusions

With implementation of a specific standard for particular application, the electroporation devices will be safer, treatments more efficient and results more reproducible, which will allow faster and more straight-forward progress of electroporation as well as treatments and therapies based on electroporation. By implementing a particular medical standard for

electrochemotherapy electroporation devices, the standard operating procedure will be improved which will result in better and more effective cancer treatment.

Acknowledgements

This study was conducted in the scope of the Slovenian-Croatian cooperation in Science and Technology (BI-HR/16-17-039; 2016-2017) and supported by the Slovenian Research Agency (ARRS) (MRIC UL IP-0510, P2-0249; 2015-2020). The investment is co-financed by the Republic of Slovenia and the European Regional Development Fund.

References

[1] T. Kotnik, L. Rems, M. Tarek, and D. Miklavčič, "Membrane Electroporation and Electropermeabilization: Mechanisms and Models," *Annu. Rev. Biophys.*, vol. 48, no. 1, pp. 63–91, May 2019.

[2] D. Miklavčič, Ed., *Handbook of Electroporation*. Cham: Springer International Publishing, 2017.

[3] D. Miklavčič, B. Mali, B. Kos, R. Heller, and G. Serša, "Electrochemotherapy: from the drawing board into medical practice," *BioMed Eng OnLine*, vol. 13, no. 1, p. 29, 2014.

[4] A. Sugrue *et al.*, "Irreversible electroporation for catheter-based cardiac ablation: a systematic review of the preclinical experience," *Journal of Interventional Cardiac Electrophysiology*, Jul. 2019.

[5] T. Kotnik, W. Frey, M. Sack, S. Haberl Meglič, M. Peterka, and D. Miklavčič, "Electroporation-based applications in biotechnology," *Trends in Biotechnology*, vol. 33, no. 8, pp. 480–488, Aug. 2015.

[6] M. Rebersek and D. Miklavcic, "Concepts of Electroporation Pulse Generation and Overview of Electric Pulse Generators for Cell and Tissue Electroporation," in *Advanced Electroporation Techniques in Biology and Medicine*, A.G. Pakhomov, D. Miklavcic, and M. S. Markov, Eds. CRC, Boca Raton, 2010, pp. 323–339.

[7] M. Rebersek, D. Miklavcic, C. Bertacchini, and M. Sack, "Cell membrane electroporation-Part 3: the equipment," *IEEE Electr. Insul. Mag.*, vol. 30, no. 3, pp. 8–18, May 2014.

[8] E. Pirc, M. Reberšek, and D. Miklavčič, "Dosimetry in Electroporation-Based Technologies and Treatments," in *Dosimetry in Bioelectromagnetics*, 1st ed., M. Markov, Ed. CRC Press, 2017, pp. 233–268.

[9] C. Bertacchini, P. M. Margotti, E. Bergamini, A. Lodi, M. Ronchetti, and R. Cadossi, "Design of an Irreversible Electroporation System for Clinical Use," *Technol Cancer Res Treat*, vol. 6, no. 4, pp. 313–320, Aug. 2007.

[10] S. Toepfl, "Pulsed electric field food processing –industrial equipment design and commercial applications," *Stewart Postharvest Review*, vol. 8, no. 2, pp. 1–7, 2012.

[11] M. Sack *et al.*, "Research on Industrial-Scale Electroporation Devices Fostering the Extraction of Substances from Biological Tissue," *Food Eng. Rev.*, vol. 2, no. 2, pp. 147–156, Jun. 2010.

[12] L. G. Campana *et al.*, "Recommendations for improving the quality of reporting clinical electrochemotherapy studies based on qualitative systematic review," *Radiology and Oncology*, vol. 50, no. 1, pp. 1–13, Jan. 2016.

[13] J. Raso *et al.*, "Recommendations guidelines on the key information to be reported in studies of application of PEF technology in food and biotechnological processes," *Innovative Food Science & Emerging Technologies*, vol. 37, pp. 312–321, Oct. 2016.

[14] M. Čemazar, G. Serša, W. Frey, D. Miklavčič, and J. Teissié, "Recommendations and requirements for reporting on applications of electric pulse delivery for electroporation of biological samples," *Bioelectrochemistry*, vol. 122, pp. 69–76, Aug. 2018.

[15] R. C. G. Martin *et al.*, "Irreversible electroporation in locally advanced pancreatic cancer: A call for standardization of energy delivery: IRE Technique in Pancreatic Cancer," *J. Surg. Oncol.*, vol. 114, no. 7, pp. 865–871, Dec. 2016.
[16] L. G. Campana *et al.*, "Electrochemotherapy – Emerging applications technical advances, new indications, combined approaches, and multi-institutional collaboration," *European Journal of Surgical Oncology*, vol. 45, no. 2, pp. 92–102, Feb. 2019.
[17] L. M. Mir *et al.*, "Standard operating procedures of the electrochemotherapy: Instructions for the use of bleomycin or cisplatin administered either systemically or locally and electric pulses delivered by the CliniporatorTM by means of invasive or non-invasive electrodes," *European Journal of Cancer Supplements*, vol. 4, no. 11, pp. 14–25, Nov. 2006.
[18] J. Gehl *et al.*, "Updated standard operating procedures for electrochemotherapy of cutaneous tumours and skin metastases," *Acta Oncologica*, vol. 57, no. 7, pp. 874–882, Jul. 2018.
[19] M. Puc, T. Kotnik, L. M. Mir, and D. Miklavčič, "Quantitative model of small molecules uptake after in vitro cell electropermeabilization," *Bioelectrochemistry*, vol. 60, no. 1–2, pp. 1–10, Aug. 2003.

Aleksandra Cvetkoska was born in Bitola, Macedonia, in 1994. She received her M.Sc. degree in biomedical engineering from the University of Ljubljana. She is currently a PhD student and a Researcher in the Laboratory of Biocybernetics, at the Faculty of Electrical Engineering, University of Ljubljana. Her research is based on development and safety of electroporation devices.

Eva Pirc was born in 1990. She received the M.Sc. degree in electrical engineering from the University of Ljubljana, Slovenia, in 2015, where she is currently working toward the Ph.D. degree. Her main research interest is development and evaluation of biomedical devices especially pulse power devices for electroporation and Health Technology Assessment.

Matej Reberšek was born in Ljubljana, Slovenia, in 1979. He received his Ph.D. degree in electrical engineering from the University of Ljubljana in 2008. He is currently an Assistant Professor at the Laboratory of Biocybernetics at the Faculty of Electrical Engineering, University of Ljubljana. He is the author of more than 20 articles in SCI-ranked journals citied more than 600 times to date. His current research interest includes electroporation, especially design of electroporation devices and investigation of biological responses to nanosecond electrical pulses.

Ratko Magjarević received his Ph.D. degree in Electrical Engineering in 1994 from the University of Zagreb. He is a Professor teaching several courses in Electronic Instrumentation and Biomedical Engineering studies. His scientific and professional interest is in the fields of electronic and biomedical instrumentation and health informatics, in particular in cardiac potentials analysis and pacing, in research of new methods for drug delivery based on electropermeabilization and recently in research of personalized intelligent mobile health systems.

Damijan Miklavčič was born in Ljubljana, Slovenia, in 1963. He received his Ph.D. degree in electrical engineering from the University of Ljubljana in 1993. He is currently a Professor at the Faculty of Electrical Engineering, University of Ljubljana, where he is also the Head of the Laboratory of Biocybernetics. His current research interests include electroporation-based treatments and therapies, including cancer treatment by means of electrochemotherapy, cardiac ablation, biological experimentation, numerical modeling of biological processes and hardware development.

3.5 Paper 4

Title: **Electronic emulator of biological load during electroporation**

Authors: Eva Pirc, Bertrand Balosetti, Damijan Miklavčič and Matej Reberšek

Publication: *IEEE Transactions on Biomedical Engineering*

Impact factor: 4.491 (2018)

Quartile: Q1 (engineering, biomedical)

Rank: 11/80 (engineering, biomedical)

Status: in preparation for submission

IEEE TRANSACTIONS ON BIOMEDICAL ENGINEERING, NOVEMBER 2019 1

Electronic emulator of biological load during electroporation

Eva Pirc, Bertrand Balosetti, Damijan Miklavčič and Matej Reberšek
University of Ljubljana, Faculty of Electrical Engineering, Tržaska 25, SI-1000 Ljubljana, SLOVENIA

***Abstract*—Electroporation is an emerging technology, with great potential in any different medical and biotechnological applications, food engineering and biomass production. Large variations of biological load characteristics, however, represent a great challenge in electroporator design, which results in different solutions. Because a medical electroporator is a medical device, it must comply with medical device regulative and standards. However, none of the existing standards directly address the operation or electroporator's performance requirements. In order to evaluate clinical, laboratory and prototype electroporation devices during the development process, or to evaluate their final performance considering at least from the perspective of output pulse parameters, we present a case study on the design of an electronic emulator of biological load during electroporation. The electronic load emulator enables constant and sustainable testing and unbiased comparison of different electroporator's operation. We developed an analog electrical circuit that has equivalent impedance to the beef liver tissue in combination with needle electrodes, during high voltage pulse delivery and or electroporation. Current and voltage measurements during electroporation of beef liver tissue "ex vivo", were analyzed and parametrized to define the analog circuit equation. An equivalent circuit was simulated, built and validated. The proposed concept of electronic load emulator can be used for classical electroporator (i.e. not nanosecond) performance evaluation and comparison of their operation. Additionally, it facilitates standard implementation, regarding the testing protocol and enables quality assurance.**

***Index Terms*—electroporation, tissue conductivity change, standard nonlinear load, biological load, electroporation emulator**

I. Introduction

Electroporation is an emerging platform technology, with great potential [1], [2]. One of the most successful electroporation-based applications in medicine is an anti-tumor therapy, called electrochemotherapy (ECT). Through the permeabilized cell membrane transport of chemotherapeutic drug, bleomycin or cisplatin is increased and considerably higher cytotoxicity is reached [8], [9], [10], [11]. According to Standard Operating Procedure for ECT [14], [15] in clinical practice, mainly eight square wave pulses of amplitudes up to 1000 V, 100 μs pulse duration each and repetition frequency of 1 Hz or 5 kHz are applied to the tissue. A medical device that enables electroporation is called an electroporator, it is a high voltage pulse generator, that generates high voltage pulses of a specific duration, pulse repetition frequency and shape [12], [13]. From the view of an electroporator, the load is a tissue defined by the electrodes [16].

Despite facts, that biological load electric properties vary considerably, tissue has an inhomogeneous composition [17], [18], [19], different electrode type/geometry (different distances between the electrodes) are used, and even though that due to electroporation [20], [21], [22], tissue conductivity changes during the pulse delivery, which results in a non-linear current response of a biological load to a square wave electric pulse [20], [23], [24], [25] the pre-set train of pulses should be generated and delivered to the load, for successful electroporation. Which represent a great challenge in electroporator design that results in different solutions. Problems mainly occur with low resistant loads, or when the pulse repetition rate is in the higher half of the device's operation range, because some pulse generators cannot deliver such high currents or energies on the output [13]. Electroporators are mainly not only pulse generators, but they also have built-in protection systems for current and energy limitation and systems for ensuring the effectiveness of the therapy (for protection of patients, operators, and electronics), the operation at boundary condition of safe operation area may deviate from the expectations. But implementation of protection systems is not straight forward, because at normal operation, high displacement current spikes occur at the beginning of the pulse (due to capacitive nature of biological tissue as the load) and ringing is sometimes present at pulse switching.

For more than a decade our group is evaluating electroporation devices [13], [37], [38] and currently, a rise in electroporation device providers, can be detected on the market. But unfortunately the transitions between devices from different manufacturers, or comparison of results obtained by different researchers using different electroporators, is becoming impossible, due to the pre-programmed electroporation procedures (The researchers do not even even know and therefore also do not report basic pulse parameters (e.g. pulse shape, repetition rate, and voltage amplitude) [26], [27].). The most stressful conditions for an electroporator are short-circuit or discharge between electrodes during the pulse delivery, which can be tested with short-circuiting or very closely placed electrodes. However until now, for the evaluation of an electroporator performance within the safe operation area mainly a resistor or more advanced, a resistor with a capacitance in parallel were used. But those evaluations are considered over-simplifie.

E. Pirc, B. Balosetti, D. Miklavčič and M. Reberšek are with the Laboratory of Biocybernetics, Faculty of Electrical Engineering at University of Ljubljana, Tržaška 25, 1000 Ljubljana, e-mail: matej.rebersek@fe.uni-lj.si

Manuscript received May, 2019; revised.

Since in the case of ECT a typical load is tissue, real tissue or its model could be used [39]. Currently, however, there are no available models on the market, which incorporates inhomogeneous characteristics of tissue or are too expensive, or for single-use and are thus inadequate, therefore some researchers rather use for example, a potato [46], [47], which might be a better solution than a resistor, because at least a conductivity increase (due to electroporation) and heating are present. We think that many problems concerning electroporation devices have evolved due to the lack of standard and evaluation protocol.

In the scope of this paper we present an idea of an analog electrical circuit that emulates beef liver tissue in combination with needle electrodes, undergoing high voltage pulse delivery and/or electroporation. Current and voltage measurements during electroporation of liver tissue *"ex vivo"*, were analyzed and parameterized in a way to fit the proposed analog circuit equation. An equivalent circuit was then simulated, built and validated as a proof of concept. The developed equivalent electronic emulator can be used for electroporators performance evaluation and comparison of the operation between different electroporators, or of an electroporator over its lifetime. The developed device emulates small volume beef liver tissue in combination with needle electrodes (IGEA, Italy). However, the proposed process/concept can be applied to any electroporation measurements and specific emulators can be developed e.g. for more conductive loads, loads with a higher threshold voltage.

II. Methods and Materials

For the development of an electronic emulation circuit, high-quality measurements of current and voltage, during pulse application to the biological tissue are needed. We used *"ex vivo"* measurements, where smaller volume, beef liver tissue, was treated because we do not have so accurate and high sample rate *"in vivo"* measurements. Unfortunately, *"in vivo"* measurements are mainly collected by means of the ECT Cliniporator device, of which sampling frequency is too low to accurately measure current spikes. Additionally, due to muscle contractions (which are triggered with pulse delivery), some noise can be present in *"in vivo"* measurements. Therefore this study is based on measurements of already published research by Langus et. al. [23]. They introduced time-dependent effects into a finite element model developed specifically for electroporation, which is able to predict the time evolution of electric current, within a 5 % error. *"Ex vivo"* experiments on beef liver samples, were done, nine different sequences of electric pulses were delivered to the tissue, by commercially available needle electrodes (IGEA, Carpi, Italy) with a 10 mm of active part, a diameter of 1.2 mm and the distance between the centers of the electrodes being 10 mm. Applied voltage and current were monitored and used for the development of the model. Current and voltage measurements of eight pulses in a sequence, 1000 V, 750 V, and 500 V voltage amplitude with a pulse duration of 100 μs, two different repetition frequencies 1 Hz and 4717 Hz, were re-processed and analyzed in order to model and emulate electroporation load with an analog electric circuit. Measurement data were imported into Matlab R2018b (MathWorks, Natick, Massachusetts, USA) computing environment, where values of optimal circuit elements were calculated and then the circuit was simulated in PSpice AD Lite (OrCAD, Cadence, California, USA). When pulsed electric fields (PEF) are applied to a biological tissue, current response has a typical time course which consists of a fast, high capacitive current, followed by a slight amplitude rise due to tissue electroporation, or more or less square wave pulse, if tissue was not yet electroporated [40], [41], [42] meaning, that applied voltage was lower than electroporation threshold voltage *(Fig. 1a)*.

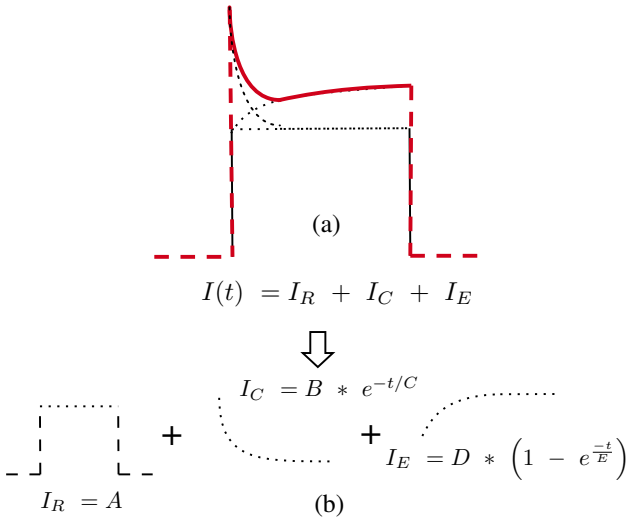


Fig. 1: (a) Typical time course during PEF application to the tissue (red) and three mathematical functions (black); (b) Current is modeled as a sum of a square wave pulse and two exponential functions, by following the equation *(Eq. 1)*

A. Mathematical model

A typical current time course, during PEF application to tissue, is shown in the figure *(Fig. 1a)*, where dynamics associated with pulse rise and fall time have not been included (indicated with the red dashed line). The dynamic of measured current, during the pulse (pulse rise and fall time are not included) can be roughly described as a sum of a square wave pulse and two exponential functions, as presented with the equation *(Eq. 1) (Fig. 1b)*, where t represents time and A, B, C, D, and E are abstract parameters that are in the circuit model substituted by applied voltage and load parameters. Such a form of the equation has been chosen, because it represents a resistive, capacitive response and increase of conductivity due to electroporation, but also because it can be easily converted to an equation, which defines an analog electric circuit.

$$I(t) = A + B * e^{-t/C} + D * (1 - e^{-t/E}), \text{when} \quad 0 < t < T_{pulse} \tag{1}$$

Where T_{pulse} is equal to the duration of the pulse. The first element of the equation, a constant *A*, represents a real (ohmic) part of the current, which is a result of resistive tissue component. But as current in tissue is also reactive, two additional elements are added. The second element $B*e^{-t/C}$, an exponential decay function, represents a capacitive current spike and can be modeled as the capacitor discharge. The third element $D*(1-e^{-t/E})$, an exponential rise function, illustrates a conductivity growth during pulse delivery, which is a result of tissue electroporation (pore formation) and tissue heating [20], [21], [22].

B. Circuit model

The mathematical model *(Eq. 1)* can be easily substituted by a circuit model *(Fig. 2)* with three parallel branches, namely, resistive, capacitive and inductive, which models increase of conductivity due to electroporation *(Eq. 2)*. When the applied voltage is lower than the electroporation threshold voltage, the tissue is not electroporated and therefore the conductivity is roughly constant. In the equivalent circuit *(Fig. 2)* a Zener diode with breakdown voltage equal to the electroporation threshold voltage, enables or disables an inductive/electroporation branch. Additionally, at the end of each applied pulse, the stored energy in the capacitor and inductor will be released and will cause the transient exponential decay current on the trailing edge of the current waveform, resulting in a short negative value current spike. The negative spike is also present in the case of biological load, but we only modeled the dynamics of the pulse to facilitate calculations, therefore we neglected the rise, fall time and post pulse dynamics.

1) Single monopolar pulse model: For a single positive monpolar pulse the following circuit is proposed *(Fig. 2)*.

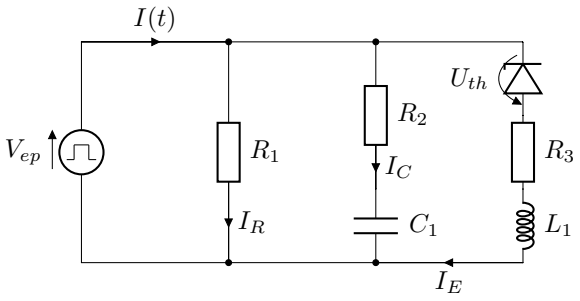


Fig. 2: Single monopolar pulse circuit emulator: equivalent circuit model of tissue during electroporation. Where V_{ep} represent applied electroporation pulse, with pulse voltage amplitude U_{amp} and U_{th} electroporation threshold voltage.

The values of the circuit elements are calculated using the following equations *(Eq. 2, 3)*.

$$I(t) = I_R + I_C + I_E$$

$$I(t) = \frac{U_{amp}}{R_1} + \frac{U_{amp}}{R_2} * e^{-t/\tau_1} + \\ + \frac{U_{amp} - U_{th}}{R_3} * (1 - e^{-t/\tau_2}), \quad \text{when} \quad 0 \quad < t < \quad T_{pulse} \tag{2}$$

$$C_1 = \frac{\tau_1}{R_2} \qquad L_1 = \tau_2 * R_3 \tag{3}$$

Where V_{ep} represents applied electroporation pulse, with pulse voltage amplitude U_{amp} and U_{th} electroporation threshold voltage. For the threshold voltage determination measurements at different applied voltages are required. While all the other values can be determined directly from the mathematical model:

$$R_1 = \frac{U_{amp}}{A}; \qquad R_2 = \frac{U_{amp}}{B} \\ \tau_1 = C; \qquad R_3 = \frac{U_{amp} - U_{th}}{D}; \quad \tau_2 = E \tag{4}$$

This solution is accurate only in case of one pulse application, or when pulse repetition frequency is low (pause between pulses is longer than 1 ms [43]) e.g. in our case 1 Hz, meaning all the applied pulses in one sequence have approximately the same current time course.

For numerical model, all measurement data (described earlier [23]), were imported into Matlab R2018b (MathWorks, Natick, Massachusetts, USA) computing environment, where values of optimal circuit elements were calculated. Five measurements of applied current and voltage for each preset voltage 500 V, 750 V and 1000 V (pulse duration: 100 μs and repetition frequency: 4.717 kHz), were averaged and downsampled for further analyses. The capacitive current spike part was due to its short duration - dynamics decimated by 10 and others by 70, though, in the end, the half of values represented a capacitive current spike and the other half an inductive/electroporation part of the pulse. At first, the real-time conductivity was calculated from applied current and voltage measurement only for the first pulse in sequence. With the Matlab's curve fitting toolbox, optimal parameters for proposed equation *(Eq. 1)* were determined, for each applied voltage separately. Those values were then used as initial values for the least square error (LSE) fit, which was performed for each applied voltage separately. That is how optimal electronic component values were calculated for each applied voltage. Then using by *(Eq. 2)*, LSE regression was done for all three applied voltages together. Due to a large number of variables and three applied voltages, each variable range was predefined. For resistive components and time constants, fifteen linearly distributed values between a minimal and maximal proposed optimal values from individual fit were used. While the threshold voltage was limited between 200 V and 400 V, because in Langus et. al. [23] optimal fitting maximum and minimum electric field magnitude limit were set to 20000 V/m and 40000 V/m . That is how all the values for single monopolar pulse circuit *(Fig. 2)* emulator were determined.

2) Multiple monopolar pulse model: With a higher repetition frequencies, in our case 4717 Hz, it was observed that

the current reaches around 75% of the final amplitude at the first pulse and percentage than exponentially rises with pulses, while all pulses in sequence have the same shape and amplitude in case of 1 Hz repetition frequency [23]. It was shown that for pulse repetition frequencies above 1 kHz a significant change in pulse shape occurs, meaning the following pulses start at higher initial value [43]. Heat generation, diffusion and pore lifetime could explain these phenomena [43], [44], [45]. Therefore in parallel to inductor L_1 an additional resistor R_4 and diode in series are added *(Fig. 3)*. A diode emulates an "electroporation" memory while resistor R_4 emulates loss of memory. For the first pulse in a pulse sequence with higher repetition frequency, the situation is the same as for first/single pulse or pulses with lower repetition frequency *(Fig. 2)*. The electroporation memory is not yet present. During the pulse, the inductor L_1 is magnetized and right after the pulse, during the pause between pulses, the L_1 current in the model regarded as I_{M0} starts to flow through the new resistor R_4 and the inductor is in a demagnetization phase until the next pulse arrival. I_E is still present when the next pulse is generated because the pause between the pulses is shorter than inductor demagnetizing time. Therefore the I_E increases, it is the sum of inductor magnetize current and I_{mem} memory current. The inductor current for second and all other pulses is calculated in accordance with equations *(Eq. 5, 6)*:

$$\mathrm{I}_L(t,N) = (\tfrac{U_{amp}-U_{th}}{R_3} - I_{mem}(N)) * (1 - e^{-(t-(N-1)*T_{per})/\tau_2}) + I_{mem}(N) \quad (5)$$

$$I_{mem}(N) = I_{M0}(N) * e^{-T_{pause}/\tau_3} \quad (6)$$

In which, N is a pulse number, I_{M0} represents a current at the end of the previous pulse, T_{pause} is equal to the duration of the pause between pulses and T_{per} in pulse period ($T_{per} = T_{pulse} + T_{pause}$).

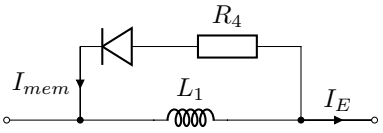


Fig. 3: Additional in series connected resistor R_4 and diode in parallel to the inductor L_1, emulate an "electroporation" memory, which enables that the following pulse starts at the higher initial value.

R_4 which enables memory current was again defined with LSE regression. In this case, the whole sequence, all eight pulses were, included in the calculation. Because only one variable R_4, was left undefined, a range of potential R_4 values was extended, meaning a lot higher number of potential values were included in the regression. Theoretical final circuit emulator *(Fig. 4)* and equations *(Eq. 7, 8, 9, 10)*, are only valid for the time of the pulse ($(N-1) * T_{per} < t < (N-1) * T_{per} + T_{pulse}$), describe well the behaviour of biological load, in our case liver tissue during monopolar ECT pulse application.

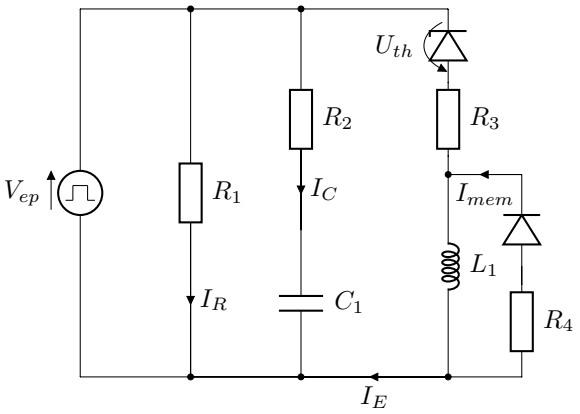


Fig. 4: Final electronic emulator of biological load during electroporation.

$$I(t,N) = \begin{cases} \frac{U_{amp}}{R_1} + \frac{U_{amp}}{R_2} * \\ *e^{-(t-(N-1)*T_{per})/\tau_1}, & U < U_{th} \\ \frac{U_{amp}}{R_1} + \frac{U_{amp}}{R_2} * e^{-(t-(N-1)*T_{per})/\tau_1} + \\ +(\frac{U_{amp}-U_{th}}{R_3} - I_{mem}(N)) * \\ *(1 - e^{-(t-(N-1)*T_{per})/\tau_2}) + \\ +I_{mem}(N), & U > U_{th} \end{cases} \quad (7)$$

$$I_{mem}(N) = I_{M0}(N) * e^{-T_{pause}/\tau_3} \quad (8)$$

$$I_{M0}(N) = \begin{cases} I((N-1) * T_{per} - T_{pause}), & N > 1 \\ 0, & N = 1 \end{cases} \quad (9)$$

$$C_1 = \frac{\tau_1}{R_2}; \qquad L_1 = \tau_2 * R_3; \qquad R_4 = \tau_3 * L_1 \quad (10)$$

Final load current for higher repetition frequencies is described with discrete equation *(Eq. 7)*, where N is the pulse number, T_{per} period (pulse duration + pause). When the applied voltage is lower than the electroporation threshold voltage, the load current consists only of the ohmic and capacitive components. However when the applied voltage is higher than the electroporation threshold voltage, additionally to an ohmic and capacitive component also the inductive/electroporation current is added, which emulates conductivity rise during electroporation *(Eq. 7)*. In the second circuit *(Fig. 4)* an additional memory current, which is pulse number dependent, is added to the inductive component. At the first pulse, the memory current is equal to zero because the inductor L_1 is not yet magnetized. Then with each successive pulse the memory current is higher until it reaches the plateau *(Eq. 8, 9)*, due to inductor magnetization and demagnetization. From the time constant also the last missing components (C_1, L_1 and R_4) are calculated and thus the circuit is fully defined *(Eq. 10)*.

C. Electronic load emulator

Since electronic components with exactly the same values as defined in the numerical model are not available, the prototype's components that have the most similar values, available on the market, were used. The circuit with available

components values was first simulated in PSpice AD Lite (OrCAD, Cadence, California, USA) and the load current compared in Matlab to the modeled and measured current. For the measurements of prototype emulator, oscilloscope (HDO6104A-MS, LeCroy, USA), high voltage differential probe (HVD3206A, LeCroy, USA) and current Hall probe (CP031A, LeCroy, USA) were used. The electric pulses, with the same pulse settings and with the same electroporation device (i.e. ELECTRO cell S20, β-teh, Leroy, France) as in Langus et. al. [23], were applied to the electronic load emulator.

III. Results

A. Numerical calculations

For each applied voltage individual LSE-fit of the mathematical model, gave a precise fit of the time evolution of electric current *(Fig. 5a)*. However the aim was to develop a prototype of electroporation load emulator, therefore the same circuit should be used for all applied voltages. Meaning a fit of a circuit model was done on all three voltages together and a slightly higher deviation from the average measurement was obtained. For both 1000 V and 750 V voltage amplitude, the modeled current is however still within the standard deviation of measurements *(Fig. 5b)*.

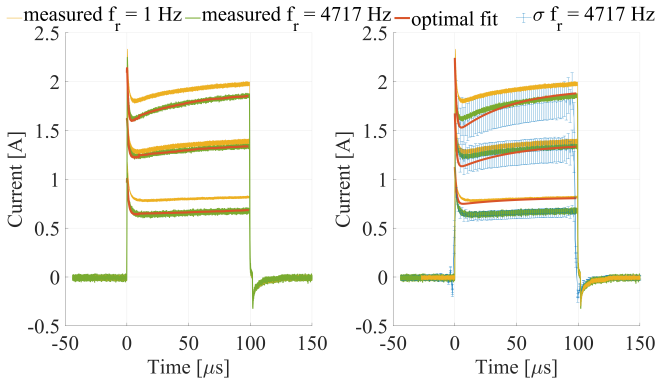


Fig. 5: Average of five current measurements of the first pulse at 1 Hz (yellow) and 4717 Hz (green) repetition frequency. (a) Modeled current (red), optimal circuit parameter values were determined for each applied voltage separately; (b) Modeled current (red), optimal circuit parameter values were determined with the LSE method, all three currents at different voltages were included into the cost function.

The first modeled pulse in a sequence with higher repetition frequency, has the same time course as in a single monopolar pulse model, while all consecutive pulses have higher initial values *(Fig. 6a, first row)*. Matching between measurement and modeled current, slightly decreases with pulse number, but it is still within the standard deviation *(Fig. 5, blue line)* of measurements for 750 V and 1000 V applied voltage amplitude. Optimal values of electronic components for a single-first pulse circuit emulator and for a sequence of pulses with higher repetition frequency are presented in table *(TABLE I)*.

TABLE I: Optimal values of electronic components for the single positive monopolar pulse model are presented in the first line and for multiple monopolar pulse model in the second.

$R_1[\Omega]$	$R_2[k\Omega]$	$R_3[k\Omega]$	$R_4[\Omega]$	$C_1[nF]$	$L_1[mH]$	$U_{th}[V]$
682.4	1.287	1.287	——	0.998	57.5	400
682.4	1.287	1.287	171.1	0.998	57.5	366.7

TABLE II: Final component value table, of components which were used in the PSpice simulation and prototype emulator.

$R_1[\Omega]$	$R_2[k\Omega]$	$R_3[k\Omega]$	$R_4[\Omega]$	$C_1[nF]$	$L_1[mH]$	$U_{th}[V]$
680	1.2	1.2	175	1	57.5	360

B. PSpice model and electronic emulator

With the help of the PSpice model, a prototype emulator was built. Available component values *(TABLE II)* were implemented into PSpice transient analysis. The analysis was run for 1.6 ms. Coil internal resistance was set to measured winded wire resistance 15 Ω. The input voltage, rise time was equal to the average rise time of all applied voltage pulses, 0.358 μs and the same was done also for fall time, which was considerably slower 2.44 μs. The pulse width was set to 99.5 μs and period to 212 μs in order to match the measurements. The PSpice simulation results of the prototype device are presented in the second row of Figure 6.

Zener diode was composed of two Zener diodes in series, one with breakdown voltage 200 V (1N5388B, MULTICOMP) and another with 160 V (1N5384B, MULTICOMP). In order to implement the most suitable inductor, that would optimally operate at the applied pulse frequency, the inductor was manufactured especially for this application. An RM14-type ferrite core made from N41 material grade with 1.9 mm gap (EPCOS/N41/RM14, 160nH, 42.2 x 34.8 x 30.2mm), was winded in a 0.25 mm thick copper wire (number of windings: 600). The inductor was analyzed with an LCR meter (E4980A, Keysight, USA), at 5 kHz the serial inductance was 57.6 mH and serial resistance 15 Ω and at 10 kHz the serial inductance was 57.8 mH and serial resistance 17.6 Ω (The inductor maximal constant current is 150 mA). CREE Silicone Carbide diode C2D05120 was used as a memory diode. Final value list of components built-in a prototype, is given in the table *(TABLE II)*. Measurements of the prototype emulator are presented in the following figure *(Fig. 6c, third row)*. An additional current measurement was added at the applied voltage set to 200 V, in order to present a current response, when the applied voltage is lower than the electroporation threshold voltage.

and in performance summary table *(TABLE III)*

IV. Discussion

The proposed numerical model slightly deviates from the measured values. The best fit was reached for the current measurement at the input voltage 750 V. This may be explained by the fact that the electric field was due to needle electrode use, slightly heterogeneous, meaning that cells that are closer to the

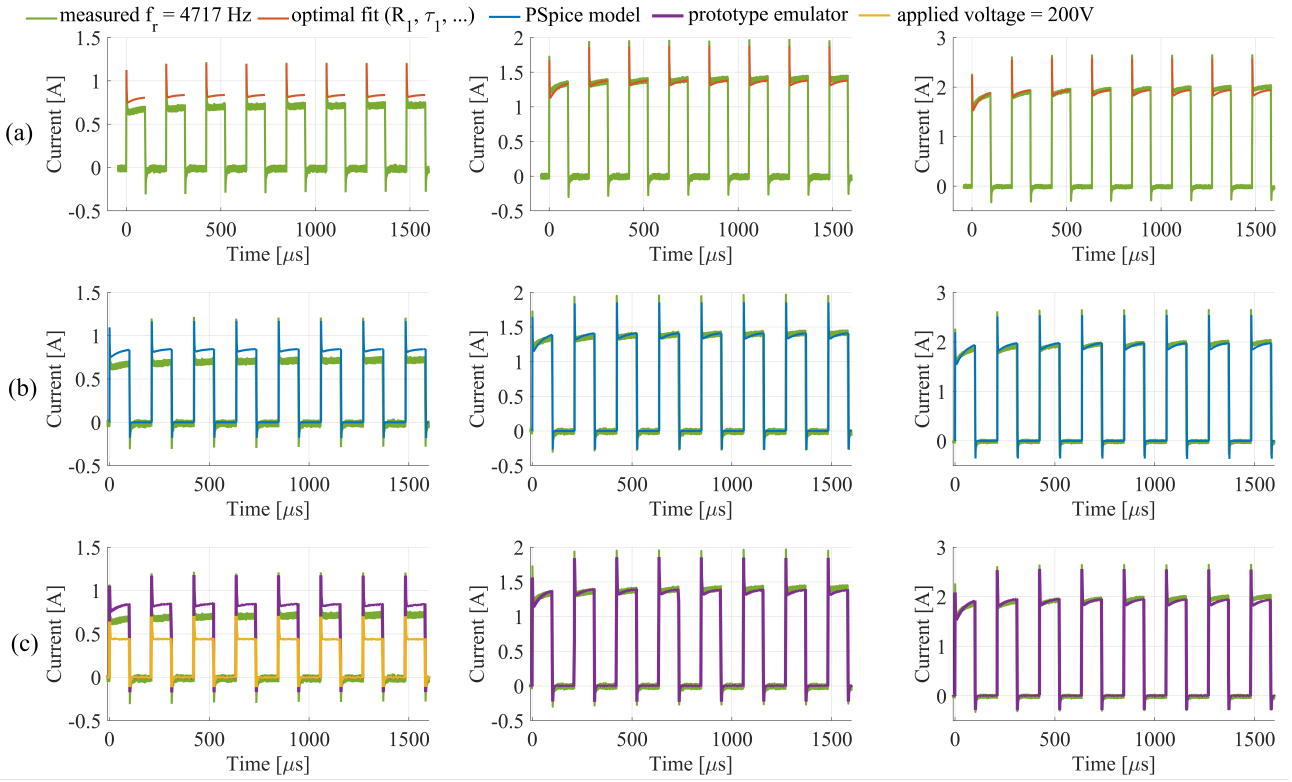


Fig. 6: Average of five current measurements at 4717 Hz repetition frequency (green), (a) numerically modeled current by Matlab (red, first row), (b) modeled current by a PSpice (blue, second row) and (c) measured current trough prototype emulator (purple, third row). An additional current measurement in the case of the emulator was added at the applied voltage set to 200 V, in order to present a current response, when the applied voltage is lower than the threshold voltage. In the first column, the current at 500 V in the second 750 V and third 1000 V applied voltage amplitude is presented.

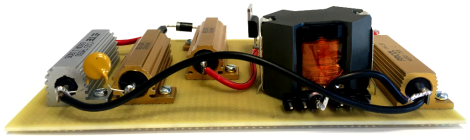


Fig. 7: A physical prototype electronic emulator of biological load during electroporation.

TABLE III: The performance summary table displays the root mean square error between measurements on beef liver and mathematical model ($RMSE_M$), or prototype emulator ($RMSE_E$) or resistor ($RMSE_R$) and mean standard deviation of five measurements on beef liver (σ_{liver}).

SET VOLTAGE REP. F	σ_{liver} $[mA]$	$RMSE_M$ $[mA]$	$RMSE_E$ $[mA]$	$RMSE_R$ $[mA]$
500 V, 1 Hz	38.5	40.3	38	101.1
500 V, 4717 Hz	46.3	125.8	132.1	101.4
750 V, 1 Hz	115.1	111.6	106.2	72.6
750 V, 4717 Hz	124.7	41.4	40.7	471.4
1000 V, 1 Hz	116.1	192.7	189.5	131.6
1000 V, 4714 Hz	177	55.7	58.1	705.7

needles, electroporate at lower voltages. In the case of plate electrodes, we believe the fit would be even better, because of a more homogeneous electric field distribution. Also the negative spike at the trailing edge of the current waveform was not modeled. The biggest challenge in the post pulse calculations is the impedance of the pulse generator. Almost every time, after pulse generation, the impedance is changed (due to the turn-off, of the switch in electroporator) and cannot be defined from the current and voltage measurements, due to high noise and low voltage.

PSpice model and the current measurement of the prototype emulator match finely. A Zener diode that defines the electroporation threshold voltage behaves as expected and therefore enables simulation of electric current before and during electroporation. Meaning the proposed mathematical, numerical and circuit models are an appropriate method for the development of an electronic emulator of biological load during electroporation. The performance summary table *(TABLE III)* presents the root mean square error values, between the measurements on beef liver and mathematical model or prototype emulator or resistor and mean standard deviation of five measurements on beef liver. For the comparison of operation, the optimal resistance value from E24 resistance value list was selected. The minimal square error criteria was used and the value of 560 Ω gave the best match. Additionally, to the three main features (capacitive spike, rise of conductivity during the pulse and electroporation memory) the developed emulator in comparison to the optimal resistor has significantly

lower RMSE. Preliminary results show that the presented circuit model could be upgraded for electroporation pulses of both polarities, named also biphasic or bipolar pulses. The last branch in the circuit should be doubled and mirrored as shown in *(Fig. 8)*. Current measurements of both polarities should be however analyzed in detail in order to determine the relation between both inductors. If there is a memory present also between a positive and negative pulse inductors should be coupled.

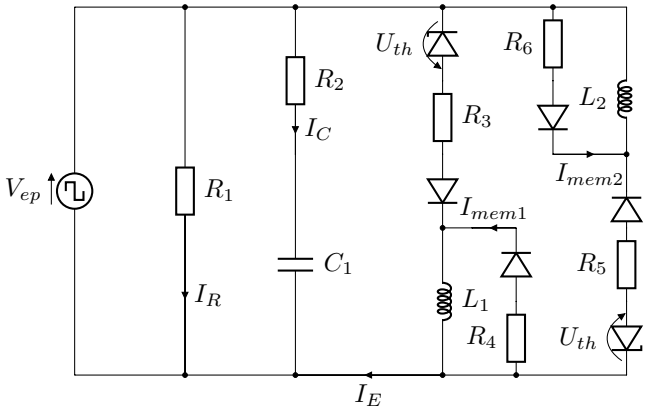


Fig. 8: Proposed emulator upgrade for electroporation with both polarity pulses, but additional current measurements should be analyzed in detail, to determine the relation between both inductors (L_1, L_2).

The solution, presented in this paper, presents a novel approach, which enables repeatable and unbiased testing and evaluation of electroporator performance. However, the proposed emulator has to be adjusted for each biological load specifically i.e. cell-suspension in cuvettes, plate electrodes, electrodes for a skin treatment or needle electrodes for deep-seated tumors. Therefore, for each biological load, a separate analysis should be done. At first, we have to collect accurate measurements, with a high sample rate, and than parametrization in accordance with this paper should be done. Finally, the circuit components should be modified accordingly to the newly calculated values. But from the prospect of standardization or evaluation and comparison of different electroporation devices, each of the components in the proposed circuit could be replaced with an adjustable value component. A series of a different capacitor and Zener diode modulation would incorporate flexibility and provide one versatile emulator. The maximal voltage and current of an emulator are defined with the maximal voltage and current of the selected components and the frequency is limited by the selected inductor. Characteristics of the ferrite core are frequency dependent and in case of saturation the inductor would not work as desired. The study in the paper is focused only on "classical" electroporation pulses. However, recently a much broader range of pulse duration is investigated and therefore the frequency spectrum of induced transmembrane potential is getting more attention [49]. With lower frequencies, the proposed concept can be used, but in case of higher frequencies, actual physical components should be thoughtfully selected with minimal parasitic values, or if not possible, the parasitic contributions should be added to the PSpice model. Also, a switch from circuital to distributed models of pulse delivery would be needed.

V. Conclusion

The proposed concept of electronic emulator of biological load during electroporation, accurately symulatethe current during electroporation. It is a proof of concept with huge potential. For each specific biological load, first voltage and current measurements should be made and a specific model should be however developed. But from the prospect of standardization or evaluation and comparison of different electroporation devices, each of the components in the proposed circuit could be replaced with an adjustable value component. A series of a different capacitor and Zener diode modulation would incorporate flexibility and provide one versatile emulator. However additional measurements on different biological loads and different electrodes should be done and analyzed for this purpose. The main innovation of the presented process/concept of an emulator is that it enables sustainable, repeatable and unbiased testing, therefore, it facilitates standard implementation. Additionally, the emulator can be used as a load during the development of an electroporator, which facilitates the development process. We believe this is the first step towards the development of an electroporator tester. Such devices are well known and indispensable in the defibrillation field [50]. Even more in case of a defibrillator, a load is well known and electrodes are well defined, while in case of electroporation the same device can be used with different electrodes and load can vary from the skin to liver, or even vegetable tissue.

Acknowledgment

This study was supported by the Slovenian Research Agency (ARRS) (MRIC UL IP-0510, P2-0249, funding for Junior Researchers to Eva Pirc). The authors would like to thank Anja Zajc for the help with coil winding and to the agreement between Esigelec and the University of Ljubljana, which enabled Bertrand Balosetti's visit to the University of Ljubljana.

References

[1] M. L. Yarmush, A. Golberg, G. Serša, T. Kotnik, and D. Miklavčič, "Electroporation-based technologies for medicine: principles, applications, and challenges," *Annu Rev Biomed Eng*, vol. 16, pp. 295–320, Jul. 2014.

[2] D. Miklavčič, "Network for development of electroporation-based technologies and treatments: COST TD1104," *J. Membr. Biol.*, vol. 245, no. 10, pp. 591–598, Oct. 2012.

[3] J. Teissie, M. Golzio, and M. P. Rols, "Mechanisms of cell membrane electropermeabilization: A minireview of our present (lack of ?) knowledge," *Biochimica et Biophysica Acta (BBA) - General Subjects*, vol. 1724, no. 3, pp. 270–280, Aug. 2005.

[4] J. C. Weaver, "Electroporation: a general phenomenon for manipulating cells and tissues," *J. Cell. Biochem.*, vol. 51, no. 4, pp. 426–435, Apr. 1993.

[5] T. Kotnik, Rems, Lea, Munir, Tarek, and D. Miklavčič, "Membrane electroporation and electropermeabilization: mechanisms and models," *Annu. Rev. Biophys*, vol. 48, 2019.

[6] M. Kranjc, F. Bajd, I. Sersa, and D. Miklavcic, "Magnetic Resonance Electrical Impedance Tomography for Monitoring Electric Field Distribution During Tissue Electroporation," *IEEE Transactions on Medical Imaging*, vol. 30, no. 10, pp. 1771–1778, Oct. 2011.

[7] S. Corovic, I. Lackovic, P. Sustaric, T. Sustar, T. Rodic, and D. Miklavcic, "Modeling of electric field distribution in tissues during electroporation," *BioMedical Engineering OnLine*, vol. 12, no. 1, p. 16, Feb. 2013.

[8] D. Miklavcic and R. V. Davalos, "Electrochemotherapy (ECT) and irreversible electroporation (IRE) -advanced techniques for treating deep-seated tumors based on electroporation," *Biomed Eng Online*, vol. 14, no. Suppl 3, p. I1, Aug. 2015.

[9] L. M. Mir, S. Orlowski, J. Belehradek, and C. Paoletti, "Electrochemotherapy potentiation of antitumour effect of bleomycin by local electric pulses," *European Journal of Cancer and Clinical Oncology*, vol. 27, no. 1, pp. 68–72, Jan. 1991.

[10] G. Serša, D. Miklavcic, M. Cemazar, Z. Rudolf, G. Pucihar, and M. Snoj, "Electrochemotherapy in treatment of tumours," *European Journal of Surgical Oncology (EJSO)*, vol. 34, no. 2, pp. 232–240, Feb. 2008.

[11] B. Mali, T. Jarm, M. Snoj, G. Serša, and D. Miklavčič, "Antitumor effectiveness of electrochemotherapy: A systematic review and meta-analysis," *European Journal of Surgical Oncology (EJSO)*, vol. 39, no. 1, pp. 4–16, Jan. 2013.

[12] M. Reberšek, D. Miklavčič, C. Bertacchini, and M. Sack, "Cell membrane electroporation-Part 3: the equipment," *IEEE Electrical Insulation Magazine*, vol. 30, no. 3, pp. 8–18, May 2014.

[13] E. Pirc, M. Reberšek, and D. Miklavčič, "12 Dosimetry in Electroporation-Based Technologies and Treatments," in *Dosimetry in Bioelectromagnetics, M. Markov, Ed. CRC Press, Taylor & Francis Group*, pp. 233–268, 2017.

[14] L.M. Mir, Gehl J, Sersa G, et al. "Standard operating procedures of the electrochemotherapy: Instructions for the use of bleomycin or cisplatin administered either systemically or locally and electric pulses delivered by the CliniporatorTM by means of invassive or non-invasive electrodes," *European Journal of Cancer Supplements*, vol. 4, pp. 14–24, 2006.

[15] J. Gehl et al., "Updated standard operating procedures for electrochemotherapy of cutaneous tumours and skin metastases," *Acta Oncol.*, vol. 57, no. 7, pp. 874–882, Jul. 2018.

[16] M. Puc, S. Reberšek and D. Miklavčič, "Requirements for a clinical electrochemotherapy device - Electroporator", *Radiology and Oncology*, vol 31, pp.368-73, 1997.

[17] A. Ivorra, "Tissue Electroporation as a Bioelectric Phenomenon: Basic Concepts," in *Irreversible Electroporation, B. Rubinsky, Ed. Berlin, Heidelberg: Springer Berlin Heidelberg*, pp. 23–61, 2010.

[18] P. J. Canatella, M. M. Black, D. M. Bonnichsen, C. McKenna, and M. R. Prausnitz, "Tissue Electroporation: Quantification and Analysis of Heterogeneous Transport in Multicellular Environments," *Biophys J*, vol. 86, no. 5, pp. 3260–3268, May 2004.

[19] K. T. Powell, A. W. Morgenthaler, and J. C. Weaver, "Tissue electroporation. Observation of reversible electrical breakdown in viable frog skin.," *Biophys J.*, vol. 56, no. 6, pp. 1163–1171, Dec. 1989.

[20] D. Sel, D. Cukjati, D. Batiuskaite, T. Slivnik, L. M. Mir, and D. Miklavčič, "Sequential finite element model of tissue electropermeabilization," *IEEE Trans Biomed Eng.*, vol. 52, no. 5, pp. 816–827, May 2005.

[21] A. Ivorra, B. Al-Sakere, B. Rubinsky, and L. M. Mir, "In vivo electrical conductivity measurements during and after tumor electroporation: conductivity changes reflect the treatment outcome," *Phys. Med. Biol.*, vol. 54, no. 19, p. 5949, 2009.

[22] R. E. Neal II, P. A. Garcia, J. L. Robertson, and R. V. Davalos, "Experimental Characterization and Numerical Modeling of Tissue Electrical Conductivity during Pulsed Electric Fields for Irreversible Electroporation Treatment Planning," *IEEE Transactions on Biomedical Engineering*, vol. 59, no. 4, pp. 1076–1085, Apr. 2012.

[23] J. Langus, M. Kranjc, B. Kos, T. Šuštar, and D. Miklavčič, "Dynamic finite-element model for efficient modelling of electric currents in electroporated tissue," *Sci. Rep.*, vol. 6, p. 26409-23, 2016.

[24] M. Pintar et al., "Time-Dependent Finite Element Analysis of In Vivo Electrochemotherapy Treatment," *Technol. Cancer Res. Treat.*, vol. 17, p. 1-9, Jan 2018.

[25] D. Cukjati, D. Batiuskaite, F. André, D. Miklavčič, and L. M. Mir, "Real time electroporation control for accurate and safe in vivo non-viral gene therapy," *Bioelectrochemistry*, vol. 70, no. 2, pp. 501–507, May 2007.

[26] L. Chicaybam et al., "An Efficient Electroporation Protocol for the Genetic Modification of Mammalian Cells," *Front Bioeng Biotechnol*, vol. 4, Jan. 2017.

[27] N. Iversen, B. Birkenes, K. Torsdalen, and S. Djurovic, "Electroporation by nucleofector is the best nonviral transfection technique in human endothelial and smooth muscle cells," *Genetic Vaccines and Therapy*, vol. 3, no. 1, p. 2, Apr. 2005.

[28] M. A. Elgenedy, A. Darwish, S. Ahmed, and B. W. Williams, "A Transition Arm Modular Multilevel Universal Pulse-Waveform Generator for Electroporation Applications," *IEEE Transactions on Power Electronics*, vol. 32, no. 12, pp. 8979–8991, Dec. 2017.

[29] V. Stankevič et al., "Electroporation system generating wide range square-wave pulses for biological applications," in *2013 IEEE Biomedical Circuits and Systems Conference (BioCAS)*, pp. 33–36, 2013.

[30] T. F. Cronjé and P. T. Gaynor, "High voltage and frequency bipolar pulse generator design for electroporation-based cancer therapy," in *2013 Australasian Universities Power Engineering Conference (AUPEC)*, pp. 1–7, 2013.

[31] M. Reberšek, E. Pirc, D. Miklavčič, and P. Kramar, "Modeling and Optimization of Blumlein Nanosecond Pulse Generator for Experiments on Planar Lipid Bilayers," in *14th International Conference on Synthesis, Modeling, Analysis and Simulation Methods and Applications to Circuit Design (SMACD)*, pp. 1–4, 2017.

[32] H. Shagoshtasbi, K. Riaz, Y.- Lee, and K. Tse, "Smartphone-based electroporator system for micro/nano electroporation chips," in *10th IEEE International Conference on Nano/Micro Engineered and Molecular Systems*, pp. 72–75, 2015.

[33] M. A. Schmitt, O. Friedrich, and D. F. Gilbert, "Portoporator©: A portable low-cost electroporation device for gene transfer to cultured cells in biotechnology, biomedical research and education," *Biosensors and Bioelectronics*, vol. 131, pp. 95–103, Apr. 2019.

[34] D. B. Kramer, S. Xu, and A. S. Kesselheim, "Regulation of Medical Devices in the United States and European Union," *New England Journal of Medicine*, vol. 366, no. 9, pp. 848–855, Mar. 2012.

[35] J. L. Martin, B. J. Norris, E. Murphy, and J. A. Crowe, "Medical device development: The challenge for ergonomics," *Applied Ergonomics*, vol. 39, no. 3, pp. 271–283, May 2008.

[36] M. Cheng and W. H. Organization, "Medical Device Regulations: Global Overview and Guiding Principles". *World Health Organization*, 2003.

[37] M. Puc, S. Corović, K. Flisar, M. Petkovsek, J. Nastran, and D. Miklavčič, "Techniques of signal generation required for electropermeabilization. Survey of electropermeabilization devices", *Bioelectrochemistry*, vol. 64, no. 2, pp. 113–124, Sep. 2004.

[38] M. Reberšek and D. Miklavčič, "Chapter 16. Concepts of Electroporation Pulse Generation and Overview of Electric Pulse Generators for Cell and Tissue Electroporation" in Advanced Electroporation Techniques in Biology and Medicine, (Ed. A.G. Pakhomov, D. Miklavčič and M.S. Markov), *CRC Press*, Boca Raton, pp. 323-339, 2010.

[39] A. Arslan-Yildiz, R. El Assal, P. Chen, S. Guven, F. Inci, and U. Demirci, "Towards artificial tissue models: past, present, and future of 3D bioprinting," *Biofabrication*, vol. 8, no. 1, p. 014103, Mar. 2016.

[40] M. Kranjc, F. Bajd, I. Serša, and D. Miklavčič, "Magnetic resonance electrical impedance tomography for measuring electrical conductivity during electroporation," *Physiol. Meas.*, vol. 35, no. 6, pp. 985–996, Jun. 2014.

[41] A. Ivorra and Boris Rubinsky. "In Vivo Electrical Impedance Measurements during and after Electroporation of Rat Liver," *Bioelectrochemistry*, vol. 70, no. 2, 287–95, May 2017.

[42] A. Ivorra, B. Al-Sakere, B. Rubinsky, and L. M. Mir. "In Vivo Electrical Conductivity Measurements during and after Tumor Electroporation: Conductivity Changes Reflect the Treatment Outcome," *Physics in Medicine and Biology*, vol. 54, no. 19, 5949–63, Oct.2009.

[43] M. Pavlin, and D. Miklavcic, "Theoretical and experimental analysis of conductivity, ion diffusion and molecular transport during cell electroporation–relation between short-lived and long-lived pores," *Bioelectrochemistry*, vol. 74, no. 1, pp. 38–46, 2008.

[44] I. Lacković, R. Magjarević, and D. Miklavčič, "Three-dimensional Finite-element Analysis of Joule Heating in Electrochemotherapy and in vivo Gene Electrotransfer," *IEEE Transactions on Dielectrics and Electrical Insulation*, vol. 16, no. 5, pp. 1338–1347, Oct. 2009.

[45] J. V. Stern, T. R. Gowrishankar, K. C. Smith, and J. C. Weaver, "Broad pore lifetime distributions: A fundamental concept for cell electroporation," *arXiv:1708.07613 [physics]*, Aug. 2017.

[46] 4 Y. Zhao, H. Liu, S. P. Bhonsle, Y. Wang, R. V. Davalos, and C. Yao, "Ablation outcome of irreversible electroporation on potato monitored by impedance spectrum under multi-electrode system," *Biomed Eng Online*, vol. 17, Sep. 2018.

[47] A. H. Ruarus, L. G. P. H. Vroomen, R. S. Puijk, H. J. Scheffer, T. J. C. Faes, and M. R. Meijerink, "Conductivity Rise During Irreversible Electroporation: True Permeabilization or Heat?," *Cardiovasc Intervent Radiol*, vol. 41, no. 8, pp. 1257–1266, 2018.

[48] L. G. Campana, M. Cesari, F. Dughiero, M. Forzan, M. Rastrelli, C. R. Rossi, and others, "Electrical Resistance of Human Soft Tissue Sarcomas: An Ex Vivo Study on Surgical Specimens", *Medical & Biological Engineering & Computing*, vol. 54, no. 5, pp. 773–87, 2016.

[49] C. Merla, A. G. Pakhomov, I. Semenov, and P. T. Vernier, "Frequency spectrum of induced transmembrane potential and permeabilization efficacy of bipolar electric pulses," *Biochimica et Biophysica Acta (BBA) - Biomembranes*, vol. 1859, no. 7, pp. 1282–1290, Jul. 2017.

[50] V. K. Sharma, Y. C. Chopra, J. S. Bajwa, B. S. Sohi, and R. K. Tiwari, "A reliable microprocessor-based defibrillator analyzer," *IEEE Transactions on Instrumentation and Measurement*, vol. IM–31, no. 1, pp. 28–31, Mar. 1982.

3.6 Paper 5

Title: **Nanosecond Pulse Electroporator with Silicon Carbide MOSFETs, Development and Evaluation**

Authors: Eva Pirc, Damijan Miklavčič and Matej Reberšek

Publication: *IEEE Transactions on Biomedical Engineering*, vol. 66, no. 12, pp. 3526 - 3533

Year: 2019

DOI: 10.1109/TBME.2019.2907165

Impact factor: 4.491 (2018)

Quartile: Q1 (engineering, biomedical)

Rank: 11/80 (engineering, biomedical)

3526 IEEE TRANSACTIONS ON BIOMEDICAL ENGINEERING, VOL. 66, NO. 12, DECEMBER 2019

Nanosecond Pulse Electroporator With Silicon Carbide MOSFETs: Development and Evaluation

Eva Pirc, Damijan Miklavčič, and Matej Reberšek

***Abstract*—Nanosecond electroporation of cell organelles is being studied since more than a decade, but it is still not entirely understood. Unique prototype hardware equipment and challenging measuring methods may also be a contributing reason for this situation. In the scope of this paper, we improve the performance of the high-voltage nanosecond pulse generator by introducing silicon carbide (SiC) MOSFETs. We developed a new high-voltage diode opening switch (DOS)—nanosecond pulse generator for laboratory use for *in vitro* experiments in electroporation cuvettes. Analysis and comparison of the most commonly used switching technologies in pulse generators were made. The device is designed by two parallel two-stage Marx-bank circuits with SiC MOSFETs that generates up to 200 A in the resonant network. A driving circuit for stable simultaneous switching of SiC MOSFETs was developed. The developed generator can deliver from 500 V to more than 6 kV, approximately 8 ns pulses to a 50 Ω load. Even though the amplitude of the output pulse is not as high as expected, the multiplication factor V_{OUT}/V_{IN} is still approximately 9, which is an improvement compared to the previously published linear DOS generator. Measurement and evaluation process is described in detail. Additionally, we emphasize on the size of an error that occurs during measurements.**

***Index Terms*—Electroporator, nanosecond pulse generator, silicon carbide power MOSFET, diode opening switch (DOS), nanosecond pulse measurement.**

I. Introduction

ELECTROPORATION is a technique in which electric pulses are applied to tissue or cells, in order to increase permeability of their membranes [1]–[3]. In case of reversible electroporation the membrane becomes permeable to molecules, that otherwise cannot pass the barrier and can subsequently fully recover, opposed to the main goal of irreversible electroporation that is cell death. Electroporation is already established in medicine and biotechnology [4], [5], electrochemotherapy (ECT) is used to introduce chemotherapeutic drug into cells [6]–[8] and IRE (irreversible electroporation) to induce cell death, thus to ablate tissue [9], [10]. Overall electroporation is a technique with great potential [5], [11]–[16]. Applied electric pulses induce voltage across the cell membrane that is pulse duration and amplitude dependent. The same amount of electroporated cells can be reached, with shorter pulse duration, if higher pulse amplitude is used [17]. The shorter the pulses are, the easier they "penetrate" into cell interior and permeabilize internal membranes of organelles [18]. The application requires electroporator to generate and electrodes to deliver electrical pulses to tissue or cells. An electroporator is a high-voltage pulse generator that generates pulses of specific shape, amplitude, duration, number and pulse repetition rate [19], [20]. It is very important to adjust pulse parameters to specific tissues, cell types, applications and desired outcomes. Most of the devices available on the market generate pulses with lengths from 10 μs to 10 ms, at an electric field strength in the range of several hundreds of volts per centimeter. Pulses from 100 to 900 μs duration, with similar electric field strengths are widely used for electrochemotherapy. Nanosecond pulses (4–600) ns long, with electric field strengths of several tens of kilo-volts per centimeter, are able to affect membranes of internal organelles [21]–[23].

There are five major concepts of electroporator design, capacitor discharge, square wave pulse generator, analog generators, pulse forming network, and diode opening switches [19], [20]. First three concepts are mostly, used to generate micro- and milli- second pulses. For nanosecond pulse generation pulse forming networks or transmission lines and DOS (Diode Opening Switch) are more appropriate solutions. All pulse power devices as well as electroporators work in two phases: charge and discharge. During charging period switches are turned off and transmission lines or capacitors are charged. When the switches are turned on, capacitors discharge to the output load [19].

In electroporation device design, we are looking for switching elements that generate maximum load current in this short on stage and are at the same time capable of fast synchronous reproducible, switching at high-voltages and currents. Semiconductor switching elements that were most often used in nanosecond pulse electroporators are radio-frequency (RF) MOSFETs in pulse-forming network concepts and power MOSFETs or IGBTs in DOS concepts. RF MOSFETs enable fast switching while

Manuscript received July 10, 2018; revised November 20, 2018 and January 12, 2019; accepted March 15, 2019. Date of publication March 25, 2019; date of current version November 20, 2019. This work was supported by the Slovenian Research Agency (ARRS) (MRIC UL IP-0510, P2-0249, funding for Junior Researchers to E. Pirc, bilateral project BI-FR/16-17-PROTEUS), and conducted within the scope of the European Associated Laboratory on the Electroporation in Biology and Medicine (LEA-EBAM). *(Corresponding author: Matej Reberšek.)*

E. Pirc and D. Miklavčič are with the Faculty of Electrical Engineering, University of Ljubljana.

M. Reberšek is with the Faculty of Electrical Engineering, University of Ljubljana, Ljubljana SI-1000, Slovenia (e-mail: matej.rebersek@fe.uni-lj.si).

Digital Object Identifier 10.1109/TBME.2019.2907165

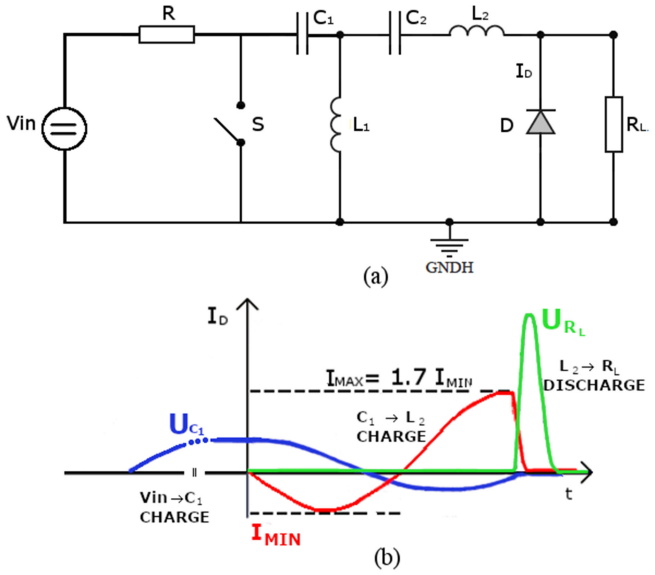


Fig. 1. Basic schematics of Diode Opening Switch – DOS and its operation. Diode D with a good repeatability transfers energy saved in the second inductor L_2 to the load R_L (a). Diode must be forward and reverse pumped, with adequate anharmonic current I_D and should stop conducting when all energy is stores in L_2, at this time a maximal reverse current through the diode I_{MAX} is equal to $1.7 * I_{MIN}$. If all the conditions are met, a short high-voltage pulse U_{RL} is induced on the load (b).

power MOSFETs and IGBTs are slower, but enable high currents. Between semiconductor materials that are used for construction of switching elements that work from 1200 V to 6500 V, which is area of our interest, wide energy band-gap materials such as silicon carbide (SiC) are more and more commonly used [24], [25]. Elements that are based on wide energy band-gap materials, have much lower leakage currents, higher operating temperatures and better radiation hardness. Therefore higher critical electric fields can be reached, consequently blocking layers can be thinner and with higher doping concentration. Therefore, SiC MOSFETs also have lower on-resistance and electrons in saturation can reach higher velocities, which results in higher operating frequencies. Additionally good thermal conductivity enables operation at higher power densities [24], [26]. However, modest transconductance and short channel effects are important to be considered, when using the device. Due to their excellent properties, SiC MOSFETs are replacing silicon IGBTs, mainly because of their faster switching times, and power MOSFETs, mainly because of their lower on-resistance.

DOS is a commonly used concept for a few nanosecond scale pulse generation, because amplitudes can reach up to several kilo-volts. The circuit operates so that the diode with a good repeatability transfers energy saved in the second inductor L_2, to the load (Fig. 1). In the first, charging period of the generation, the capacitor (C_1) is charged through the resistor (R) by DC voltage supply (V_{IN}). In the beginning of the second discharging period, the switch (S) is turned ON and the energy in the capacitor (C_1) starts circulating in the resonant network (C_1, C_2, L_1, L_2) [27]. If the diode matrix (D) is short-circuited, an aharmonic oscillation of the current through the inductor L_2 is observed. If the D is not short-circuited, the resonant network firstly pumps current through the diodes. During the forward pumping, charge is accumulated in the diodes PN-junctions [27], which is proportional to the amplitude of the current and the minority carrier lifetime. After completing the half period, the resonant network starts pumping the current through the diodes in the reverse direction. With the charge still present in the PN-junctions, the diodes resume conducting until the accumulated charge is removed. The time which takes to remove the charge is inversely proportional to the amplitude of the current. The ideal DOS abruptly stops conducting and commutates all the L_2 current into the load. The resonant network must be designed in a way that the majority of the energy, firstly stored in C_1, is stored in L_2, during the current commutation. This is achieved by doubling the reverse current in comparison to the forward current. During the current commutation the inductor L_2 induces high-voltage pulse on the load in order to maintain the current flow, which is proportional to the $R_L * I_{MAX}$, where R_L is the resistance of the load and I_{MAX} is a maximal reverse current through the diodes. The induced voltage on the load is normally much higher than the charging voltage and the ratio between them is determined by a multiplication factor. The inductors in the resonant network can have ferromagnetic saturable cores which improves the multiplication factor but the generator then operates nonlinearly and only in a small range of output voltages [28]. In contrast, the air-core inductors achieve lower multiplication factor but work linearly from zero voltage to the saturation of semiconductor switches [27].

Most of existing nanosecond pulse generators used for electroporation experiments are based on the pulse forming networks and spark gaps or RF MOSFET switches [22], [29]–[33]. Some DOS pulse generators for electroporation experiments have already been developed and have performed well [23], [22]. Among others, Sanders [27] developed a 5 kV 2.5 ns nanosecond DOS electroporator, Kuthi developed a 1200V 3.5 ns generator [28] and some simulations of the DOS generators have also been done by Kranjc [34].

Measurement of micro- and milli- second electroporation pulses is relatively straightforward as pulse reflections are normally negligible. However, with high-voltage nanosecond pulses, measurement becomes more challenging [23], [35]. Firstly, the electric losses and reflections in the wiring between the generator and the load are no longer negligible, therefore, the probe should be connected to the wiring in electrical proximity to the load. The commercial high-voltage nanosecond probes are costly and useful only in specific setups. Some probes may be incorporated into transmission lines [36] or electrodes [37], other setups may use 50 Ω probes [38] and some must use high-impedance probes [39]. High-voltage nanosecond probes can be made of wide-band resistive voltage divider [39], D-dot sensor [37], 50 Ω probe [38] or capacitive voltage divider [36].

Nanosecond electroporation is being studied for more than a decade, nevertheless the trend still clearly shows increased interest into effects of nanosecond pulses. It is also believed that further investigations of nanosecond electroporation of cells will gain important knowledge necessary for better understanding of

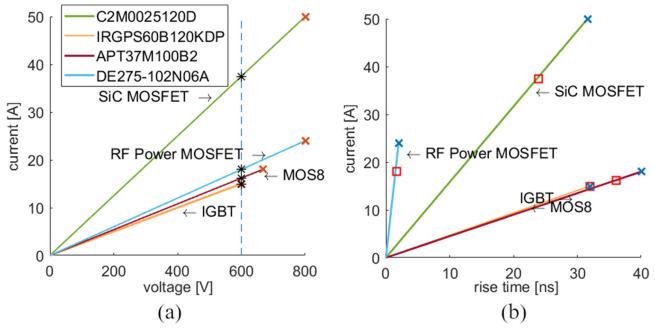


Fig. 2. Comparison of different semiconductor switching technologies which are used for generating nanosecond pulses. Compared are the amplitude (a) and the rise time (b) of the current through the switching element, derived from the data sheets. Currents generated by the switching elements are by linear approximation normalized to the same 600 V supply voltage (red star dots).

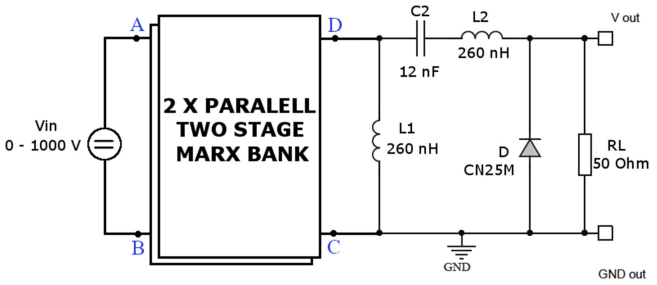


Fig. 3. Basic schematic of DOS generator with two parallel two-stage Marx- banks. Two-stage Marx-bank is used to deliver double voltage to the resonant network in comparison to the breaking voltage of the switch and power supply voltage. Two Marx-banks are used in parallel to deliver higher current to the resonant network. Resonant network (C and L) and DOS (D) are the same as in Sanders linear DOS generator [27].

dynamics and transport in classical electroporation [40]. Some studies of basic electroporation principles and nanosecond electroporation are still not confirmed or are even contradictory [23]. In case of nanosecond electroporation this can be a result of unique prototype hardware devices, with narrow scope of parameters, incorrect application, or even an incomplete description of used hardware and delivered pulse measurement and characterization [35] or inappropriate measurement equipment or inadequate delivery system. High-voltage nanosecond pulse generators for laboratory use with reproducible pulse delivery of wide range of parameters, as well as accurate high-voltage nanosecond measurement systems are needed.

II. Methods and Materials

In nanosecond pulse electroporator design, the main challenge is, to construct a generator that delivers very short and at the same time very high power pulses. The development started with the analysis and comparison between the most commonly used semiconductor switching technologies in pulse generators and silicon carbide (SiC) MOSFET technology (Fig. 2). The comparison was made with switching elements of similar breakdown voltages and sizes, with less than 50 ns rise time, namely DE275-102N06A (RF MOSFET, IXYS, USA), APT37M100B2 (Power MOSFET, Microsemi, USA), IRGPS60B120KDP (IGBT, Infineon, USA) and C2M0025120D (SiC MOSFET, CREE, USA). We have compared the rise time, specified in data sheet and the current amplitude at fixed supply voltage. As different manufacturers test their elements at different supply voltages, we have at first by normalized linear approximation, define test current at rise time data acquisition, which was 600 V (Fig. 2a). SiC technology stand out by the current amplitude. However, with the nanosecond pulses we are also interested in the rate of the current increase. Therefore, we plotted the normalized test current amplitude on the rise time axis (Fig. 2b) on which it can be observed that the current in the SiC MOSFET rises slower than in the RF MOSFET and quicker than in the power MOSFET or IGBT.

A. Pulse Generator Design

Further on we focused on DOS generator, which we believe is the most suitable topology for SiC MOSFET based nanosecond pulse generators. With the advantage of using SiC MOSFETs we aimed at developing linear DOS generator that could be used to deliver short nanosecond pulses with wide range of high-voltage amplitudes to the cuvettes. To achieve such high-voltages maximization of I_{MAX}, which is proportional to the $V_{IN} * \sqrt{C_1/L_2}$, is required. However only V_{IN} can be maximized in this equation, as L_2 defines the pulse duration, which should be optimized in accordance with desired biological effect and not for the output voltage. And as C_1 should be calculated so that the reverse recovery time, of the diodes is equal to the $\frac{\pi}{2}\sqrt{C_1 * L_2}$ which consequently enables generation of the output pulse at I_{MAX}. V_{IN} is however, always limited by the breakdown voltage of the switching elements. Therefore, in order to increase the V_{IN}, stacking of the switching elements in series is required. Not to increase the power supply voltage (V_{PS}), a two-stage Marx-bank circuit was used, to stack the switches and two Marx-banks were used in parallel to generate double current in the resonant network (Fig. 3), in comparison to the Sanders linear DOS generator [27]. As we used the same Step Recovery Diodes (SRD) for the DOS (D, CN25M, EIC, Thailand) as Sanders and we did not want to change the pulse duration, the capacitance and inductance of the elements in the resonant network are the same as in the Sanders linear DOS generator. Inductors are air coils, made specially for this application, calibrated with LCR meter (4284A, Agilent), at 1 MHz frequency and 2 V settings.

CN25M diodes have reverse recovery time of 50 ns, maximum repetitive peak reverse voltage 1000 V and maximum average forward current 25 A. Because the maximum reverse voltage of one diode is too low for our application, several diodes must be stacked together in series and as 1000 V divided by 25 A is almost 50 Ω, n × n matrix of CN25M diodes is the most appropriate for 50 Ω load, where n is the number of diodes in series and in parallel in the matrix. A compact 8 × 8 diode matrix (Fig. 4) was made by gluing the was made by gluing the diodes together by conductive adhesive (MG chemicals, 8330-19G, 8330S-21G) in order to minimize stray inductance and all parasitic properties of lines connecting the load. The diode

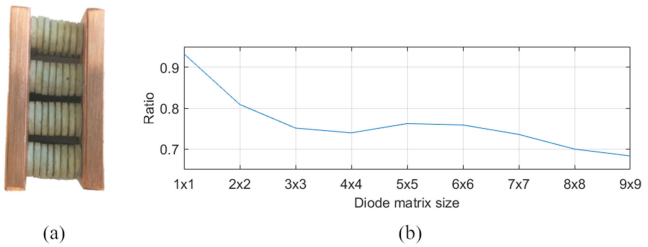


Fig. 4. (a) 8 × 8 matrix of CN25M diodes glued together with conductive adhesive used in order to minimize stray inductance. (b) Ratio between load current (calculated from the measured voltage) and L_2 current is lower with higher diode matrix size, due to their non-ideal characteristics.

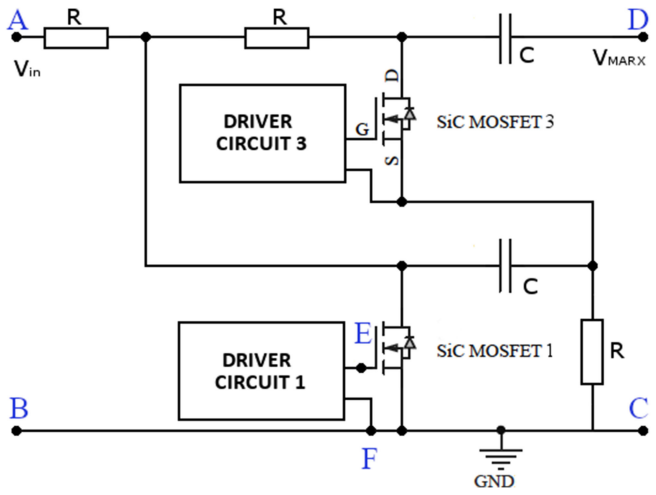


Fig. 5. Schematic of two-stage Marx-bank circuit implemented in the DOS generator. During the charging phase, capacitors (C) are charge parallelly through the resistors (R), and during the discharge phase. SiC MOSFETs connect capacitors in series and discharge them through the resonant network. Two Marx-bank circuits are implemented in parallel to generate high enough current for the resonant network. Each SiC MOSFET has its own driving circuit galvanically separated from each other.

matrix was glued as close as possible to the load and second inductor L_2. On the (Fig. 4), we can see the ratio between load current (calculated from the measured voltage) and L_2 current. The ratio is lower with higher diode matrix size, due to their non-ideal characteristics. Additionally, all diodes do not stop conducting at exactly the same moment and this may also result in a lower output pulse amplitude as expected. For DOS it would be the best to use custom made diodes.

B. Marx-Bank Circuit

Two-stage Marx-bank circuit (Fig. 5) was developed to enable charging of the resonant network with up to 2000 V. The energy storage capacitors (C) are a part of the Marx-bank circuit and also of the resonant network. During the charging phase the capacitors are parallelly charged through the resistors (R) by the external high-voltage power supply. And during the discharge phase SiC MOSFETs connect capacitors in series and discharge them through the resonant network (Fig. 3). C2M0025120D Silicon Carbide Power MOSFET with N-channel enhancement

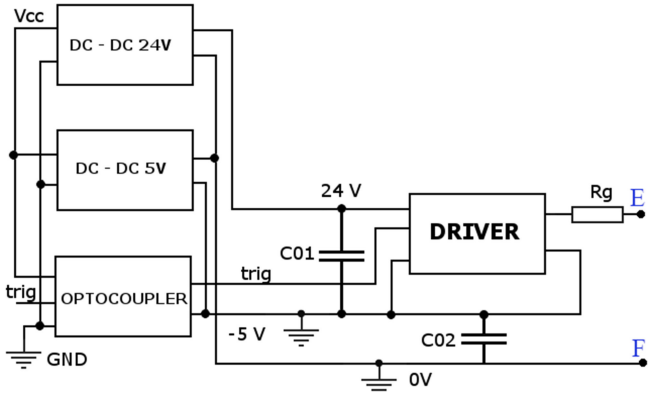


Fig. 6. Block diagram of SiC MOSFET driver circuit. Galvanic separation of the driver is made by isolated DC–DC converters and optocoupler. The driver is supplied by +24 V and −5 V. Power supply capacitors C01 and C02 stabilize the power supply voltage during switching. Gate resistor R_g is used for stable simultaneous switching of all four SiC MOSFETs.

mode (Cree, Inc., USA) were chosen because of their high drain-source breaking voltage, and their high and fast current rise (Fig. 2). It was empirically determined that at least two Marx-bank circuits must be implemented in parallel, to enable generation of high enough currents for the resonant network, before SiC MOSFET reach saturation. Charging resistors have a high value of 2 kΩ to ensure that the capacitors are in the majority discharged through the resonant network. However, due to high resistance of the charging resistors, the energy storage capacitors cannot be instantly recharged and the maximum pulse repetition rate is therefore 3.5 kHz.

C. SiC MOSFET Driving Circuit

When designing a driving circuit for SiC MOSFET (Fig. 6), it is important to consider SiC MOSFETs special characteristics [24]. Due to low transconductance, they must be driven with higher voltage difference than IGBTs or Si MOSFETs. At least 22 V voltage difference is desired for recommended operation, voltage for on stage is 20 V and for off stage from −2 V to −5 V. It is important that the upper limit of 25 V and the lower limit of −10 V are not crossed. Selected SiC MOSFETs start conducting at 2.5 V, but are not totally opened until V_{gs} reaches 16 V. Therefore, high dV/dt of the V_{gs} is needed for fast switching times that means driving circuit should have low impedance. For optimal switching of the SiC MOSFET, stray impedance of the driving circuit must be minimal, therefore, lines connecting the MOSFET driver to power supply capacitors and gate terminal should be as short as possible. Even though the stray impedance is minimized, it can still lead to excessive output oscillations, which can lead to unintentional MOSFET switching that can be suppressed by appropriate gate resistor R_g. The R_g is also important for the simultaneous switching of all four SiC MOSFETs, as asynchronous switching in Marx-bank circuit may overcharge and harm the switches. During the pulse generation, the source (S) electric potential of the first stage switches in the Marx-banks (SiC MOSFET1 and SiC MOSFET2)

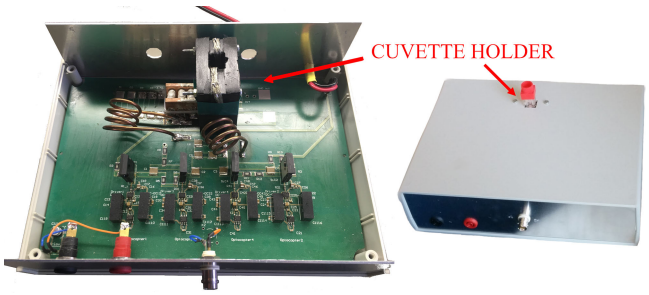


Fig. 7. Final prototype, with open (left) and closed enclosure (right). The cuvette holder is directly connected to the diode matrix in order to minimize parasitic effects.

stay on the same level as the GND (Fig. 5), while the source electric potential of the second stage switches (SiC MOSFET3 and SiC MOSFET4) have to fall to the $-V_{in}$ electric potential of the bottom capacitor (C). The second stage switches must though also charge the stray capacitance, therefore the current through the second stage switches is higher, which slows down their turning on time. To achieve simultaneous switching of the second and first stage switches, the R_g resistance, of the second stage switches, must thus be lower. However, a small difference in rise times should not affect or destroy the MOSFETS, since they operate up to 1200 V. If one of MOSFETS turns on slower, that should not be harmful, until the switching is still fast enough, to prevent the voltage rise of any MOSFET's V_{ds} over 1200 V. In our Marx-bank circuit, we have empirically determined the resistance R_g of the second stage MOSFET's to be 1 Ω, and of the first stage to be 2 Ω (Fig. 6), to achieve simultaneous switching of all MOSFET's.

Two isolated DC-DC converters (RP-0512D and RP-0505S, RECOM, Germany) are used to power supply the driving circuit and to galvanically separate it from the main power supply lines (Fig. 6). The two DC-DC converters and the source of one SiC MOSFET are connected together to form a 0 V potential for the gate driver. One DC-DC converter then supplies the driver with +24 V and the other with −5 V. Optocoulper (HCPL-0723, Avago USA) is used to galvanically separate the digital trigger signal (trig), and the SiC MOSFETs are driven by an ultrafast MOSFET driver (IXDD609SI, IXYZ,USA). Driver can be supplied with up to 35 V, it can deliver up to 9 A output current and has output resistance of up to 1 Ω. The ground of the optocoupler and the driver are connected to the −5 V. The optocoupler is then supplied with 0 V potential for the 5 V supply difference, and the driver with +24 V to have 29 V of operating voltage range. Power supply capacitors C01 and C02 are added to the power supply lines to stabilize the power supply voltage during the switching. As capacitors with different values have different resonant frequencies, C02 is composed of three different capacitors, which result in wider frequency blocking range and better stability of optocoupler's power supply.

D. Fabrication and Assembly

The device (Fig. 7) was implemented on 6U (Eurocard) sized PCB card, it is double sided with 105 μm copper layer and solder stop mask. If available, SMD electrical components were preferably chosen, trigger input connector is a 50 Ω BNC and a holder for the electroporation cuvette is made for the output. During the PCB design, special attention was given to make lines in the resonant network and in the output stage spaced enough for the high-voltage, and at the same time as short as possible and wide enough for the high currents. The device is triggered by a function generator (33220A, Agilent Technologies, USA) set to: VPP = 2.5 V, f = 1 kHz, pulse length 10 μs and offset 1.25 VDC. High-voltage generator (MCP 350-1250, FuG Elektronik GmbH, Germany) and low voltage generator (NG310, UNIWATT, Germany) that supply 5V/3A DC are used for high and low voltage power supply, respectively. Not to damage the DOS in case of too high-impedance of the load, a 100 Ω resistor (TFSF100RJE- ND, Ohmite, USA) is added in parallel to the load. If there would not be any load present at pulse generation, DOS would commutate L_2 current to high impedance and L_2 would induce very high voltage, which would damage the DOS and additionally high dv/dt could also damage the optocuplers.

E. Voltage and Current Measurements

For all the voltage and current measurements, an oscilloscope (Wavepro 7300A, LeCroy, USA) was used. Driver circuit was evaluated with differential probes LeCroy ADP305 (1 kV, 100 MHz, LeCroy, USA). The L_2 current in the resonant network was measured by high-frequency current transformer (1 kA, 500 MHz, Bezgoz, France). For the output pulse measurement several different voltage probes were used. The high-voltage output pulse was measured with different commercial probes (HVD3605, PPE20kV, PPE6kV and ADP305, LeCroy, USA), high-voltage commercial attenuators (N-type, J01026A0009, 2.5 kV 20 dB ± 0.8 dB; and BNC, J01006A0837, 20 dB ± 1 dB; both 50 Ω, 1GHz, Telegartner, Germany).

III. Results

The nanosecond pulse generator performance was evaluated in three steps. In the first step, driving circuits in the Marx- bank generators were tested for stable simultaneous switching of all four SiC MOSFETs. In the second step, the current in the resonant network was evaluated for the desired waveform and amplitude. And in the third step, the output pulse was measured.

A. SiC MOSFET Driving Circuit

Falling of the voltage V_{DS} on SiC MOSFETs, during the switching, is synchronous. Therefore, no extra voltage is redistributed to the adjacent SiC MOSFETs in the Marx- bank circuits during the switching and the probability for the SiC MOSFET breakdown during the switching is thus minimized. If the power supply voltage, V_{IN} is approximately 700 V or more, V_{DS} after reaching the zero voltage, during the switching, slightly increases because of SiC MOSFET saturation that is due to drain current (I_D) rise. Therefore, the current in the resonant network will not significantly rise with increasing the V_{IN} over 700 V. After the pulse generation, V_{DS} exponentially rises to the supply voltage in approximately 280 μs because capacitors C_1 are recharging. Therefore, the maximum pulse repetition rate of the generator that can be reached is 3.5 kHz.

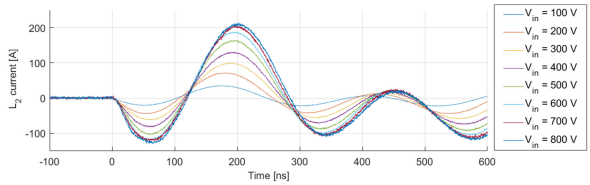


Fig. 8. The waveform of the L_2 current, in the resonant network, is a function of the power supply voltage (V_{IN}). For the analyzes the output of the generator was short circuited. At its maximum, the L_2 current reaches 200 A and the ratio between its first minimum and maximum is 1.7.

B. Resonant Network

The output of the generator was short-circuited and the L_2 current was measured (Fig. 8) to analyze the operation of the resonant network. By increasing the power supply voltage (V_{IN}) the L_2 current linearly increases up to 600 V of V_{IN} and at 700 V it saturates as predicted from the SiC MOSFET switching analyzes. At its maximum the L_2 current reaches 200 A and the ratio between its first current minimum and maximum is 1.7, which meets the expectations and is adequate for nanosecond pulse generation by diode opening switch (DOS) [27].

C. Output Pulse

Measurement of the output pulse of the generator is not straight forward, due to the short duration and at the same time high-voltage. Commercially available high impedance, high voltage probes for measuring voltage amplitudes up to 15 kV have bandwidth in range of 50–70 MHz (−3 dB). Differential 100 MHz probes that can measure up to 6 kV are a better choice for nanosecond pulse measurements, but can still be too slow, due to their rise time, which is around 3 ns. The best choice among commercially available probes for measuring nanosecond pulses are high voltage pulse attenuators that have bandwidths in GHz range and can measure up to 16 kV (Barth electronics, USA), but they are expensive compared to other previously described probes and thus not easily available to all researchers working on nanosecond electroporation field. Therefore, for the evaluation of developed device, a commercially available probes with additional calibration were used. A 50 Ω commercial attenuators were tested to measure the output pulse. The final attenuator was assembled from two N-type attenuators and one BNC attenuator to achieve 60 dB ± 2.8 dB attenuation. The factory graded accuracy of the attenuator is still not satisfying, but at least it is possible to calibrate the attenuator satisfactorily, namely, the assembled attenuator measures 31 ± 2% lower amplitude. The 50 Ω input of the attenuator of course influences the measurement, but in our case, the load was replaced by the attenuator. With such attenuator, the output pulses of the generator were measured (Fig. 9a). The shape of the pulses is Gaussian, with insignificant reflections. However, because the attenuators graded for 2.5 kV maximum voltage and in our case it broke at 4 kV. Consequently, a commercial high-voltage differential probe (HVD3605, 6 kV, 100 MHz, LeCroy, USA) was used to measure the output voltages over 4 kV. When

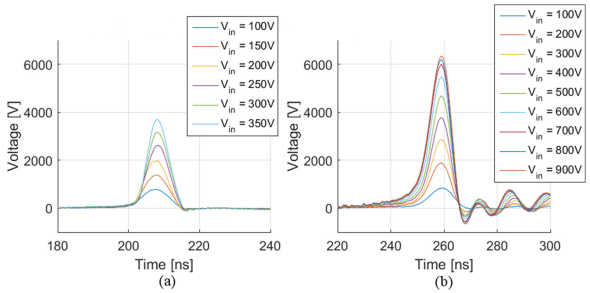


Fig. 9. Output pulse measurements by assembled attenuator (a) and HVD3605 probe (b) as a function of the power supply voltage (V_{IN}). Calculated output pulses taking into account the calibration constants 31% for the assembled attenuator and 5% for the HVD3605 probe.

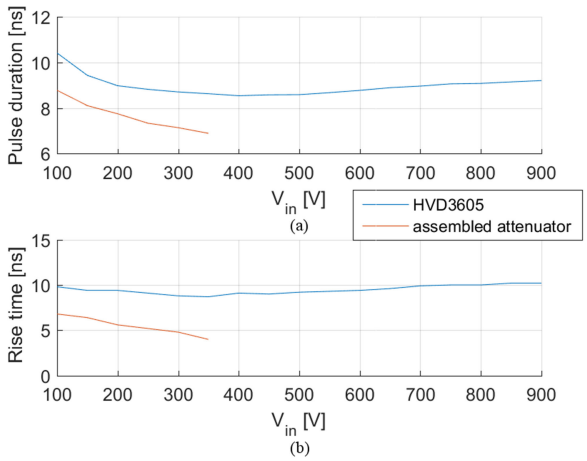


Fig. 10. Duration (a) and rise time (b) of the output pulses in case of assembled attenuator and in case of HVD3605 probe as a function of the power supply voltage (V_{IN}).

the HVD3605 probe is compared with the assembled attenuator a 5% loss in the amplitude is observed and reflections of the pulse arises (Fig. 9b). Measured pulse duration and rise time are shorter if measured with assembled attenuator in comparison to HVD3605 probe (Fig. 10). Pulse duration vary from 7 to 10 ns, while rise time vary from 4 to 10 ns. By increasing the power supply voltage V_{IN} up to 600 V, the amplitude of the output pulse linearly rises (Fig. 11). Over the 600 V of V_{IN}, due to the saturation of the SiC MOSFETs, the rise in the output pulse amplitude gradually declines by increasing the V_{IN} and at 900 V of V_{IN} the rise almost stops. The multiplication factor of the input voltage V_{OUT} /V_{IN} is around 9 in the linear space and in the saturation gradually falls to 7, at 900 V of V_{IN} (Fig. 11). On the figure (Fig. 12) we can see the output pulse amplitude and the FWHM (duration of the pulse) dependency on the load of the developed device. Our generator works as expected, also with cuvette, the amplitude is slightly lower in case of electroporation cuvette than on a 50 Ω load, while the pulse duration is a bit longer, mainly due to a smaller absolute reactance of the load at generated pulse frequency spectrum [41].

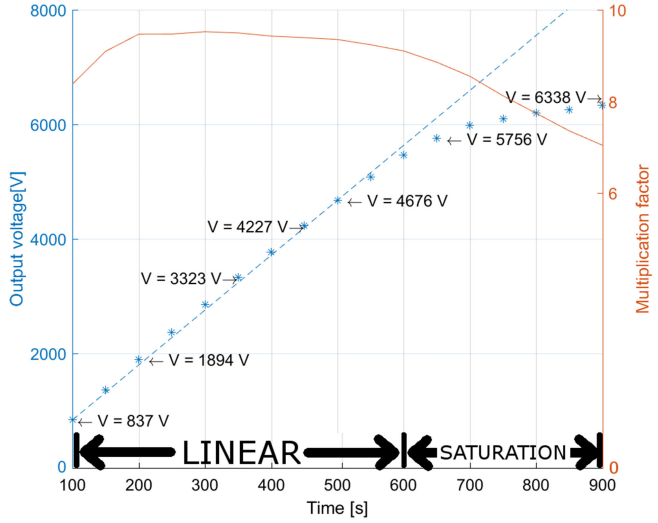


Fig. 11. Output pulse amplitude V_{OUT} as a function of the power supply voltage V_{IN} with linear and saturated space of operation. And the multiplication factor of the input voltage V_{OUT}/V_{IN}.

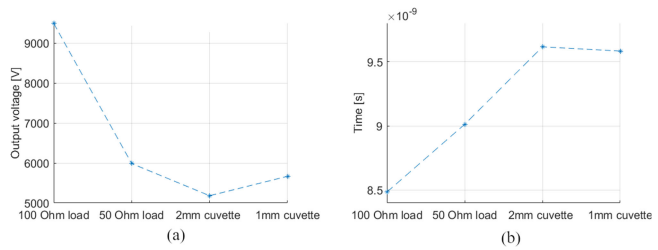


Fig. 12. The comparison of the output pulse amplitude (a) and FWHM (b) generated on different loads. In first two cases we used 100 Ω and 50 Ω load resistor (TFSF100RJE-ND, Ohmite, USA). In second case we added in parallel to 100 Ω load resistor an electroporation cuvette with 1 mm and 2 mm spacing between the electrodes, filled with KPB (Potassium phosphate buffer).

IV. Discussion

The design of the generator is based on a SiC MOSFETs, Marx-bank circuit and DOS. The RF MOSFETs, are still the most appropriate for short nanosecond pulses generated by transmission lines, which require fast rising times (Fig. 2). Whereas the SiC technology is the most suitable technology for longer nanosecond pulses or DOS generators as less elements are needed to generate higher currents. For a SiC MOSFETs a special driver circuit had to be develop that delivers the negative voltage on the gate during the off stage. For the correct operation of the Marx-bank circuit, the power supply and the control signal of the driver have to be properly galvanically isolated and the gate resistors have to be optimized for simultaneous switching of all four SiC MOSFETs. Otherwise, electrical elements could be damaged during the switching, especially the SiC MOSFETs and the optocouplers. The current in the resonant network could be increased by embedding one or two more Marx-bank circuits in parallel. Nevertheless, 200 A appeared to be enough for our aim [42] However, the resonant network and DOS configuration that were taken from the previous publication [27] did not work as expected. The rise time of the output pulse was much longer than expected, consequently, also the amplitude of the output pulse was not as high as expected. The causes for this under performance could be a mismatched frequency of the resonant network with reverse recovery time of the DOS, or the stray impedance on the generator's output. Even though, the amplitude of the output pulse was not as high as expected, the multiplication factor V_{OUT}/V_{IN} is approximately 9, which is still an improvement compared to the previously published linear DOS generator [27]. The improvement was achieved primarily through the use of SiC MOSFETs which enable more current and Marx-bank circuit which in the same time delivers double voltage to the LC oscillator. If the DOS would work as fast as in the previous publication of the linear DOS generator, it is expected that the multiplication factor would be 14. However, the biggest challenge was to adequately evaluate the output pulse. Researchers in the field of nanosecond electroporation use various methods to measure the high-voltage nanosecond pulses [36]–[39]. Due to conditional usability and high costs, of commercial probes, the researchers often develop their own high-voltage nanosecond probes. Also more costly commercial high-voltage differential probes, for example HVD3605 probe (LeCroy, USA), can be used for high-voltage nanosecond pulse measurement. These probes are very accurate in their wide bandwidth, however, at the limit of the bandwidth, due to not the most suitable connection terminals, the pulse reflection occurs and the probe loses accuracy, but not more than −3 dB. For this reason, we evaluated measuring error that occurs during the output pulse measurement. The commercial calibrated RF attenuators and the HVD3605 differential voltage probe were evaluated. Because HVD3605 is not very accurate at high-frequency signals, we would suggest that a high voltage −20 dB attenuator is made, or bought and then calibrated. And also that the commercial wide band 50 Ω attenuators are used to attenuate the signal to the oscilloscope's voltage level. Thus, each attenuator could be accurately calibrated and the measurement of the high-voltage nanosecond pulse would be more accurate. For the high-voltage attenuator, we suggest using capacitive voltage divider if we do not want to influence the measured signal, otherwise the 50 Ω attenuator should be the most accurate and appropriate.

V. Conclusion

A nanosecond pulse generator was develop for "in vitro" electroporation experiments using standard electroporation cuvettes. The generator can deliver from 500 V to more than 6 kV, approximately 8 ns pulses to a 50 Ω load. Two in parallel two-stage Marx-bank circuits can generate up to 200 A current in the resonant circuit. As the SiC MOSFETs already go into saturation at 600 V (Fig. 11). However, the biggest challenge in this study was to accurately measure the high-voltage short nanosecond pulses. We have already emphasized [23] that accurate measurement of the delivered nanosecond pulses is crucial for the unbiased nanosecond electroporation experiments. But it should be also emphasized that the bandwidth of the probes ends at −3 dB which means that the probe at the end of its bandwidth has a −29% error. A −29% error may be acceptable for electronics, however, a 29% different amplitude may have a significantly different biological effect [43], [44].

Because permeabilization and cell survival are sigmoid functions of voltage, more than 10% difference in voltage may result in a significantly different biological effect. For example, if we focus on permeabilization curve, a ±29% deviation from the real voltage amplitude result in totally different outcome. In one case the majority of cells can be intact (i.e., not permeabilized) while in other case, the majority of the cells can be permeabilized [43], [44]. Whenever we are using measuring probes, we have to be aware of the measuring error. Commercially available probes have the measuring error specified in a data sheet, but it is still sometimes overlooked, while prototype probes have to be correctly calibrated and measuring error evaluated.

Acknowledgment

The authors would like to thank Philippe Lévêque and Delia Arnaud-Cormos, from XLIM Research Institute at University of Limoges, France for the help at work that apply to nanosecond pulse measurement techniques.

References

[1] J. C. Weaver, "Electroporation of cells and tissues," *IEEE Trans. Plasma Sci.*, vol. 28, no. 1, pp. 24–33, Feb. 2000.
[2] C. Chen *et al.*, "Membrane electroporation theories: A review," *Med. Biol. Eng. Comput.*, vol. 44, no. 1–2, pp. 5–14, 2006.
[3] T. Kotnik *et al.*, "Membrane electroporation and electropermeabilization: Mechanisms and models," *Annu. Rev. Biophys.*, vol. 48, pp. 63–91, 2019.
[4] M. L. Yarmush *et al.*, "Electroporation-based technologies for medicine: Principles, applications, and challenges," *Annu. Rev. Biomed. Eng.*, vol. 16, pp. 295–320, 2014.
[5] S. Haberl *et al.*, "Cell membrane electroporation—Part 2: The applications," *IEEE Elect. Insul. Mag.*, vol. 29, no. 1, pp. 29–37, Jan./Feb. 2013.
[6] B. Mali *et al.*, "Antitumor effectiveness of electrochemotherapy: A systematic review and meta-analysis," *Eur. J. Surgical Oncol.*, vol. 39, no. 1, pp. 4–16, 2013.
[7] E. Pirc *et al.*, "Study design of a medical device pre-market assessment: A case study on electrochemotherapy," *Slovenian Med. J.*, vol. 87, no. 1–2, pp. 22–40, 2018.
[8] T. Stepisnik *et al.*, "Electrochemotherapy – an effective method for the treatment of tumors with a combination of chemotherapeutic agent and electric field," *Slovenian Med. J.*, vol. 85, no. 1, pp. 41–55, 2016.
[9] H. J. Scheffer *et al.*, "Irreversible electroporation for nonthermal tumor ablation in the clinical setting: A systematic review of safety and efficacy," *J. Vascular Interventional Radiol.*, vol. 25, no. 7, pp. 997–1011, 2014.
[10] B. Rubinsky, "Irreversible electroporation in medicine," *Technol. Cancer Res. Treatment*, vol. 6, no. 4, pp. 255–260, 2007.
[11] M. Golzio *et al.*, "Direct visualization at the single-cell level of electrically mediated gene delivery," *Proc. Nat. Acad. Sci.*, vol. 99, no. 3, pp. 1292–1297, 2002.
[12] M. Coustets *et al.*, "Flow process for electroextraction of total proteins from microalgae," *J. Membrane Biol.*, vol. 246, no. 10, pp. 751–760, 2013.
[13] I. P. Sugar *et al.*, "Model of cell electrofusion: Membrane electroporation, pore coalescence and percolation," *Biophysical Chem.*, vol. 26, no. 2, pp. 321–335, 1987.
[14] O. N. Pakhomova *et al.*, "Facilitation of electroporative drug uptake and cell killing by electrosensitization," *J. Cellular Mol. Med.*, vol. 17, no. 1, pp. 154–159, 2013.
[15] C. Luft and R. Ketteler, "Electroporation knows no boundaries: The use of electrostimulation for siRNA delivery in cells and tissues," *J. Biomolecular Screening*, vol. 20, no. 8, pp. 932–942, 2015.
[16] A. Golberg *et al.*, "Energy-efficient biomass processing with pulsed electric fields for bioeconomy and sustainable development," *Biotechnol. Biofuels*, vol. 9, no. 1, pp. 1–22, 2016.
[17] G. Pucihar *et al.*, "Equivalent pulse parameters for electroporation," *IEEE Trans. Biomed. Eng.*, vol. 58, no. 11, pp. 3279–3288, Nov. 2011.
[18] T. Kotnik and D. Miklavčič, "Theoretical evaluation of voltage inducement on internal membranes of biological cells exposed to electric fields," *Biophys. J.*, vol. 90, no. 2, pp. 480–491, 2006.
[19] M. Rebersˇek *et al.*., "Cell membrane electroporation—Part 3: The equipment," *IEEE Elect. Insul. Mag.*, vol. 30, no. 3, pp. 8–18, May–Jun. 2014.
[20] E. Pirc *et al.*, "Dosimetry in electroporation-based technologies and treatments," in *Dosimetry in Bioelectromagnetics*, M. S. Markov, Ed., Boca Raton, FL, USA: CRC Press, 2017, pp. 233–268.
[21] L. Chopinet *et al.*, "Nanosecond electric puls eeffectson gene expression," *J. Membrane Biol.*, vol. 246, no. 11, pp. 851–859, 2013.
[22] R. Sundararajan, "Nanosecond electroporation: Another look," *Mol. Biotechnol.*, vol. 41, no. 1, pp. 69–82, 2009.
[23] T. Batista Napotnik *et al.*, "Effects of high voltage nanosecond electric pulses on eukaryotic cells (in vitro): A systematic review," *Bioelectrochemistry*, vol. 110, pp. 1–12, 2016.
[24] L. D. Stevanovic *et al.*, "Recent advances in silicon carbide MOSFET power devices," in *Proc. 25th Annu. IEEE Appl. Power Electron. Conf. Expo.*, 2003, pp. 401–407.
[25] V. Novickij *et al.*, "High-frequency submicrosecond electroporator," *Biotechnol. Biotechnol. Equip.*, vol. 30, no. 3, pp. 607–613, 2016.
[26] D. M. Brown *et al.*, "Silicon carbide MOSFET technology," *Solid-State Electron.*, vol. 39, no. 11, pp. 1531–1542, 1996.
[27] J. M. Sanders *et al.*, "A linear, single-stage, nanosecond pulse generator for delivering intense electric fields to biological loads," *IEEE Trans. Dielectr. Elect. Insul.*, vol. 16, no. 4, pp. 1048–1054, Aug. 2009.
[28] A. Kuthi *et al.*, "Nanosecond pulse generator using fast recovery diodes for cell electromanipulation," *IEEE Trans. Plasma Sci.*, vol. 33, no. 4, pp. 1192–1197, Aug. 2005.
[29] J. F. Kolb *et al.*, "Nanosecond pulsed electric field generators for the study of subcellular effects," *Bioelectromagnetics*, vol. 27, no. 3, pp. 172–187, 2006.
[30] S. R. Rajulapati *et al.*, "Nanosecond biphasic pulse generators for biomedical applications," in *Proc. Abstr. IEEE Int. Conf. Plasma Sci.*, 2013, pp. 1–4.
[31] M. Reberšek *et al.*, "Blumlein configuration for high-repetition-rate pulse generation of variable duration and polarity using synchronized switch control," *IEEE Trans. Biomed. Eng.*, vol. 56, no. 11, pp. 2642–2648, Nov. 2009.
[32] T. Tang *et al.*, "Diode opening switch based nanosecond high voltage pulse generators for biological and medical applications," *IEEE Trans. Dielectr. Elect. Insul.*, vol. 14, no. 4, pp. 878–883, Aug. 2007.
[33] J. M. Sanders *et al.*, "Optimization and implementation of a solid state high voltage pulse generator that produces fast rising nanosecond pulses," *IEEE Trans. Dielectr. Elect. Insul.*, vol. 18, no. 4, pp. 1228–1235, Aug. 2011.
[34] M. Kranjc *et al.*, "Numerical simulations aided development of nanosecond pulse electroporators," in *Proc. 6th Eur. Conf. Antennas Propag.*, 2012, pp. 344–347.
[35] M. Reberšek, "Beyond electroporation pulse parameters: From application to evaluation," in *Handbook of Electroporation*, D. Miklavčič, Ed., Berlin, Germany: Springer, 2017.
[36] T. J. Camp *et al.*, "Bioelectric studies with subnanosecond pulsed electric fields," in *Proc. IEEE Pulsed Power Conf.*, 2009, pp. 876–879.
[37] A. Silve *et al.*, "Implementation of a broad band, high level electric field sensor in biological exposure device," in *Proc. IEEE Int. Power Modulator High Voltage Conf.*, 2010, pp. 711–714.
[38] P. Leveque and D. Arnaud-Cormos, "Measurement and characterization of exposure systems for high-frequency, ultrashort pulses," in *Handbook of Electroporation*, D. Miklavčič, Ed., Berlin, Germany: Springer, 2017.
[39] J. M. Sanders *et al.*, "Broadband power measurement of high-voltage, nanosecond electric pulses for biomedical applications," in *Proc. IEEE Int. Power Modulators High Voltage Conf.*, 2008, pp. 350–353.
[40] A. T. Esser *et al.*, "Mechanisms for the intracellular manipulation of organelles by conventional electroporation," *Biophysical J.*, vol. 98, no. 11, pp. 2506–2514, 2010.
[41] M. Kenaan *et al.*, "Characterization of a 50-Ω exposure setup for high-voltage nanosecond pulsed electric field bioexperiments," *IEEE Trans. Biomed. Eng.*, vol. 58, no. 1, pp. 207–214, Jan. 2011.
[42] E. B. Garon *et al.*, "In vitro and in vivo evaluation and a case report of intense nanosecond pulsed electric field as a local therapy for human malignancies," *Int. J. Cancer*, vol. 121, no. 3, pp. 675–682, 2007.
[43] I. Semenov *et al.*, "Primary pathways of intracellular Ca2+ mobilization by nanosecond pulsed electric field," *Biochimica et Biophysica Acta (BBA) – Biomembranes*, vol. 1828, no. 3, pp. 981–989, 2013.
[44] W. E. Ford *et al.*, "Nanosecond pulsed electric fields stimulate apoptosis without release of pro-apoptotic factors from mitochondria in B16f10 Melanoma," *Arch. Biochemistry Biophys.*, vol. 497, no. 1, pp. 82–89, 2010.

3.7 Paper 6

Title: **High frequency and high voltage asymmetric bipolar pulse generator for electroporation based technologies and therapies**

Authors: Eva Pirc, Damijan Miklavčič, Katja Uršič, Gregor Serša and Matej Reberšek

Publication: *Biomedical Enigineering OnLine*

Impact factor: 2.013 (2018)

Quartile: Q3 (engineering, biomedical)

Rank: 52/80 (engineering, biomedical)

Status: in preparation for submission

RESEARCH

High frequency and high voltage asymmetric bipolar pulse generator for electroporation based technologies and therapies

Eva Pirc[1], Damijan Miklavčič[1], Katja Uršič[2], Gregor Serša[2,3] and Matej Reberšek[1*]

*Correspondence:
matej.rebersek@fe.uni-lj.si
[1]University of Ljubljana, Faculty of Electrical Engineering, Tržaska 25, 1000 Ljubljana, Slovenia
Full list of author information is available at the end of the article

Abstract

Background: Currently, high-frequency irreversible electroporation studies are in full swing, but limited to a few research groups with custom made laboratory prototype electroporators. According to the review of electroporators and economic evaluations of electrochemotherapy and irreversible electroporation, we came to the conclusion, there is still a potential area of pulse parameters that needs to be investigated. The development of an asymmetric bipolar pulse generator with a maximum voltage of 4 kV and minimum duration time of few hundred nanoseconds, would enable *in vivo* evaluation of biological effects of high-frequency electroporation pulses. First high-frequency electrochemotherapy was performed in *in vitro*, by Scuderi et. al. and the developed device enables the translation to *in vivo*.

Methods: In the paper we made an analysis and comparison between the most commonly used drivers and optical isolation in high voltage pulse generators and described in detail circuit topology of the developed device. The effectiveness of electrochemotherapy with high-frequency electroporation pulses and "classical" electrochemotherapy pulses was compared.

Results: The developed device is able to generate 4 kV pulses, with theoretical maximal current 131 A and 200 ns minimal pulse duration, the maximal pulse repetition rate is 2 MHz and the burst maximal repetition rate being 1 MHz. The device was validated also *in vivo*, electrochemotherapy with high-frequency electroporation pulses was performed, which proved at least as effective as electrochemotherapy with "classic" well-established electric pulses, resulting in 86 % and 50 % of complete responses, respectively. Contrary to numerous reports, muscle contractions were comparable between the two protocols.

Conclusions: The device enables research of still unknown effects, it facilities further research of cancellation and sensitization effects *in vivo*. The desired performance was reached and first *in vivo* high-frequency electrochemotherapy was performed.

Keywords: electroporation; electroporation device; high frequency; bipolar; asymmetric; SiC MOSFET; high voltage generation; pulse generator

Background

Electroporation is a phenomenon, whereby, due to the exposure to high voltage electric pulses, biological cells become permeable to molecules, which otherwise could not cross the cell membrane [1]. We distinguish between reversible and irreversible electroporation. If the cell membrane is able to fully recover, after the electric pulse

application, the electroporation is reversible, but when the damage is too extensive, the cell dies and we name it irreversible electroporation (IRE) [2]. Electroporation is already well established in medicine and food processing [3, 4]. Additionally, the technology holds great promise also in other fields, such as biomass production [5]. Different electroporation based applications require particular pulse parameters i.e. voltage amplitudes, pulse width, a number of pulses or bursts and pulse repetition rate [6, 7, 8]. Therefore specific pulse generators, i.e. electroporators have to be designed and developed for each application. Additionally, electrical properties of biological loads considerably vary and their conductivity changes during the pulse application, due to electroporation [9, 10, 11, 12]. Therefore when designing an electroporator, one should always keep in mind that, biological sample as a load has resistive–capacitive nature and can vary from sample to sample and in addition the impedance of a biological sample decreases during pulse delivery [13, 14]. In a recent study [15] authors showed that because biological tissue is frequency-dependent, differences in pulse frequency spectrum reflect in the induced transmembrane voltage.

In medicine, electrochemotherapy (ECT), an antitumor therapy, in which electroporation introduces the chemotherapeutic drug into cells is well established [16]. According to the standard operating procedure intratumoral or intravenous delivery of the chemotherapeutic drug bleomycin or cisplatin is followed, by the application of eight high voltage pulses, which are monopolar, 100 μs long, with pulse repetition rate 1 Hz or 5 kHz and voltage amplitudes between 1000 V and 1300 V (depending on the electrode type) [17, 18, 19]. IRE is also an emerging medical application, used as non-thermal ablation of normal and tumor tissue, as cells primarily die due to membrane permeabilization and not due to increase of tissue's temperature [20, 21]. It was shown that IRE does not cause the denaturation of proteins or scarring and does not damage blood vessels. Therefore it has a potential ability to treat tumors near large blood vessels. Additionally, the rapid activation of the immune system was observed [22]. However, a local temperature increases around the electrodes can be significant at higher amplitudes and due to a high number of pulses delivered to a limited volume of tissue [23]. Also, nerve stimulation and muscle contractions are associated to high voltage pulse delivery [24, 20]. These are also observed and reported in ECT and require additional management (In order to reduce movement, muscle relaxants are administered prior to treatment, but the dosage of those relaxants should be monitored throughout the procedure [25, 26]) and synchronisation of pulse delivery with ECG, which complicates the treatment procedure [27]. Recently it was suggested that by applying high-frequency, bursts of bipolar pulses, named H-FIRE (High-Frequency IRreversible Electroporation), muscle contractions during IRE can be reduced without compromising the non-thermal mechanism of cell death [28, 29] and at the same time, render electric field distribution in tissue more homogeneous [28]. It was shown *in vitro* that the transmembrane transfer of molecules may be achieved with the same type of pulses [30]. However, H-FIRE pulses need considerably larger voltage amplitudes for cell disruption in comparison to longer monopolar pulses [30]. With the potential advantages of IRE over current ablation modalities, the technology seems uniquely suited also for cardiac ablation. Currently, the research of electoporation as a potential treatment modality of atrial

fibrillation (AF), which is the most commonly ablated arrhythmia, is in full swing [31, 32]. A term "Pulsed field ablation" (PFA) is defined as IRE that uses a train of bipolar and biphasic pulses of high voltage and short duration to create tissue injury without significant heating and therefore reduces the injury to nontargeted tissues [33] .

Recently it was also demonstrated that it is possible to use HF-EP (high-frequency electroporation) pulses in electrochemotherapy, but again, at the expense of higher electric fields than in classical ECT. Scuderi et. al. [34] determined that in high-frequency electrochemotherapy (HF-ECT) a higher electric field, equal to 3 kV/cm, has to be established than in "classical" ECT (1.3 kV/cm) to obtain comparable effectiveness *in vitro*.

Previous studies indicated, that advantage of those specific high frequency electroporation pulse characteristics might be in reducing muscle contraction and pain sensation during high voltage pulse delivery [28, 29, 35]. It was shown that the electric field threshold for muscle contraction is two times lower than the threshold for electroporation (for 100 *mu*s long pulses) [35]. It was already reported that bipolar pulses [36] in ECT and H-FIRE pulses [29] reduce muscle contraction and it was also shown that IRE can be performed with a pulse duration of 1 μs by increasing the number of electric pulses and voltage amplitude [29, 37, 38]. Also recent reports have been describing the phenomena of electrical cell sensitization [39, 40] and cancellation effect [41, 42] in the range of microsecond and sub-microsecond pulse duration. These effects i.e. sensitization, cancellation and nerve and muscle decreased excitation effects of the electroporation are still not well understood and further studies are needed in particular *in vivo*, due to inconclusive results obtained *in vitro* [43, 44]. Additionally, van Es et. al. [45] introduce a novel asymmetric, high-frequency electroporation for HF-IRE and concluded that the use of the asymmetric pulses enhances the feasibility of the HF-IRE method. Therefore, the development of an asymmetric bipolar electroporator, with variable setting of pulse duration and voltage amplitude for each half period of the pulse, would enable a new insight and interesting investigations of cancellation and sensitization effects.

For the generation of electric pulses, with high voltage amplitudes, up to several kV, there are basically three different circuit concepts used: a modular pulse generator [46]; generator with serial switches [47]; Marx generator [48].

A simple solution is a generator with serial switches, where an array of n connected switches is connected to a source. In this case, the synchronization of the switches is necessary, for proper operation, the maximum generated voltage amplitude is equal to the power supply voltage. In Marx generator topology, n capacitors are charged in parallel and then, by switching on switches simultaneously, all capacitors are discharged in series through the load. The maximal generated voltage amplitude is equal to the power supply voltage multiplied by the number of capacitors. For the generation of bipolar pulses a serial H-bridge generator is used, where in series connected array of high voltage switches is connected to the H-bridge [49]. Also a modular square wave pulse generator, can generate bipolar pulses if transistor

bridge, that enables polarity change, is added at the output. The diagonal bridge transistors in case of adequate control, change the polarity of the output voltage [50]. But for the generation of asymmetric bipolar pulses, a serial H-bridge generator is the simplest and economically most reasonable solution.

Until now in all H-FIRE studies, custom made laboratory prototype electroporators were used. Each research group has developed its own prototype device, with specific topology, which is based on an H-bridge. Therefore the research is limited only to a few research groups that have the knowledge to build such custom device. However clinical bipolar electroporator should be designed and certified before the implementation into clinical practice. In literature pulse delivery systems are most often only briefly mentioned and not described in detail. Arena et. al. were one of the first who developed a custom pulse generator, with maximal output rating of the system equal to ±450 V and with a sufficient level of charge to deliver 20 A over a 100 μs bursts [28]. In more recent studies of the same group [51], the burst consisted of a train of 100 pulses of 1 μs duration and alternating polarity, with a delay of 2 μs between each pulse, and also the amplitude was increased to 800 V. While Sano et. al. managed to reduce the minimal pulse duration to 250 ns, the minimal delay between the change of pulse polarity was still 1 μs. Their device is also able to generate asymmetric pulse durations of negative and positive half period and the voltage amplitude was increased up to 5 kV [52, 37, 53]. Yao et al. developed a IRE bipolar electroporator and electrodes, the voltage amplitude is the study changed in the range from 800 V to 2 kV. The device can generate pulses with constitutive pulse widths from 1 μs up to 100 μs, but the burst repetition rate is equal to 1 s [54], similar device was used in the first clinical study using H-FIRE pulses by Dong. et. al. [38]. Grainys et. al developed a bipolar symmetrical and asymmetrical electroporator (± 1kV, 100 A) for *in vitro* electroporation and presented it in the paper [49]. But for *in vivo* experiments, higher voltage amplitudes are required. Mirai Medicals developed a CE approved clinical electroporation generator named ePORE, it enables a simple and reliable delivery of ultra-short electrical pulses up to 250 kHz and claim it eliminates he muscular contractions and pain associated with the technology [55, 56].

According to the electroporators review [13, 57] and economic evaluations of ECT and IRE [58, 59], that have been published, we came to the conclusion, there is still a potential area of pulse parameters that needs to be investigated. The development of an asymmetric bipolar pulse generator with a maximum voltage of 4 kV and minimum duration time of few hundred nanoseconds, would enable *in vivo* evaluation of biological effects of HF-EP pulses. In the paper, the work is presented in three sections, in the methods and material part of the paper the importance and the process of electronic component selection is described. Then the concept and topology of developed electroporator are presented. In the results measurements and evaluation of the developed device and *in vivo* use, with the focus on high frequency electrochemotherapy.

Results

Device Performance

For the evaluation of developed device we first used 80 Ω resistor as load, we connected a 33 Ω resistor (AZ330KE, Ohmite, USA) and 47 Ω resistor (AZ470KE, Ohmite, USA) in series. This value was chosen because mainly *in vivo* the resistance of the tissue in combination with the needle electrodes is close to 80 Ω [60]. The measurement of monopolar and bipolar pulses with minimal pulse duration are displayed in Figure 1. The capacitor charging voltage was set to 500 V and then raised up to 4 kV with a step of 500 V. In order to reach expected 4 kV, we were forced to increase the pulse trigger width to 260 ns, meaning the electroporator's minimal pulse duration increased to 200 ns. On Figure 1 (b) also a negative half period was added, the delay between the trigger of the positive and negative half period on digital delay generator was set to 200 ns. The pulse amplitude of developed device reaches desired 4 kV, and complete symmetry between both pulse polarities can be observed.

In Figure 2. an asymmetric pulse generation is presented. The pulse width of a positive and negative half period can be independently changed from 200 ns up to 1 ms (Figure 2(a)). The same applies also to the pulse amplitude (Figure 2(b)). In Figure 2(a) the capacitor charging voltage was set to 4 kV and trigger pulse duration was set to 260, 400 and 800 ns for both half periods. It can be observed the pulse amplitude comes closed to 4 kV with the extension of pulse duration, also the pause between both half periods is reduced with the extension of pulse duration. While in Figure 2(b) the first half period was in all cases the same (4 kV and trigger duration 260 ns), and the duration of trigger (260 ns, 400 ns, and 800 ns) and charging voltage of the second half period was changed (4 kV , 2 kV and 1 kV). Figure 2 demonstrates that the minimal pulse duration and highest amplitude of the first half period has a good repeatability and can be followed by the pulse of opposite polarity of any pulse duration or amplitude from the operation range.

The monopolar pulse measurements from Figure 2 were studied in detail. The rise and fall time, as well as pulse duration and relative amplitude error, were calculated for each measured voltage. The results are displayed in Figure 3. As expected both rise and fall time of the pulse rises with pulse amplitude, however the maximal value is still under 150 ns, which allows us to generate pulses with minimal pulse duration of 200 ns at maximal voltage amplitude. The maximal relative voltage amplitude error is 5 % which confirms the desired performance is reached.

High frequency electrochemptherapy *in vivo*

Finally, the device was tested *"in vivo"*. In Figure 4 current and voltage measurement of one HF-EP are displayed. Additionally, in the Figure 5 the measurement of accelerations during the pulse delivery is presented. We compared the accelerations in case of classical ECT pulses (eight monopolar, 100 μs long, 1 Hz pulse repetition rate, and pulse amplitude 780 V) with eight burst of high frequency 1-1-1-1 μs (duration of a positive half period - pause between pulse half periods

- duration of a negative half period - pause between bipolar pulses) long bipolar pulses, with the same burst repetition rate as classical ECT pulse repetition rate, but higher voltage amplitude 1950 V. No significant difference in the muscle contractions was observed. Furthermore, the therapeutic effect of electrochemotherapy with bleomycin in combination with HF-ECT pulses was compared. Both, "classical" and HF-ECT electric pulses, significantly ($p < 0.05$) potentiated the antitumor effect of bleomycin. Namely, "classical" ECT with bleomycin resulted in 50 % (3/6 mice) of complete responses and HF-ECT with bleomycin in 86 % (6/7 mice) of complete responses. The difference between these two groups is however not significantly different. Survival of animals from these groups was significantly prolonged compared to non treated control group or only EP treated groups. HF-ECT electric pulses significantly ($p < 0.05$) prolonged the survival of animals compared to control group. However, the difference between the two types of electric pulses was not significant (Figure 6). Both, "classical" ECT and HF-ECT were well tolerated by animals. After the treatment, their body weights did not vary more than 5 % (data not shown), and no treatment-related mortality was observed.

Discussion

Device Performance

The developed device is able to generate 4 kV pulses, with theoretical maximal current 131 A and 200 ns minimal pulse duration, the maximal pulse repetition rate is 2 MHz and the burst maximal repetition rate being 1 MHz. The device was first evaluated on an 80 Ω resistor. Monopolar, as well as symmetric and asymmetric bipolar pulse generation, was successfully demonstrated (Figure 1). Rise and fall time increased with the capacitor charging voltage amplitude and therefore, also the shape of the pulse is less squared and more bell-shaped, for 200 ns pulses as pulse duration increases, i.e. FWHM decreases with voltage amplitude. The maximal relative error between the capacitor charging voltage and measured load voltage was at maximum equal to 5 %, but with the extension of pulse duration, error decreases and pulse shape is also more square shaped (Figure 2(a)). However, a slight voltage amplitude drop can be seen in Figure 2(a), which occurs due to the lack of stored energy, the capacitor bank was not yet used in this first level of evaluation. Figure 3(d) shows that relative error can be reduced also with the reduction of the applied capacitor charging voltage. Because of a poor hardware reporting of already developed bipolar electroporators comparison is difficult, however parametric comparison is possible. From the prospect of output pulse parameters, the main innovation of presented device is ability to generate bipolar asymmetric pulses. We also believe the minimal pulse duration in combination with such high voltage (4 kV) and asymertry was improved. Maximal output pulse amplitude is 4 kV, Sano et. al. [52, 53, 37] reached 5 kV, however, we believe our device, with a 131 A maximal current, can also be used for IRE.

The design proposed in this paper enables faster development and spread of the high frequency electroporation (H-FIRE, and HF-ECT). Because the developed electroporator also has an option of asymmetrical pulse delivery it enables research of still not well investigated pulse parameter space *in vitro* and due to high maximum current also *in vivo*. The developed generator consists of newest component

technologies presented in methods and material section, that enable even faster switching times $\frac{di}{dt}$ than previously described solutions [30, 49].

High frequency electrochemptherapy *in vivo*

On the second level of evaluation, the developed device was used in an *in vivo* experiment treating subcutaneous tumors on mice to evaluate tumors responses and muscle contractions present during HF-ECT. Therefore, accelerations of mice hind leg during the pulse delivery was measured. Only a half of capacitor bank (four capacitors) was used, because we used symmetric pulses. No voltage drop was detected in *in vivo* measurements in Figure 4, meaning energy storage was sufficient. *In vitro* experiments by Scuderi et. al. [34], showed that HF-EP could be used in ECT. According to the Arena et. al. [28], high-frequency fields have the potential to overcome impedance barriers posed by low conductivity tissues, which could result in more homogenous and predictable treatment outcomes in heterogeneous systems. Additionally one of the main benefits of high-frequency fields is potentially alleviated muscle contractions and pain.

In the scope of this study we present the first *in vivo* experiments of HF-EP in ECT. Scuderi et. al. [34] determined *in vitro* that in HF-ECT a higher electric field (3 kV/cm), has to be applied than in "classical" ECT (1.3 kV/cm) to obtain comparable cytotoxicity, therefore we applied the electric pulses with amplitude of 1950 V, which resulted in 3.25 kV/cm (voltage to distance ratio). ECT with bleomycin and HF-EP proved at least as effective as ECT with classic well-established electric pulses where 86 % and 50 % of complete responses were observed, respectively. Additionally, preliminary data on ECT with cisplatin and HF-ECT pulses (n=2) indicated that ECT with HF-EP is as effective as classical ECT, resulting in 100 % of complete responses. Contrary to numerous reports, muscle contractions were comparable between the two protocols. These findings, in the case of HF-EP, are not in compliance with published results of Ringel-Scaia et al. [61], where H-FIRE caused no muscle contractions in comparison to IRE pulses delivered with needle electrodes. But we are the first who used plate electrodes in combination with HF-EP. Direct translation from in *in vitro* to *in vivo* is thus not possible. In the future the voltage amplitude should be optimized for *in vivo* HF-ECT, with plate electrodes, which could result in reduction of muscle contractions.

Conclusions

The developed device operates in accordance with expectations, the maximal output voltage is 4 kV and the theoretical maximal current 131 A and 200 ns minimal pulse duration. The maximal pulse repetition frequency is 2 MHz. It generates asymmetric bipolar pulses (amplitude and duration), pulse duration asymmetry, as voltage amplitude asymmetry can be simultaneously provided. The device thus enables research of still unknown effects, in poorly investigated μs range of pulse parameters including further research of high-frequency electroporation therapies *in vivo*.

The device was first tested on a 80 Ω resistor and then *in vivo* on mouse tumor model. In both cases the desired performance was reached. Additionally, acceleration of mice leg during the pulse delivery was measured in order to evaluate muscle contractions. More *in vivo* measurements should be done, before any final conclusions can be made, but contrary to existing reports, we observed similar muscle contractions in both protocols used. Most importantly, HF-ECT with bleomycin and cisplatin proved as effective as the established "classical" ECT with bleomycin and cisplatin.

Methods and Materials

The output stage of a developed device is a custom made serial asymmetric H-bridge generator which is in detail described in this section of the paper and presented in the Figure 9. The capacitors are charged with a high voltage power supply MCP 350-1250 (FuG Elektronik GmbH, Germany) and then discharges through two series of three SiC MOSFET's connected in an H-bridge. Each MOSFET has its own driving circuit, which are galvanically separated from each other. Two digital inputs trigger the positive and negative output pulse generation. A logic circuit controls triggers, in a way that only one can be active at the same time. In our previously published paper [62] we already made an analysis and comparison between the most commonly used semiconductor switching technologies in pulse generators. We again chose the Silicon Carbide (SiC) MOSFET technology, which we believe is the most suitable for high voltage and high current short pulse duration generators. Since the manufacturer started to produce even more suitable SiC MOSFETs (C2M0045170D, Cree, USA) with a drain to source voltage 1700 V and pulse drain current 160 A. Thus only three MOSFET in series are needed in order to generate desired 4 kV pulses on the output. When designing a driving circuit for SiC MOSFET it is important to consider SiC MOSFETs special characteristics [63]. SiC MOSFET's have low transconductance, therefore must be driven with high voltage difference. For fast switching times high $\frac{dV}{dt}$ of the V_{gs} is needed, meaning driving circuit should have low impedance. For optimal switching also stray impedance of the driving circuit must be minimal, therefore, lines connecting the SiC MOSFET driver to power supply capacitors and gate terminal should be as short as possible.

The development of a versatile pulse generator started with the selection of the appropriate electronic components. In this paper we desire to upgrade the driving circuit presented in Pirc et.al [62], mainly by reaching higher $\frac{dV}{dt}$, meaning we can generate shorter pulses on the output. Therefore, we analyzed available drivers and optical isolations.

Driver and optical isolation

In order to develop a generator that would be able to generate few hundred nanoseconds wide square wave pulses, with the best possible repeatability (good time precision) and at the same time high accuracy, we made an analysis and comparison between the most commonly used drivers and optical isolation in high voltage pulse generators. We were looking for a driver and optical isolation with a short minimal FWHM (Full-Width Half-Maximum) and at the same time high common-mode transient immunity (CMTI - $\frac{dV}{dt}$), because minimal FWHM and high CMTI define

the electoporator's output minimal pulse width. Additionally, the pulse width jitter should be low for good time precision. Therefore we evaluated the minimal FWHM and calculated the pulse width jitter of each component separately.

On the basis of the following characteristics: maximum working isolation voltage, CMTI, maximum pulse width distortion, maximal propagation delay skew and maximal propagation delay, collected from the datasheets, we picked the following optical isolators: HCPL-0723(Avago Technologies, Brodcom, USA), HFBR 0508Z (Broadcom, USA), ADuM210N0BRIZ (Analog Devices, USA); and drivers: IXDN609SI(IXYS, USA), MIC4422YM (Micrel, USA), UCC27531DBVT (Texas Instruments, USA). Additionally, we evaluated also ADuM4223 (Analog Devices, USA) and Si826BAD-C-IS (Silicon labs, USA), which are a combination of a driver and optocoupler in one chip. For the evaluation we used an evaluation board offered by the manufacturer (if available), otherwise, we made a custom board in accordance with the manufacturer's instructions or similar to other designs.

All the components under test were triggered with a function generator (33220A, Agilent Technologies, USA) set to: 5 V_{pp}, with 2.5 V_{pp} offset, frequency 10 Hz, pulse rise time 5 ns, pulse width 20 ns and number of pulses 30. The minimal trigger FWHM is 20 ns if the component under test did not generate the output pulse, the trigger FWHM was increased by steps of 10 ns. In the case of optical isolation evaluation, the output was measured with a high precision differential probe TDP1000 (Tektronix, USA) and displayed on the oscilloscope MSO4104 (Tektronix, USA). While for the evaluation of driver, the MOSFET in series connected to a charged capacitor and 100 Ω load resistor (TFSF100RJE- ND, Ohmite, USA), was added and the pulse on the MOSFET was measured, with a high voltage differential probe ADP305 (Lecroy, USA) and displayed on the oscilloscope Wavepro7300A (Lecroy, USA). The capacitor was charged with a high voltage generator MCP 350-1250 (FuG Elektronik GmbH, Germany).

Minimal measured FWHM and jitter for optical solutions are shown in the Table 1 and for drivers in Table 2. The minimal FWHM in the Tables 1, 2 is defined as an average of thirty measured FWHM's at minimal trigger FWHM and jitter is defined as the difference between maximal and minimal measured FWHM at minimal trigger FWHM. Further in the paper for easier reading instead of FWHM term pulse width is used.

The test showed ADuM4223 and Si826BAD-C-IS are less suitable for our application, due to high pulse width. Therefore we excluded them from further testing. HCPL-0723 has the highest jitter and according to the datasheet, also the lowest CMTI, which is only 10 $\frac{kV}{\mu s}$. HFBR 0508Z is actually a set of fiber optic transmitter and receiver connected with a plastic fiber optic cable. Due to the higher output pulse width and design complexity of HFBR 0508Z we finally deiced to use ADuM210N0BRIZ in our electroporator, because of its high CMTI, which is 100 $\frac{kV}{\mu s}$ and its best test performance.

IXDN609SI was the only driver that can be triggered with a 20 ns pulse, however, it has the biggest jitter. Because we wanted to develop a device that would be able to generate pulses as short as 200 ns, IXDN609SI is for our goal the most appropriate component among the tested devices. Final driving circuit topology was almost the same as presented in Pirc et.al [62]. Only two isolated DC-DC converters

were replaced with one isolated DC-DC converter MGJ2D052005SC (Murata Power Solutions, Japan) and optocoupler was changed. Optimal gate resistor value was determined through testing in accordance with the performance requirements.

MOSFETs in series

After the selection of appropriate components, a monopolar prototype was built. We connected three MOSFETs in series in order to evaluate the high voltage (4 kV) switching, the feasibility of the series and selected components. On Figure 7, a basic topology of the series is presented. Capacitors C_1 (940C20W1K-F, Cornell-Dubilier, USA) are charged by an HV power supply HCP350-6K5 (FuG Elektronik GmbH, Germany) and when triggered discharged by a R_{load} 150 Ω resistor (AZ151KE, Ohmite, USA). In parallel to each SiC MOSFET, a resistor R_1 (SM102031004FE, Ohmite, USA) is added, which ensures the uniform distribution of voltage over all three MOSFETs. In order to be able to generate pulses as short as 200 ns on the output load, rise and fall time should be lower than 50 ns. Trough the testing we determent the minimal gate resistor (R_g) value that still keeps the circuit stable is 6.8 Ω. We tested also different compositions eg. turning on lower MOSFET slower than the other two, however, in the end, the optimal results were obtained when all three MOSFETs had the same value of R_g.

The circuit was triggered with a digital delay generator DG645 (Stanford Research Systems, USA), the trigger pulse width was set to 100 ns. Output pulse was measured with the oscilloscope HDO6104A-MS (Lecroy, USA), high voltage differential probe HVD3605 (Lecroy, USA) and current probe CP031A (Lecroy, USA). The outstanding performance was reached, pulse voltage amplitude was 3.98 kV and pulse current amplitude was 28.45 A, while the pulse rise time was only 40.8 ns and fall time 47 ns, meaning minimal FWHM that can be generated is 100 ns as shown in Figure 8.

Asymmetric H-bridge

Four series of three MOSFETs presented in the previous section were connected in an asymmetrical H-bridge in order to enable the generation of asymmetric bipolar pulse. In Figure 9 the topology is presented, each block marked with S represents one MOSFET series from the previous section (in Figure 7 marked with dashed line). But now the divider resistors R_1 were enlarged to 100 MΩ (SM102031006FE, Ohmite, USA), with the intention to reduce the current through resistors and therefore the voltage between the electrode in an off state, is kept bellow electrode potential. Load resistor was also enlarged to 300 Ω and capacitor bank was extended. Five $C_2 = 5$ μF (B58033I9505M001, TDK, Japan) and $C_1 = 0.25$ μF (B58031U9254M062, TDK, Japan) capacitors were connected in series in a block with $R_2 = 10$ MΩ resistors for generation of each half period, meaning altogether there are two blocks. The additional logical circuit (NOR gate (MC74AC02DG)) at the input ensured that only one trigger (for a positive or negative half period) pulse is present at the same time. Because each MOSFET has maximal pulse drain current equal to 160 A, the maximal theoretical output current of the device is evaluated to be 131 A. We reduced the 160 A for 10 % and for the current flowing through the electroporator (current flowing through the electroproator at 4 kV is $\approx$ 13 A, because load resit is equal to 300 Ω).

Fabrication, assembly and measurements

The device displayed in Figure 10 was implemented on 6U (Eurocard) sized PCB card, it is double-sided with 140 μm copper layer and solder stop mask. If available, SMD electrical components were preferably chosen, trigger input connectors are a 50 Ω SMA. During the PCB design, special attention was given to make lines spaced for the high-voltage, and at the same time as short as possible and wide enough for the high currents. As a trigger digital delay generator DG645 (Stanford Research Systems, USA), generates two trigger pulses, one for positive and one for negative half period generation Output pulse was measured with the oscilloscope HDO6104A-MS (Lecroy, USA), high voltage differential probe HVD3605 (Lecroy, USA) and current probe CP031A (Lecroy, USA). Capacitors were charged with a high voltage generator MCP 350-1250 (FuG Elektronik GmbH, Germany). The measurements were analyzed in Matlab R2018b (MathWorks, Natick, Massachusetts, USA). The rise/fall time was defined as the time required for a pulse to rise/fall from 10/90 % to 90/10 % of the maximal measured voltage. Pulse width is defined by FWHM a time passed between the first crossing and second crossing of 50 % of the maximal measured voltage. And the pulse voltage amplitude was defined as an average value above the 95 % of the maximal measured voltage. Additionally, a relative error was determined as a difference between capacitor charge voltage and calculated maximal measured pulse voltage amplitude.

In vivo experiments

Animals

Animal experiments were conducted in accordance with the principles and procedures outlined with the guidelines for animal experiments of the EU directives and the permission from The Administration of the Republic of Slovenia for food safety, veterinary and plant protection (permission No.: U34401-1/2015/43). *In vivo* experiments were performed on 8-week-old female Balb/c mice (Envigo, Udine, Italy) that were maintained under specific pathogen-free conditions at constant room temperature in a 12-hour day/night light cycle. Food and water were provided *ad libitum*. One day prior to tumor induction, mice were shaved on their right flanks. Approximately 7 days after the subcutaneous injection of $0.5 * 10^6$ CT26 cells (American Type Culture Collection, Manassas, VA, USA) or when the tumors reached 40 mm^3, mice were divided into experimental groups (6-7 animals) and electrochemotherapy with bleomycin or cisplatin was performed.

Treatment protocol

Two electrochemotherapy protocols with bleomycin or cisplatin were compared *in vivo*: electrochemotherapy with well-established "classical" ECT pulses and electrochemotherapy with HF-ECT pulses. Treatment consisted of intratumoral bleomycin (Bleomycin medac, Medac, Wedel, Germany; 5 μg; 40 μL) or cisplatin (Cisplatina Kabi, 1 mg/mL, Fresenius Kabi AG, Bad Homburg, Germany; 40 g; 40 l) injection followed by electroporation 2 minutes later. For application of electric pulses, plate parallel stainless-steel electrodes 6 mm apart were used. A water-based gel was used to ensure good conductivity at the point of contact between electrodes

and the skin overlaying the tumors. Physiological solution (40 μL) was used instead of bleomycin or cisplatin in the control group and groups with electric pulses only. During treatment mice were under inhalation anesthesia.

In case of "classical" ECT, electric pulses were delivered with a commercially available BetaTech electroporator (Electro cell B10, Leroy, France). Electric pulse parameters: 780 V, 1.3 kV/cm voltage over the distance between the electrodes, eight 100 μs long pulses were applied in two perpendicular direction (4+4) at 1 Hz repetition frequency. HF-ECT were delivered by the newly developed electroporator. One combination of parameters of electric pulses was tested, eight, 100 μs long bursts of bipolar square wave pulses 1 μs - 1 μs - 1 μs - 1 μs (duration of a positive half period - pause between pulse half periods - duration of a negative half period - pause between bipolar pulses) were applied with plate electrodes. The pulse voltage amplitude was set to 1950 V (3.25 kV/cm voltage to distance ratio). For this a capacitor bank was added, which consists of eight capacitors 947D501K112BJMSN (CDE- Cornell Dubilier, USA) connected in two independent series. Four in series connected capacitors, are discharged at positive and the other four at negative pulse generation. The capacitor bank stores a sufficient amount of energy, to prevent an increase of voltage drop over 5 % of the preset voltage.

The therapeutic effect was followed three times per week by measuring tumor volume using a vernier caliper. Tumor volume was calculated by the following formula: $V = a * b * c * \frac{\pi}{6}$, where a, b and c were three mutually orthogonal tumor diameters. Mice were humanely sacrificed when the tumor volume reached 350 mm^3. A Kaplan–Meier survival plot was constructed with the tumor volume of 300 mm^3 representing the endpoint event.

In the scope of this study we also evaluated the muscle contractions during pulse delivery. Therefore a triple axis accelerometer BMA220 (DFRobot, China), was connected to Arduino UNO (Arduino, USA) on which software captured and saved the measurements, which were analysed post festum in Matlab. The absolute acceleration was calculated and gravitation was not excluded. The accelerometer was taped with a micropore tape to the right hind foot during pulse delivery, which caused no pain.

Competing interests
The authors declare that they have no competing interests.

Acknowledgements
This research was funded by the Slovenian Research Agency (ARRS) (MRIC UL IP-0510, P2-0249,P3-0003, J2-9227 and funding for Junior Researchers to Eva Pirc)

Author details
[1]University of Ljubljana, Faculty of Electrical Engineering, Tržaska 25, 1000 Ljubljana, Slovenia. [2]Department of Experimental Oncology, Institute of Oncology Ljubljana, Zaloška 2, 1000 Ljubljana, Slovenia. [3]University of Ljubljana, Faculty of Health Sciences, Zdravstvena pot 5, 1000 Ljubljana, Slovenia.

References
1. Kotnik, T., Rems, L., Tarek, M., Miklavčič, D.: Membrane electroporation and electropermeabilization: Mechanisms and models. Annu. Rev. Biophys **48** (2019)
2. Weaver, J.C.: Electroporation: A general phenomenon for manipulating cells and tissues. Journal of Cellular Biochemistry **51**(4), 426–35 (1993)
3. Haberl, S., Miklavčič, D., Serša, G., Frey, W., Rubinsky, B.: Cell membrane electroporation-part 2: The applications. IEEE Electrical Insulation Magazine **29**(1), 29–37 (2013)

4. Yarmush, M.L., Golberg, A., Serša, G., Kotnik, T., Miklavčič, D.: Electroporation-based technologies for medicine: Principles, applications, and challenges. Annual Review of Biomedical Engineering **16**, 295–320 (2014)
5. Golberg, A., Sack, M., Teissie, J., Pataro, G., Pliquett, U., Saulis, G., Stefan, T., Miklavčič, D., Vorobiev, E., Frey, W.: Energy-efficient biomass processing with pulsed electric fields for bioeconomy and sustainable development. Biotechnology for Biofuels **9**, 94 (2016)
6. Hui, S.W.: Effects of pulse length and strength on electroporation efficiency. In: Totowa (ed.) Plant Cell Electroporation and Electrofusion Protocols, pp. 29–40. Springer, New York (1995)
7. Miklavčič, D., Towhidi, L.: Numerical study of the electroporation pulse shape effect on molecular uptake of biological cells. Radiology and Oncology **44**(1), 34–41 (2010)
8. Weaver, J.C., Smith, K.C., Esser, A.T., Son, R.S., Gowrishankar, T.R.: A brief overview of electroporation pulse strength-duration space: A region where additional intracellular effects are expected. Bioelectrochemistry **87**, 236–243 (2012)
9. Langus, J., Kranjc, M., Kos, B., Šuštar, T., Miklavčič, D.: Dynamic finite-element model for efficient modelling of electric currents in electroporated tissue. Scientific Reports **6** (2016)
10. Neal 2nd, R.E., Garcia, P.A., Robertson, J.L., Davalos, R.V.: Experimental characterization and numerical modeling of tissue electrical conductivity during pulsed electric fields for irreversible electroporation treatment planning. IEEE Transactions on Biomedical Engineering **59**(4) (2012)
11. Ivorra, A., Al-Sakere, B., Rubinsky, B., Mir, L.M.: In vivo electrical conductivity measurements during and after tumor electroporation: Conductivity changes reflect the treatment outcome. Physics in Medicine Biology **54**(19), 5949 (2009)
12. Šel, D., Cukjati, D., Batiuskaite, D., Slivnik, T., L.M. Mir, L., Miklavčič, D.: Sequential finite element model of tissue electropermeabilization. IEEE transactions on bio-medical engineering **2**(5), 816–827 (2005)
13. Reberšek, M., Miklavčič, D.: Concepts of electroporation pulse generation and overview of electric pulse generators for cell and tissue electroporation. In: Pakhomov, A.G., Miklavčič, D., Markov, M.S. (eds.) Advanced Electroporation Techniques in Biology and Medicine, pp. 323–339. CRC Press, ??? (2010)
14. Reberšek, M., Miklavčič, D.: Advantages and disadvantages of different concepts of electroporation pulse generation. Automatika **52**(1), 12–19 (2011)
15. Merla, C., Pakhomov, A.G., Semenov, I., Vernier, P.T.: Frequency spectrum of induced transmembrane potential and permeabilization efficacy of bipolar electric pulses. Biochimica et Biophysica Acta (BBA) - Biomembranes **1859**(7), 1282–1290 (2017)
16. Serša, G., Miklavčič, D., Čemažar, M., Rudolf, Z., Pucihar, G., Snoj, M.: Electrochemotherapy in treatment of tumours. European Journal of Surgical Oncology (EJSO) **34**(2), 232–40 (2008)
17. Marty, M., Serša, G., Garbay, J.R., Gehl, J., Collins, C.G., Snoj, M., Billard, V.e., Geertsen, P.F., Larkin, J.O., Miklavčič, D., Pavlovic, I., Paulin-Kosir, S.M., Čemažar, M., Morsli, N., Soden, D.M., Rudolf, Z., Robert, C., O'Sullivan, G.C., Mir, L.M.: Electrochemotherapy – an easy, highly effective and safe treatment of cutaneous and subcutaneous metastases: Results of esope (european standard operating procedures of electrochemotherapy) study. European Journal of Cancer Supplements **4**(11) (2006)
18. Mir, L.M., Gehl, J., Serša, G., Collins, C.G., Garbay, J.-R., Billard, V., Geertsen, P.F., Rudolf, Z., O'Sullivan, G.C., Marty, M.: Standard operating procedures of the electrochemotherapy: Instructions for the use of bleomycin or cisplatin administered either systemically or locally and electric pulses delivered by the cliniporatortm by means of invasive or non-invasive electrodes. EJC Supplements **4**(11), 14–25 (2014)
19. Gehl, J., Serša, G., Matthiessen, L.W., Muir, T., Soden, D., Occhini, A., Quaglino, P., Curatolo, P., Campana, L.G., C.Kunte, Clover, A.J.P., Bertino, G., Farricha, V., Odili, J., Dahlstrom, K., Benazzo, M., Mir, L.M.: Updated standard operating procedures for electrochemotherapy of cutaneous tumours and skin metastases. Acta Oncol **57**(7), 874–882 (2018)
20. Jiang, C., Shao, Q., Bischof, J.: Pulse timing during irreversible electroporation achieves enhanced destruction in a hindlimb model of cancer. Annals of Biomedical Engineering **43**(4), 887–95 (2015)
21. Rubinsky, B.: Irreversible electroporation in medicine. Technology in Cancer Research Treatment **6**(4), 255–59 (2007)
22. Rubinsky, B., Onik, G., Mikus, P.: Irreversible electroporation: A new ablation modality — clinical implications. Technology in Cancer Research Treatment **6**(1), 37–48 (2007)
23. Cornelis, F.H., Cindrič, H., Kos, B., Fujimori, M., Petre, E.N., Solomon, D.M.S.B., Srimathveeravalli, G.: Peri-tumoral metallic implants reduce the efficacy of irreversible electroporation for the ablation of colorectal liver metastases. Cardiovasc Intervent Radiol. (2019)
24. Davalos, R.V., Mir, L.M., Rubinsky, B.: Tissue ablation with irreversible electroporation. Ann Biomed Eng **33**(2), 223 (2005)
25. Ball, C., Thomson, K.R., Kavnoudias, H.: Irreversible electroporation: A new challenge in out of operating theater anesthesia. Anesthesia Analgesia **110**(5), 1305–1309 (2010)
26. Landström, F.J., Nilsson, C.O., Crafoord, S., Reizenstein, J.A., Adamsson, G.B., Löfgren, L.A.: Electroporation therapy of skin cancer in the head and neck area. Dermatologic Surgery **36**(8), 1245–50 (2010)
27. Mali, B., Jarm, T., Jager, F., Miklavčič, D.: An algorithm for synchronization of in vivo electroporation with ecg. Journal of Medical Engineering & Technology **29**(6), 288–96 (2005)
28. Arena, C.B., Sano, M.B., Rossmeisl, J.H., Caldwell, J.L., Garcia, P.A., Rylander, M.N., Davalos, R.V.: High-frequency irreversible electroporation (h-fire) for non-thermal ablation without muscle contraction. BioMedical Engineering OnLine
29. Arena, C.B., Davalos, R.V.: Advances in therapeutic electroporation to mitigate muscle contractions. Journal of Membrane Science Technology
30. Sweeney, D.C., Reberšek, M., Dermol, J., Rems, L., Miklavčič, D., Davalos, R.V.: Quantification of cell membrane permeability induced by monopolar and high-frequency bipolar bursts of electrical pulses. Biochimica et Biophysica Acta (BBA) - Biomembranes
31. Reddy, V.Y., Neuzil, P., Koruth, J.S., Petru, J., Funosako, M., Cochet, H., Sediva, L., Chovanec, M.,

Dukkipati, S.R., Jais, P.: Pulsed field ablation for pulmonary vein isolation in atrial fibrillation. Journal of the American College of Cardiology **74**(3), 315–326 (2019)
32. Sugrue, A., Vaidya, V., Witt, C., DeSimone, C.V., Yasin, O., Maor, E., Killu, A.M., S. Kapa, C.J.M., Miklavčič, D., Asirvatham, S.J.: Irreversible electroporation for catheter-based cardiac ablation: a systematic review of the preclinical experience. Journal of Interventional Cardiac Electrophysiology **55**(3), 251–265 (2019)
33. Stewart, M.T., Haines, D.E., Verma, A., Kirchhof, N., Barka, N., Grassl, E., Howard, B.: Intracardiac pulsed field ablation: Proof of feasibility in a chronic porcine model. Heart Rhythm **16**(5), 754–764 (2019)
34. Scuderi, M., Reberšek, M., Miklavčič, D., Dermol-Černe, J.: The use of high-frequency short bipolar pulses in cisplatin electrochemotherapy in vitro. Radiology and Oncology
35. Golberg, A., Rubinsky, B.: Towards electroporation based treatment planning considering electric field induced muscle contractions. Technology in Cancer Research Treatment **11**(2), 189–201 (2012)
36. Daskalov, I., Mudrov, N., Peycheva, E.: Exploring new instrumentation parameters for electrochemotherapy. attacking tumors with bursts of biphasic pulses instead of single pulses. IEEE Engineering in Medicine and Biology Magazine **18**(1), 62–66 (1999)
37. Sano, M.B., Arena, C.B., Bittleman, K.R., DeWitt, M.R., Cho, H.J., Szot, C.S., Saur, D., Cissell, J.M., Robertson, J., Lee, Y.W., Davalos, R.V.: Bursts of bipolar microsecond pulses inhibit tumor growth. Scientific Reports **5** (2015)
38. Dong, S., Wang, H., Zhao, Y., Sun, Y., Yao, C.: First human trial of high-frequency irreversible electroporation therapy for prostate cancer. Technol. Cancer Res. Treat. **17** (2018)
39. Pakhomova, O.N., Gregory, B.W., Khorokhorina, V.A., Bowman, A.M., Xiao, S., Pakhomov, A.G.: Electroporation-induced electrosensitization. PLOS ONE **6**(2), 17100 (2011)
40. Pakhomova, O.N., Gregory, B.W., Pakhomov, A.G.: Facilitation of electroporative drug uptake and cell killing by electrosensitization. Journal of Cellular and Molecular Medicine **17**(1), 154–59 (2013)
41. Pakhomov, A.G., Semenov, I., Xiao, S., Pakhomova, O.N., Gregory, B., Schoenbach, K.H., Ullery, J.C., Beier, H.T., Rajulapati, S.R., Ibey, B.L.: Cancellation of cellular responses to nanoelectroporation by reversing the stimulus polarity. Therapeutics and Clinical Risk Management **71**(22), 4431–41 (2014)
42. Ibey, B.L., Ullery, J., Pakhomova, O.N., Roth, C.C., Semenov, I., Beier, H.T., Tarango, M., Xiao, S., Schoenbach, K., Pakhomov, A.G.: Bipolar nanosecond electric pulses are less efficient at electropermeabilization and killing cells than monopolar pulses. Biochem Biophys Res Commun **443**(2), 568–573 (2014)
43. Dermol, J., Pakhomova, O.N., Pakhomov, A.G., Miklavčič, D.: Cell electrosensitization exists only in certain electroporation buffers. PLOS ONE **11**(7), 0159434 (2016)
44. Polajžer, T., Dermol-Černe, J., Reberšek, M., O'Connor, R., Miklavčič, D.: Cancellation effect is present in high-frequency reversible and irreversible electroporation. Bioelectrochemistry
45. van Es, R., Konings, M.K., Pré, B.C.D., et al.: High-frequency irreversible electroporation for cardiac ablation using an asymmetrical waveform. BioMed Eng OnLine **18**(75), 288–96 (2019)
46. Petkovšek, M., Nastran, J., Voncina, D., Zajec, P., Miklavčič, D., Serša, G.: High voltage pulse generation [electroporation]. Electronics Letters **38**(14), 680–82 (2002)
47. Novickij, V., et al.: High-frequency submicrosecond electroporator. Biotechnology Biotechnological Equipment **30**, 1–7 (2016)
48. Redondo, L.M., Zahyka, M., Kandratsyeu, A.: Solid-state generation of high-frequency burst of bipolar pulses for medical applications. IEEE Transactions on Plasma Science **47**(8), 4091–4095 (2019)
49. Elserougi, A., Massoud, A., Ahmed, S.: High-power bipolar multilevel pulsed electroporator. Instrumentation Science Technology **44**(1), 65–72 (2016)
50. Petkovšek, M., Zajec, P., Nastran, J., Voncina, D.: Multilevel bipolar high voltage pulse source - interlock dead time reduction. In: Computer as a Tool. (2003)
51. Bhonsle, S.P., Arena, C.B., Sweeney, D.C., Davalos, R.V.: Mitigation of impedance changes due to electroporation therapy using bursts of high-frequency bipolar pulses. Biomed. Eng. Online **14**(Suppl 3), 3 (2015)
52. Sano, M.B., Fan, R.E., Cheng, K., Y. Saenz, G.A.S., Hwang, G.L., Xing, L.: Reduction of muscle contractions during irreversible electroporation therapy using high-frequency bursts of alternating polarity pulses: A laboratory investigation in an ex vivo swine model. Journal of Vascular and Interventional Radiology **29**(6), 893–898 (2015)
53. Sano, M.B., Arena, C.B., DeWitt, M.R., Saur, D., Davalos, R.V.: In-vitro bipolar nano- and microsecond electro-pulse bursts for irreversible electroporation therapies. Bioelectrochemistry **100** (2014)
54. Yao, C., Dong, S., Zhao, Y., Lv, Y., Liu, H., Gong, L., Ma, J., Wang, H., Sun, Y.: Bipolar microsecond pulses and insulated needle electrodes for reducing muscle contractions during irreversible electroporation. IEEE Trans Biomed Eng **64**(12), 2924–2937 (2017)
55. Forde, P.F., Sadadcharam, M., Bourke, M.G., Conway, T.A., Guerin, S.R., de Kruijf, M., O'Sullivan, G.C., Impellizeri, J., Clover, A.J.P., Soden, D.M.: Preclinical evaluation of an endoscopic electroporation system. Endoscopy **48**(5), 477–483 (2016)
56. Mirai Medical. https://www.mirai-medical.com/. [Accessed: 25-Dec-2019]
57. Pirc, E., Reberšek, M., Miklavčič, D.: Dosimetry in electroporation-based technologies and treatments. In: Markov, M. (ed.) Dosimetry in Bioelectromagnetics, pp. 233–268. CRC Press, Taylor Francis Group, ??? (2017)
58. Colombo, G.L., Matteo, S.D., Mir, L.M.: Cost-effectiveness analysis of electrochemotherapy with the cliniporatortrade mark vs other methods for the control and treatment of cutaneous and subcutaneous tumors. Therapeutics and Clinical Risk Management **4**(2), 541–48 (2008)
59. Canadian Agency for Drugs and Technologies in Health: Irreversible Electroporation for Tumors of the Pancreas or Liver: Review of Clinical and Cost-Effectiveness. (2016). Canadian Agency for Drugs and Technologies in Health
60. Campana, L.G., Cesari, M., Dughiero, F., Forzan, M., Rastrelli, M., Rossi, C.R., Sieni, E., Tosi, A.L.: Electrical resistance of human soft tissue sarcomas: an ex vivo study on surgical specimens. Med. Biol. Eng. Comput.

54(5), 773–787 (2016)
61. Ringel-Scaia, V.M., Beitel-White, N., Lorenzo, M.F., Brock, R.M., Huie, K.E., Coutermarsh-Ott, S., Eden, K., McDaniel, D.K., Verbridge, S.S., Rossmeisl, J.H., Oestreich, K.J., Davalos, R.V., Allen, I.C.: High-frequency irreversible electroporation is an effective tumor ablation strategy that induces immunologic cell death and promotes systemic anti-tumor immunity. EBioMedicine **44**, 112–125 (2019)
62. Pirc, E., Miklavčič, D., Reberšek, M.: Nanosecond pulse electroporator with silicon carbide mosfets: Development and evaluation. IEEE Transactions on Biomedical Engineering **66**(12), 3526–3533 (2019)
63. Stevanovic, L.D., Matocha, K.S., Losee, P.A., Glaser, J.S., Nasadoski, J.J., Arthur, S.D.: Recent advances in silicon carbide mosfet power devices. In: 2010 Twenty-Fifth Annual IEEE Applied Power Electronics Conference and Exposition (APEC), pp. 401–407 (2010)

Figures

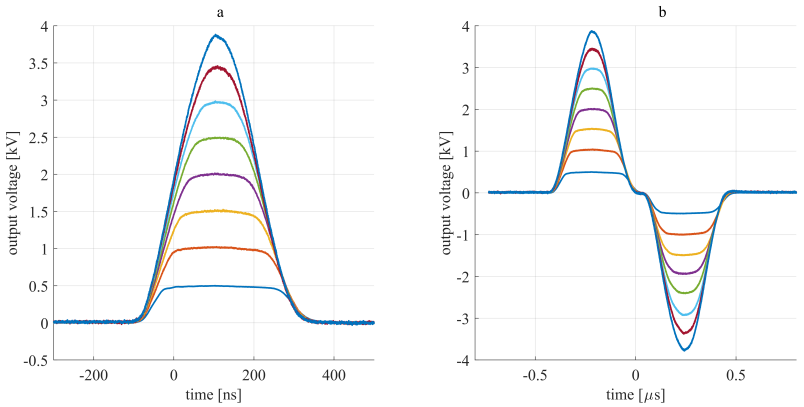


Figure 1 Output pulse measurement The measurement of monopolar (**a**) and bipolar (**b**) pulse on a 80 Ω load resistor. The capacitor charging voltage was set to 500 V and then raised to 4 kV with a step of 500 V.

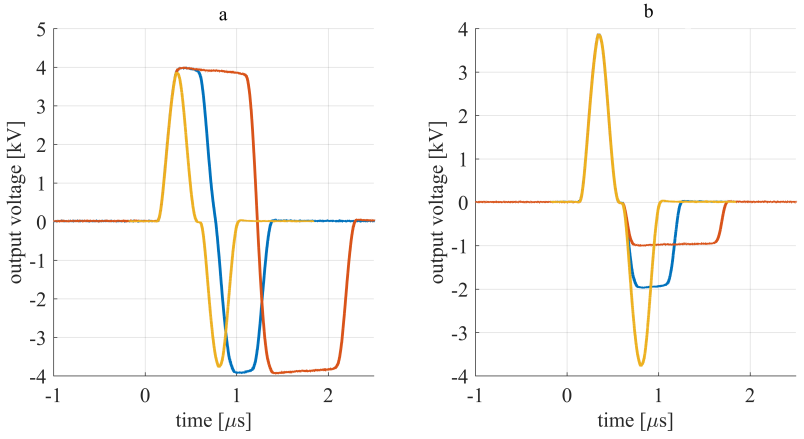


Figure 2 Output pulse measurement An example of symmetric (a) and asymmetric (equal charge) (b) bipolar pulse generation, the pulse width of a positive and negative half period can be independently changed and as well also the pulse amplitude.

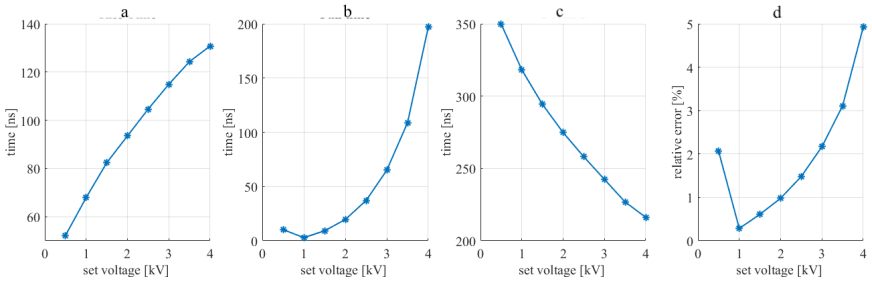


Figure 3 Output pulse measurement The rise (a) and fall time (b), as well as pulse duration (FWHM)(c) and relative amplitude error δU (d) for each measured monopolar pulse from the Figure 5(a).

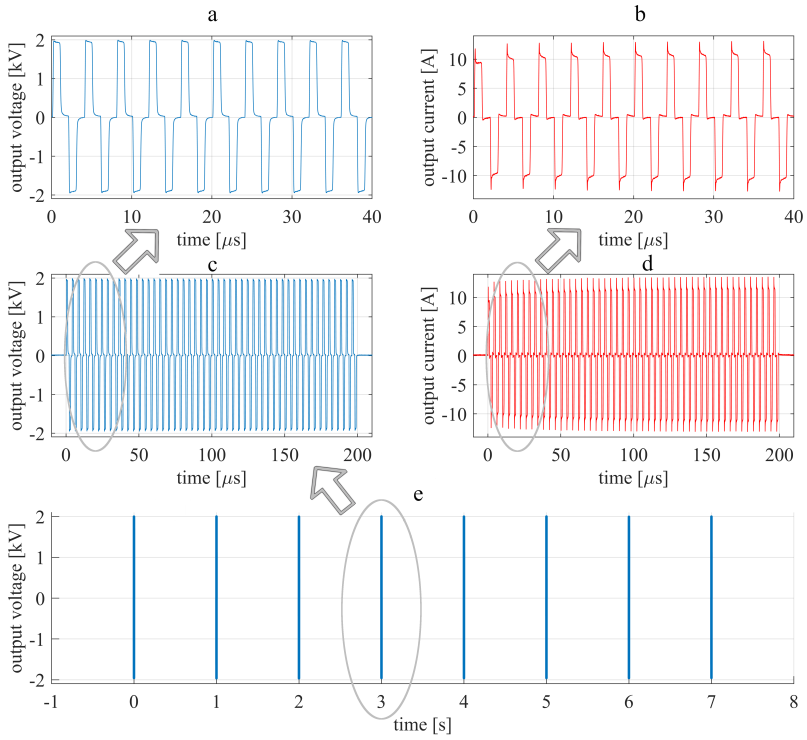


Figure 4 In vivo measurement *In vivo* measurement of applied voltage and current trough the electrodes: (e) Fourth burst of 1-1-1-1μs long bipolar pulses with the voltage amplitude set to 1950 V; (d) Measured fourth burst of current trough the electrodes; (c) Eight 100 μs long bursts were applied with the pulse repetition rate equal to 1 Hz (bursts were sequentially measured, meaning the pause between bursts was not measured). (a) and (b) are zoomed in measurements of (c) and (d), which demonstrate that, no voltage drop during the pulse was present.

Tables

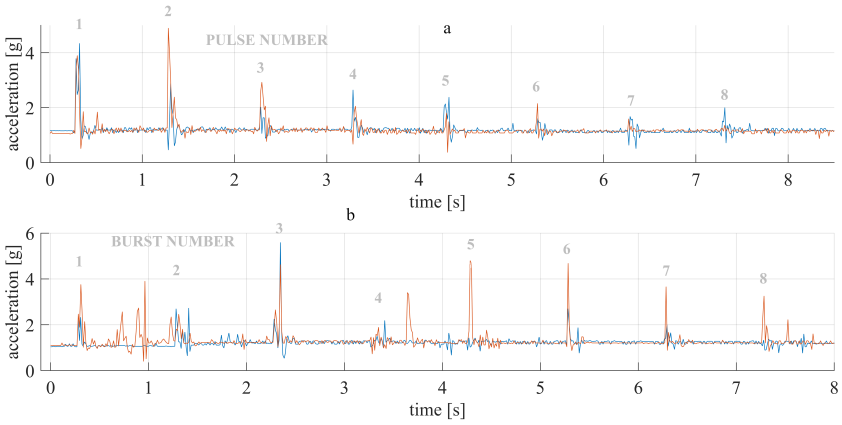


Figure 5 Mice leg acceleration measurement Measured absolute acceleration, of mice leg in case of: (a) classical ECT pulses, (b) bursts of bipolar pulses. Blue and orangre represent two individual measurements. Some noice is present in the pause between pulses, which was most likely caused by the person holding the mice and electrodes.

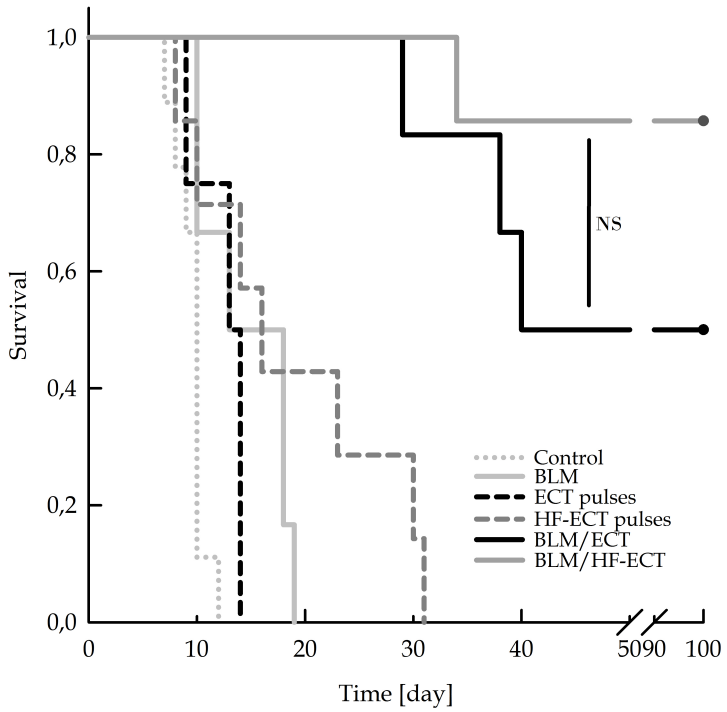


Figure 6 Animal survival Survival of animals treated with electrochemotherapy with bleomycin (BLM) using classical ECT pulses or HF-ECT pulses.

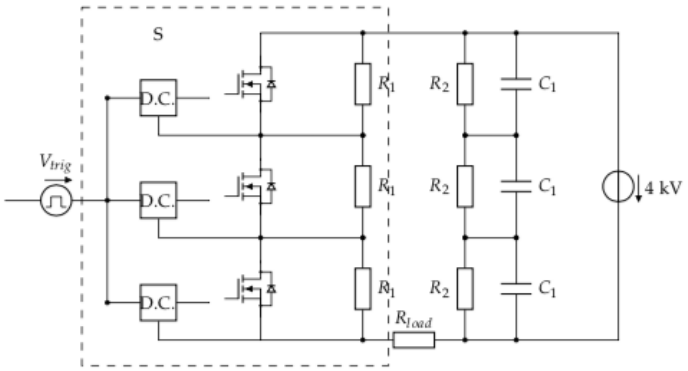


Figure 7 MOSFETs in series Basic topology of three MOSFETs in series, each MOSFET has its own driving circuit which is in the figure presented as a box marked with initials D.C.. Dashed line, defines a a box S, four of those boxes are for the purpose of bipolar pulse generation connected in H-bridge (Figure 3)

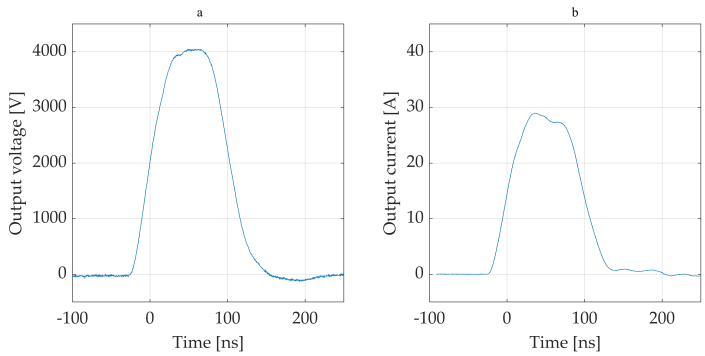


Figure 8 Measurement of the output pulse Measurement of the output pulse, generated by series of three MOSFETs: (**a**) Measured voltage on the 150 Ω load resistor. (**b**) Measured current trough the 150 Ω load resistor.

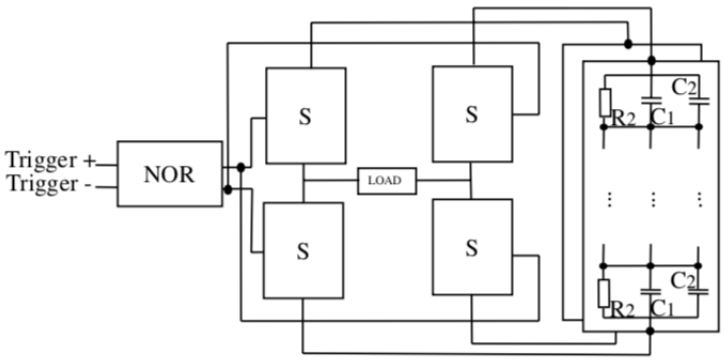


Figure 9 Basic topology of H-bridge Each block marked with S represents one MOSFET series from the previous section (in Figure 1 marked with striped line). Additional logical circuit (NOR gate (MC74AC02DG)) at the input ensured that only one trigger pulse is present at the same time.

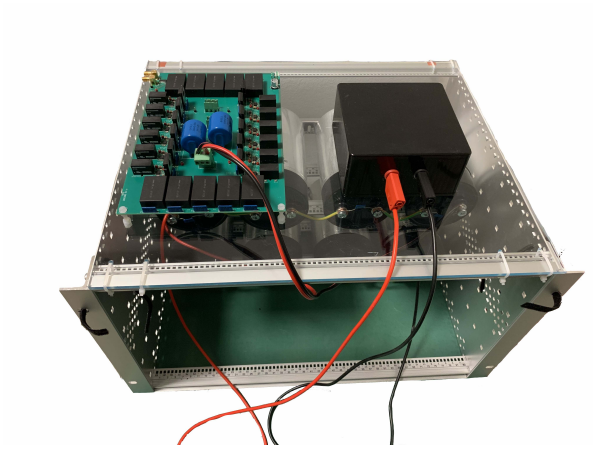


Figure 10 The developed device The developed H-bridge circuit is implemented on double-sided PCB 6U board and installed on a custom made capacitor bank.

Table 1 OPTICAL ISOLATORs: Mean values of minimal FWHM further defined as pulse width and jitter for a set of 30 measurements, for different optical isolators under test.

component	set/masured trigger width [ns]	output pulse width [ns]	jitter [ns]
HCPL-0723	20/19.10	18.53	1.04
ADuM4223	50/49.45	40.98	0.75
Si826BAD-C-IS	30/28.65	13.84	0.29
HFBR 0508Z	20/19.25	30.2	0.93
ADuM210N0BRIZ	20/21.37	18.28	0.26

Table 2 DRIVERs: Mean values of minimal FWHM and jitter for a set of 30 measurements, for different drivers under test.

component	set/measured trigger width [ns]	output pulse width [ns]	jitter [ns]
IXDN609SI	20/20.4	44.36	9.45
ADuM4223	70/69.99	67.53	1.98
UCC27531DBVT	110/109.23	57.27	3.8
MIC4422YM	40/39.82	125.26	2.55

3.8 Paper 7

Title: **Study design of a medical device pre-market assessment: a case study on electrochemotherapy**

Authors: Eva Pirc[1], Leandro Pecchia[2], Matej Reberšek[1], Gregor Serša[3], Marko Snoj[4], Aleš Grošelj[5], Damijan Miklavčič[1]

[1] University of Ljubljana, Faculty of Electrical Engineering, Ljubljana, Slovenia

[2] School of engineering, University of Warwick, Coventry, United Kingdom

[3] Institute of Oncology Ljubljana, Department of Experimental Oncology, Ljubljana, Slovenia

[4] Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia

[5] Department of Otorhinolaryngology and Cervicofacial Surgery, University Medical Centre Ljubljana, Ljubljana, Slovenia

Publication: *Zdravniški vestnik*, vol.87, no. 1-2

Year: 2018

DOI: 10.6016/ZdravVestn.2482

JAVNO ZDRAVSTVO (VARSTVO PRI DELU)

Study design of a medical device pre-market assessment: a case study on electrochemotherapy

Zasnova študije vrednotenja novih tehnologij v medicini: študija na primeru elektrokemoterapije

Eva Pirc,[1] Leandro Pecchia,[2] Matej Reberšek,[1] Gregor Serša,[3] Marko Snoj,[4] Aleš Grošelj,[5] Damijan Miklavčič[1]

Abstract

Final goal of the study is to estimate the cost-effectiveness of electrochemotherapy for the tre-atment of basal-cell carcinoma and skin melanoma. Paper consists of two parts: the first part presents basic principles and concepts of health technology assessment and cost-effectiveness analysis, and the second part reports an early cost-effectiveness analysis of electrochemothe-rapy for the treatment of basal-cell carcinoma and skin melanoma that we are developing. Few cost-effectiveness analyses of electrochemotherapy have already been done, but with a lack of information about intervention effectiveness in terms of quality of life, which may result in inac-curate or even inadequate conclusions. In order to obtain the most realistic results, two general Markov models and their reduced versions for initial calculations are presented. The models were designed specifically to assess electrochemotherapy of basal-cell carcinoma and skin me-lanoma. Also, data required for successful calculations have been identified, some of which are missing and will be collected within different studies which are still under way, including rando-mized control trials. Additionally, recommendations for data collection process and follow-up reporting are made. With this paper we want to raise awareness about the importance of nume-ric quality of life reporting and usefulness/meaning of EQ-5D questionnaire that might not be self-evident at first sight, but are crucial for cost-effectiveness analysis.

[1] Laboratory of Biocybernetics, Faculty of Electrical Engineering, University of Ljubljana, Ljubljana, Slovenia

[2] School of Engineering, University of Warwick, Coventry, United Kingdom

[3] Institute of Oncology Ljubljana, Ljubljana, Slovenia

[4] Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia

[5] Department of Otorhinolaryngology and Cervicofacial Surgery, University Medical Centre Ljubljana, Ljubljana, Slovenia

Korespondenca/ Correspondence: Eva Pirc, e: eva.pirc@fe.uni-lj.si

Ključne besede: vrednotenje tehnologij v zdravstvu (HTA); analiza stroškovne učinkovitosti (CEA); model Markova; elektroporacija; elektrokemoterapija (ECT)

Key words: health technology assessment (HTA); cost-effectiveness analysis (CEA); Markov model; electroporation; electrochemotherapy (ECT)

Izvleček

Končni cilj projekta je izdelava analize stroškovne učinkovitosti (*angl.* Cost-Effectiveness Analysis) elektrokemoterapije za zdravljenje bazalnoceličnega karcinoma in kožnega melano-ma. Prispevek je sestavljen iz dveh delov. V prvem delu prispevka so predstavljeni osnovni kon-cepti in postopki vrednotenja tehnologij v zdravstvu (*angl.* Health Technology Assessment) in analize stroškovne učinkovitosti, v drugem delu pa poročamo o zgodnji stroškovni analizi (*angl*. early Cost-Effectiveness Analysis) elektrokemoterapije, kot terapije za zdravljenje bazalnocelič-nega karcinoma in kožnega melanoma, kar je predmet naše študije. Nekaj stroškovnih analiz je že nastalo vendar zaradi pomanjkanja podatkov o učinkovitosti zdravljenja, v smislu povečanja kakovosti življenja bolnikov (*angl.* Quality of Life), zaključki ne morejo biti točni. Da bi izdelali čimbolj relevantno analizo, v članku predstavljamo dva splošna Markova modela in njuni poe-nostavljeni različici, ki ju bomo uporabili za začetne izračune. Modela sta bila zasnovana posebej za analizo elektrokemoterapije bazalnoceličnega karcinoma in kožnega melanoma. Dodatno so opredeljeni tudi potrebni podatki za uspešne izračune. Manjkajoče podatke bomo zbrali v okviru različnih študij, ki še vedno tečejo, vključno z randomiziranimi kliničnimi študijami. Predstavlje-no je tudi priporočilo za poročanje, ki bi olajšalo zbiranje podatkov. S tem prispevkom predvsem želimo dvigniti splošno ozaveščenost o pomembnosti številčnega poročanja o kakovosti življe-nja in uporabnosti oziroma pomenu vprašalnikov EQ-5D, ki na prvi pogled morda nista samou-mevna, vendar sta bistvenega pomena za analizo stroškovne učinkovitosti.

PREGLEDNI ZNANSTVENI ČLANEK

Prispelo: 12. 1. 2017
Sprejeto: 14. 11. 2017

Citirajte kot/Cite as: Pirc E, Pecchia E, Reberšek M, Serša G, Snoj M, Grošelj A, Miklavčič D. Study design of a medical device pre-market assessment. Zdrav Vestn. 2018;87(1–2):22–40.

DOI: 10.6016/ZdravVestn.2482

1. Introduction

Electroporation is an evolving technique with many applications (1-4); in this article the focus is on one of the most successful, i.e. electrochemotherapy (ECT). ECT is an antitumor therapy that increases the cytotoxicity of chemotherapeutic drugs such as bleomycin and cisplatin by the help of applied electric pulses. Electroporator is a device that generates high-voltage electric pulses, which are delivered to the tissue trough electrodes (5). If the electric field generated between the electrodes is sufficient, the permeabilization of the target (tumor) cells is triggered and entrance of the previously injected chemotherapeutic drug into cells (within the tumor) is enabled (6). As a result, electrochemotherapy is a highly efficient treatment, with complete response rates, based on a single treatment between 60 to 70 % and objective response rates up to 80 % (7).

Basal-cell carcinoma (BCC) is the most common form of skin cancer and its incidence is still increasing mainly due to the population ageing. Worldwide, every year two to three million patients are diagnosed with non-melanoma skin cancer (8). The current gold standard for the treatment of BCC (and other skin malignancies) is surgical excision (9), but for patients that are unsuitable for conventional treatments, ECT offers a good alternative. Specifically, in the treatment of BCC, the objective response rates after a single ECT session are close to 100 % (75). Upon that, the surrounding tissue remains undamaged and, consequently, a good cosmetic outcome is obtained. In most cases, ECT can be performed as an outpatient procedure under sedation or local anaesthesia. Therefore, treatment with ECT results in a considerably shorter hospital stay, faster recovery and reduced health care costs (10). ECT diminishes the need for surgery, it can be a feasible treatment option for cutaneous lesions resistant to other therapies or can serve as an adjunct to other therapies. The UK National Institute for Health and Care Excellence (NICE) deemed that ECT was a safe treatment for primary BCC, however it also warned about the limited evidence for its efficacy (11)

In western countries the incidence of melanoma has been increasing for as long as recorded (12). Melanoma spreads by lymphogenous and also by hematogenous route. ECT is a standardised procedure for the treatment of superficial metastases of melanoma resistant to other treatments. Skin metastases of melanoma occur in 2–20 % of melanoma patients (13). ECT has several advantages: the treatment can be administered in an outpatient setting under local anaesthesia and deep sedation; it can also be scheduled as a day-care or day-hospital procedure under general sedation; repeated sessions can be performed with a minimum interval of one to two weeks (14-17). Overall ECT can be considered as "patient friendly" procedure with effectiveness consistently reported throughout

the reports. Its effect can be localised, thus reducing side effects. Later in the article, the focus is mainly on the cost-effectiveness of ECT, which will facilitate a comparison of ECT to other treatment options, not only with respect to its effectiveness but also its cost effectiveness.

Screening and evaluation of medical technologies is becoming crucial, as in most of the developed countries health care expenditures are constantly increasing, while at the same time budgets are getting tighter (18). In Slovenia, health care spending is currently estimated below the European average (19,20). Between years 2002 and 2008, health care spending in Slovenia amounted to between 7.49 % and 8.08 % of GDP. It spiked in year 2009 to 8.56 % of GDP and has been between 8.5–8.73 % of GDP ever since (19,21). Unfortunately, based on demographic data, the forecast is not reassuring. In year 2017, 18.7% of Slovenian population is older than 65 years (22). The projections show that this number is going to be approximately 33.6% by 2060, what will unavoidably result in a significant increase in health care expenditure. Simulations show that health care expenditure will increase from 0.5 % up to 2.8 % of GDP per year, by the year 2060 (23). Independently of the relative or absolute expenditure per year, the health care national budgets are in any case limited. Therefore, decision makers have to consider carefully all public money spending, especially since, in limited budget scenarios, the introduction of a new technology may result in the exclusion of an old one. There are however cases where innovations were included into the medical practice, without any previous economical evaluations and had to be abandoned later due to economic inefficiency (73). The introduction of new technologies in the Slovenian public health care system is still relatively arbitrary. Each innovation is evaluated according to three criteria: i) health effectiveness (the more severe the disease is the more points it gets); ii) professional justification of the program (meta-analyses and systematic literature reviews get the most points, regardless of the results); iii) economic efficiency (only price is considered here, the cheaper the innovation is, the more points it gets). All innovations are than ranked on a priority list according to the number of points collected (24).

Therefore, an efficiency improvement of health care systems is necessary. Choices about providing health care interventions, based on available evidence regarding safety, effectiveness and cost effectiveness thus have to be made (18). Widely used in most developed countries is a Health Technology Assessment (HTA) evaluation process. HTA is a structured process aiming to inform decision makers about choices which can be used to allocate healthcare budget (25). In the majority of cases, HTA provides an estimate of the incremental cost-efficacy ratio (ICER), which informs decision makers about the gain in terms of quality of life per cost that the introduction of a new technology will produce (77). This article reports on an early cost-effectiveness analysis (eCEA) of ECT for the treatment of BCC and skin melanoma that we are developing. The first part of the paper introduces basic concepts of HTA and CEA, while the second part reports on a case study of electroporation based treatments, in particular ECT for BCC and skin melanoma.

2. Health technology assessment

According to the World Health Organization (WHO), *Health technology assessment (HTA) refers to the systematic evaluation of properties, effects, and/or impacts of health technology. It is a multidisciplinary process to evaluate the social, economic, organizational and ethical issues of a health intervention or health technology. The main purpose of conducting an assessment is to inform a policy/decision maker* (26). The evaluation can be applied to systems, general equipment, instruments, hardware and software as well as to procedures, standards, norms, staff skills, professional knowledge, drugs, public health programs, etc. However, HTA is currently manly employed for the pharmaceuticals. Slowly it is being claimed also for biomedical devices, where slightly different methods or at least some modifications of currently used methods are required (27). Main outcome of such evaluation should provide information about the costs /economic effectiveness, savings, performance, safety, ethical and social impacts and improvements of the investigated treatment/drug. Finally, the question that needs to be answered is: "Do we really need this technology and why?".

The formal proof of HTA can be found in systematic reviews, meta-analyses and randomized controlled clinical trials (28). A multi criteria decision analysis is suggested by which one can evaluate the cost-effectiveness through the benefits of quality adjusted life years (QALY). One of these analyses is the Cost-Effectiveness Analysis (CEA); its basics concepts and principles are presented in the following section.

Unlike drugs, which cannot be commercialised if sufficient evidence on their effectiveness is not collected, medical devices can be marketed after their safety has been proven. As a consequence, decision makers are called to make choices while cost-efficacy evidence is not fully available, especially for innovative technologies. Therefore, in the past years there has been a significant diffusion of early HTA (eHTA) or early economic analyses (78,79). In those cases, the limited clinical evidence that is available is projected using statistical methods, the costs are estimated assuming the worst case (i.e., max costs) and the uncertainty is quantified using statistical techniques. As a result, an eHTA informs the decision makers about the incremental risk-opportunity ratio, where risk is considered as a potential cost and the opportunity is considered as potential effectiveness. An eHTA assumes that proper HTA analyses are preformed when sufficient clinical data are available. The alternative would be to just wait and postpone the introduction of medical devices that can potentially save lives or increase significantly life quality, but this is not feasible with medical devices because their lifecycle is much shorter than that of drugs.

2.1. Cost-Effectiveness Analysis

There is a wide variety of approaches to economic evaluations of health technologies, one of them being cost-effectiveness analysis (CEA), which measures the incremental resources required for a new intervention in monetary units and the technology impact on patient health using different scales. In this paper, the focus is on the CEA, which measures

impact on health in terms of quality of life, which is known as cost - utility analysis (29).

2.1.1. Quality of life (QoL) and QALY

In HTA it is assumed that people in their life move through different health states, each of those states has a specific value attached to it - Quality of life (QoL). QoL describes a quality of individual's daily life, including social, emotional and physical aspects. For evaluating QoL, health care indices that provide information how health care influences patients, known as Health Adjusted life Years (HAYs), are used. One of them is Quality Adjusted Life Years (QALY), which is a general index (i.e., not disease specific), and a unit of health care outcome that merges length of life with quality of life. QALYs are used in the Cost Effectiveness Analysis (CEA) to determine the ratio of incremental costs (i.e., new technology versus a benchmark) to QALY gained. The index is defined as the value-weighted time-life-years, weighted by their quality-accumulated over the time (30). QoL is normalized to a standardized scale with ranges from 0.0 (death) to 1.0 (perfect health). According to some authors, negative values can also be reached, describing states worse than dead (e.g., dementia and coma are often considered equal to or worse than death (31)). Widely used and translated into most languages is the EuroQol (EQ-5D) evaluation tool (questionnaire), which is a standardized instrument for use as a measure of health outcome (The study/trial/project that use EQ-5D should be registered; see also: https://euroqol.org/support/how-to-obtain-eq-5d/) (32,33). It is also one of the measures recommended for use in cost-effectiveness analyses by the Washington Panel on Cost Effectiveness in Health and Medicine (34) and NICE (National Institute for Health and Care Excellence) (35). The health status is measured in five dimensions: mobility (walking ability); self-care (ability to wash or dress); usual activities (such as "work, study, housework, family or leisure activities"); pain/discomfort; and anxiety/depression. The respondents rate each dimension. There are two versions with a three-level (EQ-5D-3L, which rate each dimension using three values: '0', indicating no problems, '1' indicating some problems, or '2' indicating huge problems) and a five-level (EQ-5D-5L) scale. On the basis of patients' evaluation of their physical, social and cognitive functions final index – value is calculated. Therefore, the EQ-5D-3L defines 243 potential health states (i.e., 35), which together with two additional states for dead or unconscious give a total of 245 health states. For example, a year of perfect health is worth 1 and all non-perfect health years are worth less than 1, depending on the burden of the disease to the patient (36). Thus an intervention that generates six additional years in a health state valued at 0.65 will generate more QALYs than comparable technique that generate seven years in health state estimated at 0.5. There are also some other measurement instruments available that are frequently used in parallel or alternatively, such as Nottingham health profile, Quality of life Scale (QUOLS) and others (37-40). Since healthcare budget is always limited (sometimes scarce) and the introduction of a new technology may result in the exclusion of another, it is important to use a general index (e.g. QALY) in order to quantify the benefit on the whole national population (41,73).

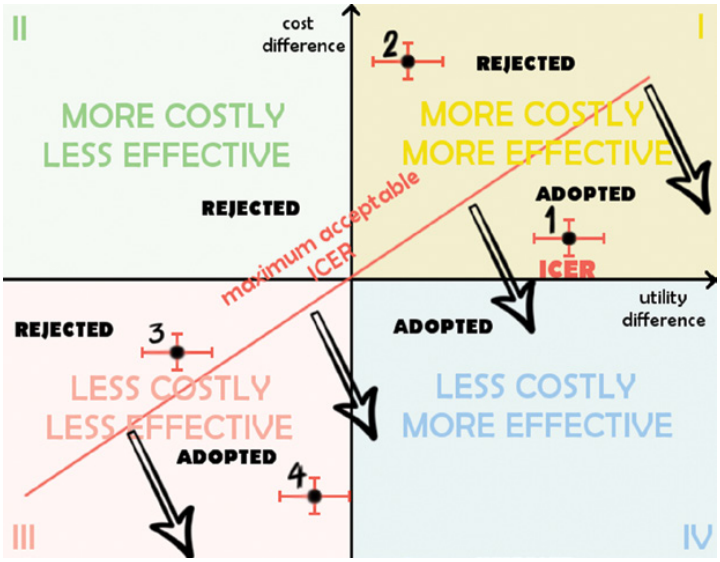


Figure 1: Incremental cost-effectiveness plane/diagram (70). The resulting ICERs are presented graphically as a ratio between costs and the effectiveness/utility or as a distribution with uncertainty in cost-effectiveness plane. Four quadrants represent all combinations of possible outcomes. The more effective outcomes are located further right on the x-axis, and with the rise of y-axis the cost of the outcome rises (69). An ICER of an innovation that is more costly and more efficient then the benchmark is located in the first quadrant; in case of a more costly and less efficient technology, ICER is in second quadrant. Other options can be derived from the figure.

2.1.2. Incremental cost ratio (ICER)

The result of CEA is presented with incremental cost ratio (ICER). ICER is defi ed as the additional cost per additional benefit/utility that is measured in QALY. Because QALY has a normal distribution and a sum of two normal variables is also normally distributed, ICER can be calculated as a ratio of two asymptotically normal variables (42). In the following equation (Eq. 1) treatment B represents a gold standard or benchmark to which new treatment X, e.g. electrochemotherapy, is compared.

$$RATIO = \frac{cost_{treatment\ X} - cost_{treatmentB}}{utility_{treatmentX} - utility_{treatmentB}} \quad (1)$$

The visualization of ICER is a distribution over a sample population, it is presented with a four quadrant cost--effectiveness plane that illustrates the relation between an incremental levels of effectiveness (utility gained) of an outcome and additional total cost of implementing this outcome (Figure 1). In case when new technology is more effective and cost efficient (fourth quadrant in Figure 1), compared to the benchmark, i.e. treatment B, than the innovation, i.e. treatment X, is for sure more suitable and worth of adopting. More commonly the outcomes of CEA of new technologies are divergent, for example the innovation is more effective but also more costly (case 1 and 2 in Figure 1). It should be decided whether the incremental utility is worth the cost and sustainable (43).

Maximum acceptable ceiling ratio (max ICER) is the threshold or the maximum amount that a decision-maker is prepared to pay for one incremental QALY. It is drawn in the cost-effectiveness plane, as a threshold line (Figure 1, red line). If the calculated ICER falls below this line, the new technology is considered cost effective and is adopted (cases 1 and 4 in Figure 1), otherwise it is rejected based on its cost-ineffectiveness (cases 3 and 2 in Figure 1) (44). Decisions, which apply to the public health care thresholds and acceptable outcomes of maximum ICER, are suggested by the WHO, however the problem remains how to apply these thresholds to each specific case. As analyses are often made by commercial entities with vested interests, the results may favor the new technology. It thus needs to be taken into account that the evaluator's subjective impact is always present. Another question that is also present is, how much the society shou-

ld pay for a QALY. According to the generally present opinion, e.g. in the UK, a QALY is worth somewhere between £20,000 and £30,000 (44). In Slovenia, rather than having a maximum value defined, each innovation is placed on a priority list of admission based on the evaluation of the following criteria: health effectiveness; quality of justification of recommended program; economic effectiveness (high scores correlate with lower costs); population perspective (more patients more points); and organizational efficiency. The quality of life is not considered in the evaluation.

CEA always includes a comparison of a new technology to the benchmark technology, if available. The innovation cannot be cost effective by itself, it has to be cost effective compared to the benchmark. In a CEA, a treatment producing an additional 0.5 QALY at an incremental cost of €3,000 per patient, is considered having a cost of €6,000 per QALY (i.e. €3,000 /0.5 QALY = €6,000 / QALY) (36).

2.1.3. Discounting

It should always be taken into account that values of costs and outcomes change with time (29). Because the CEA are projected through a certain period of time discounting is necessary. Cost and outcomes should be discounted relative to their present value at the rate of 3% or up to 5%, per year (29). The cost discount rate can be estimated from the equation (Eq. 2). Some authors suggest a common rate for costs and outcomes and others prefer a lower rate for outcomes. NICE discounting guidance for cost effectiveness analysis (45) requires that both costs and health outcomes are discounted at 3.5% (46).

$$present\ value = \sum_{n+1}^{n} \frac{future\ cost\ at\ n\ years}{(1 + annual\ discounting\ rate)^{n}} \quad (2)$$

For example, if the present value of cost is €2,500 and a 5% discount rate is used, one year in the future, the cost will be €2,375 and 5 years in the future the expense will be only €1,875. The same procedure is used for the discounting of health care outcomes.

2.1.4. Sensitivity analysis

The degree of uncertainty is a subject to variables used in CEA. To estimate plausible variations, a sensitivity analysis should always be performed. Such sensitivity analysis provides information on how a variation of a certain variable affects the result of the CEA. We distinguish between deterministic and probabilistic sensitivity analysis. Deterministic sensitivity analysis uses an approach in which one or more parameters are changed manually across a pre-specified range (range should correspond to the uncertainty defi ed in literature). Results are than analysed and the extent of the impact of input parameter variation on the output values is defined. Probabilistic sensitivity analysis captures uncertainty of all input variables, it is characterized through the use of probability distributions, translated to uncertainty and results in means and standard error for the incremental costs (47). If a correlation of input variables is present and available, it should also be considered and incorporated into the model (48).

2.1.5. Data collection and decision-making models

Most often, data collection methods used in HTA are: systematic literature review; meta-analysis; modelling; group judgment; unstructured litera-

ture review; and expert's opinions (29). Sometimes it happens that for a specific new medical device or an innovative technique there is not yet sufficient evidence available, apart from data proving its safety. When only few or low quality studies are available, potential source of bias must always be considered and documented (29). Additionally, in order to incorporate conditionality and uncertainty of data collected, decision models are used to simulate adjustments of projections of the existing primary data. With modeling patients conditions, treatment efficacy, treatment and maintenance costs and incidence of the disease, projections to a future costs and outcomes of the treatments or innovations can be made. Decision making trees, fuzzy logic and state transition modeling, such as Markov model and Monte Carlo simulation, are most often used (49). Markov models are widely used in HTA and health-economy studies, and recently Craven et al. (50) showed that this approach can also be used in pre-market HTA studies for medical devices.

In the CEA, each state in the model represents a specific state of health, or stage of disease between which the patient migrates. There is always one state that represents death, which is considered an absorbing state as it does not have a return path. Each state is associated with an average QALY, reflecting the quality of life of patients in this health status, a cost that is reflecting the cost needed to maintain the patient in this state (e.g., avoid deteriorations) and the probability to move from the current health state to another, or the stay in the same state in the following period of time (usually one year). Health states are defined according to the disease being studied. The model's structure should be as simple as possible, as it is not necessary to model the full complexity of the disease. Moreover, since the CEA focuses on incremental costs and QALY, only those states in which the technology under assessment is making real differences are relevant for the model. Model complexity (i.e. number of parameters and model order) should always be limited by the quantity and quality of data available (51). However, more aggregated structure that still includes fundamental disease process and interventions is often the best solution (47).

3. Case study on electrochemotherapy (ECT)

Electroporation is an evolving technique with many applications (1-4). In our study the focus is on electrochemotherapy of basal-cell carcinoma (BCC) and skin melanoma, as these two therapies are used for the longest time and have a very high success rate. Few cost-effectiveness analyses have already been done (52,53), but due the lack of information obtained, especially about QoL increase, the results are incomplete, inadequate and/or inaccurate. Numerical data about QoL increase are not available, QoL is reported only as "better", "highly improved", "significantly better", or using similar descriptors. Most probably the main reason that QoL was not acquired in previous studies is due to poor reporting. Our study originates from a previous one (52) and uses innovative eCEA methods (54). In order to facilitate result comparisons with benchmark technologies, cancer type specific analyses are done. For the scope of this paper, models for

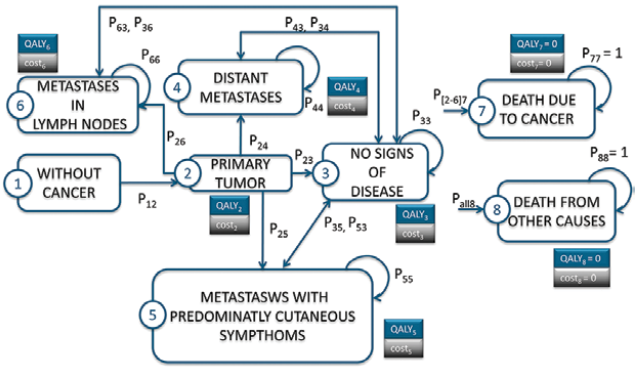


Figure 2: A general eight state Markov model of skin melanoma. The model should always reflect the essential features of the disease and its outcomes (40).

both studied cancer types are designed and presented. In addition, required and missing data are defined and presented (Table 1). In the near future we plan to collect all missing data trough randomized control trials and different studies which are still under way. With this paper we also want to raise general awareness about the importance of numeric QoL reporting and usefulness/meaning of EQ-5D questionnaires that might not be self-evident at first sight. If despite our effort the data will still not be available, simulations will be done to acquire the missing data. Our main aim is to build a general model that will allow us – with minimal changes – to also simulate/project the cost efficiency for patients with better initial survival options and to also stimulate similar studies for other cancer types treated with ECT.

Cancer patients that are treated with innovative technologies (which have not yet been introduced into clinical practice) in most cases have severe cancers and are not suitable for any other already established treatment. In fact they are usually without other treatment options. However, the goal of several emerging applications in oncology, such as ECT, is to be used in early-diagnosed patients with better survival options. Measurement of QoL increase in patients with severe cancer is more demanding and biased. QoL of a patient with severe cancer and poor survival options after the therapy cannot be equally compared with the QoL of patients, with better survival options before the procedure. Their quality of life before treatment was not the same, therefore we can assume that only a minimum increase in the quality of life could be observed (56).

Regarding the benchmark, there are different options. Some authors compare the innovative technology with the most recent cost-effective technology while others compare it with the gold standard/benchmark in order to make the analysis more replicable. For skin cancer, competitive technologies are: surgical excision, topical intralesional therapy, photochemotherapy and radiotherapy (14,55). In our study, the ECT treatment of BCC and skin melanoma will be compared to surgery and radiotherapy. We assume ECT is considerably cheaper than radiotherapy while having at least comparable effect (10,14,52).

3.1. Constructing the model

While the structure of the model should reflect the essential features of the disease and its interventions irrespective of data availability, it is expected that in some cases data availability may aff ct choices regarding model structure (29). In order to

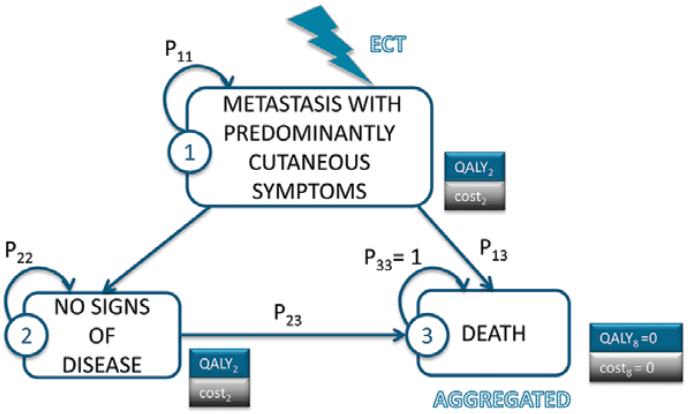


Figure 3: A reduced – initial Markov model for skin melanoma, only the states that are relevant for the new technology, in this case ECT, are included.

use the most appropriate models that would give the most realistic results, models are designed in advance and the data needed will be obtained subsequently in the near future (56). Two general models, for each cancer type, are presented in this paper (Figure 2, 4), covering whole complexity of the disease and its essential features. Those two models will be used for final simulations that could provide the cost-effectiveness evaluation of electrochemotherapy as a treatment also used in patients with less severe cancer stages. For the initial analysis, however, models are simplified/reduced (e.g. aggregating states) (Figure 3, 5), in order to facilitate the calculations and focus only on states that are relevant for the new technology (i.e. where ECT is currently used). Models for different cancer types differ from each other with respect to the number of states; their defi ition and flow through the model are conditioned by disease characteristics and possible outcomes.

Because cancer may be a recurrent disease, Markov model approach is used for modeling (57). The first general model was made for BCC (Figure 4). It is simple, it has only seven states and much less possible state transitions than the second general model that was made for skin melanoma (Figure 2) which has nine states. The initial states in both general models represent population without cancer. (These two states will be eliminated at the initial calculations, because they are not relevant for the new technology - ECT.) The following states represent different cancer stages, such as primary tumor, distant metastases, metastases in the lymph nodes, distant metastases with predominantly cutaneous symptoms and distant metastases without predominantly cutaneous symptoms in case of skin melanoma. Because all patients are not diagnosed with cancer at its initial state, some might enter the model with the disease that combines several states in the model. Clinical stages of skin melanoma are defined as follows: at stages I and II patients only have a primary tumor; stage IIIa,b means that a patient has a primary tumor and metastases in the lymph nodes; stage IIIc combines a primary tumor, local metastases and metastases with predominantly cutaneous symptoms. The last and most severe stage IV includes a primary tumor and distant metastases (76).

Electrochemotherapy is at the time of writing manly used in stage IIIc and IV patients (10,14), therefore a reduced - initial models for initial calculations consist only of states relevant for those two cancer stages. A reduced BCC model is slightly different and simpler. There are only three possible outcomes: complete response, residual disease and progressive disease. In the case of BCC, electro-

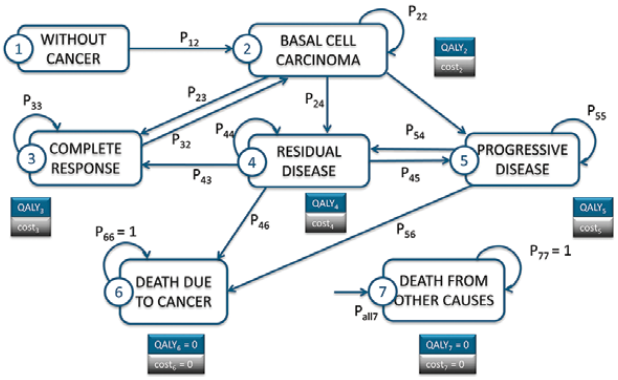


Figure 4: A seven state general Markov model of basal-cell carcinoma (BCC).

chemotherapy is used only in patients with bigger and recurrent tumors (10,14). Both general models also have the same two absorbing states, one is death due to cancer and the other is death from other causes; for initial calculations these two states will be merged into one state (patients can move to these two states from any other state, arrows are not drawn in the models for clarity (Figure 2, 4)). The probability of staying in dead state is always one and the cost zero. As seen from the model, all states are numerated and have two additional parameters: QALY and cost. The time step at which patients migrate between states is set in accordance with disease characteristics (58). Because signifi ant changes in QoL of patients treated with electrochemotherapy are detected already within few months after the treatment (34,59), the time step for the study is set to three months. In all models (Figure 2–5) numbers in the circles enumerate states, for example the state without cancer in both general models designates the state 1.The letter P in both general models (Figure 2, 4) represents a probability of transition between two states. The fi st undersigned number represents the state from which a transition is made and the second undersigned number provides the information to which state it is moving. Finally, in order to correctly include data in the model, a conditioned probability calculation will be performed (58).

3.2. Data collection approach

For each state represented in the model, three data are needed for a successful cost-effectiveness analysis:

1. The probability of transition between different states in a defi ed time step ($Example_{BCC\,g.\,model}$: P_{23} represents the probability of a complete response three months after the treatment.);
2. The cost of staying for three months in each state ($Example_{BCC\,g.\,model}$: $cost_2$ represents the sum of all expenses a health care provider has in the selected time step, such as medical examinations, medications, therapies, etc. in case patient has an untreated BCC);
3. The utility of patients in each state, expressed in QALY ($Example_{BCC\,g.\,model}$: $QALY_3$ represents the average result of an EQ-5D questionnaire filled out by the patients with complete response).

For the initial calculations, initial models that reflect only the impact of the technology under investigation (ECT) will be used. In case any of above listed information is not available, those data will have to be estimated using the best possible approach, including:

1. The transition probability between states or QoL data can be adapted from randomized control trials te-

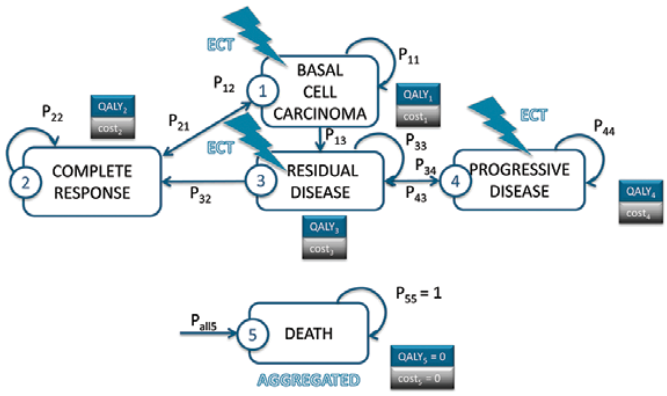


Figure 5: A reduced – initial Markov model for BCC, only the states that are relevant for the new technology, in this case ECT, are included.

sting the most similar technology in the most possible similar cancer (Figure 6: Case 1 and 2). The missing values can be estimated by fitting probabilistic distribution and then calculating mean and standard deviation, which are also useful for sensitivity analysis that will be performed later.

2. Alternatively, the missing data can be derived from interviews with expert clinicians and fitting the results with statistical distribution (Figure 6: Case 3).
3. The missing data for a general model can be simulated or predicted from initial calculations, made on reduced models that include only states relevant for the new technology. (The simulation will also provide the CEA estimation in case ECT would be used in early-diagnosed patients with greater survival options.)

Once the data for initial models will be collected, an incremental analysis will be run in order to determine the incremental cost-effectiveness ratio and its confidence interval. Finally, two steps will conclude the first stage of this study:

1. Sensitivity analysis (the model will be run using a deterministic approach by changing the input parameter value)
2. Statistical analysis (i.e. using Monte Carlo simulation, each subject will move from one state to another according to the given transition probability.)

3.3. Field of interest

1. *Probability of transition between states*: Response rates for initial calculations / probabilities for the electrochemotherapy treatments and other comparable technologies will be collected at fi st from different oncological trials in Slovenia. In case these data will have a high standard deviation, or there will not be enough of them for a representative pattern, we will expand our study requesting data and support from InspECT database (60). (Probability values will be defined from study reports; the use of a table (Table1) is suggested for easier data collection). Finally, we would also like to make some possible outcome predictions, in case that the technique will/would also be used in patients with earlier cancer stages and better survival options; for these purposes general models were developed. Cancer incidence data that will define the probability P12 (in both general models) for skin melanoma will be obtained from the Cancer Registry of the Republic of Slovenia (61). In case of BCC, the incidence should be multiplied by 1.3,because non-melanoma skin cancers are usually not properly included in the cancer registries (62). Also, the fact that the incidence of BCC continues to increase should be taken into account in general calculations.

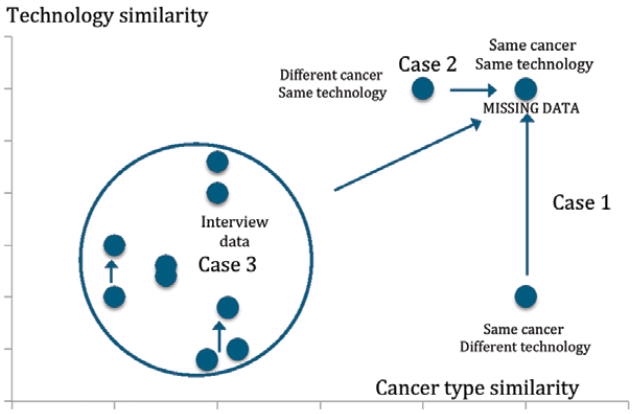


Figure 6: Visualization of data collecting and data simulating process.

Because most databases are made on an annual basis, the collected data will be projected on a three-month scale, if needed. Other probabilities will be simulated from initial calculations and expert's opinions. In case of skin melanoma, patients can be diagnosed at different cancer stages or might be in a state that is a combination of two states in the model; for example, they can have a primary tumor and distant metastases with predominantly cutaneous symptoms. In order to properly fit those probabilities in the model, statistical methods will be used.

2. *Quality of life evaluation*: already after a brief literature survey, it has become obvious that most of the relevant reports are missing data about EQ-5D results even though these should be collected as stated at clinical trial specifi ations (63,64). The quality of life is reported only as "better", "highly improved", "significantly better", or using similar descriptors (65,66,71,72) For the Markov model analysis, however, numeric data are required. This is one of the main reasons why we have decided to postpone the analysis, as we want to collect real data instead of implementing expert's estimations of QALY increase. It seems more rational to collect EQ-5D results, as only numerical values will provide us with adequate/useful results. At first we will try to collect the test results from already completed clinical trials. Most likely, if EQ-D5 were done, the results were collected at 3 and 8 months after the treatment, as specified in clinical trial documentation, so the rescaling on a time step basis will be necessary to fit into the model. Then we will ask physicians who are involved in trials including electroporation treatments to include the EQ-5D evaluation tool into their studies if they are not already included, and use the developed table template (Table 1) for reporting.

3. *Incremental cost evaluation*: costs depend on the specific application protocol, disease stages and severity. The first necessary and inevitable cost, which is common to all applications, is staff (e.g., nurses, doctors). Local or general anesthesia is also used in some procedures. In case of electrochemotherapy, the cost of intratumoral or intravenous application of chemotherapeutics cistplatinum or bleomycin must be considered. The cost of patient's hospitalization, the necessary medical examinations before the procedure and any other specific treatment that may entail side effects must also be taken into account.

The second necessary cost is the electroporator and electrodes. IGEA S.p.A. (Italy) produces a Cliniporator device, appropriate for electrochemotherapy of smaller cutaneous or subcutaneous tumors. Cliniporator VITAE is an upgrade, which has higher pulse amplitude and can also be used for electrochemotherapy of deep-seeded tumors or irreversible electroporation (IRE).

In the USA, AngioDynamics produces NanoKnife, which is mainly used for IRE. For successful application, electrodes are needed that may be for single or multiple use. IGEA offers many different electrode types. NanoKnife has only needle electrodes; from one up to six per treatment can be used. The amortization expense or annual lease of the device must also be considered. The cost of electrochemotherapy has already been evaluated for Italy by Colombo et. al. (52), a cost-effectiveness analysis has also been done, but without the quality of life consideration. All the collected data thus must be rechecked and updated for further evaluations. The authors calculated an average use of 1.3 electrode per procedure. In a further study, the number of available electrode types may significantly change because there are several new electrode configurations available. Maintenance was estimated biannually at €6,000 and annual lease of the device at that time was €12,000. The device lifespan is 8 years, and for all further calculations Colombo et. al. (52) assumed that at least 100 patients are treated per year with a single device, which may be an overly optimistic assumption. The cost is then calculated per patient. Electroporator and electrode price must be divided by its lifespan and the number of patients treated on a yearly basis. The cost of being in each state needs to be defi ed. The cost evaluation of being in initial state (having cancer) will be taken from the literature; for the USA, UK estimations of specific cancer types on a yearly basis are available (67,68). In case of expanding the data collection to other countries, we should consider the issue of different living standards, as the standard of living and money value is not the same everywhere. To align the costs, data collected from all around the world will be calibrated if needed. The adjustment will be made on the basis of living costs (74).

3.4. Data collection proposal

To facilitate initial data collection, a table template (Table 1) is proposed. The table refers to reduced models; in case of BCC there are five possible states and in case of skin melanoma only three. For each patient, every three months the clinician is supposed to:

- defi e the medical interventions;
- assess patient state by circling the appropriate number in the table;
- obtain EQ-5D questionnaire and report numerical value;
- record all medical interventions or examinations that patient had in the last period, including the type of anesthesia during ECT, days spend in hospital, biopsy, blood analysis and radiological assessment;
- report about all drugs prescribed to the patient for his/her condition, also for the management of side effects, analgesics, wound care dressing…
- report any other potential costs.

4. Conclusion

In the scope of this paper two general Markov models for CEA have been developed, one for skin melanoma and the other for BCC. Initially, both models are reduced in such a manner that they consists only of states that are relevant for the current use of new technology, i.e. electrochemotherapy. But for a cost-effectiveness analysis, the data on quality of life increase on a time step scale are also needed, which in our case represents a problem. Researchers are currently only reporting that quality of life is increased after the treatment, but

Table 1: A table template proposed for the initial data collection.

Patient code: *0001/2017* (BCC) Skin melanoma	**Pre-operative evaluation**	**Procedure**	**Follow-up at 3 months**	**Follow-up at 6 months**
Medical interventions	*1 visit at the specialist*	*Ect in general anesthesia, chest x-ray*	*2 visits at the specialist*	*3 visits at the specialist*
State (Reduced model)	(1), 2, 3, 4, 5	(1), 2, 3, 4, 5	1, 2, 3, (4), 5	1, 2, 3, (4), 5
EQ_5D	*0.45*	*0.43*	*0.38*	*0.33*
Biopsy	(Y) / N	Y / (N)	Y / (N)	Y / (N)
Blood analysis	Y / (N)	(Y) / N	(Y) / N	Y / (N)
Radiological assessment	(NO) / US / CT / MR / PET-CT	(NO) / US / CT / MR / PET-CT	(NO) / US / CT / MR / PET-CT	(NO) / US / CT / MR / PET-CT
Days of hospitalization	*0*	*2*	*0*	*2*
Wound care dressing (Product name)	*NO*	*NO*	*YES: XY©*	*NO*
Prescribed drugs	*NO*	*Paracetamol 500 mg tablets 3X1*	*NO*	*NO*
Other potential costs (Describe)	*NO*	*NO*	*NO*	*NO*

the numerical data that are crucial for successful cost-effectiveness analysis are missing (65,66,71,72) In this paper, the data needed for the evaluation of cost-effectiveness of electrochemotherapy treatment of skin melanoma and BCC are clearly identifi d. Additionally, recommendations for data collection process and follow-up reporting are made.

Because electrochemotherapy is an emerging technology, it is still not used in all patients, but as the proof of concept rises, it is gradually moving up the cancer staging scale. A realistic cost-effectiveness analysis for a specific cancer type will facilitate equipment purchase and clinical practice implementation. Additionally, the prediction of cost effectiveness can also influence the next round of fund raising. In our opinion, an additional incentive from a good cost--effectiveness analysis would definitely also benefit other electroporation-based therapies. The biggest obstacle is Quality of life data, which will be overcome by obtaining the EQ-5D questionnaires in the near future. Because the time step is set to three months, we believe a two-year data will give a representative pattern. Furthermore, specific models should be developed for each disease separately in order to include all specific phenomena in a specific cancer type. Electrochemotherapy must be compared to well established procedures that are cancer-type, location and size dependent.

5. Acknowledgment

This study was supported by COST Action BM1309 (COST EMF-MED) within the STSM (ECOST-STSM-BM1309–110416–073660) and the Slovenian Research Agency (ARRS), conducted within the scope of the European Associated Laboratory on the Electroporation in Biology and Medicine (LEA- EBAM)

References

1. Haberl S, Miklavcic D, Sersa G, Frey W, Rubinsky B. Cell membrane electroporation-Part 2: the applications. IEEE Electrical Insulation Magazine. 2013;29(1):29–37.
2. Kotnik T, Frey W, Sack M, Haberl Meglič S, Peterka M, Miklavčič D. Electroporation-based applications in biotechnology. Trends in Biotechnology. 2015;33(8):480–8.
3. Zorec B, Préat V, Miklavčič D, Pavšelj N. Active enhancement methods for intra- and transdermal drug delivery: a review. Zdrav Vestn. 2013;82(5):339–356.
4. Golberg A, Sack M, Teissie J, Pataro G, Pliquett U, Saulis G, et al. Energy-efficient biomass processing with pulsed electric fields for bioeconomy and sustainable development. Biotechnology for Biofuels. 2016;9(1).
5. Pirc E, Reberšek M, Miklavčič D. 12 Dosimetry in Electroporation-Based Technologies and Treatments. Dosimetry in Bioelectromagnetics: CRC Press; 2017. p. 233–68.
6. Stepišnik T, Jarm T, Grošelj A, Edhemović I, Djokić M, Ivanecz A, et al. Elektrokemoterapija – učinkovita metoda zdravljenja tumorjev s kombinacijo kemoterapevtika in električnega polja. Zdrav Vestn. 2016;85(1).
7. Mali B, Jarm T, Snoj M, Sersa G, Miklavcic D. Antitumor effectiveness of electrochemotherapy: A systematic review and meta-analysis. European Journal of Surgical Oncology (EJSO). 2013;39(1):4–16.
8. Narayanan DL, Saladi RN, Fox JL. Review: Ultraviolet radiation and skin cancer. International Journal of Dermatology. 2010;49(9):978–86.
9. Telfer NR, Colver GB, Morton CA. Guidelines for the management of basal cell carcinoma. British Journal of Dermatology. 2008;159(1):35–48.
10. Clover AJP, O'Mahony J, Soden D. Electrochemotherapy of Basal Cell Carcinoma. Handbook of Electroporation: Springer International Publishing; 2016. p. 1–12.
11. NICE. Electrochemotherapy for primary basal cell carcinoma and primary squamous cell carcinoma. [March 22, 2017] Available from: https://www.nice.org.uk/guidance/ipg478/resources/electrochemotherapy--for-primary-basal-cell-carcinoma-and-primary-squamous-cell-carcinoma-1899869938127557.
12. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA: a cancer journal for clinicians. 2016;66(1):7–30.
13. Savoia P, Fava P, Nardò T, Osella-Abate S, Quaglino P, Bernengo MG. Skin metastases of malignant melanoma: a clinical and prognostic survey. Melanoma Research. 2009;19(5):321–6.
14. (14) Campana LG, Sepulcri M, Valpione S, Corti L, Rossi CR. Electrochemotherapy for Superficially Metastatic Melanoma. Handbook of Electroporation: Springer International Publishing; 2017. p. 2095–112.
15. Quaglino P, Mortera C, Osella-Abate S, Barberis M, Illengo M, Rissone M, et al. Electrochemotherapy with Intravenous Bleomycin in the Local Treatment of Skin Melanoma Metastases. Annals of Surgical Oncology. 2008;15(8):2215–22.
16. Campana LG, Mocellin S, Basso M, Puccetti O, De Salvo GL, Chiarion-Sileni V, et al. Bleomycin-Based Electrochemotherapy: Clinical Outcome from a Single Institution's Experience with 52 Patients. Annals of Surgical Oncology. 2009;16(1):191–9.
17. Campana LG, Valpione S, Mocellin S, Sundararajan R, Granziera E, Sartore L, et al. Electrochemotherapy for disseminated superficial metastases from malignant melanoma. British Journal of Surgery. 2012;99(6):821–30.
18. van Tulder M. Health technology assessment (HTA) increasingly important in spine research. European Spine Journal. 2011;20(7):999–1000
19. The Organisation for Economic Co-operation and Development (OECD)"Health Resources - Health Spending - OECD Data." theOECD. [March 22, 2017] Available from: http://data.oecd.org/healthres/health-spending.htm.
20. Pallikarakis N, Moore R. Health Technology in Europe - Regulatory Framework and Industry Perspectives of the »New Approach«. IEEE Engineering in Medicine and Biology Magazine. 2007;26(3):14–7.
21. Analiza zdravstavenega Sistema v Sloveniji; Pregled izdatkov v zdravstvu; Končno poročilo, oktober 2015. [June 30, 2016] Available from: http://www.mz.gov.si/fileadmin/mz.gov.si/pageuploads/Analiza/04022016porocila SL/Report Expenditure review Slovenia FINAL FORMATTED SI 4.pdf.

22. Statistični urad republike slovenije- Starejše prebivalstvo. [March 22, 2017] Available from: http://www.stat.si/doc/StarejsePrebivalstvo.pdf.
23. Analiza zdravstvenega sistema v Sloveniji Povzetek in ključne ugotovitve. [March 22,2017] Available from: http://www.mz.gov.si/fileadmin/mz.gov.si/pageuploads/Analiza/analiza_ZS_povzetek_in_kljucne_ugotovitve_lektorirana_verzija.pdf.
24. Ministrstvo za zdravje RS. Postopek obravnave vlog za nove zdravstvene programme 2015. Available from: http://www.mz.gov.si/si/o_ministrstvu/zdravstveni_svet_in_ostala_posvetovalna_telesa/zdravstveni_svet/postopek_za_vloge/.
25. Turchetti G, Spadoni E, Geisler E. Health Technology Assessment. IEEE Engineering in Medicine and Biology Magazine. 2010;29(3):70–6.
26. World Health Organization [June 30, 2016]. Available from: http://www.who. int/medical devices/assessment/en/.
27. Pecchia L, Craven MP. Early stage Health Technology Assessment (HTA) of biomedical devices. The MATCH experience. IFMBE Proceedings: Springer Berlin Heidelberg; 2013. p. 1525–8.
28. Thokala P, Duenas A. Multiple Criteria Decision Analysis for Health Technology Assessment. Value in Health. 2012;15(8):1172–81.
29. Clifford S, Goodman. HTA 101 Introduction to Health Technology Assessment. Bethesda,MD: National Library of Medicine (US); 2014. [July 4, 2016] Available from: https://www.nlm.nih.gov/nichsr/ hta101/HTA 101 FINAL 7–23–14.pdf.
30. Weinstein MC, Torrance G, McGuire A. QALYs: The Basics. Value in Health: The Journal of the International Society for Pharmacoeconomics and Outcomes Research 12 Suppl 1:S5–9.
31. Patrick DL, Starks HE, Cain KC, Uhlmann RF, Pearlman RA. Measuring Preferences for Health States Worse than Death. Medical Decision Making. 1994;14(1):9–18.
32. EuroQol official pag. [July 4, 2016] Available from: http://www.euroqol.org/.
33. Rabin R, Charro Fd. EQ-SD: a measure of health status from the EuroQol Group. Annals of Medicine. 2001;33(5):337–43.
34. Weinstein MC, Siegel JE, Gold MR, Kamlet MS, Russell LB. Recommendations of the Panel on Cost-effectiveness in Health and Medicine. Jama. 1996;276(15):1253–8.
35. Guide to the methods of technology appraisal 2013. [April 4, 2013] Available from: https://www.nice.org.uk/process/pmg9.
36. Phillips C, Thompson G. What is a QALY Health economics 2nd ed. Newmarket: Hayward Medical Communications; 2009.
37. Burckhardt CS, Anderson KL. Health and Quality of Life Outcomes. 2003;1(1):60.
38. Hunt SM, McKenna SP, McEwen J, Williams J, Papp E. The Nottingham health profile: Subjective health status and medical consultations. Social Science & Medicine Part A: Medical Psychology & Medical Sociology. 1981;15(3):221–9.
39. Stavrianou K, Pallikarakis N. Quality of life of end-stage renal disease patients and study on the implementation of nocturnal home hemodialysis in Greece. Hemodialysis International. 2007;11(2):204–9.
40. Longworth L, Rowen D. Mapping to Obtain EQ-5D Utility Values for Use in NICE Health Technology Assessments. Value in Health. 2013;16(1):202–10.
41. Torrance GW, Feeny D. Utilities and Quality-Adjusted Life Years. International Journal of Technology Assessment in Health Care. 1989;5(04):559–75.
42. Briggs AH, Wonderling DE, Mooney CZ. Pulling cost-effectiveness analysis up by its bootstraps: A non-parametric approach to confidence interval estimation. Health Economics. 1997;6(4):327–40.
43. Hill SR. Cost-effectiveness analysis for clinicians. BMC Medicine. 2012;10(1).
44. Fenwick E, Marshall DA, Levy AR, Nichol G. Using and interpreting cost-effectiveness acceptability curves: an example using data from a trial of management strategies for atrial fibrillation. BMC Health Services Research. 2006;6(1).
45. O'Mahony JF, Paulden M. NICE's Selective Application of Differential Discounting: Ambiguous, Inconsistent, and Unjustified. Value in Health. 2014;17(5):493–6.
46. Claxton K, Paulden M, Gravelle H, Brouwer W, Culyer AJ. Discounting and decision making in the economic evaluation of health-care technologies. Health Economics. 2010;20(1):2–15.
47. Weinstein MC, O'Brien B, Hornberger J, Jackson J, Johannesson M, McCabe C, et al. Principles of Good Practice for Decision Analytic Modeling in Health-Care Evaluation: Report of the ISPOR Task Force on Good Research Practices—Modeling Studies. Value in Health. 2003;6(1):9–17.
48. Andronis L, Barton P, Bryan S. Sensitivity analysis in economic evaluation: an audit of NICE current practice and a review of its use and value in decision-making. Health Technology Assessment. 2009;13(29).

49. Rodina-Theocharaki A, Bliznakova K, Pallikarakis N. Markov Chain Monte Carlo simulation for projection of end stage renal disease patients in Greece. Computer Methods and Programs in Biomedicine. 2012;107(1):90–6.
50. Craven MP, Morgan SP. Early Stage Economic Evaluation with a Small Medical Device Start-Up Company Using a Markov Model. Journal of Medical Devices. 2011;5(2):027516.
51. Foster KR, Koprowski R, Skufca JD. Machine learning, medical diagnosis, and biomedical engineering research - commentary. BioMedical Engineering OnLine. 2014;13(1):94.
52. Colombo G. Cost-effectiveness analysis of electrochemotherapy with the Cliniporator™ vs other methods for the control and treatment of cutaneous and subcutaneous tumors. Therapeutics and Clinical Risk Management. 2008;4:541–8.
53. CADTH Rapid Response Reports. Irreversible Electroporation for Tumors of the Pancreas or Liver: A Review of Clinical and Cost-Effectiveness. Ottawa (ON): Canadian Agency for Drugs and Technologies in Health; 2016.
54. Pecchia L, Craven MP. Early stage Health Technology Assessment (HTA) of biomedical devices. The MATCH experience. IFMBE Proceedings: Springer Berlin Heidelberg; 2013. p. 1525–8.
55. Spratt DE, Gordon Spratt EA, Wu S, DeRosa A, Lee NY, Lacouture ME, et al. Efficacy of Skin-Directed Therapy for Cutaneous Metastases From Advanced Cancer: A Meta-Analysis. Journal of Clinical Oncology. 2014;32(28):3144–55.
56. Briggs AH. Handling Uncertainty in Cost-Effectiveness Models. PharmacoEconomics. 2000;17(5):479–500.
57. Komorowski M, Raffa J. Markov Models and Cost Effectiveness Analysis: Applications in Medical Research. Secondary Analysis of Electronic Health Records: Springer International Publishing; 2016. p. 351–67.
58. O'Mahony JF, Newall AT, van Rosmalen J. Dealing with Time in Health Economic Evaluation: Methodological Issues and Recommendations for Practice. PharmacoEconomics. 2015;33(12):1255–68.
59. Bianchi G, Campanacci L, Ronchetti M, Donati D. Electrochemotherapy in the Treatment of Bone Metastases: A Phase II Trial. World Journal of Surgery. 2016;40(12):3088–94.
60. Brizio M, Ribero S, Campana LG, Clover AJP, Gehl J, Kunte C, et al. International Network for Sharing Practices on Electrochemotherapy (InspECT): An Integrative Patients Treatment Consortium. Handbook of Electroporation: Springer International Publishing; 2016. p. 1–18.
61. The Cancer Registry of Republic of Slovenia. Available from: http://www.onko-i.si/rrs/.
62. Trakatelli M, Morton C, Nagore E, Ulrich C, Del Marmol V, Peris K. Update of the European guidelines for basal cell carcinoma management. European Journal of Dermatology 2014;24(3):312–29.
63. Marty M, Sersa G, Garbay JR, Gehl J, Collins CG, Snoj M, et al. Electrochemotherapy – An easy, highly effective and safe treatment of cutaneous and subcutaneous metastases: Results of ESOPE (European Standard Operating Procedures of Electrochemotherapy) study. European Journal of Cancer Supplements. 2006;4(11):3–13.
64. Campana LG, Clover AJ, Valpione S, Quaglino P, Gehl J, Kunte C, et al. Recommendations for improving the quality of reporting clinical electrochemotherapy studies based on qualitative systematic review. Radiology and oncology. 2016;50(1):1–13.
65. Larkin JO, Collins CG, Aarons S, Tangney M, Whelan M, O??Reily S, et al. Electrochemotherapy. Annals of Surgery. 2007;245(3):469–79.
66. Sersa G, Cufer T, Paulin SM, Cemazar M, Snoj M. Electrochemotherapy of chest wall breast cancer recurrence. Cancer Treatment Reviews. 2012;38(5):379–86.
67. Mariotto AB, Robin Yabroff K, Shao Y, Feuer EJ, Brown ML. Projections of the Cost of Cancer Care in the United States: 2010–2020. JNCI Journal of the National Cancer Institute. 2011;103(2):117–28.
68. National Cancer Institute, Cancer Prevalence and Cost of Care Projec- tions. Available from: https://costprojections.cancer.gov/.
69. David Parkin. Techniques of Economic Appraisal (Including Cost- Effectiveness Analysis and Modelling, Cost-Utility Analysis, Option Ap- praisal and Cost-Benefit Analysis, the Measurement of Health Benefits in Terms of QALYs and Related Measures E.g. DALYs). Health Economics 4d. Available from: https://www.healthknowledge.org.uk/public-health-textbook/medical-sociology-policy-economics/4d-health-economics/economic-appraisal.
70. Black WC. The CE Plane. Medical Decision Making. 1990;10(3):212–4.
71. Lido P, Paolino G, Feliziani A, Santurro L, Montuori M, Sanctis Fd, et al. Cutaneous metastasis of unknown primary presenting as massive and invasive abdominal lesion: an elective approach. Anais Brasileiros de Dermatologia. 2015;90(6):879–82.
72. Ribero S, Balagna E, Sportoletti Baduel E, Picciotto F, Sanlorenzo M, Fierro MT, et al. Efficacy of electrochemotherapy for eruptive legs keratoacanthomas. Dermatologic Therapy. 2016;29(5):345–8.

73. Refaat T, Choi M, Gaber G, Kiel K, Mehta M, Gradishar W, et al. Markov Model and Cost-Effectiveness Analysis of Bevacizumab in HER2-Negative Metastatic Breast Cancer. American Journal of Clinical Oncology. 2014;37(5):480–5.
74. Cost of living index. [July 4, 2016] Available from: https://www.expatistan.com/cost-of-living/index/europe.
75. Bertino G, Sersa G, De Terlizzi F, Occhini A, Plaschke CC, Groselj A, et al. European Research on Electrochemotherapy in Head and Neck Cancer (EURECA) project: Results of the treatment of skin cancer. European Journal of Cancer. 2016;63:41–52.
76. Amin MB, Edge SB, Greene FL, Byrd DR, Brookland RK, Washington MK, et al. Organization of the AJCC Cancer Staging Manual. AJCC Cancer Staging Manual: Springer International Publishing; 2016. p. 31–7.
77. Pecchia L, Bracale U, Bracale M. Health Technology Assessment of Home Monitoring for the Continuity of Care of patient suffering from congestive heart failure. IFMBE Proceedings: Springer Berlin Heidelberg; 2009. p. 184–7.
78. Ijzerman MJ, Koffijberg H, Fenwick E, Krahn M. Emerging Use of Early Health Technology Assessment in Medical Product Development: A Scoping Review of the Literature. PharmacoEconomics. 2017;35(7):727–40.
79. Fasterholdt I, Krahn M, Kidholm K, Yderstræde KB, Pedersen KM. Review of early assessment models of innovative medical technologies. Health Policy. 2017;121(8):870–9.

3.9 Paper 8

Title: **Early cost-effectiveness analysis of electrochemotherapy as a prospect treatment modality for skin melanoma**

Authors: : Eva Pirc[1], Carlo Federici[2]3, Maša Bošnjak[4], Barbara Perić[5], Matej Reberšek[1], Leandro Pecchia[2], Nebojša Glumac[5], Maja Čemažar[4], Marko Snoj[5], Gregor Serša[4] and Damijan Miklavčič[1]

[1] University of Ljubljana, Faculty of Electrical Engineering, Ljubljana, Slovenia

[2] University of Warwick, School of engineering, Coventry, United Kingdom

[3] Centre for Research on Health and Social Care Management (CERGAS), SDA Bocconi University, Milan, Italy

[4] Institute of Oncology Ljubljana, Department of Experimental Oncology, Ljubljana, Slovenia

[5] Institute of Oncology Ljubljana, Department of Surgical Oncology, Ljubljana, Slovenia

Publication:*Clinical therapeutics*

Impact factor: 2.935 (2018)

Quartile: Q2 (pharmacology & pharmacy)

Rank: 108/267 (pharmacology & pharmacy)

Status: in preparation for submission

Early cost-effectiveness analysis of electrochemotherapy as a prospect treatment modality for skin melanoma

Eva Pirc[1], Carlo Federici[2,3], Maša Bošnjak[4], Barbara Perić[5], Matej Reberšek[1], Leandro Pecchia[2], Nebojša Glumac[5], Maja Čemažar[4], Marko Snoj[5], Gregor Serša[4] and Damijan Miklavčič[1]*

*corresponding author

[1] University of Ljubljana, Faculty of Electrical Engineering, Ljubljana, Slovenia
[2] University of Warwick, School of engineering, Coventry, United Kingdom
[3]Centre for Research on Health and Social Care Management (CERGAS), SDA Bocconi University, Milan, Italy
[4] Institute of Oncology Ljubljana, Department of Experimental Oncology, Ljubljana, Slovenia
[5]Institute of Oncology Ljubljana, Department of Surgical Oncology, Ljubljana, Slovenia

Abstract

Purpose: Electrochemotherapy is increasingly entering into national and international guidelines, requiring formal evaluation of treatment costs and cost-effectiveness to ensure that their uptake actually provides value for money to budget-constrained health care systems. The study aims to conduct an early cost-effectiveness analysis of electrochemotherapy with Cliniporator™ in patients with stage IIIc and IV skin melanoma in Slovenia.

Methods: The study enrolled 23 patients, treated with electrochemotherapy at the Institute of Oncology Ljubljana. The average cost of electrochemotherapy was estimated using patient-specific cost data on electrochemotherapy procedure and subsequent follow up. Quality adjusted life years were estimated by collecting EQ-5D questionnaires at baseline; after complete or partial response following the treatment; and after a relapse of skin lesions. A discrete-time Markov model was built to estimate the lifetime costs and consequences of using electrochemotherapy compared to standard of care, from the perspective of the Slovenian health care system. The analysis was conducted separately for the whole patients' sample and for the subset of patients with bleeding lesions.

Deterministic and probabilistic sensitivity analysis were conducted to test model assumptions and characterize the uncertainty around model parameters.

Findings: In the whole patient population, electrochemotherapy of skin melanoma stage IIIc and IV is expected to increase the quality of life by 0.29 QALYs (95 % Credible interval 0.097, 0.498), at the higher cost of €6,568 (95% CrI 4,593, 8,928) in comparison to the standard of care. At the cost-effectiveness threshold of €20,000 per QALY, the probability of electrochemotherapy being cost-effective compared to standard of care is estimated to be 0.30 and 0.91 for the whole patient sample and the patients with bleeding lesions respectively. For the whole sample population, a reduction in the price of the electrodes by half is expected to increase the probability of being cost-effectiveness from 0.30 to approximately 0.64.

1. Introduction

Electroporation is a phenomenon by which the transport of otherwise impermeant molecules through the cell membrane is facilitated[1–3]. Electroporation is becoming increasingly recognized in medicine[4–6] and also in food technology and biotechnology[7–9]. In medicine, electroporation is used for the treatment of solid tumors, either in combination with chemotherapy (electrochemotherapy) or alone (irreversible electroporation). Electrochemotherapy (ECT) is a local antitumor therapy that increases the toxicity of chemotherapeutic drugs bleomycin or cisplatin[4]. Nonthermal irreversible electroporation (IRE) enables the ablation of undesirable (malignant) tissue, with minimal damage to blood vessels and nerves[10]. Additionally, electroporation is also a promising delivery method for the introduction of genetic material for DNA vaccination[11]. Published studies demonstrated that the electric field established by

applied high voltage, short-duration electric pulses, increases the plasma membrane permeability[2]. Electroporation can be performed as a reversible or irreversible electroporation. In the case of reversible electroporation, the cells fully recover after electric pulse application, while in case of irreversible electroporation after the pulse application cells die, due to the loss of the cell homeostasis. The device that generates and enables the delivery (via the application-specific electrodes) of electroporation pulses to biological tissue, is named an electroporator[12,13].

With the development of the electroporation field, new medical therapies, new clinical electroporators, and innovative delivery systems, questions regarding cost-effectiveness arise. In fact, while the technology is entering into national and international guidelines[14], however, its value for money remains largely unexplored in mostly all settings. Indeed, not many electrochemotherapy cost-effectiveness analyses have been published to date[15], and due to the lack of information obtained, especially about the quality of life increase, results are incomplete[16]. The UK National Institute for Health and Care Excellence (NICE) deemed that electrochemotherapy is a safe treatment for primary basal cell carcinoma and primary squamous cell carcinoma, however, it also warned about the limited evidence around its efficacy[17]. Electrochemotherapy treatment of skin melanoma and basal cell carcinoma has been uptaken broadly in Slovenian clinical practice and therefore there is a need to discuss whether the treatment is cost-effective and whether the price of the electrodes is appropriate. The aim of this study was to perform an economic evaluation of electrochemotherapy , with the Cliniporator™ as a treatment modality for stage IIIc and IV skin melanoma skin melanoma in Slovenia.

1.1 Electrochemotherapy of skin melanoma

From all forms of skin cancer types, cutaneous melanoma is the most deadly and sixth most common cancer type in Slovenia (3.84 % among all cancer types)[18].. Melanoma spreads by lymphogenous and also by the hematogenous route. The AJCC (American Joint Committee on Cancer) stages of skin melanoma are defined as follows: localized melanoma (stage I and II), meaning patients only have a primary tumor; stage III for regional metastatic melanoma, where IIIa/b includes patient with a primary tumor and metastases in the lymph nodes; stage IIIc combines a primary tumor, local metastases, and metastases with predominantly cutaneous symptoms. The most severe stage IV includes patients with a primary tumor and distant metastases[19]. Progression of the disease and survival rate highly depend on the stage at which melanoma is diagnosed and treated[20]. Cutaneous metastases of melanoma occur in 2–20 % of skin melanoma patients[21] and are one of the most common malignancies in the world. Electrochemotherapy is at the time of writing manly used in stage IIIc and IV patients[22,23].

Electrochemotherapy is a standardized procedure for the treatment of superficial metastases of melanoma resistant to other treatments. European Standard Operating Procedures of Electrochemotherapy (ESOPE) have been established in 2006[24], for cutaneous and subcutaneous tumors and updated in 2018[25]. National Institute for Health and Care Excellence (NICE), in the UK, has recognized electrochemotherapy as an integral part of the multidisciplinary treatment for patients with skin metastases of non-skin origin and melanoma (NICE interventional procedure guidance IPG 446)[26]. Electrochemotherapy of skin melanoma is a highly effective treatment, with complete response rates, based on a single treatment between 60 to 70 % and objective response rates up to 80 %[27,14]. The main advantages of the treatments are: i) it can be administered in an outpatient setting, under local anesthesia or deep sedation and is therefore simple

to perform; ii) the procedure is assumed as daily intervention, as it is finished within half of an hour; iii) in case of low or no response, it can be repeated after a month (however, the cumulative dosage of bleomycin should not be exceeded) or later when new metastases develop; iv) and also has a good cosmetic outcome, because the surrounding tissue remains undamaged[28,29]. Overall electrochemotherapy is considered a safe procedure with little or no side effects[27,30,31]. Electrochemotherapy diminishes the need for surgery and is, a feasible treatment option for cutaneous lesions resistant to other therapies.

2. Methods and materials

2.1 Patients

We enrolled 23 patients, treated with electrochemotherapy at the Institute of Oncology Ljubljana in Slovenia between June 2014 and March 2019. Patients were treated according to Slovenian Recommendations for the treatment of patients with cutaneous melanoma. At the time of writing 8 of the patients were still ongoing patients while the other 15 died or refused further treatment. The average patient age at the first electrochemotherapy procedure was 78.1, with a standard deviation of 12.3 years, range from 48 to 96 years. Patients were diagnosed with stage IIIc or IV malignant melanoma and were not amenable to other treatments. In Slovenian guidelines for the treatment of skin melanoma, electrochemotherapy indicated as a treatment modality for recurrence at the extremities where simple excision is not possible (> 3–5 metastases) or for recurrent relapses (sooner than 3–6 months)[32]. Patients were treated with electrochemotherapy in accordance with standard operating procedure with the Cliniporator™ device[24,25]. Follow up examinations were conducted 14 days after the intervention and after 1, 2, 4, 8 and 12 months. Altogether 38 electrochemotherapy

procedures were performed, meaning on average each patent was treated 1.6 times, range 1 to 5 procedures per patient. At each electrochemotherapy procedure, all skin lesions present at the time of procedure were treated. electrochemotherapy was repeated when new lesions were presented or when only a partial response was obtained.

2.2 Cost analysis

2.2.1 The cost of electrochemotherapy

The average cost and standard deviation of single electrochemotherapy procedure were estimated using patient-specific data on resource consumption during electrochemotherapy procedure and subsequent follow-ups and attaching the corresponding unit costs. All the costs are presented in euros and valid for Slovenia. Costs related to the hospital and procedure were obtained from the Institute of Oncology Ljubljana, whereas IGEA S.p.A. provided prices related to the medical device. The overall cost of electrochemotherapy is presented in TABLE 1.

TABLE 1. : Costs included in the evaluation of the cost of electrochemotherapy in Slovenia

Unit	Cost per unit (€)
Overnight stay in the hospital	240
(one night after electrochemotherapy + one day)	
Price of intervention and cost of staff	
(average duration: 45 minutes to1 hour)	
Staff one hour of procedure	128.25
Supplies for personnel and venue	66.13
Consumables during operation	99.25

Depreciation of apparatus in an operating room	22.57
Chemotherapeutic drug	
Bleomycin vial	30
Cisplatin bottle	23
Anesthesia	
General anesthesia	225.88
Local anesthesia	4.4
Sedation	14.60
Spinal block	15.90
Electrodes EPS series	1,200

151

152 The average cost of single electrochemotherapy procedure was estimated to €2,757
153 with the standard deviation €707.3. Additionally to the individual costs of specific items
154 reported above in TABLE 1, the Institute of Oncology also provided an exact expense
155 for eight electrochemotherapy procedures. The error between the exact amount and our
156 evaluation was less than 10 %, therefore we concluded that the assessment method was
157 appropriate.

158 General anaesthesia was used in 25 out of 38 treatments.. On average 1.5 to 2
159 bleomycin vials and one cisplatin bottle per patient was used, and the cost of bleomycin
160 was thus set to €52.5 per procedure. The cost of cisplatin is less, but was only used in
161 two electrochemotherapy procedures. Electrodes represent almost half of the price for
162 electrochemotherapy procedure and new versions of electrodes (EPSA series) are even
163 more expensive, with a price of €1600 per single electrode (VAT excluded). All
164 electrochemotherapy electrodes manufactured by IGEA S.p.A are for single-use,
165 meaning one electrode can be used for one patient, but for multiple metastases, however,

166 in some cases more than one electrode geometry is used due to the difference in
167 metastases. In the scope of our study 1.19 electrodes per patient were used, in range
168 from 1 to 2 per procedure. Also, other patient-specific costs were analysed and
169 evaluated, such as analgesics or antibiotics, but because these costs did not exceed €10
170 per treatment, they were neglected in the analysis. Follow-up specialist visits were
171 conducted after 1, 2, 4, 8 and 12 months following the intervention with an estimated
172 cost of €22.5 per examination.

173 Cliniporator^{TM} device, manufactured by IGEA S.p.A. is essential part to perform
174 electrochemotherapy. The price of the device Cliniporator^{TM} EPS02 is €100,000 (VAT
175 excluded). The maintenance cost is €3,000 and the maintenance is due every 24 or 36
176 months according to the country specific (e.g. Germany: 24 months, while Italy, U.K.
177 and Slovenia: 36 months). The Cliniporator^{TM} device is considered as a highly stable
178 device, therefore IGEA as a manufacturer requires the maintenance only every 36
179 months. The device lifetime is specified as 500 treatment sessions or 10 years (based
180 on user manual).

181 **2.2.2 Cost of skin melanoma**

182 Following an extensive literature review, we couldn't identify baseline cost data for
183 skin melanoma in the Slovenian setting. However, an extensive cost of illness study is
184 available for Croatia[33]. Because Croatia and Slovenia are neighboring countries and not
185 long ago, were even both part of the same country, data collected for Croatia were
186 considered in this study. For the calculations, the average cost per single patient for
187 stage IV melanoma was set to €4,333 per year. This value is also in accordance with the
188 European average[34] and close to value available for Italy[35]. With the help of Slovenian
189 palliative experts, we evaluated the worst-case cost of care for a patient with bleeding
190 lesions to be € 3,450 per patient per year (includes only the care of bleeding wounds).

191 This cost can be completely eliminated after the electrochemotherapy procedure,
192 however, only 5 % of melanoma patients develop bleeding lesions.

193 **2.3 Quality of life**

194 Patients were given an EQ5D questionnaire at each examination, and the following
195 results were obtained: before the procedure the average quality of life was equal to 0.65,
196 the patient that responded to electrochemotherapy had an increase in quality of life to
197 0.72 and in case of relapse of metastases, the quality of life decreased to 0.66. A
198 significant increase in quality of life was expected only in patents with bleeding nodes,
199 which after ECT gain the most, however, only 5 % of all melanoma patients have
200 bleeding lesions, meaning 1.15 patients in our study. The quality of life for patient with
201 bleeding lesions was in initial state thus reduced to minimal obtained quality of life,
202 which was equal to 0.4.

203

204 **2.4 Cost-effectiveness analysis**

205 A cost-effectiveness analysis was conducted to estimate the lifetime costs and
206 consequences of using electrochemotherapy in the target patient population from the
207 perspective of the Slovenian health care system. The model used a time horizon of 10
208 years, which was considered to appropriately reflect the average survival time in this
209 patients population. All future costs and consequences were discounted at a 3.5%
210 discount rate.

211 Patients included in the study are mostly elderly with stage IIIc and IV skin
212 melanoma and are generally not amendable to any other treatment option. Therefore, in
213 the present study, electrochemotherapy was compared to the standard of care (SOC)
214 consisting of symptomatic therapy and palliative treatment. The cost-effectiveness of

electrochemotherapy compared to standard of care was expressed in terms of incremental net health benefit (iNHB) which is calculated based on:

$$iNHB_{ECT\ vs\ SOC} = \Delta QALY - \Delta Cost/k$$

Where $\Delta QALY$ and $\Delta Cost$ are the differences in the expected QALYs and costs between electrochemotherapy and standard of care, and k is a constant value representing the cost-effectiveness threshold[36]. Electrochemotherapy is then considered cost-effective for any value of the iNHB greater than 0. The value of the cost-effectiveness threshold for Slovenia was assumed to be equal to the 2018 Slovenian real Gross Domestic Product (GDP) per capita, and set equal to €20,000 per QALY[37].

The CEA analysis was conducted separately for the whole patients' sample and for the subset of patients with bleeding lesion, because of the higher procedure costs and higher potential improvement in the quality of life was observed.

2.5 Markov model for electrochemotherapy of skin melanoma

A discrete-time Markov model was used to model patients' lifetime costs and consequences of either electrochemotherapy or standard of care. A Markov model of skin melanoma[16] was suggested, however it had to be modified to better fit the disease progression of patients with stage IIIc and IV skin melanoma after electrochemotherapy. The model is presented in Figure 1. In the initial state, patients have predominately cutaneous symptoms and are treated with electrochemotherapy, they can either not respond (i.e. they remain in the same state) or move to a response state if they experience complete or partial response (response to electrochemotherapy). Patients then remain in the response state unless they develop new metastases, then they are moving to a relapse state, where they can again receive repeated electrochemotherapy. The cycle length used in the model is two months.

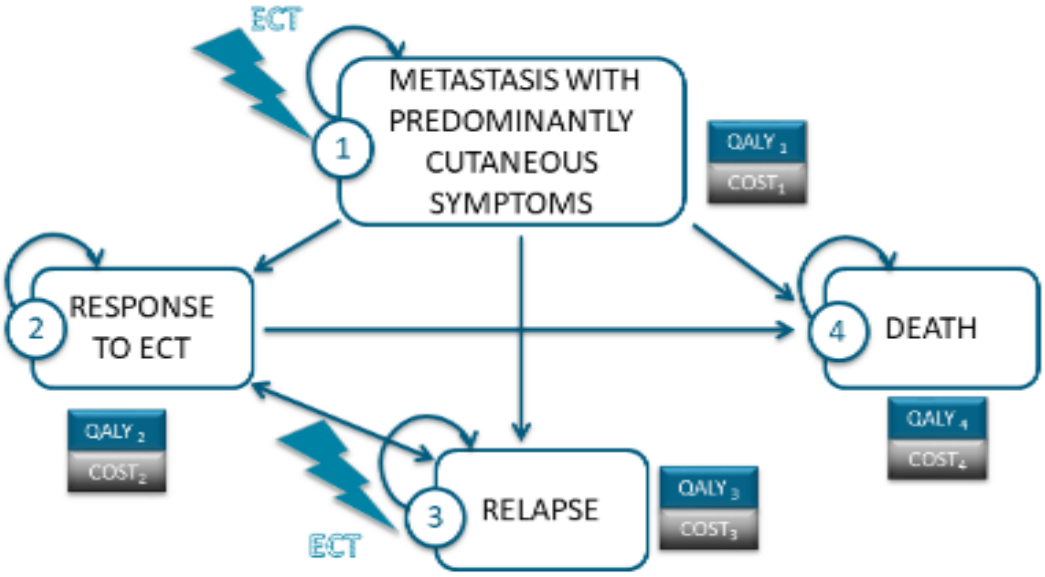


Figure 1: Four state Markov model of skin melanoma, adopted for electrochemotherapy treatment of stage IIIc and IV melanoma.

Bimonthly transitions probabilities between states were directly derived from fully observed patient-level data collected during the study. First, the total person-months of exposure in each state and the number of transitions to any other state were used to calculate a 4 by 4 transition-rate matrix *Q* in a Bayesian framework using the data from the study and uninformative prior distributions. The model was run using OpenBugs Markov Chain Monte Carlo (MCMC) software[38] (Appendix A). In the model, an initial run of 10,000 iterations was considered as 'burn in' (these values were discarded). Subsequently, two independent chains, starting from randomly assigned values were run, and convergence was monitored by looking at the ratio of the within-chain to between-chain variance to be about one, and by using Heidelberger-Welch[39] and Gelman-Rubin[40] diagnostics. Second, the transition-probability matrix *P(t)* was estimated by taking the matrix exponential $P(t) = Exp(Qt)$ using the *exmp* package

256 in Rstudio[41]. The estimated transition probabilities and their credible intervals for
257 electrochemotherapy are provided in TABLE 2.
258
259 TABLE 2: Two-month transition probabilities for patients receiving
260 electrochemotherapy

	Transition probabilities with electrochemotherapy	
	Mean	95% credible interval
From State 1 to:		
State 1	0.147	0.06 - 0.279
State 2	0.608	0.48 - 0.717
State 3	0.11	0.07 - 0.161
State 4	0.135	0.06 - 0.26
From State 2 to:		
State 1	0	-
State 2	0.726	0.64 - 0.809
State 3	0.196	0.13 - 0.268
State 4	0.079	0.04 - 0.134
From State 3 to:		
State 1	0	-
State 2	0.236	0.13 - 0.359
State 3	0.54	0.39 - 0.683
State 4	0.224	0.11 - 0.363

261

262 In the absence of electrochemotherapy, patients were assumed to remain in state 1
263 until they die, with probability, which was assumed to be equal to one of relapsed
264 patients in the electrochemotherapy group (state 3 to state 4). Lastly, transition
265 probabilities were assumed to be the same for both the whole sample and the subgroup
266 of patients with bleeding lesions.

267 **2.5.1 Scenario and sensitivity analysis**
268

269 Probabilistic sensitivity analysis (PSA) was performed by assigning probability
270 distributions to all parameters used in the cost-effectiveness model. For transition
271 probabilities, samples were taken directly from the joint posterior distribution of the

transition probability matrix, calculated with the MCMC simulation in OpenBugs. For costs and QALY data, samples were derived from Gamma and Beta distributions respectively, which were previously characterised using mean and standard deviation estimates from the study data and the literature. Mean estimates and 95% credible intervals for costs and QALY data are reported in TABLE 3. The results of the probabilistic sensitivity analysis are then reported in a cost-effectiveness plane and used to calculate cost-effectiveness acceptability curves for electrochemotherapy compared to standard of care.

In addition, the probability of electrochemotherapy being cost-effective has been also estimated as a function of the cost of the electrodes used for each electrochemotherapy procedure in both patients subgroups, because the cost of one electrode geometry represent almost half of the estimated electrochemotherapy procedure cost.

Lastly, since electrochemotherapy is usually considered to be a daily procedure, not requiring hospitalization, the results of a scenario analysis are reported, where electrochemotherapy procedures were assumed to be provided in an outpatient care setting, without any hospitalization costs.

TABLE 3: Costs and QALYs values and credible intervals used in the model (1-year values)

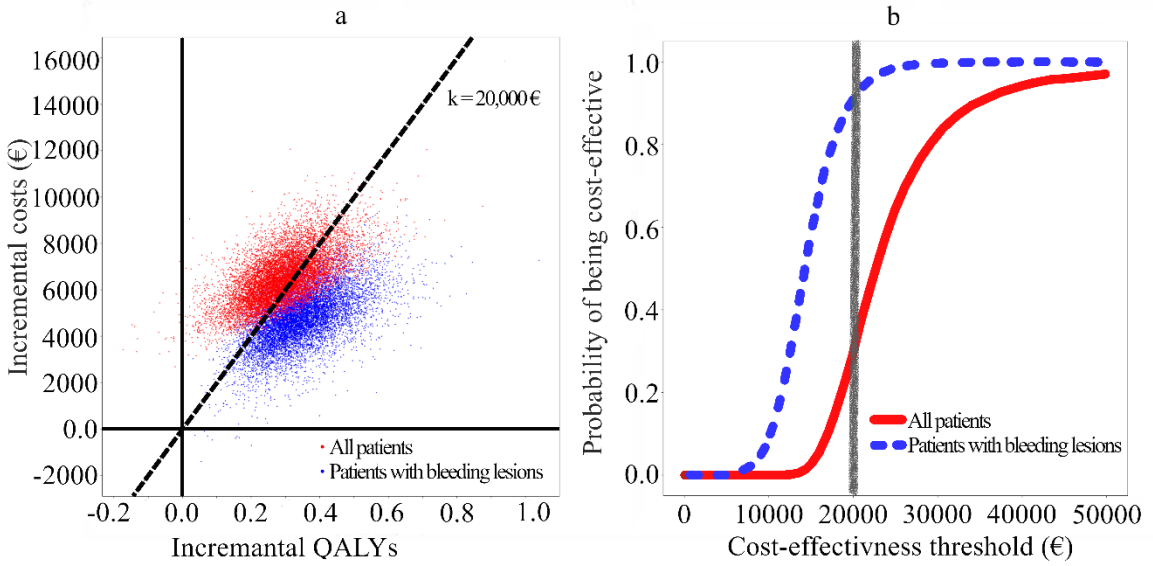
	Mean	**95% Credible interval**
Cost state 1		
All patients	4333 €	4139 - 4533
Patients with bleeding lesions	7784 €	7586 - 7978
Cost state 2	4333 €	4139 - 4533
Cost state 3	4333 €	4139 - 4533
Cost of ECT	2757 €	2095 - 3690
QALY state 1		
All patients	0.66	0.54 - 0.744
Patients with bleeding lesions	0.4	0.23 - 0.58
QALY state 2	0.72	0.66 - 0.804

QALY state 3		
All patients	0.66	0.54 - 0.744
Patients with bleeding lesions	0.4	0.23 - 0.58

3. Results

In the whole sample, electrochemotherapy is expected to improve quality of life by 0.29 QALYs (95 % CrI 0.097, 0.498) over patients lifetime, at an increased cost of 6,568 € (95 % CrI 4,593, 8,928). The expected NHB of electrochemotherapy compared to standard of care is equal -0.037 QALYs (95 % CrI -0.19, 0.11), meaning that at the used cost-effectiveness threshold of €20,000 per QALY gained, electrochemotherapy is slightly less cost-effective compared to standard of care, although the uncertainty over this estimate is quite large. For the subgroup of patients' with bleeding lesions, electrochemotherapy is expected to yield a higher quality of life by 0.34 QALYs (95 % CrI 0.18, 0.56) at a higher cost of 4,863 € (95 % CrI 2,479, 7,177). Compared to the whole sample, providing electrochemotherapy only to patients with bleeding lesions is expected to be more cost-effective with an expected iNHB of 0.10 (95 % CrI -0.06, 0.27).

The results of the probabilistic sensitivity analysis show a considerable uncertainty on the incremental costs and QALYs of electrochemotherapy for both patient groups with the simulated costs and QALYs pairs being spread widely in the cost-effectiveness plane. However it must be noted that most of the simulations remain in the first quadrant of the cost-effectiveness plane, meaning that electrochemotherapy is highly likely to be more effective and more costly compared to standard of care (Figure 2, panel a). The probability of electrochemotherapy being cost-effective is estimated to be 30% for the whole sample, and 91% in patients with bleeding lesions (Figure 2, panel b)



315

316 *Figure 2: (a) Cost-effectiveness plane; (b) The probability of electrochemotherapy being cost-effective*

317 *for all patients and patients with bleeding lesions.*

318

319 A reduction in the cost of the electrodes used in the electrochemotherapy procedure

320 is not going to greatly affect the probability of cost-effectiveness in patients with

321 bleeding lesions, since in this patient population, electrochemotherapy is already highly

322 likely to be the best treatment option even at the base case cost of the electrodes.

323 However, the cost of the electrodes has a considerable impact when considering the

324 whole patient sample (Figure 3). For example, a reduction by half in the average cost

325 of the electrodes used during the electrochemotherapy procedure would increase the

326 probability of cost-effectiveness from 0.30 to approximately 0.64.

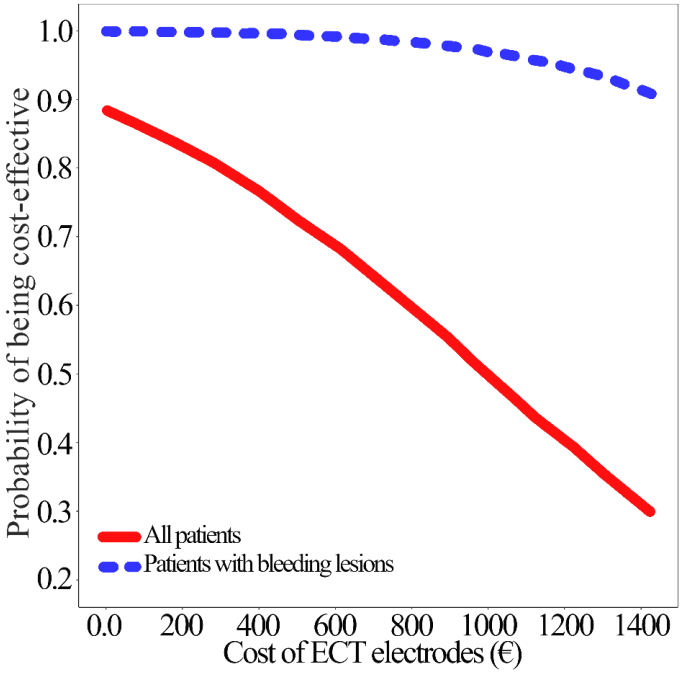


Figure 3: The probability of electrochemotherapy being cost-effective as a function of electrode cost for both patients groups.

Finaly, in the scenario without hospitalization costs, the expected incremental costs of electrochemotherapy were estimated to be 16 % lower compared to the base case analysis, which in turn resulted in a higher iNHB of 0.014 (95 % CrI - 0.12, 0.15) and a higher probability of being cost-effective equal to 0.58. (Figure 4), for the whole patient sample.

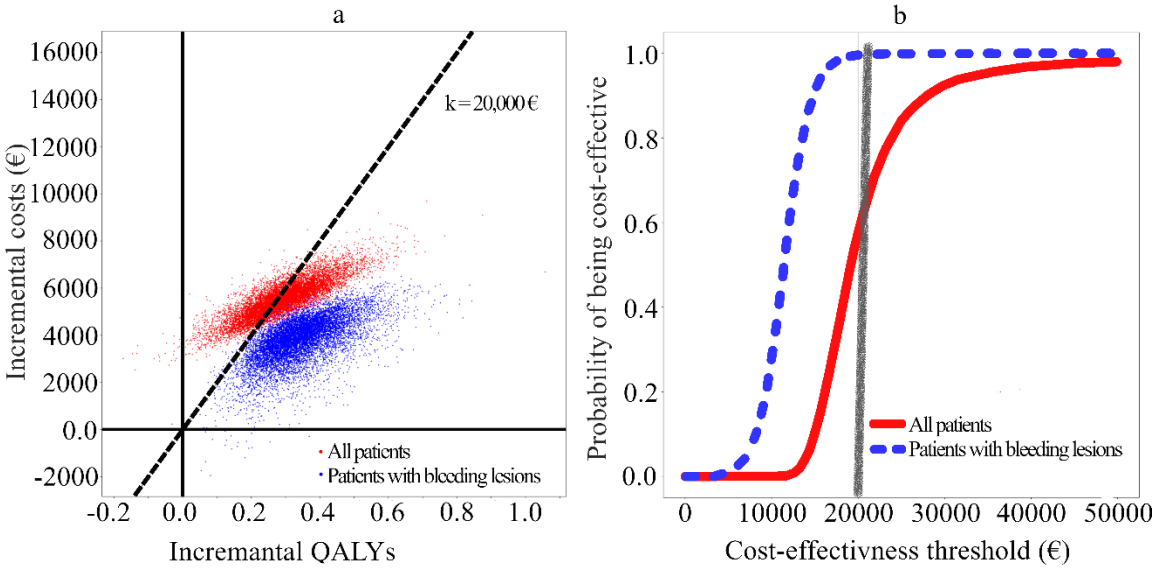


Figure 4: (a) Cost-effectiveness plane, in case of elimination of hospitalization price; (b) The probability of electrochemotherapy being cost-effective for all patients and patients with bleeding lesions, without hospitalization costs.

4. Discussion

Ten percent of skin melanoma patients develop in transit melanoma metastases during the course of cutaneous melanoma treatment. Most often in transit metastases develop on the lower limbs. Traditional methods of treatment differ among countries and are surgery, radiotherapy, topical imiquimod, isolated limb perfusion, systemic therapy or symptomatic therapy. Surgical excision is a reasonable treatment option only in case when a patient has a small number (<3) of skin lesions. However, researches show that even then, only 19% of patients remain without evidence of recurrence, within a median follow-up of 40 months[42]. Other treatment option including electrochemotherapy are thus used in case of a higher number of skin lesions. New systemic therapies are promising, nevertheless, due to the high cost, as well as to the treatment-related side effects, it is still advised to treat skin lesions locally, for as long as possible[43].

Since the average age of the patients included in our study is 78.1, those patients are often not suitable candidates for new systemic treatments due to comorbidities. For elderly patients, topical or local therapy is the most advantageous, as it is safe and has no side effects. Because our study includes mainly elderly with severe cancer, which are not amendable to any other treatment options, electrochemotherapy can improve their quality of life in comparison to symptomatic therapy or palliative treatment (natural course of the disease). However, the cost of electrochemotherapy is relatively high, the main contributor is the high cost of electrodes and electroporation device. In the future, a greater need for electrochemotherapy can be expected, despite the great

363 promise of systemic therapy. Because due to different cell clones in cutaneous
364 metastases, the management of individual strains, which do not respond to systemic
365 therapy, remains necessary.

366 In the scope of this paper, the first cost-effectiveness analysis of
367 electrochemotherapy based on the real-world study is presented. To our knowledge this
368 is the first study to report detailed primary data on the cost QALYs of
369 electrochemotherapy in this patient population. Previous studies[44–50] have reported
370 increases in quality of life after electrochemotherapy, however, quantitative information
371 is often lacking or not sufficiently detailed to allow estimation of the cost-effectiveness
372 of electrochemotherapy in patients with stage IIIc and IV skin melanoma. For example,
373 even when quality of life estimates were reported, it was not possible to differentiate
374 between cancer types and stages[51,52]. The primary data collected in this study aims to
375 fulfil this gap; however, the study has a number of limitations. First, by only collecting
376 data on patients receiving electrochemotherapy, improvements in QALYs were
377 measured using a before and after evaluations, which is prone to biases. Second, the
378 estimation of the relative effectiveness of electrochemotherapy compared to standard of
379 care is based on the assumption that patients not receiving treatment would remain with
380 the same baseline utility values for the rest of their life, which may not be the case.
381 Third, EQ_5D questionnaires were collected at each examination, but some patient data
382 were missing. Nevertheless, the QALY estimates are consistent with the existing
383 literature and were judged plausible by the clinicians who collected the data in the first
384 place.

385 When considering all patients with stage IIIc and IV skin melanoma,
386 electrochemotherapy is expected to be less cost-effective compared to palliative care
387 and symptomatic treatment (iNHB -0.037 QALYs, with probability of being the most

cost-effective strategy equal to 0.32). Conversely, electrochemotherapy is expected to be more cost-effective in patients with bleeding lesions as both the relative improvement in QALYs after successful electrochemotherapy and the expected savings in management costs are expected to be higher, although with a considerable uncertainty in the model estimates. It should be also noted that the NHB estimates are calculated using a cost-effectiveness threshold of €20,000 per QALY, equal to approximately the 2018 Slovenian per capita GDP. While the use of thresholds based on GDP has been recommended by the WHO[37], other estimates have also been proposed in the literature. For example Woods et al. provide country specific values using empirical estimates of the threshold for the UK; estimates of the relationship between country GDP per capita and the value of a statistical life; and a series of explicit assumptions[53]. For Slovenia the authors estimate a threshold in a range between $11,374 and $15,690 purchasing power parity (PPP) which correspond to a range in Euro between €6,710 and €9,257. It is expected that electrochemotherapy wouldn't be considered cost-effective in any case at these lower thresholds.

Electrochemotherapy is in theory, a daily procedure, but due to the age of some of patients involved in this study, most of them were hospitalized for a day or two, some patients had even longer hospitalizations up to 8 days, due to other disease related complications (not related to electrochemotherapy). Also, one of the advantages of electrochemotherapy is that it can be performed under local anesthesia, but in our study 65.8% of patient received general anesthesia, due to the high number of metastases treated in a patient.

In case electrochemotherapy would be used also on younger patients or patients with less severe cancer stage, the number of procedures would increase and an increase in quality of life after electrochemotherapy might be higher than for patients included

in our study. However, in this case, the cost-effectiveness analysis of electrochemotherapy should include other treatment options for this patient population, including surgery, radiotherapy, and immunotherapy. The cost of immunotherapy is significantly higher than the costs of electrochemotherapy procedures while the quality of life increase might be comparable. For example, it was estimated that the cost of immunotherapy in Slovenia may be up to €60,000 per year. Electrochemotherapy in case of reasonable electrode price, is also expected to be cheaper than surgery, because it is a daily procedure is lasting no more than 45 minutes in case of less severe cancer stages. Increases in quality of life and treatment response rates are also likely to be higher in this patient population. Therefore, one could arguably assume that the probability of electrochemotherapy being cost effective is potentially higher for less severe cancer stages compared to their more severe peers.

Nevertheless, the cost of electrodes remains a critical issue that may hinder a broader adoption of electrochemotherapy in clinical practice. Indeed, electrodes represent almost half of the procedure costs, and this study showed the extent to which their price is likely to affect electrochemotherapy's value for money. This study also shows that cost-effectiveness of electrochemotherapy is highly dependent on which patients' subgroups are considered in the analysis, suggesting that the optimal price of the device is likely to be indication specific. More research is needed to estimate the cost-effectiveness of electrochemotherapy in other less severe patient populations. Systematic collection of EQ_5D questioners or any other quantitative reporting of quality of life during the electrochemotherapy treatment are essential for further economic evaluations of electrochemotherapy. It seems that even if it is obligatory to collect quality of life data, it is not done on a regular basis, as it is not considered important.

438

439 ## 5. Conclusions

440 Electrochemotherapy of skin melanoma stage IIIc and IV increases the quality of life
441 after the procedure. The probability of electrochemotherapy (with hospitalization) being
442 cost-effective for the patient with stage IIIc and IV skin melanoma is just above 30 %,
443 which implies the prices of the device and electrodes should be reduced for successful
444 implementation into clinical practice. However, if patients have bleeding lesions than
445 electrochemotherapy is more likely to be cost-effective (probability rises to 0.91). In
446 order to simulate the probability of electrochemotherapy being cost-effective for
447 patients with less severe cancer stages, the hospitalization cost was removed from the
448 cost of the procedure, as electrochemotherapy can be performed as a daily procedure.
449 The probability of being cost effective for all patients included in the study raised,
450 however, the cost effectiveness can be easily increased with the reduction of the
451 electrode price, which represents almost half of the whole procedure cost.

452

453 ## 6. Acknowledgments

454 This study was supported by COST Action BM1309 (COST EMF-MED) within the
455 STSM (ECOST-STSMBM1309–110416–073660) and the Slovenian Research Agency
456 (ARRS) (P2-0249, P3-0003, 379J2-9227 and funding for Junior Researchers to Eva
457 Pirc).

458

459 ## 7. References

460
461 1. Weaver, J. C. Electroporation: a general phenomenon for manipulating cells and tissues. *J. Cell.*
462 *Biochem.* **51**, 426–435 (1993).
463 2. Kotnik, T., Rems, Lea, Munir, Tarek & Miklavčič, D. Membrane electroporation and
464 electropermeabilization: mechanisms and models. *Annu. Rev. Biophys* **48**, (2019).
465 3. Kotnik, T., Kramar, P., Pucihar, G., Miklavcic, D. & Tarek, M. Cell membrane electroporation- Part
466 1: The phenomenon. *IEEE Electrical Insulation Magazine* **28**, 14–23 (2012).

4. Campana, L. G. *et al.* Electrochemotherapy – Emerging applications technical advances, new indications, combined approaches, and multi-institutional collaboration. *European Journal of Surgical Oncology* **45**, 92–102 (2019).
5. Reddy, V. Y. *et al.* Pulsed Field Ablation for Pulmonary Vein Isolation in Atrial Fibrillation. *J Am Coll Cardiol* **74**, 315–326 (2019).
6. Bart Geboers *et al.* High-voltage electrical pulses in oncology: irreversible electroporation, electrochemotherapy, gene electrotransfer, electrofusion, and electro-immunotherapy. *RADIOLOGY* **in press**,.
7. Mahnič-Kalamiza, S., Vorobiev, E. & Miklavčič, D. Electroporation in Food Processing and Biorefinery. *J Membrane Biol* **247**, 1279–1304 (2014).
8. Kotnik, T. *et al.* Electroporation-based applications in biotechnology. *Trends Biotechnol.* **33**, 480–488 (2015).
9. Golberg, A. *et al.* Energy-efficient biomass processing with pulsed electric fields for bioeconomy and sustainable development. *Biotechnology for Biofuels* **9**, 94 (2016).
10. Davalos, R. V., Mir, L. M. & Rubinsky, B. Tissue Ablation with Irreversible Electroporation. *Ann Biomed Eng* **33**, 223 (2005).
11. Lambricht, L. *et al.* Clinical potential of electroporation for gene therapy and DNA vaccine delivery. *Expert Opinion on Drug Delivery* **13**, 295–310 (2016).
12. Rebersek, M., Miklavcic, D., Bertacchini, C. & Sack, M. Cell membrane electroporation-Part 3: the equipment. *IEEE Electrical Insulation Magazine* **30**, 8–18 (2014).
13. Pirc, E., Rebersek, M. & Miklavcic, D. 12 Dosimetry in Electroporation-Based Technologies and Treatments. in *Dosimetry in Bioelectromagnetics* (ed. Markov, M.) 233–268 (CRC Press, Taylor & Francis Group, 2017). doi:10.1201/9781315154572-13.
14. Campana, L. G. *et al.* Electrochemotherapy of superficial tumors – Current status:: Basic principles, operating procedures, shared indications, and emerging applications. *Seminars in Oncology* **46**, 173–191 (2019).
15. Colombo, G. L., Matteo, S. D. & Mir, L. M. Cost-effectiveness analysis of electrochemotherapy with the Cliniporator^{TM} vs other methods for the control and treatment of cutaneous and subcutaneous tumors. *Ther Clin Risk Manag* **4**, 541–548 (2008).
16. Pirc, E. *et al.* Study design of a medical device pre-market assessment: a case study on electrochemotherapy. *1* **87**, 22–40 (2018).
17. Electrochemotherapy for primary basal cell carcinoma and primary squamous cell carcinoma | Guidance | NICE. https://www.nice.org.uk/guidance/IPG478.
18. http://www.who.int/choice/en/.
19. Gershenwald, J. E. *et al.* Melanoma Staging: Evidence-Based Changes in the American Joint Committee on Cancer Eighth Edition Cancer Staging Manual. *CA Cancer J Clin* **67**, 472–492 (2017).
20. Butina, M. R., Švab, I., Perić, B. & Bartenjev, I. Health behaviour changes of cutaneous melanoma survivors in Slovenia – a qualitative study. *Slovenian Journal of Public Health* **58**, 70–77 (2019).
21. Savoia, P. *et al.* Skin metastases of malignant melanoma: a clinical and prognostic survey. *Melanoma Research* **19**, 321 (2009).
22. Brizio, M. *et al.* International Network for Sharing Practices on Electrochemotherapy (InspECT): An Integrative Patients Treatment Consortium. in *Handbook of Electroporation* (ed. Miklavcic, D.) 1–18 (Springer International Publishing, 2017). doi:10.1007/978-3-319-26779-1_95-1.
23. Campana, L. G., Sepulcri, M., Valpione, S., Corti, L. & Rossi, C. R. Electrochemotherapy for Superficially Metastatic Melanoma. in *Handbook of Electroporation* (ed. Miklavcic, D.) 1–19 (Springer International Publishing, 2016). doi:10.1007/978-3-319-26779-1_223-1.
24. Mir, L. M. *et al.* Standard operating procedures of the electrochemotherapy: Instructions for the use of bleomycin or cisplatin administered either systemically or locally and electric pulses delivered by the CliniporatorTM by means of invasive or non-invasive electrodes. *EJC Supplements* **4**, 14–25 (2006).
25. Gehl, J. *et al.* Updated standard operating procedures for electrochemotherapy of cutaneous tumours and skin metastases. *Acta Oncol* **57**, 874–882 (2018).
26. NICE | Guidance | Electrochemotherapy for metastases in the skin from tumours of non-skin origin and melanoma. https://www.nice.org.uk/guidance/IPG446.
27. Mali, B., Jarm, T., Snoj, M., Sersa, G. & Miklavcic, D. Antitumor effectiveness of electrochemotherapy: A systematic review and meta-analysis. *European Journal of Surgical Oncology (EJSO)* **39**, 4–16 (2013).
28. Sersa, G., Cemazar, M. & Rudolf, Z. Electrochemotherapy: Advantages and drawbacks in treatment of cancer patients. *Cancer Ther* **1**, (2003).
29. Landström, F. J. *et al.* Electrochemotherapy - possible benefits and limitations to its use in the head and neck region. *Acta Otolaryngol.* **135**, 90–95 (2015).

30. Girelli, R. *et al.* Feasibility and safety of electrochemotherapy (ECT) in the pancreas: a pre-clinical investigation. *Radiol Oncol* **49**, 147–154 (2015).
31. Probst, U., Fuhrmann, I., Beyer, L. & Wiggermann, P. Electrochemotherapy as a New Modality in Interventional Oncology: A Review. *Technol Cancer Res Treat* **17**, (2018).
32. Hočevar, M. *et al.* Priporočila za obravnavo bolnikov s kožnim melanomom. https://www.onko-i.si/fileadmin/onko/datoteke/Smernice/Priporocila_za_obravnavo_bolnikov_s_koznim_melanomom_2018.pdf.
33. Benčina, G., Buljan, M., Šitum, M., Stevanović, R. & Benković, V. Health and Economic Burden of Skin Melanoma in Croatia - Cost-of-illness Study. *Acta Dermatovenerol Croat* **25**, 1–7 (2017).
34. Krensel, M., Schäfer, I. & Augustin, M. Cost-of-illness of melanoma in Europe – a modelling approach. *Journal of the European Academy of Dermatology and Venereology* **33**, 34–45 (2019).
35. Maio, M. *et al.* The cost of unresectable stage III or stage IV melanoma in Italy. *Journal of Experimental & Clinical Cancer Research* **31**, 91 (2012).
36. Thokala, P., Ochalek, J., Leech, A. A. & Tong, T. Cost-Effectiveness Thresholds: the Past, the Present and the Future. *Pharmacoeconomics* **36**, 509–522 (2018).
37. Choosing interventions that are cost-effective. *Geneva: World Health Organization; 2014* http://www.who.int/choice/en/.
38. Welton, N. J. & Ades, A. E. Estimation of Markov Chain Transition Probabilities and Rates from Fully and Partially Observed Data: Uncertainty Propagation, Evidence Synthesis, and Model Calibration: *Medical Decision Making* (2016) doi:10.1177/0272989X05282637.
39. Heidelberger, P. & Welch, P. D. A Spectral Method for Confidence Interval Generation and Run Length Control in Simulations. *Commun. ACM* **24**, 233–245 (1981).
40. Gelman, A. & Rubin, D. B. Inference from Iterative Simulation Using Multiple Sequences. *Statist. Sci.* **7**, 457–472 (1992).
41. Jones, E., Epstein, D. & García-Mochón, L. A Procedure for Deriving Formulas to Convert Transition Rates to Probabilities for Multistate Markov Models. *Med Decis Making* **37**, 779–789 (2017).
42. Racz, J. M., Block, M. S., Baum, C. L. & Jakub, J. W. Management of local or regional non-nodal disease. *Journal of Surgical Oncology* **119**, 187–199 (2019).
43. Nelson, D. W. & Faries, M. B. ASO Author Reflections: What Role Do Surgeons Play in the Era of Effective Systemic Therapy for Melanoma? *Ann Surg Oncol* **26**, 4619–4620 (2019).
44. Cabula, C. Neoadjuvant electochemotherapy for breast cancer. The first case to be treated in Italy: Results after 25 months of follow-up. *European Journal of Surgical Oncology* **38**, 1004 (2012).
45. Solari, N., Spagnolo, F., Queirolo, P., Ponte, E. & Cafiero, F. Electrochemotherapy for cutaneous metastases: The IRCCS San Martino Hospital and National Cancer Research Institute of Genoa's experience. *European Journal of Surgical Oncology* **38**, 1004 (2012).
46. Caracò, C. *et al.* Long-lasting response to electrochemotherapy in melanoma patients with cutaneous metastasis. *BMC Cancer* **13**, 564 (2013).
47. Valpione, S., Campana, L. G., Pigozzo, J. & Chiarion-Sileni, V. Consolidation electrochemotherapy with bleomycin in metastatic melanoma during treatment with dabrafenib. *Radiol Oncol* **49**, 71–74 (2015).
48. Bianchi, G., Campanacci, L., Ronchetti, M. & Donati, D. Electrochemotherapy in the Treatment of Bone Metastases: A Phase II Trial. *World Journal of Surgery* **40**, 3088–3094 (2016).
49. Plaschke, C. C. *et al.* The DAHANCA 32 study: Electrochemotherapy for recurrent mucosal head and neck cancer. *Head & Neck* **41**, 329–339 (2019).
50. Kaehler, K. C., Egberts, F. & Hauschild, A. Electrochemotherapy in Symptomatic Melanoma Skin Metastases: Intraindividual Comparison with Conventional Surgery. *Dermatologic Surgery* **36**, 1200 (2010).
51. Campana, L. G. *et al.* Bleomycin-Based Electrochemotherapy: Clinical Outcome from a Single Institution's Experience with 52 Patients. *Ann Surg Oncol* **16**, 191–199 (2009).
52. Campana, L. G. *et al.* Treatment efficacy with electrochemotherapy: A multi-institutional prospective observational study on 376 patients with superficial tumors. *Eur J Surg Oncol* **42**, 1914–1923 (2016).
53. Woods, B., Revill, P., Sculpher, M. & Claxton, K. Country-Level Cost-Effectiveness Thresholds: Initial Estimates and the Need for Further Research. *Value Health* **19**, 929–935 (2016).

Appendix A: Code used in OpenBugs to estimate the transition-rate matrix

```
model{
#estimate rates from fully observed data
#loop for any state except for the death state
```

```
585 for (i in 1:3){
586 # model transitions from each state to any other state as a
587 poisson distribution
588 temp[i]<-lambda[i]*E[i]
589 m[i]~dpois(temp[i])
590 #estimate conditional probability to go to one of the two
591 possible other states (conditional on leaving state i)
592 tr[i,1]~ dbin(condP[i],m[i])
593 }
594 #define rate-transition matrix
595 G[1,1]<--G[1,2]-G[1,4]
596 G[1,2]<-lambda[1]*condP[1]
597 G[1,3]<-0
598 G[1,4]<-lambda[1]*(1-condP[1])
599 G[2,1]<-0
600 G[2,2]<--G[2,3]-G[2,4]
601 G[2,3]<-lambda[2]*condP[2]
602 G[2,4]<-lambda[2]*(1-condP[2])
603 G[3,1]<-0
604 G[3,3]<--G[3,2]-G[3,4]
605 G[3,2]<-lambda[3]*condP[3]
606 G[3,4]<-lambda[3]*(1-condP[3])
607 #define priors for rates and conditional probabilities
608 for (s in 1:3){
609 lambda[s]~dgamma(0.1,0.1)
610 condP[s] ~dbeta(1,1)}
611 }
612 #data
613 list(m=c(24,27,19),
614       E=c(724,4271,1635),
615       tr=structure(.Data=c(22,24,11,2,3,8),.Dim=c(3,2)))
```

4 Conclusions

Electroporation is a platform technology, which is already established in many different fields. When dealing with electroporation, measuring is crucial for achieving effective electroporation, because quality assurance can only be provided by appropriate measurements, i.e., measuring the voltage and current using an oscilloscope. The biggest challenge is to accurately measure the high-voltage short nanosecond long pulses. It should be emphasized that the bandwidth of the probes ends at -3 dB which means that the probe at the end of its bandwidth has a -29 % error. A -29 % error may be acceptable for electronics, however, a 29 % different amplitude may have a significantly different biological effect [103, 104]. Because permeabilization and cell survival are sigmoid functions of voltage, more than 10 % difference in voltage may result in a significantly different biological effect [103, 104]. Whenever we are using measuring probes, we have to be aware of the measuring error. Commercially available probes have the measuring error specified in a data sheet, but it is still sometimes overlooked, while prototype probes have to be correctly calibrated and measuring error evaluated.

Unfortunately in the field of electroporation, failed efforts to confirm other published paperwork are increasing [30]. We believe the main reason for this situation is, that methods in many papers describing/using electroporation are reported in insufficient details, and quite often measurements of output pulses are not reported [18, 31]. The field of electroporation is in need of promoting reproducible research that can only be achieved by adequate measurements, standardized reports, and proper use of electroporators and electrodes. For example, skin electroporation is a promising modality for treating different conditions with transdermal drug delivery, gene electrotransfer, electrochemotherapy and irreversible electroporation. A considerable amount of *in vitro* and *in vivo* studies exists, which use significantly different electrode configurations, waveforms, and pulse generators. Results from studies with different parameters are sometimes difficult (if not impossible) to be compared. The reasons for slow translation of skin electroporation into clinics are non-standard pulse parameters, non-standard electrode configurations, generators not complying with their technical specifications or having no technical specifications at all, lack of reporting on the delivered waveforms and electric field

distribution, not performing the current-voltage measurements, and significantly different skin structure of animals and humans. Nevertheless, it was shown that electroporaton can reduce cost and facilitate treatment procedures. According to the ongoing studies the electroporation based therapies are safe, with little or no side effects. The clinical data published on electroporation-based applications are quite encouraging, therefore we believe, that in the future, electroporation will be indispensable in gene therapy, cancer treatment, infection disease treatment, intracardiac ablation, and vaccination. However, further development of standard electroporation treatment protocols, regulation of electroporator development and user training, remain essential for successful incorporation into clinical practice. Electroporation based therapies have a huge potential for implementation into clinical practice also in socioeconomically disadvantaged populations. Currently, the price of electroporation treatments is relatively high, mainly due to costly electroporators and electrodes, but custom low budget devices can be developed. It is not technically difficult to develop an electroporator, more challenging is the quality and safety assurance, because biological loads characteristics vary considerably from sample to sample and even more from tissue to tissue. Therefore, due to the huge variation in biological load characteristics, delivered pulses may significantly deviate from the pre-set. Low impedance of a load (tissue or cell suspension) requires large power/currents, which quite often leads to significant voltage drop on the load. Protocols in which a larger number of pulses (or long pulses) are delivered, can result in reduced amplitude of pulses. With implementation of a particular standard for particular application, the electroporation devices will be safer, treatments more efficient and results more reproducible, which will allow faster and more straight-forward progress of electroporation as well as treatments and therapies based on electroporation. By implementing a particular medical standard for clinical electroporation devices, the quality of clinical devices will be improved which will result in better and more effective cancer treatment. But in order to propose particular standard for electroporator, first technical specifications and tolerances have to be determined, then, testing protocols have to be established. We proposed the tolerances in the scope of the paper 2 and 3 and a possible solution for establishment of testing protocol in paper 4. Because biological loads are nonlinear resistive capacitive loads, ad hoc biological loads (e.g. potato tuber) and resistors are not appropriate for electroporation device evaluation, because they are not adequate approximation of therapeutic biological load. Therefore, we develop an electronic emulator of a biological load during electroporation, which allows sustainable and constant testing of electroporation devices and an objective

comparison of the operation of different electroporators or of an electroporator over its lifetime. We believe the developed emulator represents the first step toward the development of standard and the testing or evaluation protocol, which we believe will contribute to scientific and overall progress in the field of electroporation. Defining the load is an important step in electroporator development, which was/is often neglected. Illdefined load makes the development process more challenging and can result in non-optimal solutions. Now, with high-quality measurements, a load can be defined and an emulator can be developed, which raises the quality of the development process.

The electroporation device developed in the scope of this Ph.D thesis operates in accordance with expectations, the maximal output voltage is 4 kV and the theoretical maximal current 131 A and 200 ns minimal pulse duration, the maximal pulse repetition frequency is 2 MHz. It generates asymmetric bipolar pulses, as well as pulse duration asymmetry, as voltage amplitude asymmetry can be simultaneously delivered. Therefore, the device enables research of still unknown effects, it facilities further research of cancellation and sensitisation effects, high frequency electroporation and due to high maximal current also *in vivo* experiments with bursts of bipolar pulses. The device was tested *in vivo* on mice. In both cases the desired performance was reached. Additionally, acceleration of mice lag during the pulse delivery was measured in order to evaluate muscle contractions. More *in vivo* measurements should be done, before any final conclusions can be made, but for now, we can only summarize, that muscle contractions were present also in case of high frequency bursts and were significant. Importantly, HF-ECT with bleomycin proved as effective as well established "classical" ECT with bleomycin.

Two general Markov models for CEA have been developed, one for skin melanoma and the other for Basal cell carcinoma (BCC). Initially, both models are reduced in such a manner that they consist only of states that are relevant for the current use of new technology, i.e. electrochemotherapy. Because electrochemotherapy is an emerging technology, it is still not used in all patients, but as the proof of concept rises, it is gradually moving up the cancer staging scale. A realistic cost-effectiveness analysis for a specific cancer type facilitates equipment purchase and clinical practice implementation. Additionally, the prediction of cost-effectiveness can also influence the next round of fund raising. Furthermore, specific models should be developed for each disease separately in order to include all specific phenomena in a specific cancer type. Electrochemotherapy must be

compared to well established procedures that are cancer-type, location and size dependent.

As part of the doctoral dissertation, cost-effectiveness for electrochemotherapy of stage IV and IIIc skin melanoma was evaluated. The results show that electrochemotherapy increases the quality of life of patients after the intervention, but the probability of being cost-effective is quite low (approximately 50 %), which means that the price should be reduced for successful implementation in clinical practice. However, if patients have bleeding lesions the ECT can be assumed cost-effective (probability rises to 0.97). Cost-effectiveness would be best achieved by reducing the cost of the electrode, which accounts for almost half of the total cost of the intervention.

5 Original Scientific Contributions

Evaluation of electroporators and recommendations for standardization of electroporator as a standalone medical device

The electroporation device manufacturers are currently hindering the development of electroporation field by concealing the output pulse parameters of their devices, designing them in a way to disable output pulse measuring, specifying characteristics which device cannot deliver, and making devices that do not warn the user when pulse delivery fault occurs. Consequently, reports of electroporation studies are missing data that are essential for the reproduction of studies. In order to properly understand the current situation and trends of development on the electroporation field, a new review of commercially available devices, their characteristics, limitations, and weaknesses was made. Also an expert opinion about the electroporation devices used in skin electroporation was provided. The quality of the pulse delivery was evaluated, by checking if the delivered pulses were adequately addressed and measured in the electroporation studies, with the focus on the field of nanosecond electroporation, where the delivery and measuring is the most challenging. With the evaluation of commercially available devices and their use, we would like to raise the awareness of the importance of measuring, and influence on the manufacturers to stop obscuring pulse parameters and enable measuring – quality control. Additionally, recommendations for standardization, mainly focusing on the evaluation of electroporators proper or improper operation were proposed and electronic emulator of the biological load during electroporation, which enables constant and sustainable testing and unbiased comparison of different electroporators operation, was developed.

Development of a novel bipolar high-frequency high-voltage pulse generator - electroporator

Recent insights on high-frequency irreversible electroporation (H-FIRE), and sensitization and cancellation effects drive us to develop high frequency, high voltage, bipolar, square wave pulse generator. We developed such a generator by using and improving the latest pulse generator designs. The developed 4 kV pulse generator, generates high voltage pulses with minimized switching time between positive and negative pulse. The minimal pulse duration is 200 ns and maximal repetition rate

2 MHz. The new generator allows us to study uptake and excitation effect *in vivo* by high frequency pulses and test hypothesis related to more uniform tissue electroporation and reduction of nerve and muscle excitation and pain sensation. It is also able to generate asymmetrical bipolar pulses that enable us to study in detail the sensitization and cancellation effects. The developed device was tested in first *in vivo* high frequency electrochemotherapy (HF-ECT), in which HF-ECT with bleomycin and cisplatin was proved to be as effective as well established "classical" ECT with bleomycin and cisplatin.

Cost-effectiveness analysis of electrochemotherapy for treatment of basal cell carcinoma and skin melanoma

Cost-Effectiveness Analysis (CEA) has already been made for some ECT and IRE, nevertheless, they were made without acquiring the quality of life (QoL) experienced by the patients, which is crucial for adequate and accurate Health Technology Assessment (HTA). The main reason that QoL was not acquired in previous studies is most likely poor reporting, numerical data are not available. In our study, we focused on electrochemotherapy of skin melanoma and basal cell carcinoma (BCC) as these two therapies are used for the longest time and are also very successful. We developed two Markov models, one for each therapy. For each of our Markov models, we defined which data are needed. We collect these data (by data collection methods) for the skin melanoma and conducted a CEA of ECT of stage IV and IIIc skin melanoma. The cost-effectiveness of electrochemotherapy of stage IV and IIIc skin melanoma was calculated for patients treated at the Institute of Oncology in Ljubljana using the Cliniporator device and associated electrodes. We also provide suggestions on how to improve the cost-effectiveness of the therapy. The realistic CEA enriches health technology developers, with a better understanding of the field, and provides a cost framework for a successful implementation in clinics. The HTA also provides a basis for assessment of allowable costs for developing a new product i.e. medical device.

References

[1] D. Miklavčič, "Network for development of electroporation-based technologies and treatments: Cost td1104," *J. Membr. Biol.*, vol. 245, no. 10, pp. 591–598, 2012.

[2] M. L. Yarmush, A. Golberg, G. Serša, T. Kotnik and D. Miklavčič, "Electroporation-based technologies for medicine: Principles, applications, and challenges," *Annual Review of Biomedical Engineering*, vol. 16, pp. 295–320, 2014.

[3] S. Haberl, D. Miklavčič, G. Serša, W. Frey and B. Rubinsky, "Cell membrane electroporation-part 2: The applications," *IEEE Electrical Insulation Magazine*, vol. 29, no. 1, pp. 29–37, 2013.

[4] T. Kotnik, W. Frey, M. Sack, S. H. Meglič, M. Peterk and D. Miklavčič, "Electroporation-based applications in biotechnology," *Trends Biotechnol.*, vol. 33, no. 8, pp. 480–488, 2015.

[5] A.-R. Denet, R. Vanbever and V. Préat, "Skin electroporation for transdermal and topical delivery," *Advanced Drug Delivery Reviews*, vol. 56, no. 5, pp. 659–674, 2014.

[6] J. C. Weaver, "Electroporation: A general phenomenon for manipulating cells and tissues," *Journal of Cellular Biochemistry*, vol. 51, no. 4, pp. 426–35, 1993.

[7] T. Kotnik, P. Kramar, G. Pucihar, D. Miklavčič and M. Tarek, "Cell membrane electroporation — part 1: The phenomenon," *IEEE Electrical insulation magazine*, vol. 28, no. 5, pp. 14–23, 2012.

[8] E. Pirc, M. Reberšek and D. Miklavčič, *Dosimetry in Bioelectromagnetics*, ch. Dosimetry in Electroporation-Based Technologies and Treatments, pp. 233–268. CRC Press, Taylor Francis Group, 2017.

[9] L. G. Campana and et al., "Electrochemotherapy – emerging applications technical advances, new indications, combined approaches, and multi-institutional collaboration," *European Journal of Surgical Oncology*, vol. 45, no. 2, pp. 92–102, 2019.

[10] E. Sokołowska and A. U. Błachnio-Zabielska, "A critical review of electroporation as a plasmid delivery system in mouse skeletal muscle," *International Journal of Molecular Sciences*, vol. 20, no. 11, pp. 2776, 2019.

[11] B. Rubinsky, "Irreversible electroporation in medicine," *Technology in Cancer Research Treatment*, vol. 6, no. 4, pp. 255–59, 2007.

[12] S. Mahnič-Kalamiza, E. Vorobiev and D. Miklavčič, "Electroporation in food processing and biorefinery," *J. Membrane Biol.*, vol. 247, no. 12, pp. 1279–1304, 2014.

[13] D. W. Deamer and J. Bramhall, "Permeability of lipid bilayers to water and ionic solutes," *Chemistry and Physics of Lipids*, vol. 40, no. 2, pp. 167–188, 1986.

[14] T. Kotnik, L. Rems, M. Tarek and D. Miklavčič, "Membrane electroporation and electropermeabilization: Mechanisms and models," *Annu. Rev. Biophys*, vol. 48, 2019.

[15] M. Tarek, "Membrane electroporation: A molecular dynamics simulation," *Biophysical Journal*, vol. 88, no. 6, pp. 4045–4053, 2005.

[16] T. Kotnik, G. Pucihar and D. Miklavčič, "Induced transmembrane voltage and its correlation with electroporation-mediated molecular transport," *J. Membr. Biol.*, vol. 236, no. 1, pp. 3–13, 2010.

[17] M. Isokawa, "Membrane time constant as a tool to assess cell degeneration," *Brain Research Protocols*, vol. 1, no. 2, pp. 114–116, 1997.

[18] T. B. Napotnik, M. Reberšek, P. T. Vernier, B. Mali and D. Miklavčič, "Effects of high voltage nanosecond electric pulses on eukaryotic cells (in vitro): A systematic review," *Bioelectrochemistry*, vol. 110, pp. 1–12, 2016.

[19] O. N. Pakhomova, B. W. Gregory, V. A. Khorokhorina, A. M. Bowman, S. Xiao and A. G. Pakhomov, "Electroporation-induced electrosensitization," *PLOS ONE*, vol. 6, no. 2, pp. e17100, 2011.

[20] O. N. Pakhomova, B. W. Gregory and A. G. Pakhomov, "Facilitation of electroporative drug uptake and cell killing by electrosensitization," *Journal of Cellular and Molecular Medicine*, vol. 17, no. 1, pp. 154–59, 2013.

[21] C. Jiang, Q. Shao and J. Bischof, "Pulse timing during irreversible electroporation achieves enhanced destruction in a hindlimb model of cancer," *Annals of Biomedical Engineering*, vol. 43, no. 4, pp. 887–95, 2015.

[22] J. Dermol, O. N. Pakhomova, A. G. Pakhomov and D. Miklavčič, "Cell electrosensitization exists only in certain electroporation buffers," *PLOS ONE*, vol. 11, no. 7, pp. e0159434, 2016.

[23] M. Reberšek, D. Miklavčič, C. Bertacchini and M. Sack, "Cell membrane electroporation-part 3: The equipment," *IEEE Electrical Insulation Magazine*, vol. 30, no. 3, pp. 8–18, 2014.

[24] M. Reberšek, *Handbook of Electroporation*, ch. Beyond Electroporation Pulse Parameters: From Application to Evaluation, pp. 977–997. Springer International Publishing, 2017.

[25] M. Reberšek and D. Miklavčič, "Advantages and disadvantages of different concepts of electroporation pulse generation," *Automatika*, vol. 52, no. 1, pp. 12–19, 2011.

[26] M. Reberšek, E. Pirc, D. Miklavčič and P. Kramar, "Modeling and optimization of blumlein nanosecond pulse generator for experiments on planar lipid bilayers," v *2017 14th International Conference on Synthesis, Modeling, Analysis and Simulation Methods and Applications to Circuit Design (SMACD)*, 2017.

[27] E. Pirc, D. Miklavčič and M. Reberšek, "Nanosecond pulse electroporator with silicon carbide mosfets: Development and evaluation," *IEEE Transactions on Biomedical Engineering*, vol. 66, no. 12, pp. 3526–3533, 2019.

[28] J. M. Sanders, A. Kuthi, Y.-H. Wu, P. T. Vernier and M. A. Gundersen, "A linear, single-stage, nanosecond pulse generator for delivering intense electric fields to biological loads," *IEEE Transactions on Dielectrics and Electrical Insulation*, vol. 16, no. 4, pp. 1048–1054, 2009.

[29] J. M. Sanders, A. Kuthi and M. A. Gundersen, "Optimization and implementation of a solid state high voltage pulse generator that produces fast rising nanosecond pulses," *IEEE Transactions on Dielectrics and Electrical Insulation*, vol. 18, no. 4, pp. 1228–1235, 2011.

[30] J. Kaiser, "Calling all failed replication experiments," *Science*, vol. 351, no. 6273, pp. 548, 2016.

[31] L. G. Campana, P. Clover, S. Valpione, P. Quaglino, J. Gehl, C. Kunte, M. Snoj, M. Čemažar, C. R. Rossi, D. Miklavčič and G. Serša, "Recommendations for improving the quality of reporting clinical electrochemotherapy studies based on qualitative systematic review," *Radiology and Oncology*, vol. 20, no. 1, pp. 1–13, 2016.

[32] A. G. Pakhomov, B. L. Ibey, A. M. Bowman, F. M. Andre and O. N. Pakhomova, *Nanosecond-Duration Electric Pulses Open Nanometer-Size Pores in Cell Plasma Membrane.* Munich, Germany, Berlin, Heidelberg: World Congress on Medical Physics and Biomedical Engineering, 2009.

[33] B. L. Ibey, A. G. Pakhomov, B. W. Gregory, V. A. Khorokhorina, C. C. Roth, M. A. Rassokhin, J. A. Bernhard, G. J. Wilmink and O. N. Pakhomova, "Selective cytotoxicity of intense nanosecond-duration electric pulses in mammalian cells," *Biochim Biophys Acta*, vol. 1800, no. 11, pp. 1210–1219, 2010.

[34] D. Sel, D. Cukjati, D. Batiuskaite, T. Slivnik, L. M. Mir and D. Miklavčič, "Sequential finite element model of tissue electropermeabilization," *IEEE Transactions on Biomedical Engineering*, vol. 52, no. 5, pp. 816–827, 2005.

[35] D. Miklavčič, S. Čorović, G. Pucihar and N. Pavšelj, "Importance of tumour coverage by sufficiently high local electric field for effective electrochemotherapy," *European Journal of Cancer Supplements*, vol. 4, no. 11, pp. 45–51, 2006.

[36] I. Lacković, R. Magjarević and D. Miklavčič, "Three-dimensional finite-element analysis of joule heating in electrochemotherapy and in vivo gene electrotransfer," *IEEE Transactions on Dielectrics and Electrical Insulation*, vol. 16, no. 5, pp. 1338–47, 2009.

[37] B. Kos, B. Valič, T. Kotnik and P. Gajšek, "Occupational exposure assessment of magnetic fields generated by induction heating equipment—the role of spatial averaging," *Physics in Medicine and Biology*, vol. 57, no. 19, pp. 5943–53, 2012.

[38] D. Miklavčič, M. Snoj, A. Županič, B. Kos, M. Čemažar, M. Kropivnik, M. Bracko, T. Pecnik, E. Gadzijev and G. Serša, "Towards treatment planning and treatment of deep-seated solid tumors by electrochemotherapy," *Biomedical Engineering Online*, vol. 9, 2010.

[39] I. Edhemovic, E. M. Gadzijev, E. Brecelj, D. Miklavčič, B. Kos, A. Županič, B. Mali, T. Jarm, D. Pavliha, M. Marčan, G. Gasljevic, V. Gorjup, M. Music, T. P. Vavpotic, M. Čemažar, M. Snoj and G. Serša, "Electrochemotherapy: A new technological approach in treatment of metastases in the liver," *Technology in Cancer Research & Treatment*, vol. 10, no. 5, pp. 475–85, 2011.

[40] D. Miklavčič. and R. V. Davalos, "Electrochemotherapy (ect) and irreversible electroporation (ire)-advanced techniques for treating deep-seated tumors based on electroporation," *Biomedical Engineering Online*, vol. 14, no. Suppl 3, pp. I1, 2015.

[41] M. Hjouj and B. Rubinsky, "Magnetic resonance imaging characteristics of nonthermal irreversible electroporation in vegetable tissue," *The Journal of Membrane Biology*, vol. 236, no. 1, pp. 137–46, 2010.

[42] D. Pavliha, B. Kos, A. Županič, M. Marčan, G. Serša and D. Miklavčič, "Patientspecific treatment planning of electrochemotherapy: Procedure design and possible pitfalls," *Bioelectrochemistry*, vol. 87, 2012.

[43] D. Miklavčič, K. Beravs, D. Semrov, M. Čemažar, F. Demšar and G. Serša, "The importance of electric field distribution for effective in vivo electroporation of tissues," *Biophysical Journal*, vol. 74, no. 5, pp. 2152–58, 1998.

[44] D. Pavliha, B. Kos, M. Marčan, A. Zupanič, G. Serša and D. Miklavčič, "Planning of electroporation-based treatments using webbased treatment-planning software," *The Journal of Membrane Biology*, vol. 246, no. 11, pp. 833–42, 2013.

[45] B. Kos, A. Županič, T. Kotnik, M. Snoj, G. Serša and D. Miklavčič, "Robustness of treatment planning for electrochemotherapy of deep-seated tumors," *The Journal of Membrane Biology*, vol. 236, no. 1, pp. 147–53, 2010.

[46] A. Grošelj, B. Kos, M. Čemažar, J. Urbancic, G. Kragelj, M. Bosnjak, B. Veberic, P. Strojan, D. Miklavčič and G. Serša, "Coupling treatment planning with navigation system: A new technological approach in treatment of head and neck tumors by electrochemotherapy," *Biomedical Engineering Online*, vol. 14, no. Suppl 3, pp. S2, 2015.

[47] A. Županič and D. Miklavčič, "Tissue heating during tumor ablation with irreversible electroporation," *Elektrotehniški vestnik*, vol. 78, no. 1-2, pp. 42–47, 2011.

[48] M. Kranjc, B. Markelc, F. Bajd, M. Čemažar, I. Serša, T. Blagus and D. Miklavčič, "In situ monitoring of electric field distribution in mouse tumor during electroporation," *Radiology*, vol. 274, no. 1, pp. 246–50, 2015.

[49] D. Miklavčič, B. Mali, B. Kos, R. Heller and G. Serša, "Electrochemotherapy: From the drawing board into medical practice," *BioMedical Engineering OnLine*, vol. 13, no. 29, 2014.

[50] T. Stepišnik, T. Jarm, A. Grošelj, I. Edhemović, M. Djokić, A. Ivanecz, B. Trotovšek and et al., "Elektrokemoterapija – uČinkovita metoda zdravljenja tumorjev s kombinacijo kemoterapevtika in elektriČnega polja," *Zdravniški Vestnik*, vol. 85, no. 1, 2016.

[51] D. M. Soden, J. O. Larkin, C. G. Collins, M. Tangney, S. Aarons, J. Piggott, A. Morrissey, C. Dunne and G. C. O'Sullivan, "Successful application of targeted electrochemotherapy using novel flexible electrodes and low dose bleomycin to solid tumours," *Cancer Letters*, vol. 232, no. 2, pp. 300–310, 2006.

[52] B. Mali, T. Jarm, M. Snoj, G. Serša and D. Miklavčič, "Antitumor effectiveness of electrochemotherapy: A systematic review and meta-analysis," *European Journal of Surgical Oncology: The Journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology*, vol. 39, no. 1, pp. 4–16, 2006.

[53] D. E. Spratt, E. A. G. Spratt, S. Wu, A. DeRosa, N. Y. Lee, M. E. Lacouture and C. A. Barker, "Efficacy of skin-directed therapy for cutaneous metastases from advanced cancer: A meta-analysis," *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology*, vol. 32, no. 28, pp. 3144–55, 2014.

[54] L. M. Mir, J. Gehl, G. Serša, C. G. Collins, J.-R. Garbay, V. Billard, P. F. Geertsen, Z. Rudolf, G. C. O'Sullivan and M. Marty, "Standard operating procedures of the electrochemotherapy: Instructions for the use of bleomycin or cisplatin administered either systemically or locally and electric pulses delivered by the cliniporatortm by means of invasive or non-invasive electrodes," *EJC Supplements*, vol. 4, no. 11, pp. 14–25, 2014.

[55] J. Gehl, G. Serša, L. Matthiessen, T. Muir, D. Soden, A. Occhini, P. Quaglino, P. Curatolo, L. Campana, C.Kunte, A. Clover, G. Bertino, V. Farricha, J. Odili, K. Dahlstrom, M. Benazzo and L. Mir, "Updated standard operating

procedures for electrochemotherapy of cutaneous tumours and skin metastases," *Acta Oncol*, vol. 57, no. 7, pp. 874–882, 2018.

[56] Priporočila za obravnavo bolnikov s kožnim melanomom: `https://www.onko-i.si/fileadmin/onko/datoteke/Smernice/Priporocila_za_obravnavo_bolnikov_s_koznim_melanomom_2018.pdf`.

[57] G. Serša, S. Kranjc, J. Scancar, M. Krzan and M. Čemažar, "Electrochemotherapy of mouse sarcoma tumors using electric pulse trains with repetition frequencies of 1 hz and 5 khz," *The Journal of Membrane Biology*, vol. 236, no. 1, pp. 155–62, 2010.

[58] A. Županič, S. Ribaric and D. Miklavčič, "Increasing the repetition frequency of electric pulse delivery reduces unpleasant sensations that occur in electrochemotherapy," *Neoplasma*, vol. 54, no. 3, pp. 246–50, 2007.

[59] B. Mali, T. Jarm, F. Jager and D. Miklavčič, "An algorithm for synchronization of in vivo electroporation with ecg," *Journal of Medical Engineering & Technology*, vol. 29, no. 6, pp. 288–96, 2005.

[60] B. Mali, V. Gorjup, I. Edhemovic, E. Brecelj, M. Čemažar, G. Serša, B. Strazisar, D. Miklavčič and T. Jarm, "Electrochemotherapy of colorectal liver metastases—an observational study of its effects on the electrocardiogram," *Biomedical Engineering Online*, vol. 14, no. Suppl 3, pp. S5, 2015.

[61] J. F. Edd, L. Horowitz, R. V. Davalos, L. M. Mir and B. Rubinsky, "In vivo results of a new focal tissue ablation technique: Irreversible electroporation," *IEEE Transactions on Biomedical Engineering*.

[62] B. Kos, P. Voigt, D. Miklavcčič and M. Moche, "Careful treatment planning enables safe ablation of liver tumors adjacent to major blood vessels by percutaneous irreversible electroporation (ire)," *Radiology and Oncology*.

[63] P. A. Garcia, R. V. Davalos and D. Miklavčič, "A numerical investigation of the electric and thermal cell kill distributions in electroporation-based therapies in tissue," *PloS One*.

[64] N. Jourabchi, K. Beroukhim, B. A. Tafti, S. T. Kee and E. W. Lee, "Irreversible electroporation (nanoknife) in cancer treatment," *Gastrointestinal Intervention*, vol. 3, no. 1, pp. 8–18, 2014.

[65] C. Bertacchini, P. M. Margotti, E. Bergamini, A. Lodi, M. Ronchetti and R. Cadossi, "Design of an irreversible electroporation system for clinical use," *Cancer Research & Treatment*, vol. 6, no. 4, pp. 313–20, 2007.

[66] K. R. Thomson, W. Cheung, S. J. Ellis, D. Federman, H. Kavnoudias, D. Loader-Oliver, S. Roberts, P. Evans, C. Ball and A. Haydon, "Investigation of the safety of irreversible electroporation in humans," *Journal of Vascular and Interventional Radiology*, vol. 22, no. 5, pp. 611–21, 2011.

[67] C. B. Arena, M. B. Sano, J. H. Rossmeisl, J. L. Caldwell, P. A. Garcia, M. N. Rylander and R. V. Davalos, "High-frequency irreversible electroporation (h-fire) for non-thermal ablation without muscle contraction," *BioMedical Engineering OnLine*.

[68] M. Kandušer, D. Miklavčič and M. Pavlin, "Mechanisms involved in gene electrotransfer using high- and low-voltage pulses—an in vitro study," *Bioelectrochemistry*, vol. 74, no. 2, pp. 265–71, 2009.

[69] M.-A. Shibata, Y. Ito, J. Morimoto, K. Kusakabe, R. Yoshinaka and Y. Otsuki, "In vivo electrogene transfer of interleukin-12 inhibits tumor growth and lymph node and lung metastases in mouse mammary carcinomas," *The Journal of Gene Medicine*, vol. 8, no. 3, pp. 335–52, 2006.

[70] P. Chiarella, V. M. Fazio and E. Signori, "Application of electroporation in dna vaccination protocols," *Current Gene Therapy*, vol. 10, no. 4, pp. 281–86, 2010.

[71] R. K. Grewal, M. Lulsdorf, J. Croser, S. Ochatt, A. Vandenberg and T. D. Warkentin, "Doubled-haploid production in chickpea (cicer arietinum l.): Role of stress treatments," *Plant Cell Reports*, vol. 28, no. 8, pp. 1289–99, 2009.

[72] G. Serša, J. Teissié, M. Čemažar, E. Signori, U. Kamensek, G. Marshall and D. Miklavčič, "Electrochemotherapy of tumors as in situ vaccination boosted by immunogene electrotransfer," *Cancer Immunology, Immunotherapy*, vol. 64, 2015.

[73] M. Golzio, J. Teissié and M.-P. Rols, "Direct visualization at the single-cell level of electrically mediated gene delivery," *Proceedings of the National Academy of Sciences*, vol. 99, no. 3, pp. 1292–97, 2002.

[74] S. Šatkauskas, M. F. Bureau, M. Puc, A. Mahfoudi, D. Scherman, D. Miklavčič and L. M. Mir, "Mechanisms of in vivo dna electrotransfer: Respective contributions of cell electropermeabilization and dna electrophoresis," *Molecular Therapy: The Journal of the American Society of Gene Therapy*, vol. 5, no. 22, pp. 133–40, 2002.

[75] M. Pavlin, S. Haberl, M. Reberšek, D. Miklavčič and M. Kandušer, "Changing the direction and orientation of electric field during electric pulses application improves plasmid gene transfer in vitro," *Journal of Visualized Experiments: JoVE*, vol. 55, no. 3309, 2011.

[76] S. J. Beebe, J. White, P. F. Blackmore, Y. Deng, K. Somers and K. H. Schoenbach, "Diverse effects of nanosecond pulsed electric fields on cells and tissues," *DNA and Cell Biology*, vol. 22, no. 12, pp. 785–96, 2003.

[77] WHO, Health technology assessment', WHO. [Online, Accessed: 28-Nov-2019]]. Available:: `https://www.who.int/medical_devices/assessment/en/`.

[78] L. Pecchia and M. P. Craven, "Early stage health technology assessment (hta) of biomedical devices. the match experience," *World Congress on Medical Physics and Biomedical Engineering May 26-31, 2012, Beijing, China, Berlin, Heidelberg*, 2013.

[79] P. Thokala and A. Duenas, "Multiple criteria decision analysis for health technology assessment," *Value in Health*, vol. 15, no. 8, pp. 1172–81, 2012.

[80] M. J. IJzerman, H. Koffijberg, E. Fenwick and M. Krahn, "Emerging use of early health technology assessment in medical product development: A scoping review of the literature," *Pharmacoeconomics*, vol. 35, no. 7, pp. 727–40, 2017.

[81] I. Fasterholdt, M. Krahn, K. Kidholm, K. B. Yderstræde and K. M. Pedersenn, "Review of early assessment models of innovative medical technologies," *Health Policy*, vol. 121, no. 8, pp. 870–9, 2017.

[82] C. S. Goodman, *HTA 101 INTRODUCTION TO HEALTH TECHNOLOGY ASSESSMENT*. The Lewin Group, Falls Church, 2014.

[83] M. C. Weinstein, G. Torrance and A. McGuire, "Qalys: The basics," *Value in Health: The Journal of the International Society for Pharmacoeconomics and Outcomes Research*, vol. 12, no. 1, pp. S5–9, 2009.

[84] D. L. Patrick, H. E. Starks, K. C. Cain, R. F. Uhlmann and R. A. Pearlman, "Measuring preferences for health states worse than death," *Med. Decis. Making*, vol. 14, no. 1, pp. 9–18, 1994.

[85] EuroQol official page: `http://www.euroqol.org/`.

[86] R. Rabin and F. de Charro, "Eq-5d: a measure of health status from the euroqol group," *Ann. Med.*, vol. 33, no. 5, pp. 337–343, 2001.

[87] C. Phillips and G.Thompson, "What is a qaly," *Health economics 2nd ed.*, 2009.

[88] A. H. Briggs, D. E. Wonderling and C. Z. Mooney, "Pulling cost-effectiveness analysis up by its bootstraps: a non-parametric approach to confidence interval estimation," *Health. Econ.*, vol. 6, no. 4, pp. 327–40, 1997.

[89] S. R. Hill, "Cost-effectiveness analysis for clinicians," *BMC Medicine*, vol. 10, no. 1, pp. 10, 2012.

[90] E. Fenwick, D. A. Marshall, A. R. Levy and G. Nichol, "Using and interpreting cost-effectiveness acceptability curves: an example using data from a trial of management strategies for atrial fibrillation," *BMC Health Services Research*, vol. 6, no. 1, 2006.

[91] A. G. Pakhomov, I. Semenov, S. Xiao, O. N. Pakhomova, B. Gregory, K. H. Schoenbach, J. C. Ullery, H. T. Beier, S. R. Rajulapati and B. L. Ibey, "Cancellation of cellular responses to nanoelectroporation by reversing the stimulus polarity," *Therapeutics and Clinical Risk Management*, vol. 71, no. 22, pp. 4431–41, 2014.

[92] K. Dymek, L. Rems, B. Zorec, P. Dejmek, G. Gómez and D. Miklavčič, "Modeling electroporation of the non-treated and vacuum impregnated heterogeneous tissue of spinach leaves," *Innov. Food Sci. Emerg. Technol.*, vol. 29, pp. 55–64, 2016.

[93] M. Reberšek and D. Miklavčič, *Advanced Electroporation Techniques in Biology and Medicine*, ch. Concepts of electroporation pulse generation and overview of electric pulse generators for cell and tissue electroporation, pp. 323–339. CRC Press, 2010.

[94] M. Puc, S. Corović, K. Flisar, M. Petkovšek, J. Nastran and D. Miklavčič, "Techniques of signal generation required for electropermeabilization. survey of electropermeabilization devices," *Bioelectrochemistry*, vol. 64, no. 2, pp. 113–124, 2004.

[95] G. L. Colombo, S. D. Matteo and L. M. Mir, "Cost-effectiveness analysis of electrochemotherapy with the cliniporatortrade mark vs other methods for the control and treatment of cutaneous and subcutaneous tumors," *Therapeutics and Clinical Risk Management*, vol. 4, no. 2, pp. 541–48, 2008.

[96] C. A. for Drugs and T. in Health, "Irreversible electroporation for tumors of the pancreas or liver: Review of clinical and cost-effectiveness," 2016.

[97] C. B. Arena and R. V. Davalos, "Advances in therapeutic electroporation to mitigate muscle contractions," *Journal of Membrane Science Technology*.

[98] A. Golberg and B. Rubinsky, "Towards electroporation based treatment planning considering electric field induced muscle contractions," *Technology in Cancer Research Treatment*, vol. 11, no. 2, pp. 189–201, 2012.

[99] C. Ball, K. R. Thomson and H. Kavnoudias, "Irreversible electroporation: A new challenge in out of operating theater anesthesia," *Anesthesia Analgesia*, vol. 110, no. 5, pp. 1305 – 1309, 2010.

[100] F. J. Landström, C. Nilsson, S. Crafoord, J. A. Reizenstein, G. Adamsson and L. A. Löfgren, "Electroporation therapy of skin cancer in the head and neck area," *Dermatologic Surgery*, vol. 36, no. 8, pp. 1245–50, 2010.

[101] I. Daskalov, N. Mudrov and E. Peycheva, "Exploring new instrumentation parameters for electrochemotherapy. attacking tumors with bursts of biphasic pulses instead of single pulses," *IEEE Engineering in Medicine and Biology Magazine*, vol. 18, no. 1, pp. 62–66, 1999.

[102] M. Scuderi, M. Reberšek, D. Miklavčič and J. Dermol-Černe, "The use of high-frequency short bipolar pulses in cisplatin electrochemotherapy in vitro," *Radiology and Oncology*.

[103] I. Semenov, S. Xiao and A. G. Pakhomov, "Primary pathways of intracellular Ca2+ mobilization by nanosecond pulsed electric field," *Biochimica et biophysica acta*, vol. 1828, no. 3, pp. 981–989, 2013.

[104] W. E. Ford., R. Wei, P. F. Blackmore, K. H. Schoenbach and S. J. Beebe, "Nanosecond pulsed electric fields stimulate apoptosis without release of pro-apoptotic factors from mitochondria in B16f10 melanoma," *Archives of Biochemistry and Biophysics*, vol. 497, no. 1, pp. 82–89, 2010.

[105] M. Pavlin, M. Kandušer, M. Reberšek, G. Pucihar, F. X. Hart, R. Magjarević and D. Miklavčič, "Effect of cell electroporation on the conductivity of a cell suspension," *Biophysical Journal*, vol. 88, no. 6, pp. 4378–90, 2005.

[106] M. Puc, T. Kotnik, L. M. Mir and D. Miklavčič, "Quantitative model of small molecules uptake after in vitro cell electropermeabilization," *Bioelectrochemistry*, vol. 60, no. 1, pp. 1–10, 2003.

[107] M.-P. Rols and J. Teissié, "Electropermeabilization of mammalian cells to macromolecules: Control by pulse duration," *Biophysical Journal*, vol. 75, no. 3, pp. 1415–1423, 1998.

[108] V. Novickij, I. Girkontaitė, A. Grainys, A. Zinkevičienė, E. Lastauskienė, J. Švedienė, A. Paškevičius, S. Markovskaja and J. Novickij, "Measurement of transient permeability of sp2/0 myeloma cells: Flow cytometric study," *Measurement Science Review*, vol. 16, no. 6, pp. 300–304, 2016.

[109] S. A. Freeman, M. A. Wang and J. C. Weaver, "Theory of electroporation of planar bilayer membranes: Predictions of the aqueous area, change in capacitance, and pore-pore separation.," *Biophysical Journal*, vol. 67, no. 1, pp. 42–56, 1994.

[110] J. C. Weaver and Y. A. Chizmadzhev, "Theory of electroporation: A review.," *Bioelectrochemistry and Bioenergetics*, vol. 41, no. 2, pp. 135–60, 1996.

[111] G. Pucihar, T. Kotnik, D. Miklavčič and J. Teissié, "Kinetics of transmembrane transport of small molecules into electropermeabilized cells," *Biophysical Journal*, vol. 95, no. 6, 2008.

[112] P. Kramar, L. Delemotte, A. M. Lebar, M. Kotulska, M. Tarek, and D. Miklavčič, "Molecular-level characterization of lipid membrane electroporation using linearly rising current," vol. 245, no. 10, pp. 651–659, 2013.

[113] A. Polak, D. Bonhenry, F. Dehez, P. Kramar, D. Miklavčič and M. Tarek, "On the electroporation thresholds of lipid bilayers: Molecular dynamics simulation investigations," *Journal of Membrane Biology*, vol. 246, no. 11, pp. 843–850, 2013.

[114] A. Golberg, M. Sack, J. Teissie, G. Pataro, U. Pliquett, G. Saulis, T. Stefan, D. Miklavčič, E. Vorobiev and W. Frey, "Energy-efficient biomass processing with pulsed electric fields for bioeconomy and sustainable development," *Biotechnology for Biofuels*, vol. 9, pp. 94, 2016.

[115] S. Becker, B. Zorec, D. Miklavčič and N. Pavšelj, "Transdermal transport pathway creation: Electroporation pulse order," *Mathematical Biosciences (Multiscale models and methods in biomedicine)*, vol. 257, pp. 60–68, 2014.

[116] B. Zorec, V. Préat, D. Miklavčič and N. Pavšelj., "Energy-efficient biomass processing with pulsed electric fields for bioeconomy and sustainable developmentactive enhancement methods for intra- and transdermal drug delivery: A review," *Zdravniški Vestnik*, vol. 82, no. 5, 2013.

[117] G. Serša, D. Miklavčič, M. Čemažar, Z. Rudolf, G. Pucihar and M. Snoj, "Electrochemotherapy in treatment of tumours," *European Journal of Surgical Oncology (EJSO)*, vol. 34, no. 2, pp. 232–40, 2008.

[118] D. C. Sweeney, M. Reberšek, J. Dermol, L. Rems, D. Miklavčič and R. V. Davalos, "Quantification of cell membrane permeability induced by monopolar and high-frequency bipolar bursts of electrical pulses," *Biochimica et Biophysica Acta (BBA) - Biomembranes*.

[119] J. C. Weaver, K. C. Smith, A. T. Esser, R. S. Son and T. R. Gowrishankar, "A brief overview of electroporation pulse strength-duration space: A region where additional intracellular effects are expected," *Bioelectrochemistry*, vol. 87, pp. 236–243, 2012.

[120] A. Ivorra, B. Al-Sakere, B. Rubinsky and L. M. Mir, "In vivo electrical conductivity measurements during and after tumor electroporation: Conductivity changes reflect the treatment outcome," *Physics in Medicine Biology*, vol. 54, no. 19, pp. 5949, 2009.

[121] M. Sack, C. Schultheiss and H. Bluhm, "Triggered marx generators for the industrial-scale electroporation of sugar beets," *IEEE Transactions on Industry Applications*, vol. 41, no. 3, pp. 707–714, 2005.

[122] M. A. Elgenedy, A. Darwish, S. Ahmed and B. W. Williams, "A transition arm modular multilevel universal pulse-waveform generator for electroporation applications," *IEEE Transactions on Power Electronics*, vol. 32, no. 12, pp. 8979–8991, 2017.

[123] A. Elserougi, A. Massoud and S. Ahmed, "Conceptual study of a bipolar modular high voltage pulse generator with sequential charging," *IEEE Transactions on Dielectrics and Electrical Insulations*, vol. 23, no. 6, pp. 3450–3457, 2016.

[124] V. Novickij and et al., "High-frequency submicrosecond electroporator," *Biotechnology Biotechnological Equipment*, vol. 30, pp. 1–7, 2016.

[125] A. Elserougi, A. Massoud and S. Ahmed, "High-power bipolar multilevel pulsed electroporator," *Instrumentation Science Technology*, vol. 44, no. 1, pp. 65–72, 2016.

[126] M. Cheng and W. H. Organization, "Medical device regulations: Global overview and guiding principles," *Overview and Guiding Principles. World Health Organization*, 2003.

[127] J. L. Martin, B. J. Norris, E. Murphy and J. A. Crowe, "Medical device development: The challenge for ergonomics," *Applied Ergonomics*, vol. 39, no. 3, pp. 271–283, 2008.

[128] D. B. Kramer, S. Xu and A. S. Kesselheim, "Regulation of medical devices in the united states and european union," *New England Journal of Medicine*, vol. 366, no. 3, pp. 848–855, 2012.

[129] M. van Tulder, "Health technology assessment (hta) increasingly important in spine research," *European Spine Journal*, vol. 20, no. 7, pp. 999–1000, 2011.

[130] Analiza zdravstvenega sistema v Sloveniji, Povzetek in ključne ugotovitve: `http://www.mz.gov.si/fileadmin/mz.gov.si/pageuploads/Analiza/analiza_ZS_povzetek_in_kljucne_ugotovitve_lektorirana_verzija.pdf`.

[131] G. Turchetti, E. Spadoni and E. Geisler, "Health technology assessmen," *IEEE Engineering in Medicine and Biology Magazine*, vol. 29, no. 3, pp. 70–76, 2010.

[132] F. R. Kenneth, R. Koprowski and J. D. Skufca, "Qalys: The basics," *BioMedical Engineering OnLine*, vol. 13, pp. 94, 2014.

[133] G. W. Torrance and D. Feeny, "Utilities and quality-adjusted life years," *International Journal of Technology Assessment in Health Care*, vol. 5, no. 4, pp. 559–75, 1989.

[134] M. C. Weinstein, B. O'Brien, J. Hornberger, J. Jackson, M. Johannesson, C. McCabe and B. R. Luce., "Principles of good practice for decision analytic modeling in health-care evaluation: Report of the ispor task force on good research practices—modeling studies," *Value in Health*, vol. 6, no. 1, pp. 9–17, 2003.