UNIVERZA V LJUBLJANI
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DOKTORSKA DISERTACIJA

Katja Balantič

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DOKTORSKA DISERTACIJA

Elektrokemijske reakcije, sproščanje kovinskih ionov in oksidacija lipidnih molekul pri elektroporaciji

Electrochemical reactions, dissolution of metal ions and lipid oxidation in electroporation

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ELEKTROKEMIJSKE REAKCIJE, SPROŠČANJE KOVINSKIH IONOV IN OKSIDACIJA LIPIDNIH MOLEKUL PRI ELEKTROPORACIJI

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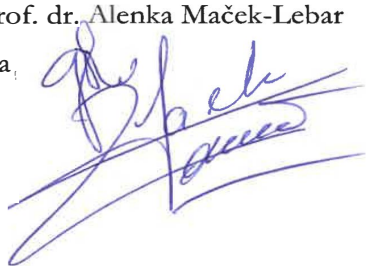

I hereby declare that I am the author of the doctoral thesis titled **"Electrochemical reactions, dissolution of metal ions and lipid oxidation in electroporation"**. The work was carried out during the doctoral studies at the Laboratory of Biocybernetics, Faculty of Electrical Engineering, University of Ljubljana. Scientific papers that are part of this thesis were not used for the same purpose by any other co-author. I declare that the printed version of the doctoral thesis is identical to the electronic version. The results of the presented work were published in the following articles in international journals:

- **Paper 1:** K. Balantič, D. Miklavčič, I. Križaj and P. Kramar, **"The good and the bad of cell membrane electroporation"**, *Acta Chimica Slovenica*, vol. 68, no. 4, pp. 753-764, December 2021.
- **Paper 2:** K. Balantič, P. Kramar and D. Miklavčič, **"In silico numerical model of aluminium and iron dissolution during electric pulse application for electroporation"**, *Bioelectricity*, submitted on July 17, 2023.
- **Paper 3:** K. Balantič, G. Allmaier, V.U. Weiss and P. Kramar, **"Calcium ion effect on phospholipid bilayers as cell membrane analogues"**, *Bioelectrochemistry*, vol. 143, no. 107988, February 2022.
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ZA MAMI IN OČITA

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Abstract

Exposure of biological cells to electric pulses is a useful tool for manipulating the permeability of the cell membrane. It is believed that even a brief exposure to the electric field causes structural changes in lipid membrane through the formation of hydrophilic pores. This temporarily makes the membrane more permeable to molecules that normally lack the mechanism to overcome the membrane's hydrophobic barrier. The chemical and physical processes that occur at the molecular level during electroporation are relatively well studied. However, some processes are still less known and will be investigated in this doctoral dissertation.

Electroporation can be described as an application of high voltage electric pulses passing direct electric current through electrodes in contact with the tissue. Application of electric pulses unavoidable causes electrochemical reactions at the electrode-electrolyte interface, specifically, metal release from the electrodes. Metal release can have adverse effects on the electroporation process, equipment and biological tissue leading to lipid oxidation. The processes described can be studied with *in silico* numerical models or *in vitro* cell membrane models such as liposomes and planar lipid bilayers.

In silico numerical models are based on a set of differential equations and can be used to describe a physical problem, for example the occurrence of electrochemical reactions at the electrode-electrolyte interface during electroporation. By solving these equations, concentration profiles of dissolved substances in dependence of applied pulse amplitude and pulse polarity are obtained. Developed and validated numerical model is therefore a very useful tool, to study the effects of different electroporation protocols on the extent of electrochemical reactions, in a fast and reliable way.

In vitro models such as liposomes and planar lipid bilayers can be used to study various processes at the molecular level. The artificial lipid bilayer is a simple model of the cell membrane with less complexity compared to the biological cell membrane. We can form liposomes with structure that is very similar to the cell, but the composition of the membrane is greatly simplified. Electroporation processes can also be studied with planar lipid bilayers. The composition of the planar lipid bilayer can be arbitrarily changed to mimic the composition of a real cell membrane. Thus, in a simple membrane model, we can study how the cell membrane is affected by metal ions that are physiologically present in the biological environment or released from electrodes during the application of high-voltage electric pulses. What is more, using planar lipid bilayers, we can also study the influence of lipid oxidation on electrical properties of cell membranes.

In this doctoral dissertation, we were able to develop *in silico* numerical model describing the dissolution of metal ions from aluminium and iron electrodes during the application of high-voltage electric pulses. The numerical model was validated using experimental results from the study of electrolyte contamination by Kotnik *et al.* Furthermore, *in vitro* membrane models were used to study the effect of metal ions on membrane structure. Using planar lipid bilayers and liposomes, changes in the phospholipid membrane, namely increased membrane thickness, were observed due to the addition of metal ions, such as calcium, aluminium, and iron. Finally, lipid oxidation was studied using planar lipid bilayers. An increase in electric conductivity and capacitance was observed, leading us to believe that lipid oxidation indeed plays a role in prolonged membrane permeability after electroporation.

Key words: Electroporation, electrochemistry, numerical modelling, cell membrane models

Povzetek

Izpostavitev bioloških celic visokonapetostnim električnim pulzom se je izkazalo kot uporabno orodje za povečanje prepustnosti celične membrane. Že kratkotrajna izpostavitev električnemu polju povzroči strukturne spremembe v membrani, v obliki hidrofilnih por. Tako postane membrana začasno bolj prepustna za molekule, ki običajno nimajo mehanizmov za prehajanje te hidrofobne pregrade. Kemijski in fizikalni procesi, kot na primer nastanek hidrofilnih por, ki potekajo na molekularni ravni pri elektroporaciji, so razmeroma dobro raziskani, različni procesi pa še vedno ostajajo neznani in so predmet preučevanja te doktorske disertacije.

Eden od procesov, ki potekajo pri elektroporaciji, so zagotovo elektrokemijske reakcije na stiku elektrode in elektrolita, ki povzročajo sproščanje kovinskih ionov z elektrod ter nastajanja mehurčkov v obliki plinastega kisika in vodika. Kovinski ioni, ki se sproščajo z elektrod med uporabo visokonapetostnih električnih pulzov, lahko vplivajo na biološke aktivnosti v celici in povzročijo oksidacijo lipidov v celični membrani. Opisane procese lahko preučujemo z numeričnimi modeli in modeli celičnih membran, kot so liposomi in ravninski lipidni dvosloji.

In silico numerični modeli temeljijo na nizu diferencialnih enačb, ki opisujejo fizikalni problem, na primer elektrokemijske reakcije na stiku med elektrodo in elektrolitom pri elektroporaciji. Z reševanjem teh enačb je mogoče simulirati koncentracijske profile raztopljenih kovinskih ionov v odvisnosti od uporabljene napetosti in oblike pulzov. Validirani numerični modeli so tako zelo uporabni za hitro in zanesljivo testiranje različnih protokolov elektroporacije, ter vsaj deloma nadomeščajo eksperimentalno delo.

Modele celične membrane, kot so liposomi in ravninski lipidni dvosloji, pa

lahko uporabljamo za preučevanje različnih procesov na molekularni ravni. Ti umetni lipidni dvosloji so preprosti, a še vedno zadovoljivi modeli celične membrane. Liposomi so oblikovno zelo podobni celici; a sestava membrane je močno poenostavljena. Elektroporacijo lahko proučujemo tudi z uporabo ravninskih lipidnih dvoslojev. Prednost ravninskih lipidnih dvoslojev je, da so kemično in električno dostopni z obeh strani. Tako lahko v enostavnem modelu preučujemo, kako na celično membrano vplivajo kovinski ioni, ki so fiziološko prisotni v biološkem okolju ali pa se sproščajo z elektrod med aplikacijo visokonapetostnih električnih pulzov. Proučujemo lahko tudi vpliv oksidacije lipidov na spremembe električnih lastnosti lipidnega dvosloja.

V doktorski disertaciji smo razvili numerični model, ki opisuje sproščanje kovinskih ionov iz aluminijevih in železovih elektrod pri elektroporaciji. Numerični model smo validirali z uporabo eksperimentalnih rezultatov študije kontaminacije elektrolita opisane v članku Kotnika s sodelavci. Poleg tega sta bila uporabljena dva različna modela celične membrane za preučevanje vpliva kovinskih ionov in lipidne oksidacije na strukturo lipidnega dvosloja. Z uporabo ravninskih lipidnih dvoslojev in liposomov smo opazovali posledice konformacijskih sprememb, bolj specifično, povečanje debeline lipidne membrane ob prisotnosti kovinskih ionov, kot so kalcij, aluminij in železo. Nazadnje smo preučili vpliv lipidne oksidacije na spremembo električnih lastnosti ravninskih lipidnih dvoslojev in zaznali povečanje električne prevodnosti in kapacitivnosti lipidnega dvosloja, kar lahko pojasni dlje časa trajajočo prepustnost celične membrane pri elektroporaciji.

Ključne besede: Elektroporacija, elektrokemija, numerično modeliranje, modeli celične membrane

Razširjen povzetek v slovenskem jeziku

I Uvod

Vse biološke celice se za pravilno delovanje in nadzor homeostaze zanašajo na električne lastnosti celične membrane [1]. Zanimivo pa je, da se biološke celice odzivajo tudi na zunanje dovedene električne pulze. Dandanes obstaja širok spekter aplikacij, ki to dovzetnost izkoriščajo na različnih področjih, v medicini [2]–[4], biotehnologiji [5] in prehrambni industriji [6], [7]. Z uporabo visokonapetostnih električnih pulzov je namreč mogoče spremeniti strukturo celične membrane in s tem vplivati na njeno prepustnost. To tehniko imenujemo elektroporacija, ali elektropermeabilizacija [8].

Elektroporacija je metoda, kjer z izpostavljenostjo bioloških celic visokonapetostnim električnim pulzom, povzročimo strukturne spremembe celične membrane v obliki hidrofilnih por. Posledično postane membrana kratkotrajno bolj prepustna za molekule, ki običajno ne morajo prehajati hidrofobne pregrade celične membrane [8], [9].

Kemični in fizikalni procesi, ki potekajo na molekularni ravni med in po elektroporaciji, kot na primer nastanek hidrofilnih por, so razmeroma dobro poznani. Veliko slabše so poznani spremljajoči procesi, kot so elektrokemijske reakcije, sproščanje kovinskih ionov in lipidna oksidacija, le-ti so predmet preučevanja te doktorske disertacije.

I.1 Celična membrana in električno polje

Vsaka celica je obdana s celično membrano, ki jo lahko z električnega vidika opišemo kot tanko izolacijsko plast, obdano z elektrolitom [10]. Učinki ele-

ktričnega polja na celično membrano so zelo raznoliki in kompleksni, zato je preučevanje in razumevanje le-teh ključnega pomena za razvoj novih in izboljšanih načinov zdravljenja z elektroporacijo.

I.1.1 Celična membrana

Celična membrana je 6 -10 nm debel sloj, ki sestavlja in obdaja vsako celico in predstavlja selektivno pregrado med znotrajceličnim in zunajceličnim okoljem [1]. Njena glavna naloga je, da zadržuje sestavine celice v notranjosti, hkrati pa preprečuje vstop neželenih snovi v celico [11]. Celični membrani funkcijo pregrade omogoča njena struktura iz amfifilnih fosfolipidnih molekul ter membranskih proteinov. Fosfolipidi so sestavljeni iz molekule glicerina, na katero sta pritrjeni dve dolgi verigi maščobnih kislin, ki predstavljata hidrofobni del molekule. Tretjo pozicijo pa zavzema fosfatna skupina, ki predstavlja hidrofilni del molekule. Tako lahko fosfolipidi tvorijo neprekinjeno dvojno plast, z izjemno hidrofobnim jedrom in hidrofilno zunanjostjo. Fosfatno skupino lahko dodatno modificiramo z različnimi molekulami in tako spremenimo lastnosti fosfolipida. Najpogostejša modifikacija fosfatne skupine je dodatek holina, etanolamina ali serina [12]. Različne verige maščobnih kislin privedejo do različnih lastnosti fosfolipidov. Verige maščobnih kislin v fosfolipidih so lahko nasičene ali nenasičene, kar pomeni, da lahko vsebujejo eno ali več dvojnih vezi. Dolžina verige maščobne kisline in število dvojnih vezi, ki jih vsebuje, močno vplivata na notranjo energijo celične membrane [1]. Dvojne vezi v verigah maščobnih kislin so območja s posebnimi lastnostmi; v strukturi maščobnih kislin ustvarjajo pregibe in tako povečujejo fluidnost membrane [13].

Dandanes poznamo različne *in vitro* modele, ki posnemajo strukturo celične membrane in služijo kot poenostavljene, a učinkovite platforme za raziskovanje celičnih procesov na molekularni ravni. Najpogostejši modeli celičnih membran so ravninski lipidni dvosloji in liposomi. Ravninski lipidni dvosloj je preprost, vendar še vedno zadovoljiv model celične membrane in prav ravninski lipidni dvosloji ponujajo priložnost za preučevanje lastnosti fosfolipidnih molekul v nadzorovanem okolju [14]. Prednost ravninskih lipidnih dvoslojev je, da so kemično in električno dostopni z obeh strani. Sestavo lipidnega dvosloja lahko poljubno spreminjamo, da posnemamo sestavo prave celične membrane. Po drugi strani

pa so liposomi sferični mehurčki oziroma vezikli, s steno, ki jo gradi ukrivljen lipidni dvosloj [15]. Njihova podobnost z biološko celico je boljša prav zaradi te ukrivljenosti. Analiza ravninskega lipidnega dvosloja in liposomov je razmeroma preprosta in poteka pod dobro nadzorovanimi eksperimentalnimi pogoji, zato so priljubljeni modeli za raziskovanje osnovnih lastnosti in funkcij celičnih membran, brez dodane kompleksnosti, ki jo najdemo v živih celicah.

I.1.1.2 Molekularni transport preko celične membrane

Prenos molekul preko celične membrane je lahko pasiven ali aktiven. Pasivni transport ne potrebuje energije, njegova hitrost pa je odvisna od fizikalno-kemijskih lastnosti membrane in molekul, ki se prenašajo. Majhne, hidrofobne in nevrtalne molekule ter tudi plini, lahko prosto difundirajo skozi lipidno membrano zaradi elektrokemijskega gradienta [16]. Večje molekule z nabojem, kot so aminokisline, nukleozidi, ogljikovi hidrati in ioni, se lahko zaradi svojih koncentracijskih gradientov premikajo skozi membrano, če jim pri tem pomagajo specifični transportni proteini ali kanali. Po drugi strani pa prenos molekul in ionov skozi biološke membrane proti elektrokemijskemu gradientu zahteva vnos energije in se zato imenuje aktivni transport. Na splošno je prenos prek lipidne membrane ključnega pomena za pravilno delovanje celic in vzdrževanje homeostaze [1], [17].

Kot opisano, je selektivnost lipidne membrane za prehajanje molekul ali ionov zelo visoka, zato je za številne medicinske in biotehnološke procese zelo pomemben razvoj tehnik, ki omogočajo manipulacijo transmembranskega transporta, po možnosti na razmeroma nadzorovan način.

I.1.2 Uporaba električnega polja ali elektroporacija

Elektroporacija je metoda, kjer z uporabo električnih pulzov povečamo prepustnost celične membrane. Izraz elektroporacija so leta 1982 prvič opisali Neumann in sodelavci [18]. Z mehanističnega vidika lahko elektroporacijo opišemo s tako imenovano teorijo nastanka hidrofilnih por. Ko na celično membrano dovedemo dovolj visoko električno polje, pride do reorientacije fosfolipidnih molekul v lipidnem dvosloju [19]–[21]. Ta strukturna sprememba povzroči prodiranje molekul

vode v hidrofobno domeno lipidnega dvosloja. Posledično pride do preoblikovanja sosednjih lipidnih molekul, tako da njihovi polarni (hidrofilni) deli sledijo smeri vdora molekul vode. Tako nastanejo hidrofilne pore. Zaradi nastalih por se poveča prepustnost celične membrane, ki lahko traja od milisekund do minut [22], [23].

I.1.2.1 Transmembranska napetost in elektroporacija

Celična membrana ima v naravnem stanju tako imenovano transmembransko napetost, pogosto imenovano transmembranski potencial. Transmembranska napetost je posledica razlike električnih potencialov znotraj in zunaj celice, zaradi razlike med koncentracijo znotrajceličnih in zunajceličnih ionov. V stanju mirovanja dobi celična membrana tako imenovan "mirovni (trans)membranski potencial", ki ima gledano na zunanjost celice običajno vrednost med -40 mV in -70 mV [8]. Zaradi odpiranja ali zapiranja ionskih kanalov v celični membrani se lahko mirovni potencial premakne v bolj negativne ali bolj pozitivne vrednosti, tj. membrana postane hiperpolarizirana ali depolarizirana [24].

Ko izpostavimo celico električnemu polju, tj. ko nanjo delujejo električni pulzi, se obstoječem mirovnem transmembranskem potencialu prišteje še inducirani transmembranski potencial [25]. Zaradi induciranega transmembranskega potenciala se lokalno spremenita struktura in delovanje celične membrane, kar povzroči povečano prepustnost za praktično vse molekule. Ker je celična membrana dvodimenzionalna tekočina, se lahko vrne v stanje pred izpostavitvijo električnemu polju, ter tako celica preživi [26], [27].

Na ravni celice lahko opredelimo tri nivoje elektroporacije: (i) območje kjer elektroporacije ni mogoče zaznati, (ii) ombočje reverzibilne elektroporacije in (iii) ireverzibilne elektroporacije (IRE). Posamezna območja so opredeljena z jakostjo električnega polja (V/cm) in trajanjem izpostavljenosti električnemu polju. Za doseganje elektroporacije je pri daljših trajanjih električnih pulzov potrebna manjša jakost električnega polja. Na primer, pri električnem pulzu, dolgem 1 milisekundo, od 0 V/cm do 250 V/cm ni zaznavne elektroporacije, med 250 V/cm in 1750 V/cm se pojavi reverzibilna elektroporacija, nad 1750 V/cm pa se pojavi IRE [7], [23]. V prvem območju, kjer elektroporacije ne zaznamo, so pore,

če nastanejo, premajhne in/ali preveč nestabilne, da bi jih bilo mogoče zaznati. Pri reverzibilni elektroporaciji lahko pore zagotovijo začasno pot za molekularni transport skozi membrano, čeprav se po koncu električnega pulza ta prenos postopoma preneha, in večina teh celic preživi ter ostane vitalnih. Pri IRE pa se nekatere pore ne zaprejo ali pa se zapirajo prepočasi, da bi se ohranila sposobnost preživetja celic. Te celice nato izgubijo svojo celovitost in nazadnje odmrejo [27].

I.1.3 Aplikacije elektroporacije v medicini

Različne klinične aplikacije izkoriščajo elektroporacijo za povečanje prepustnosti celične membrane in vnos različnih molekul v celico, kot na primer za vnos citostatikov pri elektrokemoterapiji ali genskega material pri genski elektrotransfekciji. Ireverzibilna elektroporacija pa se uporablja predvsem za kontrolirano induciranje celične smrti.

I.1.3.1 Elektrokemoterapija

Elektrokemoterapija (EKT) je način tumorskega zdravljenja, kjer povečamo vnos kemoterapevtika z dovajanjem električnih pulzov [2], [9], [28]. Uporablja se predvsem za zdravljenje lokaliziranih ali površinskih tumorjev. Električni pulzi se dovajajo prek kovinskih elektrod, vstavljenih neposredno v tumor in okoliško tkivo. Elektroporacija nato povzroči večjo prepustnost celične membrane. S tem se poveča sprejem zdravila, ki je bilo vbrizgano pred uporabo električnih pulzov [9], [29]. Običajno sta za kemoterapevtsko učinkovino izbrana bleomicin ali cisplatin, saj sama slabo prehajata celično membrano. Enkratni odmerek kemoterapevtika se aplicira lokalno na mesto tumorja ali sistemsko, sledi pa uporaba električnih pulzov, kar poveča citotoksični učinek zdravila. Nenazadnje pa pri EKT tarčne celice umrejo bolj nadzorovano, tumor pa se krči počasi in z manjšim tveganjem okužb [30]–[33]. Poleg tega lahko elektrokemoterapija dandanes cilja tudi na tumorje, ki jih je težko zdraviti z drugimi metodami [30].

I.1.3.1 Genska elektrotransfekcija

Pri genski elektrotransfekciji (GET) se uporabljajo visokonapetostni električni pulzi za prenos molekul DNK ali RNK (deoksiribonukleinske kisline ali ribonukleinske kisline) v celice. Gre za večstopenjski proces, ki vključuje pritrditev molekule DNK ali RNK na celično membrano, prenos preko celične membrane, migracijo skozi citoplazmo, prenos preko jedrske ovojnice in končno izražanje genov v jedru za DNK ali citoplazmi celice za RNK [34], [35]. Gre za nevirusno metodo prenosa genov z različnimi terapevtskimi aplikacijami. V onkologiji se uporablja za genski inženiring rakavih celic [5], [36]. GET se lahko uporablja tudi za cepljenje proti nalezljivim boleznim, za povečanje vnosa DNK in RNK molekul ali za gensko terapijo [37]. Genska elektrotransfekcija ne povzroča nezaželene specifične imunosti in zmanjšuje tveganje za integracijo terapevtskih nukleinskih kislin v genom gostitelja ali njihovo širjenje v okolje [38]. GET velja za eno najbolj obetavnih nevirusnih metod za prenos genov v celice zaradi varnosti, učinkovitosti, prilagodljivosti in enostavne uporabe [35], [39]. Še več, z njo je mogoče različne biomolekule vnesti v milijone celic tekom enega samega postopka [37].

I.1.3.1 Ablacija tkiva

Po drugi strani pa je pri ireverzibilni elektroporaciji (IRE) poškodba celične membrane zaradi uporabe električnega polja veliko večja kot pri zgoraj omenjenih aplikacijah in cilj IRE je, da celica odmre. IRE se večinoma uporablja kot minimalno invazivna kirurška tehnika za ablacijo tkiva [4]. S to tehniko je mogoče nadzorovano in natančno odstraniti tkivo, ne da bi poškodovali okoliške strukture [4], [40]. Obstajajo različne minimalno invazivne metode za ablacijo tkiva, vendar ima IRE pred njimi nekatere prednosti. IRE ne temelji na toploti, zato lahko ciljno tkivo uničimo brez pregrevanja okoliških tkiv. IRE je enostavna za uporabo, lokalni pretok krvi ne vpliva na njeno učinkovitost in ne zahteva uporabe podpornih zdravil. Vpliva le na membrane živih celic, medtem ko zunajcelične strukture ostanejo nedotaknjene. Rezultat je manj brazgotinjenja in hitrejše celjenje zdravljenega tkiva [41]–[43]. Trenutno je ena izmed najbolj obetavnih aplikacij IRE v medicini ablacija srca po atrijski fibrilaciji [44], [45]. Gre za katetrsko ablacijo,

ki je zaradi svojih prednosti uporabljena tudi v humani kardiologiji [46]–[49].

I.2 Učinki elektroporacije

Zaradi vse pogostejše uporabe elektroporacije tako v biotehnologiji kot tudi v medicini, je zelo pomembno natančno preučiti vse možne procese, ki se odvijajo na celični membrani med uporabo visokonapetostnih električnih pulzov, saj lahko s tem znanjem zagotovimo še bolj varno uporabo elektroporacije.

Elektroporacija se običajno izvaja z aplikacijo visokonapetostnih električnih pulzov preko kovinskih elektrod, vstavljenih v tkivo. Pri uporabi kovinskih elektrod se je potrebno zavedati, da določeni elektrokemijski procesi potekajo takoj, ko so elektrode v stiku s tkivom ali potopljene v raztopino elektrolita. Ob dodatku električnih pulzov pa lahko pride do nadaljnjega raztapljanja elektrod in posledičnega sproščanja kovinski ionov v okolico. Sproščeni kovinski ioni so lahko škodljivi za celico, saj spremenijo strukturo celične membrane in delujejo kot katalizatorji v reakcijah lipidne oksidacije. Poleg tega je znano, da izpostavljenost električnim pulzom povzroča nastanek reaktivnih kisikovih radikalov, ki lahko še dodatno povzročijo oksidativne poškodbe nenasičenih lipidnih molekul.

I.2.1 Elektrokemijske reakcije

Elektroporacija, tj. dovajanje visokonapetostnih električnih pulzov, poteka z uporabo dveh ali več elektrod v stiku z elektrolitom [50]. Običajno se za izdelavo elektrod uporabljajo prevodni materiali, kot so kovine. Pri elektrokemijskih reakcijah pride do tako imenovanih redoks reakcij oziroma prenosa elektronov med elektrodo in elektrolitom oziroma ionsko zvrstjo v raztopini [51], [52].

Ko elektrode potopimo v elektrolit, se ustvari tako imenovana dvojna plast (ang. *double layer*). Ob uporabi električnega polja, se med elektrodama pojavi potencialna razlika, ki ji sledi akumulacija naboja v dvojni plasti. Če je razlika potencialov večja od pragovne napetosti, znane tudi kot ravnotežni potencial, začnejo na meji med elektrodo in elektrolitom potekati elektrokemijske reakcije [50], [53]. Anoda se oksidira kar povzroči nastajanje ionov na meji med elektrodo

in elektrolitom. Katoda se reducira kar povzroči manjko ionov na stiku med elektrodo in elektrolitom [54]. Na hitrost elektrokemijske reakcije vplivajo številni dejavniki, kot so uporabljena napetost, frekvenca električnih pulzov, koncentracija ionov v raztopini elektrolita, temperatura in vrsta elektrodnega materiala [52], [53], [55].

Vrsta elektrodnega materiala določa tudi vrsto elektrodnih reakcij. Če je anoda narejena iz topnega materiala, je glavna reakcija na anodi sproščanje kovinskih ionov [55], [56]. Elektrokemijske reakcije povzročajo tudi sproščanje plinov in nastajanje plinskih mehurčkov, kot sta kisik in vodik, kar je lahko nevarno pri ablaciji tkiva, na primer v srcu, zlasti v levem atriju [57], [58]. Nenazadnje pa reakcija sproščanja kovinskih ionov tudi drastično skrajša življenjsko dobo elektrod [55].

Najpomembnejša elektrokemijska reakcija na anodi je reakcija nastajanja kisika (enačba I.1):

$$2H_2O_{(l)} \Rightarrow O_{2(g)} + 4H^+ + 4e^- \tag{I.1}$$

Na anodi nastajajo tudi kovinski ioni. Če z M označimo kovino lahko zapišemo:

$$M_{(s)} \Rightarrow M^{x+} + Xe^- \tag{I.2}$$

V enačbi X predstavlja valenco kovinskega iona in število elektronov. Glavna reakcija na katodi je nastajanje vodika:

$$2H_2O_{(l)} + 2e^- \Rightarrow H_{2(g)} + 2OH^- \tag{I.3}$$

Homogena reakcija v elektrolitu je protoliza vode:

$$H^+ + OH^- \Rightarrow H_2O_{(l)} \tag{I.4}$$

Opisane elektrokemijske reakcije so nezaželen pojav pri elektroporaciji, še posebaj pri ablaciji tkiva v srcu. Znano je, da je za zmanjšanje obsega elektrokemijskih reakcij potrebno prilagajanje elektroporacijskih parametrov. Z nižjo napetostjo, krajšimi električnimi pulzi in manj prevodnim medijem je mogoče zmanjšati elektrokemijske procese. Poleg tega uporaba bifaznih električnih pulzov [59] ter

različna medfazna in medpulzna zakasnitev vplivajo na koncentracijo sproščenih kovinskih ionov [60]. Eden od izzivov pri razvoju elektroporacije je tako optimizacija dejavnikov, ki povzročajo elektrokemijske reakcije.

Velik potencial za hitro in zanesljivo optimizacijo elektroporacijskih postopkov predstavljajo numerični modeli. Z numeričnimi modeli je mogoče napovedati potek zdravljenja, optimalno postavitev elektrod in nenazadnje tudi obseg elektrokemijskih reakcij pri uporabi različnih elektroporacijskih parametrov. V numeričnem modelu lahko z diferencialnimi enačbami opišemo procese, ki potekajo na stiku elektroda-elektrolit med uporabo električnih pulzov. Z reševanjem teh enačb dobimo numerične rezultate, ki jih lahko primerjamo z eksperimentalnimi in s tem validiramo model [61]. Prvi numerični model za elektrokemijsko zdravljenje tumorjev je razvila Nilsson s sodelavci [51]. Preučevali so eno-dimenzionalni model sproščanje platine z elektrode. Ta osnovni model sedaj služi za razvoj bolj kompleksnih modelov za opis elektrokemijskih reakcij pri uporabi visokonapetostnih električnih pulzov.

I.2.2 Sproščanje kovinskih ionov

Preučevanje vpliva kovinskih ionov na lipidne membrane je dandanes vse bolj pomembno, saj je znano, da interakcija fosfolipidnih molekul s kovinami povzroči spremembe v sami strukturi fosfolipidnih molekul ter posledično v celični membrani [62], [63]. Posebno pomembno biofizikalno vprašanje je interakcija lipidnih membran z dvovalentnimi in trivalentnimi kovinskimi kationi. Za preučevanje so zanimivi tako biološko prisotni ioni, kot sta kalcij in železo, kot aluminijevi ioni, ki so sicer razširjeni v okolju, vendar ne igrajo vloge pri presnovnem procesu živih bitij.

V povezavi z elektroporacijo je bil kalcij predlagan kot način zdravljenja tumorjev. Pri tem protitumorskem zdravljenju se med postopkom elektroporacije v celico internalizirajo velike količine kalcija [64]. Menijo, da kalcijevi ioni zaradi izčrpavanja adenozin trifosfata (ATP) povzročijo nekrozo tumorja [65]–[67]. Protitumorska učinkovitost kalcijeve elektroporacije je bila dokazana *in vitro*, *in vivo* in v kliničnih testiranjih [31], [65], [68]–[72].

Na celično membrano pa lahko vplivajo tudi kovinski ioni, ki se sproščajo z elektrod pri elektroporaciji. Dandanes so v poskusih *in vitro* najpogosteje uporabljene kivete za elektroporacijo izdelane iz aluminija [73], ki je nestabilna kovina in lahko hitro korodira. V študiji Loomis-Husselbeeja s sodelavci je bilo dokazano, da lahko aluminij, ki se sprošča iz elektroporacijskih kivet, spremeni signalne poti inozitol fosfatov [74]. Pomemben je tudi vpliv železovih ionov na lipidno membrano, saj je železo glavna sestavina elektrod iz nerjavnega jekla, ki se pogosto uporabljajo pri zdravljenju z elektroporacijo. Stapulionis s sodelavci je preučeval sproščanje železovih ionov z elektrod med uporabo visokonapetostnih električnih pulzov in ugotovil, da lahko železovi ioni spremenijo strukturo nukleinskih kislin in beljakovin [75].

Pretekle študije so pokazale, da vezava kovinskih ionov povzroči konformacijsko spremembo hidrofilnih delov fosfolipidov [76], [77], kar povzroči zmanjšanje površine lipidne molekule [78]–[82]. V visokih koncentracijah lahko kovinski ioni povečajo rigidnost dvosloja in zmanjšajo njegovo fluidnost [78], [79]. Poleg tega je več študij molekularne dinamike (MD) pokazalo zaporedno vezavo kovinskih ionov na fosfolipide, in posledične strukturne spremembe lipidne membrane, v obliki konformacijskih sprememb lipidne glave [83], [84]. Interakcija pozitivno nabitih kovinskih ionov z negativnimi fosfolipidnimi skupinami lahko povzroči tudi spremembe v prepustnosti celične membrane [85], kar vpliva na prenos molekul v celico in iz nje [63], [86].

Kovinski ioni se lahko vežejo tudi na druge strukture v celični membrani, kot so membranski proteini, in spremenijo njihovo aktivnost ter tako spremenijo celične signalne poti in delovanje ionskih kanalov [87], [88]. Znano je na primer, da imajo kalcijevi ioni pomembno vlogo pri uravnavanju krčenja mišic in sproščanju nevrotransmiterjev, saj se vežejo na posebne receptorje v celični membrani [89]–[91]. Vendar pa je lahko prekomerno kopičenje kalcijevih ionov škodljivo in povzroči celično smrt [27], [92]. Poleg tega se je izkazalo, da imajo tako aluminijevi kot tudi železovi ioni vlogo pri oksidaciji lipidov, kar vodi do sprememb v prepustnosti membrane in celičnem delovanju [93]–[98].

I.2.3 Lipidna oksidacija

Oksidacija lipidov je verižna reakcija, ki povzroča degredacijo lipidnih molekul. Reakcija oksidacije se prične z napadom radikalov na verigo maščobne kisline, ki vsebuje šibko C-H vez ob dvojni vezi [99]. Prav te dvojne vezi v verigah maščobnih kislin nenasičenih fosfolipidov so namreč nagnjene k oksidativnim poškodbam. Z odstranitvijo vodika iz skupine C-H ostane na ogljikovem atomu neparen elektron. Tako nastane lipidni radikal s središčem v ogljiku, ki se lahko stabilizira s premikom dvojnih vezi. S tem nastane konjugirani dien ali peroksidni radikal [100], [101]. Nadalje ta proces ustvari še več radikalov, ki širijo škodo na bližnje molekule, saj lahko lipidni peroksidni radikali odvzamejo vodik iz bližnje nenasičene lipidne molekule in tvorijo primarni produkt oksidacije, hidroperoksid [102].

Zaporedna redukcija molekularnega kisika, ki je prisoten v atmosferskem zraku, vodi do nastanka kisikovih radikalov, kot je superoksidni radikal (enačba I.6) [103], [104]:

$$O_{2(g)} + e^- \Rightarrow O_2^-\cdot \tag{I.5}$$

Superoksidni radikal lahko tvori vodikov peroksid H_2O_2

$$O_2^-\cdot + e^- + 2H^+ \Rightarrow H_2O_{2(l)} \tag{I.6}$$

Vodikov peroksid lahko disocira v HO·.

$$H_2O_{2(l)} + e^- \Rightarrow OH^- + HO\cdot \tag{I.7}$$

Zelo pomemben radikal je singletni kisik, ki nastane s fotolizo tripletnega kisika.

$$^3O_2 \Rightarrow {}^1O_2 \tag{I.8}$$

Singletni kisik lahko reagira z vodno paro v ozračju in tvori hidroksilne radikale.

Hidroksilni radikali, ki so zelo reaktivni, se lahko združijo s še eno molekulo O_2 v hidroperoksilni radikal (HOO·).

Poleg tega kovinski ioni, kot so železovi kationi, ki so močni oksidanti, povzročajo nastanek hidroksilnih in hidroperoksilnih radikalov. Železo Fe^{2+} reagira s H_2O_2 in se oksidira v Fe^{3+}, pri čemer nastane hidroksilni radikal (HO·). Fe^{3+} lahko nato nadalje reagira z drugo molekulo H_2O_2 in povzroči nastanek hidroperoksilnega radikala (HOO·) [105], [106].

Znano je, da visokonapetostni električni pulzi lahko povzročijo oksidativno poškodbo celične membrane. Opravljenih je bilo več študij, ki so potrdile, da elektroporacija povzroči oksidacijo lipidov [107]–[109], kar se je dejansko pokazalo pri bakterijah [110], liposomih [111] in na celicah [108], [109], [112], [113]. Obseg oksidacije lipidov se povečuje s številom električnih pulzov, njihovim trajanjem in amplitudo [108], [109], [114], [115]. Z uporabo električnih pulzov lahko radikali nastajajo v zunajceličnem mediju zaradi elektrokemijskih reakcij ali znotrajcelično zaradi destabilizacije mitohondrijskih membran [115]–[117]. V nedavni študiji [22] je bilo dokazano, da se prepustnost in prevodnost membrane povečata zaradi oksidacije lipidov. Ob prisotnosti oksidiranih lipidov se debelina dvosloja zmanjša, medtem ko se površina fosfolipida poveča [118]–[122]. Ker lahko uporaba visokonapetostnih električnih pulzov povzroči oksidacijo lipidnih molekul, se prevodnost membrane poveča, kar pomeni, da pri neselektivnem prenosu med elektroporacijo in po njej ne igrajo vloge le hidrofilne pore, temveč tudi oksidacija lipidov. Oksidirane lipidne molekule je treba v celici encimsko popraviti ali nadomestiti, da se ohranita struktura in delovanje membrane [123], kar zahteva čas in lahko pojasni, kako prepustnost celične membrane ostane povečana tudi po končanem dovajanju električnih pulzov.

S simulacijami molekularne dinamike, so pokazali, da se urejenost lipidov v dvosloju ob prisotnosti oksidacijskih produktov zmanjša. Ob večinski oksidaciji fosfolipidov, pride do samodejnega nastanka por. To omogoča še dodatni vnos prostih radikalov v celico, ki lahko poškodujejo znotrajcelične strukture. Preučevanje sekundarnih oksidacijskih produktov [124] je pokazalo, da je zaradi prisotnosti aldehidov prevodnost oksidirane membrane še večja in ustreza opaženim spremembam prevodnosti v celicah, ki so bile izpostavljene električnim pulzom. Pri tem se je pokazalo, da lahko že majhno število oksidacijskih pro-

duktov povzroči dramatično povečanje prepustnosti membrane. Zato moramo za nadaljnjo optimizacijo elektroporacijskih tehnologij in zdravljenj preučiti povezavo med oksidacijo lipidne membrane in elektroporacijo.

II Namen dela

Namen te doktorske disertacije je izboljšati razumevanje elektroporacijskih tehnologij in zdravljenj s preučevanjem spremljajočih procesov, ki potekajo med uporabo visokonapetostnih električnih pulzov, kot so elektrokemijske reakcije, sproščanje kovinskih ionov in oksidacija lipidnih molekul. Preverili smo naslednje hipoteze:

- Numerični model, za opis elektrokemijskih reakcij na stiku med elektrodo in elektrolitom pri elektroporaciji, se lahko uporabi za optimizacijo elektroporacijskih parametrov.
- Kovinski ioni, ki so fiziološko prisotni v našem telesu ali se sproščajo z elektrod med elektroporacijo, lahko vplivajo na strukturo in stabilnost lipidnih dvoslojev.
- Oksidacija lipidnih molekul lahko povzroči merljive spremembe električnih lastnosti ravninskih lipidnih dvoslojev.

Uporaba visokonapetostnih električnih pulzov vodi do elektrokemijskih reakcij na stiku med elektrodo in elektrolitom. Elektrodna napetost in električni tok sprožita reakcije, ki povzročajo sproščanje kovinskih ionov in nastajanje prostih radikalov. Elektrokemijske reakcije vodijo tudi do hitrejše obrabe elektrod in nastanka plinskih mehurčkov, kar je še posebaj nevarno pri ablaciji srčne mišice. Prav zato je zelo pomembno poznavanje in nadzorovanje obsega elektrokemijskih reakcij pri elektroporaciji. Za zmanjšanje obsega elektrokemijskih reakcij se uporabijo različne modifikacije elektroporacijskega protokola, kot so znižanje napetosti, skrajšanje trajanja električnih pulzov, uporaba bifaznih pulzov in spreminjanje frekvence pulzov. Naš namen je bil razvoj numeričnega modela za opis

sproščanja kovinski ionov s katerim bomo lahko preučili uporabo različnih protokolov električnih pulzov na pojav elektrokemijskih reakcij na stiku med elektrodo in elektrolitom. Z validiranim numeričnim modelom bo mogoče optimizirati elektroporacijske protokole in omejiti neželene stranske učinke, kot je sproščanje kovin z elektrod, deloma brez potrebe po obsežnem eksperimentalnem delu.

Kovinski ioni, ki so fiziološko prisotni v našem telesu ali se sproščajo z elektrod pri elektroporaciji, imajo različne vplive na strukturo celične membrane. Kalcij se uporablja kot aktivna molekula pri kalcijevi elektrokemoterapiji, tj. elektroporaciji v kombinaciji z visoko koncentracijo kalcija, ki povzroči celično smrt zaradi pomanjkanja molekul ATP. Poleg tega lahko med uporabo visokonapetostnih električnih pulzov pride do sproščanja kovinskih ionov z elektrod. Najpogosteje uporabljeni kovini pri elektroporacijskih postopkih sta aluminij in nerjavno jeklo katerega glavna sestavina je železo. Znano je, da aluminijevi in železovi ioni vplivajo na strukturo in stabilnost lipidnih membran, ter katalizirajo reakcijo lipidne oksidacije. Pokazali smo, da lahko spremembe celične membrane zaradi prisotnosti kovinskih ionov zaznamo tudi na preprostih membranskih modelih. Za preučevanje vpliva kovinskih ionov na strukturo celične membrane smo tako uporabili ravninske lipidne dvosloje in liposome. Ti umetni lipidni dvosloji so preprost, vendar še vedno zadovoljiv model celične membrane, ki se je izkazal kot zelo uporaben način za preučevanje interakcij med fosfolipidi in kovinskimi ioni.

Nenazadnje pa smo raziskali vpliv prisotnosti oksidiranih lipidov na električne lastnosti ravninskih lipidnih dvoslojev. Številne študije so namreč pokazale, da prepustnost membrane traja dlje kot uporaba električnih pulzov, kar pomeni, da morajo poleg strukturnih sprememb membrane v obliki nastanka hidrofilnih por, potekati tudi druge, bolj kemične spremembe. Oksidacija lipidov je proces, ki lahko pojasni to dolgotrajno prepustnost celične membrane. Lipidna oksidacija je namreč verižna reakcija, ki povzroči strukturne spremembe in degredacijo fosfolipidnih molekul. Do oksidacije lipidnih molekul lahko pride pri elektroporaciji, zaradi elektrokemijskih reakcij, nastanka prostih radikalov ali destabilizacije mitohondrijskih membran. Poleg tega lahko na oksidacijo lipidnih molekul vplivajo tudi kovinski ioni, na primer železovi ioni, ki se sproščajo z elektrod, saj je znano, da kovinski ioni katalizirajo verižno reakcijo oksidacije. Za preučevanje vloge oksidacije lipidov na prepustnost lipidne membrane smo razvili eksperimentalni protokol za detekcijo sprememb električnih lastnosti neoksidiranih in oksidiranih

ravninskih lipidnih dvoslojev.

Z numeričnimi modeli in modeli celične membrane želimo preučiti vpliv elektrokemijskih reakcij, kovinskih ionov in oksidacije lipidov na lipidno membrano pri elektroporaciji. S tem bomo razširili znanje o spremljajočih procesih, ki potekajo na stiku med elektrodo in elektrolitom pri uporabi visokonapetostnih električnih pulzov, in opozorili na negativne učinke, ki jih imajo te procesi na lipidno membrano.

III Pregled in sklepne misli

Delo, opravljeno v tej doktorski disertaciji, je predstavljeno v treh člankih, objavljenih v mednarodnih znanstvenih revijah s faktorjem vpliva. Rezultati posameznih prispevkov so podrobno predstavljeni in obravnavani v člankih, zato so v tem poglavju samo povzete ugotovitve, opisane v objavljenih člankih, in podrobneje predstavljeni še neobjavljeni rezultati.

III.1 Učinki elektroporacije na lipidno membrano

V prvem članku z naslovom *"The good and the bad of cell membrane electroporation"*, je opisana elektroporacija, kot metoda za povečanje prepustnosti celične membrane. V članku smo opisali ključne celične strukture na katere vpliva elektroporacija, kot so na primer celična membrana, membranski proteini in citoskelet. Kritično smo primerjali elektroporacijo z drugimi metodami, ki se prav tako uporabljajo za povečanje prepustnosti celične membrane. Opisane so tudi glavne aplikacije elektroporacije ter prednosti in slabosti klinične uporabe elektroporacije.

Za razumevanje elektroporacije je pomembno poznavanje strukture celične membrane. Celično membrano lahko opišemo kot tanko dvojno plast fosfolipidov, ki obdaja vsako celico in predstavlja selektivno pregrado med znotrajceličnim in zunajceličnim okoljem. Njena glavna naloga je, da zadržuje sestavine celice v notranjosti, hkrati pa preprečuje vstop neželenih snovi v celico. S tem je omogočen selektivni prenos bistvenih hranilnih snovi v celico in odpadnih produktov v nasprotni smeri.

Prenos molekul skozi celično membrano je lahko pasiven ali aktiven [1]. Velike in nabite molekule, kot so beljakovine, nukleinske kisline in sintetična zdravila, ne morejo prehajati celične membrane, saj za to nimajo ustreznih mehanizmov [125]. Tudi številne terapevtske molekule so takšne narave, zato so bile za njihovo vstopanje v celice, kjer delujejo, razvite različne tehnike za povečanje prenosa skozi celično membrano.

Elektroporacija vpliva na številne celične strukture, kot na primer celično membrano, membranske proteine in celo na citoskelet. Znano je, da elektroporacija povzroča oksidacijo fosfolipidov, ki gradijo celično membrano [8]. Vsi prosti radikali, ne glede na njihov izvor, lahko med elektroporacijo povzročijo oksidacijo lipidov [126]; ker pa so ti radikali kratkoživi, bodo poškodbe lipidov povzročili le tisti, ki nastanejo v neposredni bližini celične membrane in imajo dostop do lipidnih repov. Dokazano je bilo, da radikali oksidirajo samo dele membrane, ki so elektropermeabilizirani [20]. Te reakcije dosežejo vrh nekaj sekund po uporabi električnih pulzov, nato pa se postopoma zmanjšujejo. Lipidna oksidacija povzroči kemične spremembe v fosfolipidnih molekulah, predvsem v regiji maščobnih kislin. Ta degredacija vodi do povišane prepustnosti lipidnega dvosloja.

Poleg tega elektroporacija celične membrane lahko vpliva na membranske proteine, pri čemer v najslabšem primeru pride do njihove denaturacije zaradi lokalnega povečanja temperature, ki ga povzročijo električni pulzi [127]. Izpostavljenost celic električnim pulzom poveča prevodnost transmembranske Na^+/K^+-ATPaze in zmanjša transmembranske ionske tokove skozi napetostno krmiljene ionske kanale [8].

Elektroporacija ima posledice tudi na celovitost citoskeleta. Izpostavljenost celic električnim pulzom lahko poruši mrežo mikrofilamentov in mikrotubulov [128]. Ti učinki so odvisni od napetosti in reverzibilni, saj se citoskelet lahko v nekaj urah popolnoma obnovi. Izkazalo se je, da prekinitev mikrofilamentov celo zaščiti celico pred uničenjem zaradi električnih pulzov [129]. Elektroporacija veziklov z aktinskimi filamenti je pokazala, da prisotnost aktinskih filamentov poveča rigidnosti membrane, kar blokira vsako večjo deformacijo veziklov in preprečuje nastanek velikih membranskih por [130]. Celostni vpliv električnih pulzov na citoskelet pa dandanes še ni popolnoma raziskan.

Nenazadnje pa smo v članku predstavili glavne aplikacije elektroproacije v medicini, kot so elektrokemoterapija, genska elektrotransfekcija in ablacija tkiva. Menimo, da ima elektroporacija številne prednosti pred drugimi metodami, ki prav tako služijo za neselektiven vnos molekul skozi celično membrano. Elektroporacija deluje na vseh vrstah celic, je pa učinkovitost elektroporacije odvisna od velikosti celice, večja kot je celica, višja električna napetost bo potrebna za nastanek hidrofilnih por. Elektroporacija je preprosta za uporabo in cenovno ugodna tehnika, brez zahteve po kompleksni opremi, za izvedbo postopka namreč potrebujemo elektroporator in elektrode.

Kljub vsemu pa so potrebne nadaljne raziskave na področju elektroporacije, da zagotovimo njeno varno uporabo v medicini. Uporaba ireverzibilne elektroporacije za ablacijo tkiva se uporablja predvsem za ablacijo srčne mišice, kjer je še kako pomembno poznavanje vseh stranskih procesov uporabe električnih pulzov. Visokonapetnosti električni pulzi lahko povzročijo sproščanje kovinskih ionov z elektrod in nastajanje mehurčkov zaradi elektrokemijskih reakcij. Nastajanje prostih radikalov pa vodi do lipidne oksidacije, kar prav tako privede do sprememb v strukturi celične membrane. Opisane procese smo preučevali v tej doktorski disertaciji.

III.2 Numerični model za opis elektrokemijskih reakcij

Razvili smo numerični model za opis sproščanja kovinskih ionov pri uporabi visokonapetnostih električnih pulzov, ki je opisan v članku z naslovom *"numerical model of aluminium and iron dissolution during electric pulse application for electroporation"*. Z uporabo tega modela je možno preučevati obseg elektrokemijskih reakcij, bolj specifično, koncentracijo sproščenih kovinski ionov pri uporabi različnih elektroporacijskih protokolov.

Sproščanje kovinskih ionov je spremljajoči proces uporabe električnih pulzov na kovinskih elektrodah v stiku z elektrolitom in poteka predominantno na anodi. Elektrode so pogosto izdelane iz aluminija ali nerjavnega jekla, katerega glavni gradniki so železovi ioni. Prav aluminijevi in železovi ioni pa imajo lahko nezaželene učinke na celice, vplivajo na celično delovanje in povzročajo lipidno

oksidacijo. Poleg tega pa sproščanje kovinskih ionov povzroči tudi hitrejšo obrabo elektrod in poveča prevodnost elektrolita, kar lahko vpliva na potek elektroporacije.

Zaradi razmeroma preprostega eksperimentalnega določanja koncentracije kovinskih ionov z masno spektrometrijo in dostopnih rezultatov iz študije Kotnika s sodelavci, smo se odločili, da bomo razvili numerični model, ki bo opisoval sproščanje kovinskih ionov pri uporabi različnih elektroporacijskih protokolov in bo lahko validiran z eksperimentalnim rezultati pridobljenimi iz omenjene študije. V študiji Kotnika s sodelavci so bili uporabljeni tako monofazni kot tudi bifazni električni pulzi z napetostjo med 0 V in 400 V za monofazne pulze in med 0 V in 280 V za bifazne pulze. Izmerili so koncnetracijo sproščenih aluminijevih in železovih ionov v rastnem mediju celične kulture ter pokazali, da se pri uporabi bifaznih pulzov močno zmanjša kontaminacija elektrolita s kovinskimi ioni.

Numerični model za opis elektrokemijskih reakcij in sproščanja kovinskih ionov smo razvili s programom COMSOL Multiphysics 6.0 (Comsol Inc., Burlington, MA, USA). Osnovne elektrokemijske reakcije uporabljene v numeričnem modelu so opisane v poglavju I.2.1. Uporabili smo Nernst-Planck-ovo enačbo za opis masnega toka kovinskih ionov v elektrolitu. Sproščanje kovinskih ionov je opisano s Faraday-evim zakonom, za opis elektrodne kinetike pa smo uporabili Butler-Volmer-jevo enačbo. V modelu smo upoštevali tudi reverzibilno kemijsko reakcijo protolize vode v elektrolitu.

Dvo-dimenzionalna geometrija modela je sestavljena iz domene elektrolita, v našem primeru NaCl, kjer potekajo reakcije in štirih robov. Dva robova predstavljata elektrodi in sicer anodo in katodo. Preostala dva robova pa sta izolirana. V modelu je bilo upoštevanih šest neznanih spremenljivk, in sicer koncentracije posameznih ionov (H^+, Na^+, Cl^-, OH^- in kovinskih ionov) ter električno polje. Rezultat numeričnega modela je koncentracijski profil za vsak ion v odvisnosti od uporabljene amplitude in polarnosti pulza. V modelu smo implementirali funkcijo monofaznih in bifaznih električnih pulzov. Uporabili smo različne napetosti in sicer med 0 V in 400 V za monofazne pulze in med 0 V in 280 V za bifazne pulze. Molarno koncentracijo sproščenih kovinskih ionov smo izračunali s površinsko integracijo koncentracije posameznega iona v domeni elektrolita.

Z naraščajočo napetostjo tako monofaznih kot tudi bifaznih pulzov se je koncentracija sproščene kovine povečevala. Najvišja koncentracija sproščenih kovinskih ionov, tako aluminijevih kot tudi železovih, je bila pričakovano ugotovljena pri monofaznih električnih pulzih z napetostjo 400 V. Pri bifaznih pulzih je bilo sproščanje kovinskih ionov manjše v primerjavi z monofaznimi pulzi. Natančneje, petkrat manjše za aluminij in skoraj štirikrat manjše za železove ione.

Rezultati numeričnega modela kažejo na dobro ujemanje med eksperimentalnimi podatki iz študije Kotnika s sodelavci za napetosti monofaznih pulzov do 160 V. Z naraščanjem napetosti nad 160 V pa naš numerični model ne opisuje več ustrezno vseh elektrokemijskih reakcij, zato je koncentracija sproščenih kovinskih ionov v modelu nižja od tiste, dobljene v eksperimentalnih poskusih. Neskladja med numeričnimi in eksperimentalnimi rezultati pri višjih napetostih so lahko posledica dodatnih elektrokemijskih procesov, ki potekajo na stiku elektroda-elektrolit in niso upoštevani v sedanjem numeričnem modelu. Na primer, hrapavost površine elektrode se s sproščanjem kovinskih ionov povečuje, zato je med poskusi večja površina izpostavljena elektrolitu, kar lahko privede do večje koncentracije sproščenih kovinskih ionov.

Zanimivo pa je, da se modelirana koncentracija raztopljene kovine veliko bolje ujema z eksperimentalnimi rezultati, če so uporabljeni bifazni pulzi. Amplituda bifaznih pulzov je bila modelirana kot "peak-to-peak"vrednost, zato so bile napetosti med posamezno elektrodo nižje, kar je privedlo do boljše korelacije med numeričnimi in eksperimentalnimi rezultati.

Razvili in validirali smo model za opis elektrokemijskih reakcij, bolj natančno sproščanje kovinskih ionov z elektrod pri elektroporaciji. Z validiranim numeričnim modelom smo pokazali, da amplituda in polarnost električnih pulzov močno vplivata na sproščanje kovinskih ionov. Model se lahko v prihodnosti uporablja za preučevanje optimalnih elektroporacijskih parametrov, za zagotovljanje manjše kontaminacija elektrolita in večje stabilnost elektrod in vsaj deloma nadomesti obsežno eksperimentalno delo.

III.3 Učinek kovinskih ionov na lipidno membrano

V drugem članku z naslovom *Čalcium ion effect on phospholipid bilayers as cell membrane analogues"*, smo preučevali vpliv kovinskih ionov na spremembe lipidne membrane. Z uporabo dveh različnih modelov celične membrane smo opisali vpliv različnih kovinskih ionov na velikost liposomov in na električne lastnosti ravninskih lipidnih dvoslojev.

Za preučevanje smo izbrali fiziološko prisotne kalcijeve ione, ki se uporabljajo v novi tehniki elektroporacijskega zdravljenja z internalizacijo visoke koncnetracije kalcija. Preučevanje vpliva kovinskih ionov pa je pomembno tudi z vidika varnosti elektroporacije. Kot smo pokazali v prejšnjem poglavju, uporaba električnih pulzov namreč povzroči sproščanje kovinskih ionov z elektrod. Sproščeni kovinski ioni vplivajo na strukturo celične membrane saj povzročijo spremembe v debelini lipidnega dvosloja. Prav tako pa lahko vodijo do oksidacije lipidnih molekul.

Za preučevanje vpliva kovinskih ionov smo v zunanjo okolico liposomov oziroma na eno stran ravninskega lipidnega dvosloja dodali raztopino kovinskih ionov v koncentracijah od 0 mM do 50 mM. Z metodo plinske elektroforeze nES GEMMA (nano-electrospray gas-phase electrophoretic mobility molecular analyzer) smo izmerili spremembe v velikosti liposomov v odvisnosti od koncentracije dodanih kovinskih ionov. Plinska elektroforeza je analizna tehnika, ki omogoča kvantifikacijo velikost delcev v vzorcu na podlagi njihove elektroforetske mobilnosti. Z uporabo elektrosprej procesa in ekvilibracije naboja smo zagotovili, da imajo vsi delci isti naboj in so posledično ločeni v električnem polju izključno po velikosti.

Za analizo električnih lastnosti ravninskih lipidnih dvoslojev smo uporabili merilnik impedance, ter izmerili električno upornost in kapacitivnost ravninskih dvoslojev v odvisnosti od koncnetracije dodanih kovinskih ionov. Električna upornost podaja lastnost snovi, v našem primeru fosfolipidnega dvosloja, preko katerega teče električni tok. Ravninski lipidni dvosloji so dobri izolatorji, ki imajo visoko električno upornost. Prav tako pa imajo ravninski lipidni dvosloji možnost shranjevanja električnega naboja kar opiše izmerjena električna kapacitivnost. Iz-

merjena kapacitivnost pa je odvisna od več dejavnikov, vključno z geometrijo in debelino dvosloja ter dielektrično konstanto dvosloja. Dodatek pozitivno nabitih kovinskih ionov privede do konformacijskih sprememb v strukturi ravninskega lipidnega dvosloja ter posledično v izmerjenih vrednostih električne upornost in kapacitivnosti. Dodatno smo izmerili tudi porušitveno napetost in življensko dobo ravninskega lipidnega dvosloja s sistemom, ki smo ga razvili v našem laboratoriju. Porušitvena napetost predstavlja minimalno napetost, ki je potrebna, da se lipidni dvosloj poruši in omogoči prehod molekul ali snovi skozi membrano. Porušitveno napetost smo izmerili z linearno naraščajočim signalom napetostne rampe 4.8 kV/s. Življenska doba ravninskega lipidnega dvosloja pa predstavlja čas ob porušitvi ravninskega lipidnega dvosloja.

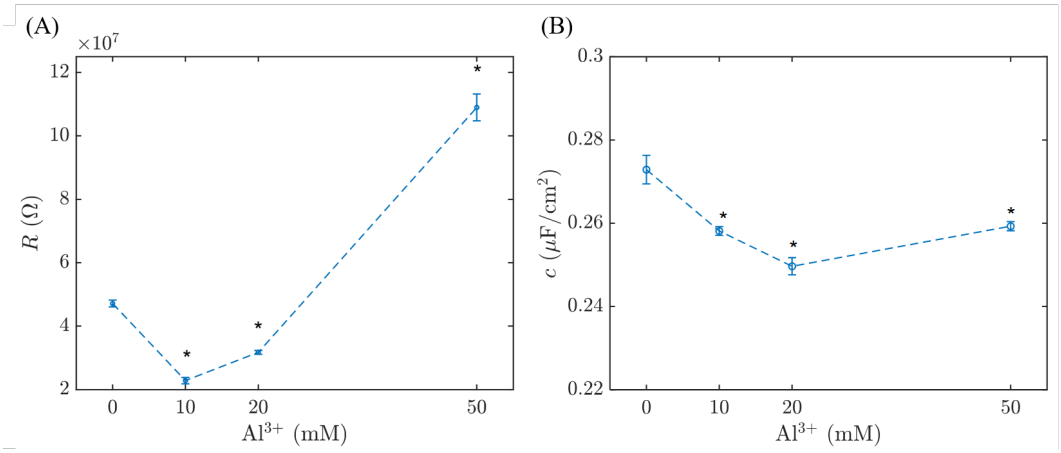
Kalcijevi ioni

S plinsko elektroforezo liposomov smo pokazali, da se velikost veziklov zmanjšuje z naraščajočo koncentracijo kalcija v zunanjem okolju delcev. Izmerjeno krčenje liposomov je verjetno posledica zmanjšanja površine fosfolipida zaradi interakcije s kalcijevimi ioni. Poleg tega bi lahko bilo od kalcijevih ionov odvisno zmanjšanje velikosti veziklov povezano tudi s tesnejšim povezovanjem fosfolipidov v dvosloju in s tem manjšo velikostjo veziklov.

Električne lastnosti ravninskih lipidnih dvoslojev, in sicer upornost in kapacitivnost, smo prav tako preučevali v odvisnosti od koncentracije kalcijevih ionov. Ob dodatku kalcijevih ionov na eno stran ravninskega lipidnega dvosloja smo opazili povečanje upornosti in zmanjšanje specifične kapacitivnosti ravninskega lipidnega dvosloja, kar povezujemo s povečanjem debeline ravninskega lipidnega dvosloja ob prisotnosti kalcijevih ionov. Dobljeni rezultati so skladni s povečano togostjo ravninskih lipidnih dvoslojev ob prisotnosti dvovalentnih kalcijevih kationov zaradi tesneje povezane strukture, kot smo opazili pri analizi liposomov. Prav tako pa lahko vezava pozitivno nabitih kalcijevih ionov na glave fosfolipidnih molekul vodi do spremembe v dielektrični konstanti dvosloja.

Aluminijevi ioni

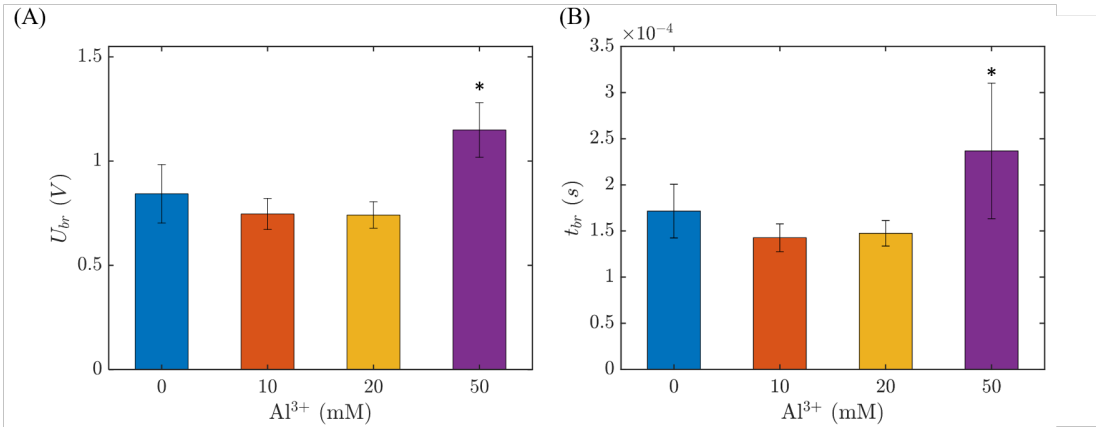
Pri dodatku aluminijevih ionov v elektrolit na eno stran ravninskega lipidnega dvosloja prav tako pride do spremembe v izmerjenih električnih lastnostih dvosloja. Električne lastnosti dvoslojev, in sicer upornost, kapacitivnost, porušitveno napetost in življenjsko dobo dvoslojev, smo merili v odvisnosti od koncentracije aluminijevih ionov. Ko smo v elektrolit dodali majhno koncentracijo (20 mM) kovinskih ionov, se je upornost celotnega sistema zmanjšala, saj se prevodnost elektrolita zaradi večje koncentracije ionov v raztopini povečala. Z naraščajočo koncentracijo kovinskih ionov (50 mM) se je upornost znatno povečala, predvidevamo, da zaradi povečane debeline dvoslojev, saj se kovinski ioni vežejo na lipidne glave, ter tako ustvarijo dodatni sloj na površini lipidnih molekul. Pozitivno nabiti kovinski ioni ob vezavi na hidrofilne dele lipidnih molekul povzročijo konformacijske spremembe v lipidni molekuli in s tem tesnejšo povezavo med samimi molekulami.



Slika III.1: Električne lastnosti ravninskih lipidnih dvoslojev v odvisnosti od koncentracije aluminijevih ionov. (A) Upornost in (B) specifična kapacitivnost ravninskega lipidnega dvosloja. Podatki so prikazani kot povprečna vrednost ± standardna deviacija (n = 10 meritev). (*) predstavljajo signifikantno razliko ($p \leq 0.05$) med 0 mM koncnetracijo kovine in višjimi koncentracijami (10 mM, 20 mM, 50 mM).

Slika III.1 prikazuje meritve upornosti (A) in specifične kapacitivnosti (B) ravninskih lipidnih dvoslojev v prisotnosti aluminijevih ionov. Specifična kapacitivnost ravninskega lipidnega dvosloja se ob dodatku kovinskih ionov zmanjša, najverjetneje zaradi povečanja debeline dvosloja. Izmerili smo tudi porušitveno

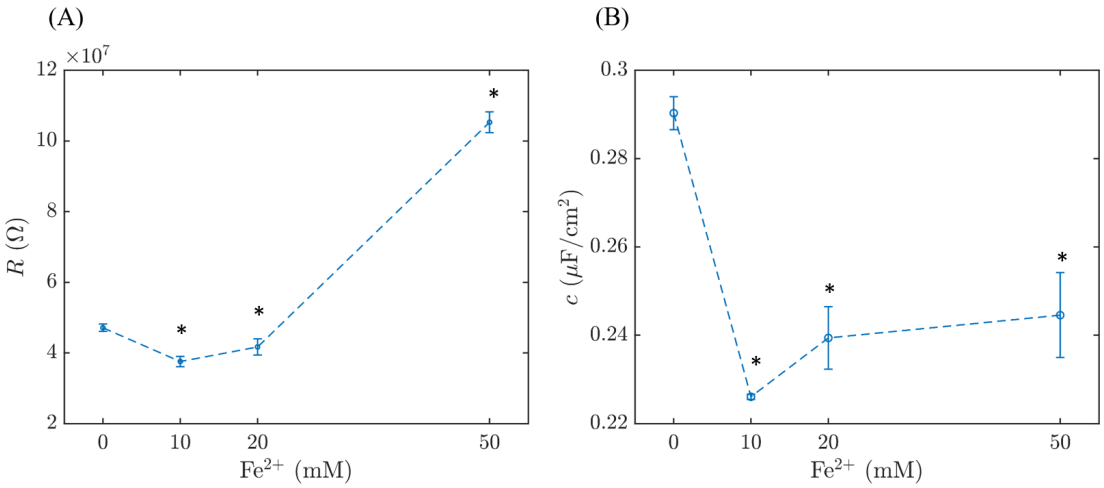
napetost in življenjsko dobo ravninskih lipidnih dvoslojev, dobljeni rezultati pa so prikazani na sliki III.2 (A) oziroma (B). Pri dodatku 50 mM koncentracije aluminijevih ionov se je napetost, potrebna za porušitev dvosloja, povečevala, življenjska doba dvosloja pa je bila daljša, zaradi česar lahko domnevamo, da prisotnost aluminija povečuje debelino dvoslojev.



Slika III.2: Porušitvena napetost in življenska doba ravninskih lipidnih dvoslojev v odvisnosti od koncentracije aluminijevih ionov. (A) U_{br} in (B) t_{br} ravninskega lipidnega dvosloja. Podatki so prikazani kot povprečna vrednost ± standardna deviacija (n = 10 meritev). (*) predstavljajo signifikantno razliko ($p \leq 0.05$) med 0 mM koncnetracijo kovine in višjimi koncentracijami (10 mM, 20 mM, 50 mM).

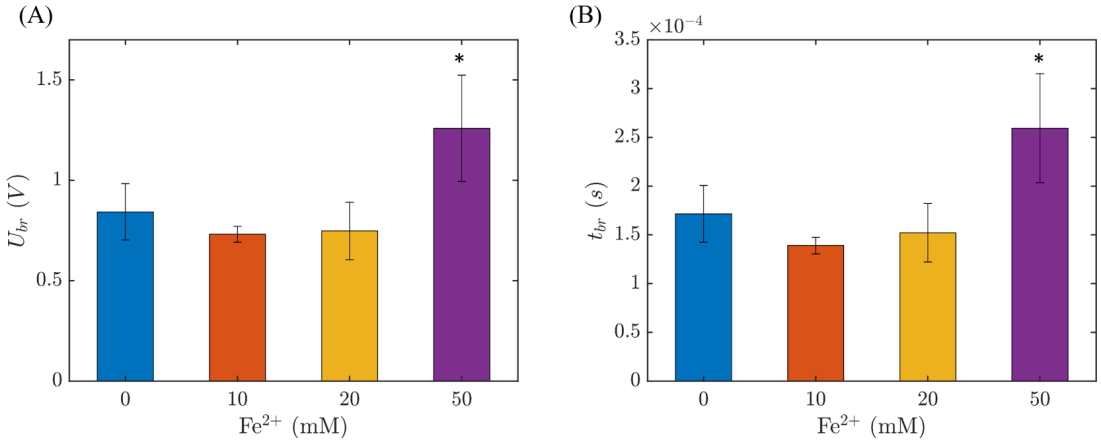
Železovi ioni

Spremembe električnih lastnosti ravninskih lipidnih dvoslojev zaradi dodajanja železovih ionov v elektrolit, na eno stran ravninskega lipidnega dvosloj so predstavljene na sliki III.3, in sicer sta bili izmerjeni upornost (A) in specifična kapacitivnost (B). Opazili smo povečanje upornosti pri višji koncentraciji železa (50 mM), zaradi česar menimo, da prisotnost železovih ionov poveča debelino dvosloja. Po drugi strani pa se je specifična kapacitivnost pri višji koncentraciji kovinskih ionov zmanjšala, kar bi lahko bila posledica debelejšega dvosloja oziroma spremembe v dielektrični konstanti dvosloja. Porušitvena napetost in življenjska doba ravninskih lipidnih dvoslojev sta prikazani na sliki III.4 (A) oziroma (B). Z naraščajočo koncentracijo železovih ionov se je napetost, potrebna za porušitev dvosloja, povečevala, življenjska doba dvosloja pred porušitvijo pa je bila daljša.



Slika III.3: Električne lastnosti ravninskih lipidnih dvoslojev v odvisnosti od koncentracije železovih ionov. (A) Upornost in (B) specifična kapacitivnost ravninskega lipidnega dvosloja. Podatki so prikazani kot povprečna vrednost ± standardna deviacija (n = 10 meritev). (*) predstavljajo signifikantno razliko ($p \leq 0.05$) med 0 mM koncnetracijo kovine in višjimi koncentracijami (10 mM, 20 mM, 50 mM).

Naši rezultati kažejo, da prisotnost kovinskih ionov privede do sprememb v velikosti liposomov in v električnih lastnostih ravninskih lipidnih dvoslojev. Pri visoki koncentraciji kovinskih ionov pride do povečane debeline lipidne membrane in zmanjšane površine lipidne molekule, kar se kaže kot zmanjšanje premera liposomov ter povečanje upornosti in zmanjšanje specifične kapacitivnosti ravninskih lipidnih dvoslojev. Dobljene spremembe lahko vplivajo na strukturo celične membrane pri elektroporaciji in povzročijo dodatne spremembe v debelini lipidnega dvosloja, zato je pomembno, da te spremembe identificiramo ter razumemo njihov vpliv na potek elektroporacije.



Slika III.4: Porušitvena napetost in življenska doba ravninskih lipidnih dvoslojev v odvisnosti od koncentracije železovih ionov. (A) U_{br} in (B) t_{br} ravninskega lipidnega dvosloja. Podatki so prikazani kot povprečna vrednost ± standardna deviacija (n = 10 meritev). (*) predstavljajo signifikantno razliko ($p \leq 0.05$) med 0 mM koncnetracijo kovine in višjimi koncentracijami (10 mM, 20 mM, 50 mM).

III.4 Vpliv lipidne oksidacije na ravninske lipidne dvosloje

V tretjem članku z naslovom *"The role of lipid oxidation on electrical properties of planar lipid bilayers and its importance for understanding electroporation"*, smo raziskovali vpliv oksidacije fosfolipidnih molekul na električne lastnosti ravninskih lipidnih dvoslojev. Znano je, da elektroporacija povzroči oksidacijo lipidnih molekul, preko elektrokemijskih reakcij, ki lahko povzročijo sproščanje kovinskih ionov in nastajanje prostih radikalov ali preko destabilizacije mitohondrijskih membran. Pojavljajo se tudi ugibanja, da lahko z lipidno oksidacijo pojasnimo dlje časa trajajočo prepustno celične membrane tudi po prenehanju uporabe električnih pulzov.

Zaradi kompleksnosti verižne reakcije lipidne oksidacije smo se odločili ta pojav preučevati na preprostem modelu celične membrane - ravninskem lipidnem dvosloju. Tako se izognemo tudi dodani kompleksnosti celice, ki vsebuje antioksidante, ki zavirajo pojav lipidne oksidacije. Cilj raziskave je bil preučevanje kompleksnega pojava oksidacije na preprostem modelu celične membrane z uporabo metode merjenja električnih lastnosti ravninskih lipidnih dvoslojev.

Fosfolipidne molekule z različno stopnjo nenasičenosti smo kemično oksidirali na zraku, z uporabo $KMnO_4$ in pa s Fentonovimi reagenti ($FeCl_2$ in H_2O_2). Oksidacijske produkte smo analizirali z masno spektrometrijo, ter pridobili spekter različnih oksidacijskih produktov za posamezno fosfolipidno molekulo in tip oksidacije. Nadalje so bili iz neoksidiranih ali oksidiranih lipidnih molekul zgrajeni ravninski lipidni dvosloji in izmerjene njihove električne lastnosti. Izmerili smo specifično kapacitivnost in električno upornost, ki je obratno sorazmerna električni prevodnosti in meri sposobnost električnega toka, tj. ionov, da prehajajo skozi dvosloj. Izmerili smo tudi porušitveno napetost in življensko dobo ravninskih lipidnih dvoslojev s sistemom izdelanim v našem laboratoriju.

Pri lipidnih dvoslojih, zgrajenih iz oksidiranih lipidov, smo zaznali povečanje prevodnosti in kapacitivnosti, zaradi česar menimo, da so oksidirani dvosloji bolj prevodni od neoksidiranih. Z naraščajočo oksidacijo lipidov bo jedro dvosloja zaradi kemičnih sprememb postalo bolj polarno in posledično bolj prepustno. Poleg tega se v ravninskem lipidnem dvosloju zaradi prisotnosti oksidiranih vrst in dovajanja električnih pulzov lahko pojavijo prehodne pore, ki prav tako povečajo prevodnost dvosloja. Povečanje kapacitivnosti ravninskega lipidnega dvosloja lahko razložimo z nastankom polarnih območij v prej nepolarnem okolju in prisotnosti kratkoverižnih oksidacijskih produktov, kar povzroči povečanje dielektričnega koeficienta dvosloja in zmanjšanje debeline dvosloja. Vendar so spremembe kapacitivnosti pri lipidnih molekulah z isto stopnjo nenasičenosti in pri uporabi istih oksidacijskih protokolov manj izrazite kot spremembe prevodnosti. Poudariti je treba, da so spremembe strukture dvosloja zaradi prisotnosti oksidiranih vrst kompleksne. Oksidacijsko povzročene spremembe v maščobnih verigah lahko privedejo tudi do modifikacij v območju glav fosfolipidov. Poleg tega lahko pojav dolgoverižnih oksidacijskih produktov poveča debelino dvosloja. Vsi ti učinki lahko povzročijo manjše spremembe kapacitivnosti med neoksidiranimi in oksidiranimi dvosloji.

Za izmerjeno porušitveno napetost in življensko dobo nismo našli statistično signifikantne razlike med neoksidiranimi in oksidiranimi ravninskimi lipidnimi dvosloji. Povsem mogoče je, da uporaba našega sistema za merjenje porušitvene napetosti in življenjske dobe ni dovolj občutljiva metoda za odkrivanje razlik med neoksidiranimi in oksidiranimi lipidnimi dvosloji. Možno pa je tudi, da oksidacija lipidov nima ključne vloge pri stabilnosti ravninskih lipidnih dvoslojev.

V študiji smo pokazali, da oksidacija fosfolipidnih molekul signifikantno vpliva na električne lastnosti ravninski lipidnih dvoslojev. Znano je, da uporaba visokonapetostnih električnih pulzov povzroči oksidacijo lipidnih molekul, kar pomeni, da pri neselektivnem transportu med elektroporacijo in po njej nimajo vloge le hidrofilne pore, temveč tudi oksidacija lipidov. Oksidirane lipidne molekule mora celica encimsko popraviti ali nadomestiti, da se ohranita struktura in delovanje membrane, kar zahteva čas, in to lahko pojasni, zakaj lahko prepustnost celične membrane ostane povečana tudi do nekaj minut po prenehanju uporabe električnih pulzov.

IV Izvirni prispevki k znanosti

Razvili smo numerični model za opis elektrokemijskih reakcij, ki potekajo na stiku med elektrodo in elektrolitom pri elektroporaciji.

Numerični modeli lahko predvidijo izid, učinkovitost in morebitne neželene učinke elektroporacije. V ta namen smo oblikovali numerični model, ki opisuje elektrokemijske reakcije na stiku med elektrodo in elektrolitom, za preučevanje sproščanja kovinskih ionov iz aluminijastih kivet in elektrod iz nerjavnega jekla. Z validiranim modelom lahko tako predvidimo obseg elektrokemijski reakcij in izračunamo koncentracijo sproščenih kovinskih ionov. Dvo-dimenzionalni numerični model smo razvili z uporabo programa COMSOL Multiphysics, ter ga validirali z eksperimentalnimi rezultati pridobljenimi iz študije Kotnika s sodelavci. V modelu so uporabljeni monofazni in bifazni električni pulzi z različnimi amplitudami. Numerični model omogoča optimizacijo elektroporacijskih protokolov, deloma brez potrebe po obsežnem eksperimentalnem delu. V numeričnem modelu je namreč mogoče enostavno implementirati različne parametre pulzov, ki se običajno uporabljajo v elektroporaciji in s tem preučevati vpliv različnih elektroporacijskih protokolov na koncentracijo sproščenih kovinskih ionov.

Dokazali smo, da kovinski ioni, ki so fiziološko prisotni v našem telesu ali pa se sproščajo iz elektrod med elektroporacijo, vplivajo na strukturo lipidnih dvoslojev.

Kovinski ioni, kot na primer fiziološko prisotni kalcij ali pa aluminij in železo, ki se sproščata z elektrod pri elektroporaciji, vplivajo na strukturo fosfolipidne membrane, kar smo preučevali z uporabo membranskih modelov *in vitro*. Vpliv kovinskih ionov na lipidne membrane smo merili s plinsko elektroforezo liposomov in električnimi meritvami ravninskih lipidnih dvoslojev. Ugotovili smo, da

so kovinski ioni, dodani k lipidni membrani, odgovorni za povečanje debeline lipidne membrane, kar povzroči zmanjšanje površine lipidne molekule in posledično zmanjšanje velikosti liposomov. Prav tako smo izmerili signifikantno spremembo v električnih lastnostih ravninskih lipidnih dvoslojev ob dodatku kovinskih ionov, in sicer povečanje upornosti in zmanjšanje kapacitivnosti, kar povezujemo s povečanjem debeline ravninskega lipidnega dvosloja. Kovinski ioni namreč tvorijo elektrostatične vezi z nabitimi fosfolipidnimi molekulami, zaradi česar so te tesneje povezane. Poleg tega prisotnost kovinskih ionov povzroči povečano porušitveno napetost in daljšo življenjsko dobo ravninskega lipidnega dvosloja, kar nakazuje na povečano debelino lipidne membrane. Posledično je preučevanje vpliva kovinskih ionov pomembno z vidika elektroporacije, saj smo pokazali, da sproščeni kovinski ioni vplivajo na strukturne spremembe lipidne membrane. Nenazadnje pa kovinski ioni lahko delujejo tudi kot katalizatorji v oksidacijskih reakcijah lipidov in povzročajo oksidativne poškodbe lipidnih membran.

Dokazali smo, da oksidacija lipidnih molekul povzroči merljive spremembe električnih lastnosti ravninskih lipidnih dvoslojev.

Oksidacija lipidov povzroči kemijske spremembe fosfolipidnih molekul, kar lahko pojasni dlje časa trajajočo prepustnost celične membrane po uporabi visokonapetostnih električnih pulzov, na kar nakazujejo študije opravljene na celicah. Znano je, da lahko električno polje pospeši proces oksidacije s spodbujanjem nastajanja prostih radikalov preko elektrokemijskih reakcij ali preko destabilizacije mitohondrijskih membran. Preučevanje oksidacije lipidov je zahtevna naloga, zato smo se odločili uporabiti ravninske lipidne dvosloje, da bi lahko v nadzorovanem okolju opazovali spremembe v električnih lastnostih lipidnih dvoslojev ob prisotnosti oksidiranih lipidnih molekul. Fosfolipidne molekule smo kemijsko oksidirali in oksidacijske produkte analizirali z masno spektrometrijo. Z merilnim sistemom razvitim v našem laboratoriju smo izmerili razlike v električnih lastnostih neoksidiranih in oksidiranih ravninskih lipidnih dvoslojev. Osredotočili smo se na meritve upornosti, kapacitivnosti, porušitvene napetosti in življenske dobe ravninskega lipidnega dvosloja. Dokazali smo, da lipidne membrane, ki so običajno električni izolatorji, postanejo bolj prevodne zaradi prisotnosti oksidiranih molekul in posledično večje polarnosti hidrofobne notranjosti dvosloja. Poleg tega se poveča tudi kapacitivnost oksidiranih lipidnih dvoslojev zaradi zmanjšanja debeline dvosloja in povečanega dielektričnega koeficienta ob prisotnosti oksidacijskih

funkcionalnih skupin. Pojav lipidne oksidacije tako spremeni kemične lastnosti lipidnega dvosloja, ter s tem poveča prepustnost celične membrane. Z dobljenimi rezultati lahko pojasnimo zakaj, zaradi kemičnih sprememb v obliki lipidne oksidacije, ostane prepustnost celične membrane povečana tudi do nekaj minut po prenehanju uporabe električnih pulzov.

Electrochemical reactions, dissolution of metal ions and lipid oxidation in electroporation

1 Introduction

All living things, animals and plants, depend on the inherent electric properties of their cells to function properly and control their homeostasis [1]. Interestingly, all biological cells also respond to externally applied electric pulses, and there exists a wide range of applications that take advantage of this susceptibility in various fields such as medicine [2]–[4], biotechnology [5], and in the food industry [6], [7]. By applying high-voltage electric pulses, it is possible to change the structure of the cell membrane and manipulate its permeability. This can be achieved by a technique referred to as electroporation, electropermeabilization, or pulsed electric field (PEF) treatment [8].

Electroporation is a method that describes the exposure of biological cells to high-voltage electric pulses leading to structural changes in cell membrane and the formation of hydrophilic pores. This temporarily makes the cell membrane more permeable to molecules that normally lack the mechanism to cross the membrane's hydrophobic barrier [8], [9].

The chemical and physical processes that occur at the cell membrane level during and after electroporation are relatively well understood. However, several underlying processes, such as electrochemical reactions, dissolution of metal ions and lipid oxidation are still less known and are the subject of study in this doctoral dissertation.

1.1 Cell membrane in applied electric field

Every cell is enclosed by a cell membrane, which from an electrical point of view can be described as a thin insulating layer surrounded on both sides by an electrolyte [10]. The main function of the cell membrane is to control ionic gradients and electrolyte homeostasis.

1.1.1 Cell membrane

Cell membrane is a 6–10 nm thick biological structure that surrounds every cell and forms a selective barrier between the intracellular and extracellular environments [1]. Its main function is to keep the cell's components inside while preventing unwanted substances from entering the cell. Consequently, it mediates the selective transport of essential nutrients into the cell and waste products in the opposite direction [11]. The cell membrane provides a selective barrier because of its unique structure, which consists mainly of amphiphilic phospholipid molecules with embedded proteins. Phospholipids have a glycerol backbone to which two long fatty acid chains are attached creating a hydrophobic region, and a phosphate head group occupies the third position creating the hydrophilic region. The phosphate head group can be further modified with various molecular attachments, which changes the properties of the lipid. The most common modification of the phosphate group is the addition of a molecule such as choline, ethanolamine, or serine [12].

Different fatty acid chains result in different phospholipid properties. The fatty acid chains in phospholipids may be saturated or unsaturated, i.e., they may contain one or more double bonds. The length of a fatty acid chain and the number of double bonds it contains have profound effects on the internal energy of the cell membrane [1]. The double bonds in the fatty acid chains are regions with specific properties; they create kinks in the fatty acid structure, increasing the fluidity of the molecules [13]. Proteins embedded in (i.e., integral to) or associated with (i.e., peripheral to) the bilayer structure are also an important part of the cell membrane structure, as they confer specific functions to it, such as selective passage of molecules and ions [11], [131].

Various *in vitro* models have been developed to mimic the structure of the cell membrane and serve as simplified but effective platforms for studying cell processes at the molecular level. The most common cell membrane models are planar lipid bilayers and liposomes. The artificial lipid bilayer is a simple but still a satisfactory model of the cell membrane. Planar lipid bilayers offer an opportunity to measure the properties of phospholipid molecules in a controlled environment [14]. The advantage of planar lipid bilayers is that they are chemically and electrically accessible from both sides. The composition of the planar lipid bilayer can be arbitrarily changed to mimic the composition of a real cell membrane.

Liposomes, on the other hand, are spherical vesicles surrounded by a phospholipid bilayer encapsulating an aqueous lumen [15]. Their similarity to a biological cell membrane is greater than that of a planar lipid bilayer due to their shape and curvature. The analysis of planar lipid bilayers and liposomes is relatively simple, straightforward, and performed under well-controlled experimental conditions. Therefore, they are popular models for studying the properties and functions of cell membranes without the overwhelming complexity found in living cells.

1.1.1.2 Molecular transport across cell membrane

Transport of molecules across the cell membrane can be passive or active. Passive transport requires no energy, and its rate is determined by the physicochemical properties of the membrane and the molecules being transported. Small hydrophobic and uncharged molecules, as well as gasses, are called permeant molecules because they can diffuse unimpeded through lipid membranes along their electrochemical gradient [16].

Charged molecules such as amino acids, nucleosides, carbohydrates, and ions can be induced by their concentration gradient to move across the membrane when assisted by specific transporter proteins or channels, in the process known as 'facilitated diffusion'. On the other hand, transport of molecules and ions across biological membranes against their electrochemical gradient requires the input of energy and is therefore referred to as active transport. Overall, transport

across the lipid membrane is critical for the proper functioning of cells and the maintenance of homeostasis in the body [1], [17].

The selectivity of the lipid membrane for the passage of molecules or ions is very high. Therefore, for therapeutic or biotechnological reasons, the goal is to develop techniques that allow manipulation of transmembrane transport, ideally in a relatively controlled manner.

1.1.2 Applied electric field or electroporation

Electroporation is a technique used for increasing the permeability of the cell membrane through the application of electric pulses. A sufficiently high electric field leads to the formation of hydrophilic pores in the cell membrane and consequently increases the transmembrane transport of impermeable ions and molecules.

The term electroporation was coined by Neumann and colleagues in 1982 [18]. It originally described the process of electrically induced hydrophilic pore formation in the lipid bilayer [19]–[21]. Electroporation is thought to be initiated by the penetration of water molecules into the hydrophobic core domain of the lipid bilayer, which then causes a realignment of the neighbouring lipid molecules, whereby their polar head groups will follow the direction of the penetrating water molecules. The pores formed increase the permeability of the cell membrane, a process that can last from milliseconds to minutes. [22], [23].

1.1.2.1 Transmembrane voltage and electroporation

Living cells in their natural state have a so-called transmembrane voltage, often referred to as a potential. The transmembrane voltage results from the difference between the electric potentials inside and outside the cell, which is due to the difference between the intracellular and extracellular ion concentrations. In the resting state, the cell membrane assumes the so-called ‘resting (trans)membrane potential’, which for the exterior of the cell, typically ranges between -40 mV and -70 mV [8]. The opening or closing of ion channels in the cell membrane can shift

the resting potential to more negative or more positive values, i.e., the membrane becomes hyperpolarized or depolarized [24].

When a cell is placed in an external electric field, i.e., when electric pulses are applied to the cell, an induced transmembrane potential overlaps the existing resting transmembrane potential [25]. Due to this induced transmembrane potential, the structure and function of the cell membrane are locally altered. The membrane undergoes a breakdown via formation of hydrophilic pores, resulting in increased permeability to virtually all molecules. Since the cell membrane is a two-dimensional fluid, it can return to its pre-breakdown state and the cell can survive [26], [27].

In electroporation, three general steps have been defined: (i) no detectable electroporation; (ii) reversible electroporation; and (iii) irreversible electroporation (IRE). The range over which each of these steps occurs is usually characterized by the strength of the applied external electric field (V/cm) and the duration of exposure to that electric field. To achieve electroporation, longer pulse durations require lower electric field strengths. For example, for a pulse of 1 millisecond, no electroporation is detectable from 0 V/cm to 250 V/cm, reversible electroporation occurs between 250 V/cm and 1750 V/cm, and IRE occurs above 1750 V/cm[7], [23]. If pores form in the first range for non-detectable electroporation, they are too small and/or too unstable to be detected. In reversible electroporation, the pores can provide a temporary pathway for transport of molecules across the membrane. However, once the electric pulse ceases, the pores gradually close again, the induced transport stops, and most of these cells survive and remain viable. At IRE, some pores do not close or they reseal too slowly to maintain cell viability. These cells then lose their integrity, releasing their contents, and eventually die [27].

1.1.3 Applications of electroporation in medicine

Various clinical applications take advantage of increased cell membrane permeability through electroporation, which allows impermeant or poorly permeant molecules to enter the cell.

Electroporation can increase the uptake of chemotherapeutic agents by application of electric pulses to tumor tissue, rendering the cell membranes permeable to anticancer drugs. This facilitates a potent local cytotoxic effect. What is more, electroporation routinely faciliates *in vitro* gene transfection in microbiology laboratories. Recent discoveries show that electroporation can also enhance *in vivo* gene transfection. Through the application of much higher electric fields, irreversible electroporation is achieved, which cause excessive damage to the cell membrane, resulting in cell death and is used for tissue ablation. The potential of electroporation to ablate tissues in a non-thermal mode has promoted its use for cancer treatments and in cardiac tissue ablation.

1.1.3.1 Electrochemotherapy

Electrochemotherapy (ECT) is a medical treatment that involves chemotherapy and application of electric pulses to increase drug transport into the cells [2], [9], [28] It is primarily employed for the treatment of localized or superficial tumors, due to ease of application. electric pulses are applied via metal plate or needle electrodes inserted directly into the tumor and surrounding tissue to permeabilize cell membranes, thereby increasing the uptake and efficacy of the drug injected prior to the application of the electric pulses [29], [132]. Bleomycin or cisplatin are usually chosen as chemotherapeutic agents because they do not penetrate the cell membrane well by themselves. A single dose of the chemotherapeutic agent is administered locally to the tumor or systemically, followed by the application of electric pulses that increase the uptake and cytotoxic effect of the drug. With ECT, the targeted cells die in a more controlled manner, and the tumor slowly shrinks with fewer risks of infections [30]–[33]. In addition, electrochemotherapy can nowadays target tumors that are difficult to treat with other treatment modalities [30].

1.1.3.2 Gene electrotransfer

Gene electrotransfer (GET) uses high-voltage electric pulses to introduce DNA or RNA (deoxyribonucleic acid or ribonucleic acid) molecules into cells. It is a multi-step process involving attachment of the DNA or RNA molecule to the cell

membrane, translocation across the cell membrane, migration through the cytoplasm, translocation through the nuclear envelope, and finally gene expression in the nucleus in the case of DNA or in the cytoplasm of the cell in the case of RNA [35], [39]. It is a non-viral method of gene delivery that has various therapeutic applications. In oncology, it is used to induce anti-cancer effects in tumor cells by genetic modification of cancer cells [5], [36]. GET can also be used for DNA or RNA vaccination against infectious diseases or for gene therapy, as it increases the expression of specific proteins to treat cardiovascular or autoimmune diseases [37]. GET does not induce unwanted specific immunity and reduces the risk of integration of therapeutic nucleic acids into the host genome or their dissemination in the environment [38]. GET is considered one of the most promising non-viral methods for gene delivery to cells, as it is safe, effective, flexible, and easy to use [35], [39].

1.1.3.3 Tissue ablation

In irreversible electroporation (IRE), on the other hand, membrane damage is more severe compared to reversible electroporation, and the cell dies. Irreversible electroporation is mainly used as a minimally invasive surgical technique for tissue ablation [4]. With this technique, it is possible to remove tissue in a controlled and precise manner without damaging the surrounding critical structures [4], [40]. There are several minimally invasive methods for tissue ablation, but IRE has certain advantages over them. IRE is not temperature dependent, so the target tissue can be destroyed without overheating the surrounding tissue. IRE is easy to apply, local blood flow does not affect its effectiveness, and no supportive drugs are required. It acts only on the membranes of living cells, leaving the extracellular structures intact. The result is less scarring and faster healing of the treated tissue [41]–[43]. Currently, one of the most promising applications of IRE in medicine is cardiac ablation for atrial fibrillation [45], [133]. This is a catheter-based ablation that has recently been used in human cardiology because of its advantages over current ablation procedures [46]–[49].

1.2 Electroporation effects

Due to the increasing use of electroporation in various fields, it is of great importance to carefully study all possible processes occurring during the application of high-voltage electric pulses either on the cell membrane or the surrounding solution to ensure its safe use.

Electroporation usually involves the application of high-voltage electric pulses via metal electrodes inserted in the tissue, which may cause electrochemical reactions and dissolution of metal ions. The released metal ions can be toxic for the cell, as they can alter the structure of the cell membrane or act as catalysts in lipid oxidation reactions. In addition, exposure to electric pulses can induce the formation of reactive oxygen species, which can also cause oxidative damage to unsaturated lipid molecules.

1.2.1 Electrochemical reactions

Electroporation i.e., the delivery of high-voltage electric pulses, is carried out with two or more electrodes in contact with tissue or an electrolyte [50]. Usually, conductive materials such as metals are used to build electrodes so that electric current flows through them via the movement of electrons. Once we place an electrode in an aqueous electrolyte or tissue, an electrode-electrolyte interface is formed as the movement of electrons in the electrode is transferred to the movement of ions in the electrolyte [51], [52]. This process is known as oxidation-reduction reaction.

As soon as an electrode is immersed in an electrolyte, a double layer is formed, even without an external voltage being applied [52]. When an electric field is applied, a potential difference is created between the two electrodes, followed by charge accumulation across the double layer. When this potential difference is higher than the threshold voltage, also known as the equilibrium potential, electrochemical reactions begin to take place at the electrode-electrolyte interface [50], [53]. This leads to immediate chemical reactions and electron transfer between the electrode and the electrolyte [134].

Anode is oxidized and donates electrons to the electrolyte solution. This leads to the formation of ions at the interface between the electrode and the electrolyte. Cathode is reduced and accepts electrons from the electrolyte solution. This leads to the consumption of ions at the electrode-electrolyte interface [54]. The overall electrochemical reaction at the electrode-electrolyte interface is determined by the balance of the anodic and cathodic reactions. The rate of the electrochemical reaction is affected by a number of factors, including the applied voltage, pulse repetition rate, concentration of ions in the electrolyte solution, temperature, and type of electrode material [52], [53], [55].

The type of electrode material also determines the nature of electrode reactions. If the anode consists of a soluble material, a significant portion of the anodic current is due to dissolution of the metal. Subsequently, the metal ions are released into the electrolyte where they can form reactive species depending on the type of metal and electrolyte used [55], [56]. In addition, local distortions and arching of the electric field can occur due to the metal release, drastically shortening the life of the electrodes [55]. Anodic electrochemical reactions consist of oxygen production and metal dissolution. Therefore, electrochemical reactions also lead to the release of gases and the formation of gas bubbles such as oxygen and hydrogen, which can be dangerous during ablation of tissue, e.g., in the heart, especially in the left atrium. Hydrogen ions are formed at the anode and hydroxide ions are released at the cathode. This creates a more acidic environment at the anode and a more basic environment at the cathode [57], [58].

The most important electrochemical reaction at the anode is the oxygen evolution reaction (Eq. 1.1):

$$2H_2O_{(l)} \Rightarrow O_{2(g)} + 4H^+ + 4e^- \tag{1.1}$$

When using a metal electrode, the dissolution of the material from the anode occurs as well, if M denotes the metal ion and X the valence number of the metal ion, we can write:

$$M_{(s)} \Rightarrow M^{x+} + Xe^- \tag{1.2}$$

The main reaction at the cathode is the hydrogen evolution:

$$2H_2O_{(l)} + 2e^- \Rightarrow H_{2(g)} + 2OH^- \tag{1.3}$$

A homogeneous chemical reaction in the electrolyte is the water proteolysis reaction:

$$H^+ + OH^- \Rightarrow H_2O_{(l)} \tag{1.4}$$

Several measures have been proposed to reduce the magnitude of electrochemical reactions. Using a lower voltage, shorter pulses, and a less conductive medium can minimize the electrochemical processes. In addition, the use of biphasic pulses [59] and different interphase and interpulse delays between biphasic pulses (i.e., pulse repetition rate) affects the concentration of metal ions released [60]. Therefore, a challenge in developing high-voltage electric pulses is to predict and optimize the factors that cause electrochemical reactions.

Numerical models are a good tool for fast and reliable optimization of different processes. For example, numerical models can be used to simulate electrochemical reactions at the electrode-electrolyte interface. With the use of differential equations we can describe metal dissolution from the electrodes during electroporation. Solving these equations yields concentration profiles of dissolved metal ions as a function of used pulse protocol [61]. The first numerical model for the electrochemical treatment of tumors was developed by Nilsson *et al.* [51], where a one-dimensional model of a platinum electrode was studied. Nowadays this model serves as the basis for further development of electroporation related numerical models, since numerical models are a powerful tool for further optimization of electroporation protocols in terms of increased safety and electrode stability.

1.2.2 Dissolution of metal ions

An up-and-coming area of research today is the interaction of phospholipid membranes with divalent and trivalent metal cations. It is known, that the presence of metal ions leads to structural modifications of the cell membrane, with different effects on the cell physiology [62], [63]. Metal ions of interest can be either physiologically present in biological environments, such as calcium, or in relation to electroporation, released from electrodes during the application of high-voltage electric pulses, such as aluminum and iron. Since electroporation leads to structural changes in the cell membrane it is important to examine if the presence of metal ions plays a role in electroporation efficiency.

What is more, calcium in combination with electroporation has recently been proposed for the treatment of tumors. In this novel anti-tumor treatment, large amounts of calcium are internalized by the cell during the electroporation process [64]. Calcium ions are thought to induce tumor necrosis due to adenosine triphosphate (ATP) depletion [65]–[67]. The anti-tumor efficacy of calcium electroporation has been demonstrated *in vitro*, *in vivo* and in clinical studies [31], [65], [68]–[72].

In relation to electroporation, the most commonly used cuvettes are made from aluminium [73]. It has already been shown that aluminium is a very unstable metal and can corrode quickly. In a study by Loomis-Husselbee *et al.*, it was shown that aluminium released from electroporation cuvettes can alter inositol phosphate signalling pathways [74]. Released Al^{3+} can also affect the pH of the electrolyte [73]. Last but not least, Fe^{2+} and Fe^{3+} ions are the main components of stainless steel electrodes commonly used in electroporation-based treatments. Stapulionis *et al.* studied the iron ions released from electrodes during the application of high-voltage electric pulses and concluded that iron ions can alter the structure of nucleic acids and proteins [75].

Lately, the effects of metal ions on the stability and structure of cell membrane have been studied extensively, and numerous studies have been performed to better understand the interactions between metals and phospholipids. It has been observed that the binding of metal ions causes a conformational change in the polar phospholipid headgroups. [76], [77], leading to a reduction in phospholipid area per molecule [78]–[82]. It has also been shown that metal ions, when adsorbed on a membrane, decrease the surface charge density of phospholipids [135] and cause partial dehydration of the bilayer [62], [136]. At high concentrations, metal ions can increase the rigidity of the bilayer and decrease its fluidity [78], [79]. This is because the ions can form electrostatic bonds with the negatively charged head groups, which can cause them to be packed more densely [137]. What is more, several molecular dynamics (MD) studies have shown that sequential binding of metal ions to phospholipids leads to structural changes in the lipid membrane [83], [84], namely a decrease in surface area per lipid. The interaction of positively charged metal ions with negative phospholipid headgroups can also lead to changes in membrane permeability [85], which affects the transport of molecules in and out of the cell [63], [86].

Metal ions can also bind to other structures in the cell membrane, such as membrane proteins, and alter their activity, thereby altering cellular signaling pathways and ion channel function [87], [88]. For instance, calcium ions are known to play an important role in regulating muscle contraction and neurotransmitter release by binding to specific receptors in the cell membrane [89]–[91]. However, excessive accumulation of calcium ions can be toxic and lead to cell death by activating enzymes that damage cell membranes and lead to apoptosis [27], [92]. Furthermore, aluminum ions have also been shown to play a role in metal-induced lipid oxidation, leading to changes in membrane permeability and cell signaling [93]–[98]. Iron ions can also damage the cell membrane due to their interaction with membrane lipids, resulting in iron-induced lipid oxidation [138], [139]

Metal ions are ubiquitous in the environment and can lead to changes in phospholipid structure, catalyze lipid oxidation reactions, and consequently alter cell membrane properties. By studying the effects of metal ions on the cell membrane, we can gain a better understanding of the mechanisms by which metal ions cause membrane deformation and potentially develop ways to mitigate these effects, particularly in electroporation treatments.

1.2.3 Lipid oxidation

Lipid oxidation is a free radical chain reaction that causes the degradation of lipid molecules and is initiated by a radical attack on the fatty acid chain containing a weak C-H bond doubly bonded to another carbon atom [99]. These double bonds in the fatty acid chains of unsaturated phospholipids are susceptible to oxidative damage. Removal of hydrogen from the C-H group leaves an unpaired electron on the carbon atom. This creates a carbon-centered lipid radical that can be stabilized by shifting the double bonds to a conjugated diene or by reacting with molecular oxygen to form the lipid peroxide radical [100], [101]. As this process continues, more radicals are formed, transferring the damage to neighboring molecules as lipid peroxide radicals can abstract hydrogen from a nearby unsaturated lipid molecule, forming a primary oxidation product, hydroperoxide [102]. Oxidation processes can lead to various products with truncated lipid tails, as primary products decay further to produce aldehydes and carboxylic acids as secondary products [140]–[142].

Lipids can be oxidized by enzymatic or non-enzymatic pathways. Lipoxygenases and cyclooxygenases are enzymes that catalyze the oxidation process of lipids, generating lipid peroxyl radicals. Non-enzymatic lipid oxidation by free radicals is initiated by the presence of reactive oxygen species (ROS) and begins with the removal of hydrogen or the addition of oxygen radicals to the unsaturated fatty acid [143]. Sequential reduction of molecular oxygen present in atmospheric air results in the formation of oxygen radicals such as the superoxide radical [103], [104]:

$$O_2 + e^- \Rightarrow O_2^- \cdot \tag{1.5}$$

The superoxide radical can further react to form hydrogen peroxide H_2O_2.

$$O_2^- \cdot + e^- + 2H^+ \Rightarrow H_2O_2 \tag{1.6}$$

Hydrogen peroxide can further dissociate into the hydroxyl radical HO·.

$$H_2O_2 + e^- \Rightarrow OH^- + HO\cdot \tag{1.7}$$

Another important ROS is the singlet oxygen, which is formed by the photolysis of the triplet oxygen.

$$^3O_2 \Rightarrow {}^1O_2 \tag{1.8}$$

Singlet oxygen can react with water vapour in the atmosphere to form hydroxyl radicals. Hydroxyl radicals, being highly reactive, can then combine with another O_2 molecule to form a hydroperoxyl radical HOO·.

Furthermore, metal ions, such as iron cations, which are strong oxidising agents, lead to the formation of hydroxyl and hydroperoxyl radicals. Iron Fe^{2+} reacts with H_2O_2 and is oxidised to Fe^{3+}, forming a hydroxyl radical (HO·). Fe^{3+} can then further react with another molecule of H_2O_2 to form a hydroperoxyl radical (HOO·) [105], [106].

The application of high-voltage electric pulses is a stressful event for the cell, leading to oxidative damage of its membrane. Several studies have been performed to confirm that lipid oxidation occurs during electroporation [107]–[109], which has indeed been shown in bacteria [110], vesicles [111] and cells [108], [109], [112], [113]. The extent of lipid oxidation increases with the number of pulses, pulse duration, and pulse amplitude [108], [109], [114], [115]. By applying electric pulses, ROS can be generated either in the extracellular medium by electrochemical reactions or intracellularly by destabilizing mitochondrial membranes [115]–[117]. In a recent MD study [22], membrane permeability and conductivity were shown to increase due to lipid oxidation. In the presence of oxidized lipids, the thickness of the bilayer decreases while the area per lipid increases [118]–[122]. Because the application of high-voltage electric pulses can lead to oxidation of lipid molecules, membrane conductivity increases, implying that not only hydrophilic pores but also lipid oxidation plays a role in non-selective transport during and after electroporation. Oxidized lipid molecules must be enzymatically repaired or replaced in the cell to maintain membrane structure and function [123], which takes time and may explain why cell membrane permeability can be increased even after the application of electric pulses has ceased.

Detection and measurement of lipid oxidation has proven to be a difficult task because many different pathways can lead to lipid oxidation, each resulting in different products and byproducts. The techniques commonly used for the detection of lipid oxidation are mostly qualitative, as lipid oxidation products are unstable, making their quantification hard [144]. Breton and Mir observed the chemical consequences of electroporation on giant unilamellar vesicles (GUV) using mass spectrometry and absorption methods. They showed that the application of electric pulses causes lipid oxidation and that the degree of oxidation depends on the duration of the pulses [111].

Computer simulations were also performed to improve the understanding of the chemical mechanisms involved in lipid oxidation of phospholipid molecules. Van der Paal *et al.* [145] performed molecular dynamics simulations to illustrate the effects of lipid oxidation on the structural and dynamic properties of the cell membrane. They demonstrated that the order of lipids in the lipid bilayer decreases with the introduction of oxidation products. When all phospholipids were oxidized, pore formation occurred. This allows ROS to enter the cell, which can

further damage intracellular membranes and macromolecules. Secondary oxidation products were studied as well [124] showing that the increase in conductivity due to the formation of aldehydes was even higher, corresponding to the observed conductivity changes in cells exposed to electric fields. In addition, an increase in passive diffusion can be observed, as well as spontaneous pore formation due to an increased concentration of oxidized lipids in the bilayers [122]. In this context, it has been shown that even a small number of oxidation products can cause a dramatic increase in membrane permeability.

The studies show that the level of ROS clearly increases after the application of electric pulses in the area of the membrane under the influence of the electric field [108], [109] leading to lipid oxidation. Therefore, to further optimize the efficiency of electroporation-based technologies and treatments, we need to investigate the relationship between lipid membrane oxidation and its susceptibility to electroporation.

2 Aims of the dissertation

The aim of this doctoral dissertation was to improve electroporation-based technologies and treatments by studying the concomitant processes that occur when high-voltage electric pulses are applied, such as electrochemical reactions, dissolution of metal ions, and lipid oxidation. The following hypotheses were tested:

- A numerical model describing electrochemical reactions taking place at the electrode-electrolyte interface during electric pulse application, can be used to optimize electroporation parameters for increased electrode stability and minimization of unwanted side effects.
- Metal ions, which are physiologically present in our body or are released from the electrodes during electroporation, can affect the structure and stability of lipid bilayers.
- Oxidation of lipid molecules leads to measurable changes in electrical properties of planar lipid bilayers.

The application of high-voltage electric pulses leads to electrochemical reactions at the electrode-electrolyte interface, causing metal dissolution from the electrodes and production of reactive oxygen species. To reduce the extent of the electrochemical reactions, various protocol changes can be made, such as reducing the voltage, shortening the pulse duration, using biphasic pulses, and changing the pulse repetition rate. Nowadays, numerical models present a fast and reliable way to simulate and study electroporation protocols and treatments. Our aim was to develop a numerical model describing metal dissolution during application of high-voltage electric pulses. Concentration of released metal ions calculated

from the simulation can be used to validate the model using experimental data. Finally, with a validated numerical model for description of metal dissolution we can further optimize electroporation parameters and limit undesirable electrochemical processes. What is more, optimization of electroporation protocols via numerical modelling can be achieved without extensive experimental work.

Metal ions, either physiologically present in our body or released from the electrodes during electroporation, can have different effects on the structure and stability of lipid membranes. Calcium ions were investigated since they are used as active molecules in calcium electrochemotherapy, i.e., electroporation associated with high calcium concentration that leads to cell death due to depletion of ATP molecules. Aluminium and iron ions were studied as well since aluminium cuvettes and stainless steel electrodes are commonly used in electroporation research and treatments. It is known that high concentrations of aluminium and iron ions can modify cell physiology, what is more they can act as catalyzers in lipid oxidation reactions. For this reason, we thoroughly investigated whether metal ions affect the structure of lipid membranes using planar lipid bilayers and liposomes as *in vitro* cell membrane models. These artificial bilayers are a simple but satisfactory model of the cell membrane and can be used to study the interactions between phospholipids and metal ions.

Furthermore, we investigated the role of lipid oxidation on the long-lasting permeability of the cell membrane even after the application of electric pulses is ceased. Numerous studies have shown that membrane permeability persists longer than the application of electric pulses, suggesting that not only structural changes in the form of hydrophilic pores but also chemical changes, such as lipid oxidation must occur in the lipid membrane. Oxidation of lipid molecules can be initiated due to the application of high-voltage electric pulses, resulting in electrochemical reactions and the formation of reactive oxygen species. In addition, the oxidation of lipid molecules can also be affected by metal ions, e.g., iron released from the electrodes, as metal ions are known to catalyse the chain reaction of lipid oxidation. The aim of our study was to investigate the role of lipid oxidation on membrane permeability, to that end we developed an experimental protocol for detection of changes in the electrical properties of planar lipid bilayers due to lipid oxidation.

Thus, with the *in silico* numerical model and *in vitro* cell membrane models, we are able to study electrochemical reactions, dissolution of metal ions, and lipid oxidation in electroporation to better understand the processes occurring during the application of high-voltage electric pulses for further optimization of electroporation protocols.

3 Research papers

The work carried out in this thesis is presented in three papers published in international scientific journals. The results of each contribution are presented and discussed in detail in the papers; therefore, the Results section consists of the published papers.

Chapter 3.1 presents the first paper, *"The good and the bad of cell membrane electroporation"*, which gives an overview of electroporation, its applications, and effects on the cell membrane. We first describe the structure of the cell membrane and the transport mechanisms used to maintain cell homeostasis. Different techniques to affect the permeability of the cell membrane are critically presented, with emphasis on electroporation. The effects of the electric field on various cell membrane structures, such as phospholipids, proteins, and the cytoskeleton, are described as well. Finally, the most relevant challenges of this promising research area are discussed, in particular, the contribution of electrochemical reactions as well as the effects of released metal ions, and lipid oxidation on the cell membrane.

Chapter 3.2 presents the paper (submitted on July 17, 2023) *"In silico numerical model of aluminium and iron dissolution during electric pulse application for electroporation"* where we have developed a numerical model to describe the electrochemical reactions and metal dissolution at the electrode-electrolyte interface during the application of high-voltage electric pulses. Developed numerical model allowed us to describe a physical problem using basic transport differential equations, thereby introducing, for the first time, a mathematical framework for optimization of electroporation parameters, such as pulse amplitude and polarity. Aluminium and iron ions were modelled due to a common use of aluminium cuvettes and stainless steel electrodes in electroporation research and treatments.

Monophasic and biphasic electric pulses were employed in the simulation and concentration profiles of dissolved metal ions were obtained as a function of applied voltage. We were able to validate the model using experimental results reported in the work of Kotnik *et al.* [19]. With the validated numerical model, we observed an increase in released metal ions when monophasic electric pulses were used compared to biphasic electric pulses, as well as at higher pulse amplitudes. In the future, the metal dissolution model can be used to optimise electroporation protocols, without the need for extensive experimental work.

Chapter 3.3 presents the paper *"Calcium ion effect on phospholipid bilayers as cell membrane analogues"*, where the effect of divalent cations on phospholipid bilayers as cell membrane models was studied. We used liposomes and planar lipid bilayers, to study the metal ion effects on cell membrane models in a well controlled environment. Calcium was selected as a divalent cation because of its key role in cell membrane signaling. In addition, calcium electroporation is used as a novel treatment in electrochemotherapy. Calcium ions were added to external environment of liposomes and planar lipid bilayers at different concentrations. Changes in liposome diameter were measured via gas-phase electrophoresis, and we observed a decrease in liposome size. The electrical properties of the planar lipid bilayers, namely resistance and capacitance, were also studied. The addition of calcium increased the resistance while the capacitance decreased, leading us to believe that the bilayers become more rigid due to the increasing concentration of calcium ions. Not yet published results are presented in Chapter 4.3., where the effect of aluminium and iron ions on lipid membranes was studied. We demonstrated that the increasing concentration of metal ions leads to membrane rigidification, which could affect the efficiency of electroporation treatments. In addition, released metal ions may act as catalysts in the cascade of chemical reactions leading to lipid oxidation, which will be discussed in the last chapter.

Chapter 3.4 presents the last paper *"The role of lipid oxidation on electrical properties of planar lipid bilayers and its importance for understanding electroporation"* in which the effects of lipid oxidation on the permeability of planar lipid bilayers are described. Lipid molecules with varying degrees of unsaturation were chemically oxidized and the corresponding oxidation products were analyzed by mass spectrometry. Since iron ions can be released from electrodes during electroporation, Fenton reagents (Fe^{2+} and H_2O_2) were used as one of the chemical

oxidants employed in the study. Planar lipid bilayers were constructed from either non-oxidized or oxidized lipid molecules and their electrical properties were measured. We detected an increase in the conductivity and specific capacitance of the oxidized bilayer, which led us to believe that oxidized bilayers are indeed more permeable than their non-oxidized counterparts. Moreover, oxidized bilayers are thinner and their dielectric coefficient increases, which is due to the formation of polar regions in the previously non-polar interior. From the results obtained in oxidation study, we can conclude that the presence of oxidized species increases the electric conductivity of planar lipid bilayers, which can be associated with prolonged membrane permeability after electroporation as observed in experiments on cells.

3.1 Paper 1

Title: **The good and the bad of cell membrane electroporation**

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Povzetek: Elektroporacija je metoda, ki z uporabo visokonapetostnih električnih pulzov povečuje prepustnosti celične membrane. Dandanes se elektroporacija že uporablja na različnih področjih, kot na primer v medicini, biotehnologiji in prehrambni industriji. Elektroporacija povzroči nastanek hidrofilnih por v lipidnem dvosloju celične membrane, kar omogoča transport molekul, ki sicer ne morejo prehajati hidrofobne pregrade, ki jo predstavlja celična membrana. V članku smo opisali osnovne principe elektroporacije ter prednosti in slabosti te metode. Razprava govori o učinkih elektroporacije na ključne komponente bioloških celic, kot so lipidna membrana, membranski proteini in citoskelet, ter o glavnih aplikacijah elektroporacije v medicini, kot so elektrokemoterapija, elektro-transfekcija in ablacija tkiva. Na koncu pa smo izpostavili še najpomembnejše izzive tega perspektivnega raziskovalnega področja.

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Feature article

The Good and the Bad of Cell Membrane Electroporation

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Abstract

Electroporation is used to increase the permeability of the cell membrane through high-voltage electric pulses. Nowadays, it is widely used in different areas, such as medicine, biotechnology, and the food industry. Electroporation induces the formation of hydrophilic pores in the lipid bilayer of cell membranes, to allow the entry or exit of molecules that cannot otherwise cross this hydrophobic barrier. In this article, we critically review the basic principles of electroporation, along with the advantages and drawbacks of this method. We discuss the effects of electroporation on the key components of biological membranes, as well as the main applications of this procedure in medicine, such as electrochemotherapy, gene electrotransfer, and tissue ablation. Finally, we define the most relevant challenges of this promising area of research.

Keywords: Electroporation; cell membrane; electrochemotherapy; gene electrotransfer; tissue ablation; nanotechnology

1. Introduction

Cell membrane electroporation, also known as electropermeabilization,[1] is an effective method for internalization of various molecules into biological cells, with increasing number of applications in oncology,[2,3] gene therapy,[4–6] tissue ablation,[7–9] food technology[10,11] and nanotechnology.[12]

Electroporation depends on the nature of the molecular constituents of biological membranes and their behavior in electric field. The first part of this article thus dissects out the structure of the cell membrane and describes the main transport mechanisms across this barrier. In the second part, the mechanistic principles of electroporation are presented, followed by a description of the influence of an externally applied electric field on specific cell-membrane components, such as lipids and proteins, as well as the cytoskeleton. Finally, the advantages, disadvantages, and remaining challenges of electroporation are critically discussed.

1. 1. Structure of the Cell Plasma Membrane

The plasma membrane is basically a 6–10 nm-thick biological structure that surrounds every living cell, and it provides a selective barrier between the intracellular and extracellular environments.[13] Plasma membrane thickness of some cells can however be much larger than this basic value, for example due to glycocalyx, a highly charged layer of membrane-bound biological macromolecules attached to the membrane (e.g., endothelial and epithelial cells), or the membrane skeleton, a specialized part of the cytoskeleton closely coupled to the plasma membrane. The main function of the plasma membrane is to keep the constituents of the cell inside, while preventing unwanted substance to enter the cell. At the same time, it mediates the selective transport of essential nutrients into the cell, and of waste products in the opposite direction.[14]

The cell membrane provides a selective barrier due to its unique structure, which consists mainly of amphiphilic phospholipid molecules. These form a continuous double layer (the 'phospholipid bilayer') that has a profoundly hydrophobic core. The proteins embedded in or associated with this structure endow it with specific functions, such as the selective passage of molecules and ions. Cell shape is primarily determined by interactions between the cell-membrane components, the cytoskeleton, and the extracellular matrix,[14,15] however factors contributing to the cell shape are much more complex.[16–19]

As well as the major lipid constituents of the plasma membrane, the phospholipids, there are two other lipid species that are very important: sterols and glycolipids.[20] Cholesterol is the main sterol-based lipid molecule in the plasma membrane. It is intercalated between the lipid tails of the adjacent phospholipid molecules in the phospholipid bilayer thus increasing their ordering. In this way, it reduces membrane fluidity.[15,21] Glycolipids (e.g., gangliosides) are very important cell-surface markers that serve as specific determinants for cellular recognition and cell-to-cell communication, and as receptors for different biomolecules. The fatty acid chains in the phospholipids and glycolipids usually contain an even number of carbon atoms, and can be saturated or unsaturated, i.e., they can contain one or more double bonds. The length of a fatty-acid chain and the number of double bonds that it contains have profound effects on the internal energy of the cell membrane; i.e., on its order and fluidity.[13]

1. 2. Molecular Transport Through the Plasma Membrane

The cell plasma membrane is selectively permeable, whereby the passage into the cell of molecules needed for its survival is highly regulated. The transport of molecules through the plasma membrane can be passive or active. Passive transport does not require energy, and its rate is governed by the physicochemical properties of the cell membrane, visco-elastic properties on both sides of the membrane, physicochemical and electrical properties of the media on both sides of the membrane, and the molecules to be transported.[22–24] Small hydrophobic and uncharged molecules, and also gasses, are termed as permeant molecules[25], as these can diffuse through biological membranes freely down their electrochemical gradient.[26] Charged molecules, such as amino acids, nucleosides, carbohydrates, and ions, can be driven by difference in electric potential or their concentration differences to move through the membrane when assisted by specific transporter proteins, or channels, in the process known as 'facilitated diffusion'. On the other hand, the transport of molecules and ions across biological membranes against their electrochemical gradient requires the input of energy, and is therefore referred to as active transport.[13] The build-up of concentration gradients of molecules and ions across biological membranes proceeds exclusively through transmembrane protein systems, such as ion pumps and the ATP-binding cassette (ABC) transporters, which are usually powered by ATP hydrolysis.[27] Large and charged molecules, such as proteins, nucleic acids (e.g., DNA, RNA), and diverse synthetic drugs, cannot cross cell membranes *per se* at all. Numerous therapeutic molecules are of this nature, and therefore to get them into cells, where they function, different techniques have been developed to increase the plasma membrane permeability.

1. 3. Ways to Increase the Permeability of the Plasma Membrane

The main physiological role of the cell plasma membrane is to control and regulate the flux of molecules or ions into and out of the cell. The selectivity of the plasma membrane for the passage of molecules or ions is very high, and therefore for therapeutic or biotechnological reasons, the aim is to create procedures that enable the manipulation of transmembrane transport, ideally in a relatively controlled fashion. Caution is however needed, as treatments to increase the permeability of the plasma membrane can also result in increased molecular efflux, which can then induce cell death. On the other hand, the efflux of molecules from cells can also be exploited under certain conditions in biotechnology, to extract bioproducts.[28]

Several methods to increase the permeability of biological membranes have been described. Table 1 gives the main characteristics and applications of the main biochemical (lipid and polymer particles, microbubbles), biological (viral), and physical (ultrasound, electroporation) methods for plasma-membrane permeabilization.

In this article, the focus is on electroporation, as the alteration of the cell membrane permeability induced by exposure to an externally applied electric field. Due to the membrane exposure to pulsed electric field, pores are formed in the cell membrane and increase its conductance for various hydrophilic molecules, such as peptides, nu-

Table 1. Different methods used to manipulate cell-membrane permeability.

Method	Main characteristics	Applications	References
Sonoporation mediated by microbubbles	Transient perforation of the plasma membrane; noninvasive	Drug and gene delivery	29–31
Lipid or lipid-like vesicle fusion	Oral delivery; protects a loaded drug; release of a drug in a controlled way	Drug delivery	32,33
Virus fusion	Injection; can trigger an immune response	Gene delivery	34
Cytolytic toxins	Bacterial cytotoxic proteins	Virulence-targeted therapies	35
Ultrasound	High intensity focused ultrasound; generation of cavities due to ultrasound oscillation	Drug and gene delivery; tissue ablation	36–38
Electroporation	High voltage electric pulses; formation of hydrophilic pores in the plasma membrane	Drug and gene delivery; tissue ablation	2,7,39

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cleic acids, and drug molecules. Electroporation is used in medicine and biotechnology for the delivery of drugs or genes into cells, for tissue ablation, for extraction of bioproducts from cells, and for microbial inactivation in food preservation.[3,40–42]

2. Principles of Plasma-Membrane Electroporation

Electroporation leads to increased permeability of the cell membrane as a consequence of the application of electric pulses. The term electroporation was coined by Neumann and colleagues in 1982.[43] It originally described the process of electrically induced hydrophilic pore formation in the lipid bilayer (Figure 1).

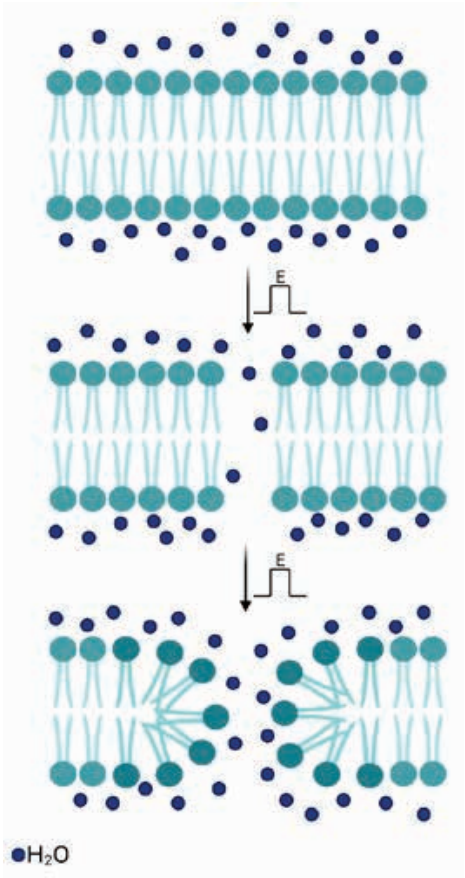

Figure 1. Formation of a hydrophilic pore in the membrane lipid bilayer. Exposure of the membrane to the electric field (E) allows the penetration of water molecules into the lipid bilayer. This induces reorientation of the polar headgroups of the lipids towards the penetrating water molecules, which ultimately leads to the formation of a hydrophilic pore, i.e. electroporation.

From the electrical point of view, the cell membrane can be regarded as a thin insulation sheet that is surrounded on both sides by an electrolyte. The transmembrane voltage is the difference in the electric potentials between outside and inside of the cell, which is due to the difference between the intracellular and extracellular ion concentrations. Specifically, different ions are present on either side of the membrane plane and have a concentration gradient across it, which results in formation of the transmembrane voltage. An electrical double layer is formed when a charged membrane plane is in contact with an electrolyte solution consisting of charged ions and oriented dipoles, resulting in accumulation of oppositely charged ions (counter-ions) and depletion of ions with the same charge (co-ions).[44–46] Membrane itself has a net charge, which is dependent on the lipid composition, due to charged lipid head groups. The hydrophobic region of the membrane has a zero net charge. When the membrane is surrounded by an electrolyte, an interface forms, due to the separation of charged ions on either side of the membrane causing the formation of electrical double layer and consequently the transmembrane voltage.[47–49] In the resting state, the cell membrane acquires the so called 'resting (trans)membrane voltage, which is typically between –40 mV and –70 mV.[1]

Due to the opening or closing of ion channels in the cell membrane, the resting voltage can shift to more negative or more positive values, i.e., the membrane becomes hyperpolarized or depolarized.[13] When a cell is exposed to an external electric field, an induced transmembrane voltage is superimposed on the existing resting transmembrane voltage. The resting transmembrane voltage is always different from zero, and is equal all around the cell since the membrane is an isotropic dielectric medium with constant dielectric permittivity. On the other hand, the induced transmembrane voltage is present only for the duration of the external electric pulse, and it is anisotropic, or dependent on the position on the cell membrane.[50] Due to this induced transmembrane voltage, the structure and function of the cell membrane is locally modified.[1] The membrane undergoes electrical breakdown, which results in increased permeability for virtually all molecules. As the cell membrane behaves as a two-dimensional liquid, it can return to its pre-breakdown state, and thus the cell can survive. In such a case, we talk about reversible electroporation. However, when the exposure of the cell membrane to an electric field is very intensive, the cell will die, even if the membrane manages to reseal. This type of electroporation is referred to as irreversible (IRE).[40,51]

In electroporation, three general levels have been defined: (1) no detectable electroporation; (2) reversible electroporation; and (3) IRE. The range over which each of these occur is characterized by the strength of the external electric field applied (V/cm) and the duration of exposure (seconds) to it. To achieve electroporation, longer pulse durations require lower electric field strengths. For example, for a pulse of 1 millisecond, no detectable elec-

troporation is seen from 0 V/cm to 250 V/cm, reversible electroporation occurs between 250 V/cm and 1750 V/cm, and IRE occurs above 1750 V/cm.[52] In the first range for no detectable electroporation, if pores are formed, they are too small and/or too unstable to be detected. For reversible electroporation, the pores can provide a temporary pathway for molecular transport across the membrane, although once the electric pulse ceases, the membrane gradually reseals, the induced transport stops and most of these cells will survive and remain viable. For IRE, the membrane may not reseal or will reseal too slowly for cell to maintain its viability. These cells then lose their integrity, with the release of their contents, and ultimately die.[51,53]

From a mechanistic point of view, electroporation is best described by the theory of hydrophilic pore formation. The external electric field induces a drop in the electric potential across the lipid bilayer, which leads to the formation of hydrophilic pores in the bilayer.[43] Both, theoretical considerations and molecular dynamics simulations suggest that electroporation is initiated by the penetration of water molecules into the hydrophobic core domain of the lipid bilayer, which then causes a re-orientation of the adjacent lipid molecules, whereby their polar headgroups will follow the direction of the invading water molecules (Figure 1).[54] First, single water molecules penetrate the hydrophobic core of the bilayer due to local defects in the lipid headgroup region. Then, these so-called water fingers expand into the hydrophobic core of the bilayer, and firstly form a hydrophobic pore.[1,54,55] Subsequently, these pores are stabilized by reorientation of the lipid headgroups adjacent to the water molecules, thus stabilizing the pore into its hydrophilic state and allowing more water, as well as other polar molecules and ions, to enter.[40,55,56] After the electric field is eliminated, the pores that are formed and stabilized have lifetimes from milliseconds to minutes (Table 2).[1] As indicated experimentally and theoretically, stability of the pores can be increased by intercalation of different molecules in the lipid bilayer.[57–59]

Furthermore, membrane tension and mechanical stress can also play a role in formation of hydrophilic pores in the lipid bilayer.[60–62] Applied electric field can cause lateral stress to the membrane influencing interfacial tension and pore formation.[55] With a reduction in membrane tension in the lateral plane a decrease in the interaction between the phospholipid molecules occurs and with it an increase in ion permeability.[63]

3. Effects of an Electric Field on Cellular Structures

Cells consist of many different components, and an external electric field can affect these in different ways. Some of these alterations are necessary for the cell membrane electroporation to occur. However, others are not wanted, as they can induce cell death. Thus, attempts are made to reduce the unwanted effects as much as possible. We are focusing here on the effects of an external electric field on three main cellular structures: the lipids that form the plasma membrane; the proteins associated with the plasma membrane; and the cytoskeleton that lies under the plasma membrane and imposes shape to the cell (Figure 2).

3. 1. Effects of an Electric Field on the Lipid Bilayer

Application of electric pulses induces the formation of transient hydrophilic transmembrane pores in lipid bilayers. However, this does not fully describe the sustained increased permeability of the lipid bilayer, which can last long after the electric field has been removed. One possibility to explain such effects is peroxidation of lipids during the electroporation, which changes the chemical structure of the membrane to remain permeable.[64,65]

Lipid peroxidation is a chemical reaction between lipid molecules and oxygen that results in the formation of unstable lipid peroxides. This can occur for lipid structures under stress, such as in the presence of reactive oxygen species (ROS). Lipid peroxidation is a free-radical chain reaction that can generate various products, most of which are harmful for the cell.[66,67] The unsaturated fatty acid chains of the lipid molecules are the main targets of the peroxidation. Oxidized lipid tails become more polar and can also shorten in length. These changes can disrupt the structure of the lipid bilayer, to thus alter its fluidity, and consequently increase the permeability of the cell membrane.[68] The membrane becomes thinner, less densely

Table 2. Steps in the formation of hydrophilic pores during electroporation of a lipid bilayer.[1]

Step	Main characteristics	Duration
Initiation	Membrane electrical conductivity and permeability start to increase	Nanoseconds (conductivity for electric current); microseconds (permeability for ions and molecules)
Expansion	Conductivity and permeability persist and intensify	Until the end of the pulse (up to milliseconds)
Partial recovery	After the external voltage ceases, membrane conductivity and permeability decrease rapidly, but not to zero (i.e. not to the pre-poration state)	Microseconds (conductivity for electric current); milliseconds (permeability for ions and molecules)
Resealing	The membrane recovers to its physiological state of impermeability	Seconds to minutes
Memory	The cell can show alterations to stressors before finally returning to its normal state	Hours

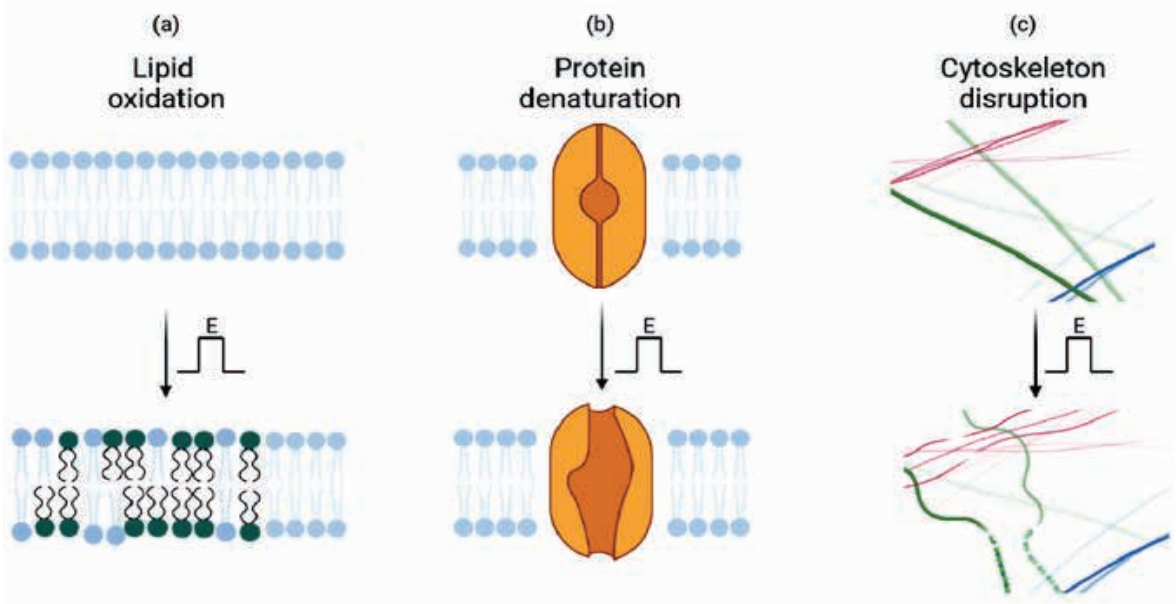

Figure 2. The effects of electric field (E) on the main cellular components. (**a**) The process of electroporation can induce oxidation of the lipids in the cell membrane. (**b**) An external electric field can induce localized heating in membrane proteins, which can lead to their reversible or irreversible denaturation, with a temporary or permanent loss of their function, respectively. (**c**) During electroporation, the cytoskeleton often depolymerizes and detaches from the plasma membrane (Figure adapted from reference 1).

packed, and with lower internal order. Such lipid bilayers are no longer stable and are prone to undergo lateral phase separation. The cumulative result here is that the physiological functions of the cell membrane are altered, which can lead to cell damage, and even to cell death.[69–71]

It has been reported that electroporation induces lipid peroxidation in bacteria, plant cells, and mammalian cells, as well as in liposomes made from polyunsaturated phospholipids.[1] The origins of ROS are diverse. It has been suggested that electric pulses can generate ROS by triggering redox reactions in the water medium, on the membrane surface, and at the electrode–electrolyte interface.[72,73] However, electric pulses initiate creation of ROS also inside the lipid bilayer and in the cell. In addition, there are always some ROS already present in the system.[74,75] All ROS, no matter their origin, can result in peroxidation of lipids during electroporation; however, as ROS are short-lived, only those generated in close proximity to the cell membrane will cause lipid damage. It has been demonstrated that ROS peroxidize only the parts of the membrane that are electropermeabilized. These reactions reach their peak a few seconds after application of electric pulses, and then gradually diminish.[76]

3. 2. Effects of an Electric Field on the Membrane Proteins

Membrane proteins are molecules associated with (i.e., peripheral) or embedded in (i.e., integral) the lipid bilayer of the cell membrane, and they are mainly responsible for all of the specific functions of the biological membranes.

Cell membrane electroporation affects membrane proteins to different extents, where the worst case scenario leads to their inactivation by denaturation, due to the local increase in temperature induced by the electric pulses.[77] For example, it was shown that exposure of cells to electric pulses increased the conductivity of transmembrane Na^+/K^+-ATPases[1] and decreased transmembrane ionic currents through voltage-gated ion channels.[78] Gating potentials of voltage-gated ion channels are in the range of 50 mV. Therefore, when electric pulses are applied, these channels will open and can experience very large ion currents. This can also inflict irreversible damage to the channel proteins as a result of the local Joule heating or chemical modifications.[79] The recovery of damaged membrane proteins is much slower than their opening and closing. While channel closing occurs in microseconds, their opening can take even tens of minutes.[79] The consequences for the cell can therefore be serious, and even fatal.

3. 3. Effects of an Electric Field on the Cytoskeleton

The cytoskeleton is a cytoplasmic protein structure that is attached under the cell plasma membrane. As it is attached to the plasma membrane, it shapes the cell and has important roles in cell adhesion and migration. The

main components of the cytoskeleton are microfilaments, intermediate filaments, and microtubules.[13]

The application of electric pulses can affect the integrity of the cytoskeleton. Exposure of cells to electric pulses can disrupt the network of microfilaments and microtubules. These effects are voltage-dependent and reversible, as the cytoskeleton can fully recover within hours without significant loss of cell viability.[1,80] The disruption of microfilaments was shown to even protect the cell from being killed by external electric pulses.[81] Electroporation of vesicles with actin filaments showed that membrane rigidification occurs, which blocks any large deformation of the vesicles, and prevents the formation of large membrane pores.[82] The mechanism of cytoskeleton disruption includes conformational changes and electromechanical processes, although it remains not entirely clear to date.[83,84] Atomic force microscopy has revealed a decrease in membrane stiffness, leading to the rippling and destabilization of microfilaments. The main reason for the morphological changes observed was shown to be the impaired attachment of the cytoskeleton to the cell membrane. Electroporation often results in cell swelling due to the induced osmotic imbalance, and the resulting swelling force is an important factor in the dislocation of the cytoskeleton from the membrane.[1]

4. Advantages and Disadvantages of Cell Electroporation

Electroporation is an efficient method for the manipulation of cell membrane permeability. It can be applied to all types of cells, and no matter which stage of the cell cycle they are in. Its efficiency depends on the size of the cell, as stronger electric fields are required for induction of pore formation in smaller cells than in larger cells. Moreover, the electrical properties of the tissue also greatly influence the electroporation process, such as its conductivity.[85] As the transport of materials into and out of electroporated cells is not specific, an ionic imbalance can occur, which can be harmful for the cell. Thus, for each specific application of electroporation, the electric pulse parameters need to be appropriately adjusted to minimize unwanted cell damage, or even cell death.[53]

The most widely used applications of electroporation in medicine, electrochemotherapy (ECT), electro-transfer of genes (GET), and irreversible electroporation (IRE) for tissue ablation are illustrated in Figure 3.

4. 1. Electrochemotherapy

Electrochemotherapy (ECT) is a local treatment that includes chemotherapy followed by tumor-directed elec-

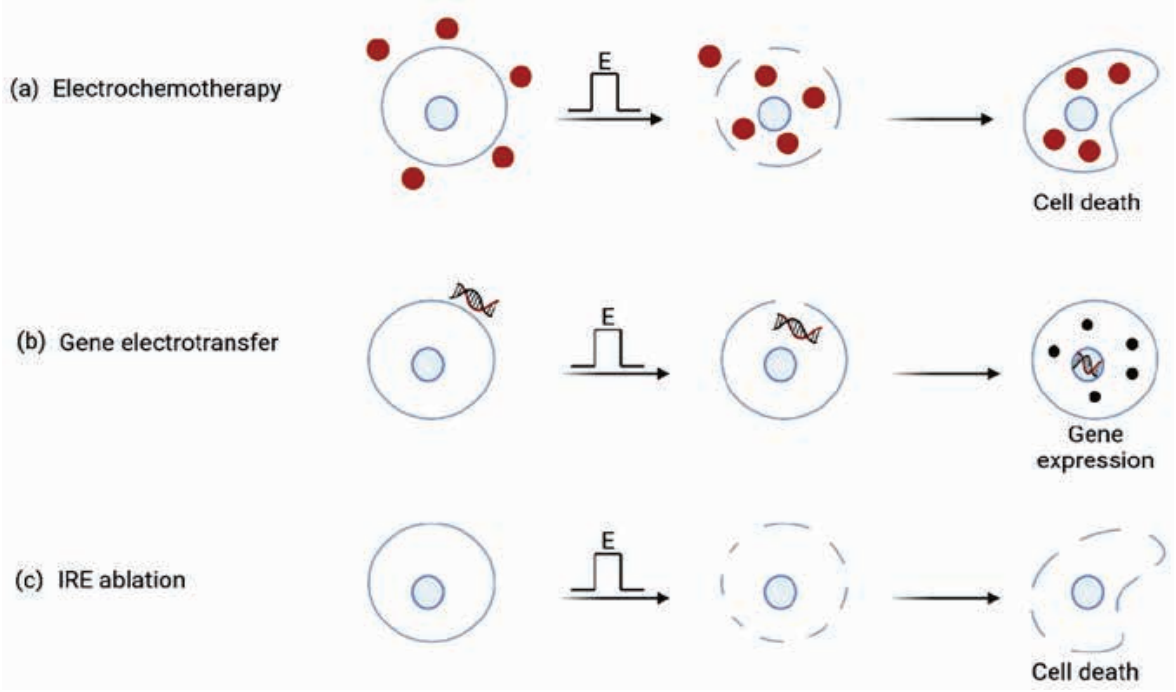

Figure 3. The main applications of electroporation in medicine. (**a**) Electrochemotherapy uses electroporation to increase the uptake of chemotherapeutic drugs into cells, thus boosting their cytotoxic effects. (**b**) Gene electrotransfer uses electroporation to transfer DNA or RNA molecules into cells, to induce expression of the desired proteins. (**c**) Irreversible electroporation (IRE) causes cell death and is used to nonthermally ablate tissue (Figure adapted from reference 86).

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tric pulses, to increase the drug delivery into the malignant cells. Electric pulses are applied through metal plate or needle electrodes, to permeabilize the membranes of the cells, and hence to increase the uptake and effectiveness of the drug that was injected prior to the application of the electric pulses.[87]

Electrochemotherapy is simple and easy to perform. It is also a relatively inexpensive treatment. To perform ECT, we need an electric pulse generator (i.e., an electroporator) and suitable electrodes. The treatment can be performed on practically any part of the body. After the treatment, patients do not require special care, nor post-treatment medication. The main advantage of ECT when compared to other techniques is that it combines chemotherapy and the application of electric pulses. The targeted cells die in a more controlled manner, which results in slower shrinkage of the tumor, without development of massive necrosis that represents a major burden for patients and is accompanied by the risk of complications, such as infections.[88]

As well as these advantages, ECT also has some disadvantages. One of these is the pain that patients can experience during the application of the electric pulses as well as muscle contraction.[85] The factors that can limit the use of ECT include the size of the tumor[89] and difficult accessibility of a tumor by electrodes For safety reasons, ECT is currently contraindicated for patients with cardiac pacemakers and patients on anticoagulant therapy.[3,90]

4. 2. Gene Electrotransfer

Gene electrotransfer (GET) uses high-voltage electric pulses to deliver DNA or RNA molecules into cells. In oncology, this is used to induce anticancer effects in tumor cells.[4,6] GET can also be used for DNA or RNA vaccination, or for gene therapy, as it improves the expression of pertinent proteins.[91] GET can be used to treat cardiovascular, autoimmune, and infectious diseases. Two specific benefits of GET are that it does not induce unwanted specific immunity, and that it lowers the risk of integration of therapeutic nucleic acids into the host genome, or their environmental spread.[6] Nowadays GET is among the most promising nonviral methods for gene delivery to cells, due to its safety, efficacy, flexibility, ease of application, and relatively low cost.[4,92]

The main obstacle against the more widespread use of GET, particularly in human medicine[2], is that when applied *in vivo*, there can be substantial increases in the local temperature and large changes in the pH close to the electrodes, both of which reduce the efficacy of the therapy.[4,92]

4. 3. Irreversible Electroporation Ablation

Electroporation, as IRE, is used as a minimally invasive surgical technique for tissue ablation.[7] With this procedure, it is possible to ablate undesirable tissue in a controlled and precise manner, without damaging the surrounding critical structures.[93]

There are different minimally invasive methods for tissue ablation, but IRE has certain advantages over these. IRE is not temperature based, and therefore the target tissue can be destroyed without overheating of the tissue. IRE is easy to apply, the local blood flow does not influence its efficacy, and it does not require the use of supportive drugs. It affects only the membranes of living cells, while the extracellular structures remain intact. The result is less scarring and faster healing of the treated tissue.[94–96] One of the most promising applications of IRE in medicine is for cardiac ablation after atrial fibrillation.[97,98] This is a catheter-based ablation, and due to its advantages over the contemporary ablation procedures, it has also been recently transferred to human cardiology.[99,100]

On the other hand, IRE can damage the entire tissue that is exposed to the electric pulses if the operating parameters are not correctly selected. Therefore, meticulous treatment planning and setting of the correct electroporation parameters are important, to avoid such damage.[101]

5. The Challenges Ahead

Electroporation of biomembranes has been studied and developed over the past 40 years; nevertheless, there remain some challenges for further improvement of this methodology.

One fundamental challenge that remains to be resolved for biomembrane electroporation is to identify the underlying molecular mechanisms. Only full understanding of the phenomenon at molecular level will allow unraveling its full potential and its reliable control. For example, the contribution of electric pulses to increased cell membrane permeability due to lipid peroxidation and protein modifications are far from being well understood today.

Preclinical and clinical trials have confirmed the great potential for electroporation-based treatments for cancer and gene therapy, as well as in tissue ablation. However, it is evident that there remains room for further technical improvements to increase the precision and specificity of these treatments, one of the possibilities is through the use of nanoparticles for enhanced electroporation efficiency.[102] Furthermore, the reduction or elimination of the serious side effects that sometimes occur is of great importance.[6,85,103] In this context, the processes that occur directly at the electrodes inserted into the tissue during pulse applications need to be better controlled, such as the electrochemical reactions, bubble formation, and local large changes in pH.

Last, but not least, a major problem for the use of electroporation in medicine that awaits resolution is reduction of the intensity and the extent of muscle contraction during the treatments. This would attenuate or even

eliminate the pain that treated patients experience today, without the need for muscle relaxants.[87] In this respect, trials that are investigating high-frequency bipolar electroporation pulses appear to be very promising.[104–107]

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Povzetek

Elektroporacija je metoda, s katero povečamo prepustnost celične membrane z uporabo visokonapetostnih električnih pulzov. Metodo uporabljamo na različnih področjih: v medicini, biotehnologiji in v živilski industriji. Visokonapetostni električni pulzi izzovejo nastanek hidrofilnih por v lipidnem dvosloju celične membrane, ki omogočijo prehajanje molekul, ki sicer membrane ne prehajajo. V članku podajamo pregled osnovnih principov electroporacije ter kritično spregovorimo o prednostih in slabostih te metode. Razpravljamo o učinkih electroporacije na ključne komponente bioloških membran, kot tudi o glavnih uporabah te metode v medicini, o elektrokemoterapiji, vnosu genov v celice in odstranjevanju tkiv. V zaključku predstavimo še najbolj relevantne izzive tega obetavnega področja raziskav.

3.2 Paper 2

Title: ***In silico* numerical model of aluminium and iron dissolution during electric pulse application for electroporation**

Authors: **Katja Balantič**, Peter Kramar and Damijan Miklavčič

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Povzetek: Elektroporacija je tehnika, ki z uporabo visokonapetostnih električnih pulzov poveča prepustnost celične membrane, kar pa lahko privede tudi do elektrokemijskih reakcij na stiku elektroda-elektrolit, zlasti sproščanja kovin z elektrod. Elektrokemijske reakcije lahko privedejo do hitrejše obrabe elektrod, sprememb v pH vrednosti elektrolita in sproščanja plinastih mehurčkov. Vsi te procesi so nezaželeni pri zdravljenju z elektroporacijo. Eno od možnih orodij za optimizacijo protokolov elektroporacije in posledično zmanjšanje elektrokemijskih reakcij, je numerično modeliranje reakcij, ki potekajo v bližini elektrod. Cilj tega dela je bil razviti numerični model za opis elektrokemijskih reakcij, predvsem sproščanja kovin, ki potekajo na stiku elektrode in elektrolita med uporabo visokonapetostnih električnih pulzov. Uporabljen je bil dvodimenzionalni model z Nernst-Planckovimi enačbami za prenos ionov in Butler-Volmerjevimi enačbami za opis elektrodne kinetike. Numerični model je bil validiran z eksperimentalnimi rezultati. Študija je pokazala, da amplituda električnega pulza, tako pri monofaznih kot tudi pri bifaznih pulzih močno vpliva na sproščanje kovinskih ionov iz aluminijastih kivet in elektrod iz nerjavnega jekla. Razviti model se lahko nadalje uporablja za preučevanje optimalnih parametrov električnih pulzov za uporabo elektroporacije v medicini in biotehnologiji.

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In silico numerical model of aluminium and iron dissolution during electric pulse application for electroporation

Abstract

Electroporation is a technique that increases the cell membrane permeability by application of electric pulses and has a widespread use in different fields such as medicine, biotechnology as well as in the food industry. Electric pulses unavoidable cause electrochemical reactions at the electrode-electrolyte interface among others, metal release from the electrodes. Consequently, a challenge in developing electroporation treatments is in predicting and optimizing the factors affecting electrochemical reactions. Efficient tool for optimization of electroporation protocols is by modelling the reactions that take place close to the electrodes. The aim of this work was to develop and validate a numerical model to describe electrochemical reactions, mainly metal dissolution taking place at the electrode-electrolyte interface during the application of electric pulses. The analysis was focused on modelling aluminium cuvette and stainless steel plate electrodes, as they are commonly used in electroporation research. A two dimensional model was used with Nernst-Planck equations for ion transport and Butler-Volmer equations to describe electrode kinetics thus for the first time giving the possibility to implement different electroporation protocols, i.e. pulse waveforms as an input function to the numerical model. The developed model was validated using experimental study by Kotnik *et al*. Numerical model shows that the pulse amplitude and polarity (monophasic vs. biphasic) greatly affects the dissolution of aluminium and iron ions from the electrodes. The presented model requires further improvements but can with its limitations be used to optimize electroporation pulse waveforms in medicine and biology.

Keywords: Electroporation, Electrochemical reaction, Numerical simulation, Mathematical modelling

1. INTRODUCTION

Exposure of biological cells to electric field has proven to be a useful tool for manipulating the cell membrane permeability and has a widespread use in many medical[1–4] and biotechnological applications[5,6] as well as in the food industry.[7] Even short-term exposure to electric field can induce structural changes in biological membranes due to the formation of hydrophilic pores. As a result, the membrane becomes temporarily more permeable for molecules, which usually lack the mechanism to cross the membrane's hydrophobic barrier.[8] This phenomenon is known as electroporation.[9]

In medicine and biology, electroporation is used in numerous applications including electrochemotherapy [10], gene therapy[11], and tissue ablation[12]. When using reversible electroporation the cell remains viable after exposure to electrical pulses and is used primarily for introduction of chemotherapeutic drugs or genes into the cell. On the other hand, in irreversible electroporation (IRE), the membrane can reseal, but the cell dies nevertheless.[2] Irreversible electroporation is mainly used for tissue ablation of abdominal tumours and heart muscle.[3,13–15] It is believed that tissue destruction is caused mainly by necrosis and/or apoptosis.[16] Due to application of electric pulses, electrochemical reactions occur at the electrode-electrolyte interface, potentially contributing to cell death as suggested recently.[17–21] These reactions cause electrolysis, which results in pH changes, generation of radicals, and the release of metal ions from the electrodes, as well as formation of bubbles in the form of gaseous oxygen and hydrogen.[7,22] Due to the increasing use of electroporation in biology and medicine and in particular in intracardiac ablation[13–15], it is important to carefully examine all possible electrochemical processes and thus ensure minimization of unwanted electrode chemical reactions to guarantee its safe and best use.

Electroporation can be electrochemically described as an application of electric pulses passing electric current through electrodes in contact with the tissue.[23] Application of electric pulses unavoidably causes electrochemical reactions at the electrode-electrolyte interface, specifically, metal release from the electrodes, which can have adverse effects on the electroporation process, equipment and biological tissue. Aluminium cuvettes and stainless steel electrodes are commonly used in electroporation based experiments and treatments.[24] However, aluminium is known to be toxic for biological environment.[25–27] Whereas excess iron ions can also lead to modifications of the cell membrane [28,29] and cause lipid oxidation.[30,31] What is more, electrochemical reactions also cause electrode wear due to corrosion, and their surface roughness can increase due to metal dissolution.[32] This, in turn, can lead to distortions of the electric field and arcing, which shortens the lifetime of the electrodes.[33] Electrochemical reactions are

therefore important aspect of electroporation process, but not well described yet.

Limited research is available on electrode's reactions and their consequences. It has been found that the amount of dissolved metal depends on material of the electrode, and pulse parameters such as its shape, amplitude, polarity, and duration as well as the composition and chemical-physical properties of the electrolyte.[34–36] Different strategies are employed to reduce the intensity of electrochemical reactions, for example lowering the current and the conductance of the electrolyte, shortening the pulses, or use of biphasic pulses. Studies also showed that different pulse repetition rate affects the concentration of released metal ions.[22,37]

One possible tool for optimization of electroporation protocols is by modelling the electrochemical processes and reactions that take place close to the electrodes. Numerical models can be used to predict the course of electroporation, electrochemical processes occurring at the electrode-electrolyte interface, pH changes, and any possible side effects.[22,23,33,35,38] With a set of differential equations, we can describe electrochemical reactions and by solving these mathematical models, concentration profiles of dissolved substances, and the potential profile can be simulated as a function of used electroporation protocol.[39–41]

The aim of this work was to develop a numerical model to describe electrochemical processes taking place at the electrode-electrolyte interface, for minimization of unwanted processes such as undesirable metal release from aluminium cuvettes[24,34,42,43] and from stainless steel electrodes[41,44–47] during electroporation. A two dimensional numerical model was developed with Nernst–Planck equations for ion transport and Butler-Volmer equations to describe electrode kinetics. The model was solved with COMSOL Multiphysics (Comsol Inc., Burlington, MA, USA).[48] The results were compared with experimental data from the study of cell suspension contamination carried out by Kotnik *et al.*[34] The emphasis of the study was on the use of two different electroporation protocols, namely monophasic and biphasic electric pulses, which were employed in the model using COMSOL's built in function. The study shows that not only the pulse amplitude but also the polarity of the electric pulses greatly influences the dissolution of metal ions. The model can be further used to study optimal electroporation parameters with the novel possibility to implement the electric pulse waveform as an input function to the numerical model. Specifically, we can use the pulse waveforms measured during experiments and treatments as an input function to the model.

2. THEORY

Electroporation i.e. electric pulse delivery can be represented as an electrochemical cell, where two or more electrodes are immersed in electrolyte.[38,49] Most often metals are used to

build electrodes, consequently electric current flows through them via the movement of electrons. Once we place an electrode in the (aqueous) electrolyte, the movement of electrons in the electrodes is converted to the movement of ions in the electrolyte. Consequently an electrode-electrolyte interface will form, where electrical energy in the form of electrons is transferred to chemical energy in the form of ions.[45,50,51]

Two electrodes with different polarities and processes are distinguished, the positive anode where oxidation or loss of electrons takes place, and the negative cathode where reduction or gain of electrons occurs. If NaCl is used as an aqueous electrolyte then anodic electrochemical reactions consist mainly in production of oxygen, gaseous chlorine and protons, while gaseous hydrogen and hydroxide ions are released at the cathode. This results in acidification (lowering pH) at the anode and alkalinisation (increasing pH) at the cathode.[22,23,35,38] The most important electrochemical reactions at the anode are the oxygen evolution and chlorine evolution reactions:

$$2H_2O \leftrightarrow O_{2(g)} + 4H^+ + 4e^- \quad (1)$$

$$2Cl^- \leftrightarrow Cl_{2(g)} + 2e^- \quad (2)$$

When using a metal electrode, the dissolution of the material from the anode occurs as well:

$$M_{(s)} \leftrightarrow M^{x+} + Xe^- \quad (3)$$

The main reaction at the cathode is the hydrogen evolution:

$$2H_2O + 2e^- \leftrightarrow H_{2(g)} + 2OH^- \quad (4)$$

The only homogeneous chemical reaction in the electrolyte that is accounted for in our model is the water proteolysis reaction:

$$H^+ + OH^- \leftrightarrow H_2O \quad (5)$$

Important phenomenon of electrochemical cells is the so-called double layer.[52] Electric double layer refers to the region of charged particles that forms at the electrode-electrolyte interface even if no external voltage is applied. It consists of two layers: the first layer comprises ions that are strongly attracted to the electrode surface and form a condensed layer, called the Stern layer; the second layer consists of ions that are less strongly attracted to the electrode surface and form a diffuse layer, called the Gouy-Chapman layer.[39] Once the potential drop across the double layer overcomes the threshold voltage of the reaction potential of electrode material, electrochemical reactions start to occur.[41,45]

The electrochemical reactions that occur at each of the two electrodes are also determined by the choice of material. When an electrochemically soluble material, such as aluminium[53], is used, the majority of the anodic current results from the dissolution of the metal. Metal ions are then transported to the surrounding electrolyte, where they may produce harmful effects to cells, depending on the type of metal used.[23,36,42,49,53,54]

The amount of chemical reactions and released metal ions is proportional to the charge in the form of electrons that is transferred across the electrode-electrolyte interface, during the oxidation or reduction i.e. to the amplitude and duration of the electric current. The metal ions and other ionic species released at the anode and cathode are transported to the surrounding electrolyte mainly by diffusion, due to existing concentration gradients, and migration during the electric pulse, due to electric potential gradient.[22,47] Furthermore, the reaction products, specifically the H^+ and OH^- ions produced around the electrodes, are believed to be the main cause of tissue ablation by low level direct current (electrochemical treatment) due to electrolysis and strong changes in pH.[35,51,55,56] If one of the mechanism leading to cell death during electric pulse application is related to the electrochemical reactions at the electrode-electrolyte interface such as electrolysis, metal dissolution and pH changes, then a numerical model describing these processes can be a useful tool to optimize the electroporation protocols. Such a model should be able to calculate concentration profiles of substances dissolved in the electrolyte as well as the potential profile in dependence of different electroporation parameters.[57]

3. MODELLING

A finite element method based software COMSOL Multiphysics 6.0 (Comsol Inc., Burlington, MA, USA) with *Electrochemistry module* was used for all numerical computations. The electrochemical model consists of two electrodes, namely the anode and the cathode surrounded by the electrolyte as in previous studies.[23,35,51,58] The analysis was focused on a model of either aluminium cuvette or stainless steel plate electrodes, as they were used in the experiments we used for model validation.[34] As an approximation, the cell suspension was treated as an aqueous solution of 0.16 M NaCl at pH 7.

The following simplifications were used in the model, namely, convection was neglected. Also it was assumed that the gas bubbles formed on the electrode surface do not influence the overall conductivity of the electrolyte. The solution domain to the problem is the electrolyte phase, where transport equations take place. There are two boundaries; the anode surface and the cathode surface, where reactions take place. All of the kinetic parameters were obtained from the literature and can be found in Table 1. The transport equations and electrode kinetics used in the mathematical model are presented below.

3.1. Geometry

The model consists of two *Rectangle* 2D geometries, the first geometry represents the aluminium cuvette (Figure 1A), and the second geometry represents the stainless steel plate electrodes (Figure 1B). The dimensions used in the model are:

(A) Aluminium cuvette	(B) Stainless steel electrodes
h = 20 mm	h = 20 mm
y = 11.3 mm	y = 9 mm
x = 2 mm between the electrodes	x = 2 mm between the electrodes

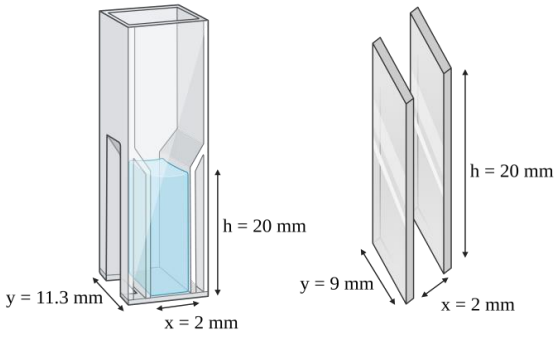

Figure 1*: 3D geometries that were converted to 2D geometries used in the numerical model: (A) aluminium cuvette and (B) stainless steel electrodes with the corresponding dimensions.*

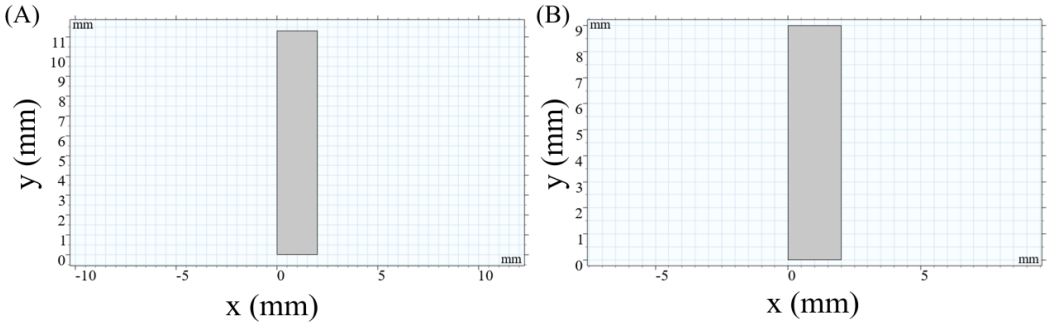

Figure 2: *2D geometries implemented in the numerical model. (A) The 2D rectangle geometry for aluminium cuvette and (B) stainless steel electrodes. the distance between the electrodes (x) was 2 mm in both cases.*

3.2. Governing equations

The main equations used in the COMSOL numerical model are described below. The COMSOL *Electrochemistry* module provides users with ready-made, user-friendly interface for modelling electrochemical processes. The model uses the *Tertiary current distribution, Nernst-Planck* interface to describe the transport and reactions during application of electric pulses.

Using Nernst-Planck equation, the problem was solved for several variables, such as the concentration of individual ions, e.g. Na^+, Cl^-, H^+, OH^- and metal ions according to the material used for the electrode in the model. Therefore, metal release can be described as a mass transport process using Nernst-Planck equation:

$$\frac{\partial c_i}{\partial t} = -\nabla N_i + R_i \quad (6)$$

Where c_i is the concentration and R_i the reaction rate of the ionic species *i*. N_i is the molar flux of the ionic species *i*. Sodium-based electro neutrality was used as a charge conservation

model, e.g. Na^+ concentration was obtained by Equation 7 where z_i is the valence number of the ion:

$$\sum_{i=1}^{5} z_i c_i = 0 \quad (7)$$

Transport of ionic species takes place by diffusion, due to concentration gradients, and electric migration, caused by the presence of electric potential gradients. Therefore, the molar flux, N_i, of the ionic species *i* can be expressed as:

$$N_i = -D_i \nabla c_i - z_i u_i c_i \nabla \phi \quad (8)$$

Where D_i is the diffusion coefficient, u_i is the mobility and z_i is the number of charges carried by the ion i (valence number). ϕ is the electric potential in the electrolyte, therefore, $\nabla\phi$ is electric field in electrolyte. Faradic current density, j in the electrolyte is calculated from the flux of charged species, and is given by Faraday's law:

$$j = F \sum_{i=1}^{5} z_i N_i \quad (9)$$

Where F is the Faraday constant. Therefore, the equation for conservation of electric charge $\nabla j = 0$, is used to solve the electric field:

$$\sum_{i=1}^{5} z_i D_i \nabla^2 c_i + \nabla \sum_{i=1}^{5} z_i u_i c_i \nabla \phi = 0 \quad (10)$$

In addition to the transport equations, kinetics of the electrode reactions are introduced at the anode and cathode boundary. The expressions for molar fluxes at the boundaries are based on the electrode reaction currents based on Equation 8 and Equation 9:

$$N_i = \frac{-v_i j_i}{nF} \quad (11)$$

Where v_i represents the stoichiometric coefficient, n the number of electrons transferred in the electrochemical reaction and j_i the Faradic current density for reaction *i*. The partial current density expression for each reaction was calculated using the *Butler-Volmer* type of kinetics expression with *From kinetics expression* option in COMSOL; therefore, the current density for electrochemical reactions is calculated as:

$$I = I_o(exp^{(\frac{\alpha a F z}{RT})} - exp^{(\frac{\alpha c F z}{RT})}) \quad (12)$$

Where I_o is the exchange current density. The first exponential term in Equation 12 represents the rate of the anodic process, while the second term is that of the cathodic process. αa and αc are the anodic and cathodic transfer coefficients respectively. Transfer coefficients describe the likelihood of electrochemical reactions occurring at the electrodes. These coefficients are used in the framework of the Butler-Volmer equation, describing the relationship between the rate of the electrochemical reaction and the electrode potential. For reactions taking place at the anode (Equation 1, 2, 3) the αa was set to 1 and αc was 0. The same is true for reactions taking palce at the cathode (Equation 4) where αc was 1 and αa was 0. R is the gas constant and T is temperature.

Electrodes were introduced as boundary conditions to the left and right side of the studied geometry. *Electrode surface* node was used to model anode and cathode with specific reactions. For upper and lower boundary condition e.g. insulation, there was no flux $-n * N_i = 0$ and no electric potential $-n * j_i = 0$. In the electrolyte, the *Reactions node* was used to model the water proteolysis reaction.

Electric potential was applied to the *Electrode surface* node as an *External electric potential* condition. Monophasic and biphasic electric pulses were used in the model. For eight monophasic pulses the pulse duration was 1 ms, frequency was 1 Hz. The electric pulse was obtained by subtracting two Heaviside functions using COMSOL's built in function *flc1hs*.[59,60] The pulse rise time was set to 0.001 ms to ease the sharpness of the square pulse for better convergence of the model. The values of electric potential applied were from 40 V to 400 V. For eight biphasic pulses, each pulse was of 1 ms total duration (500 µs of positive polarity followed by 500 µs of negative polarity). For biphasic pulses the electric pulse was obtained in similar way to monophasic one. The positive polarity pulse was obtained subtracting two Heaviside functions using COMSOL's built in function *flc1hs*. The negative polarity pulse was obtained by multiplying the positive one with (-1). The pulse rise time was set to 0.001 ms and the delay time between positive and negative pulse was 0.1 ms.[59,60] The polarities were exchanged in such a way that the first 500 µs of pulse was applied to the anode surface and the opposite polarity for the duration of 500 µs was applied to the cathode surface. In such a way, anodic reactions were exchanged for cathodic reactions mid pulse. The values of electric potential applied were from 40 V to 280 V (peak-to-peak amplitude). The function of electric pulses was implemented in the *Definitions* section, under *Function*, *Analytic*.

Electrochemical reactions were applied to the anode (Equation 1, 2 and 3) and cathode (Equation 4) electrode surface. For each specific reaction the stoichiometric coefficients, equilibrium potential E_{eq} and electrode kinetics were applied. For the electrolyte reactions, the

water proteolysis reaction was considered with the following reaction rates (Equation 13) for H^+ and OH^-.

$$R_H = R_{OH} = k_b \cdot c_{H_2O} - k_f \cdot c_H \cdot c_{OH} \quad (13)$$

Where, k_b and k_f are the rate constants of the water proteolysis reaction in the backward and forward direction.

3.3. Initial and boundary conditions

Inputs to the model are the electric potential, width (y) of the electrodes and the function to model the waveforms of monophasic and biphasic pulses. Thermodynamic and kinetic parameters used in the model are listed in Table 1. Standard electrode potentials are given relative to standard hydrogen electrode (SHE) at 25°C. The partial pressures of oxygen and chlorine, produced at the anode, are assumed to be constant and equal to 1 atm.

Name	**Value**	**Description**	**Name**	**Value**	**Description**
x	2 mm	Geometry x	z_Fe	2	Charge number, Fe
y	9 mm / 11.3 mm	Geometry y	z_Al	3	Charge number, Al
D_Na	0.89^{-5} cm^2/s	Diffusivity, Na	z_OH	-1	Charge number OH
D_H	$6.25e^{-5}$ cm^2/s	Diffusivity, H	i_I0	$1e^{-6}$ A/m^2	Exchange current density, reaction 1
D_Cl	$1.36e^{-5}$ cm^2/s	Diffusivity, Cl	i_II0	10 A/m^2	Exchange current density, reaction 2
D_Fe	$1.98e^{-5}$ cm^2/s	Diffusivity, Fe	i_III0	0.1 A/m^2	Exchange current density, reaction 3 Fe
D_OH	$3.52e^{-5}$ cm^2/s	Diffusivity, OH	i_III0	$1e^{-4}$ A/m^2	Exchange current density, reaction 3 Al
T	298 K	Temperature	i_IIII0	1 A/m^2	Exchange current density, reaction 4
Na0	0.16 mol/liter	Initial concentration, Na	E_eqI	1.23 V	Equilibrium potential, reaction H
H0	$1e^{-7}$ mol/liter	Initial concentration, H	E_eqII	1.36 V	Equilibrium potential, reaction Cl
Cl0	0.16 mol/liter	Initial concentration, Cl	E_eqIII	-0.44 V	Equilibrium potential, reaction Fe
Fe0	$1e^{-4}$ mol/liter	Initial concentration, Fe	E_eqIII	-1.66 V	Equilibrium potential, reaction Al
Al0	$1e^{-4}$ mol/liter	Initial concentration, Al	E_eqIIII	-0.828 V	Equilibrium potential, reaction OH
OH0	$1e^{-7}$ mol/liter	Initial concentration, OH	kf	$1.5e^{11}$ $dm^3/(mol\ s)$	Backward rate constant water hydrolysis
H2O0	55.5 mol/liter	Initial concentration H2O	kb	$2.7e^{-5}$ s^{-1}	Forward rate constant water hydrolysis
z_Na	1	Charge number, Na	z_Cl	-1	Charge number, Cl
z_H	1	Charge number, H	D_Al	$3.65e^{-5}$ cm^2/s	Diffusivity, Al

***Table 1**: Input parameters of the mathematical model, all input parameters were obtained from Nilsson et.al.[23] For Al^{3+} and Fe^{2+} the values were obtained from Electrochemical Thermodynamics and Kinetics (Landolt-Börnstein: Numerical Data and Functional Relationships in Science and Technology).*[61]

3.4. Computational methods

The set of partial differential equations introduced in the previous paragraphs, with their relative initial and boundary conditions were solved using the commercial software package COMSOL Multiphysics 6.0 (Comsol Inc., Burlington, MA, USA).

Events physics was used to solve the model. For monophasic pulses 6 explicit events were added to describe a single pulse, while for biphasic pulses 12 explicit events were added to describe a single pulse. The event was made periodic to include all 8 pulses.

A user defined mesh was implemented with custom element size. Mapped distribution with linear growth rate was used to discretize the electrolyte domain. Sufficiently dense boundary layer elements close to the electrodes was implemented to resolve the sharp concentration gradients and suppress oscillations in the electrolyte domain until no further noticeable changes in the model results were observed. Number of elements was 200 and element ratio was 400.

A time dependent study with current distribution initialization was used to solve the equations. The studied time range was 9 s, with 0.0001 s time-step. A user-controlled tolerance was employed with relative tolerance 0.0001. All the simulations were performed using a PC running on Windows 10 with Intel ® Core ™ i5-8259U CPU @ 2.30 GHz with 16 GB RAM, which allowed for solving the model in approximately 10 min computation time.

3.5. Model validation

The experimental data for metal release used to validate the model was found in the study by Kotnik *et al*.[34] In the experimental study, the contamination of cell growth medium during application of electroporation pulses with varying voltage and pulse polarities was measured. A mass spectrometry analysis of the cell growth medium was carried out to obtain the concentration of released aluminium and iron ions.[34] The concentration values from the experimental study were obtained from the corresponding graphs using “graph reader” application (http://www.graphreader.com/). In order to obtain the concentration (mM) of the released metal ions from the COMSOL numerical model, the surface integration of the electrolyte domain was performed for each specific concentration of studied ions. Surface integration was performed using *Derived values* option in COMSOL. The obtained values for each specific ion concentration were then multiplied by the domain height (h) and divided by the electrolyte volume (50 µl) as used in the study by Kotnik *et al*.[34]

4. RESULTS AND DISCUSSION

The time-dependent numerical model of electrochemical reactions at the electrode-electrolyte interface during the application of electric pulses was developed to observe the release of metal ions, e.g. aluminium and iron from the electrodes, using different pulse waveforms functions as input parameters to the model. By applying the transport equations of ionic species in dilute solutions, and the equations of electrode kinetics, concentration profiles of metal ions and potential distribution were calculated as functions of used pulse waveforms.

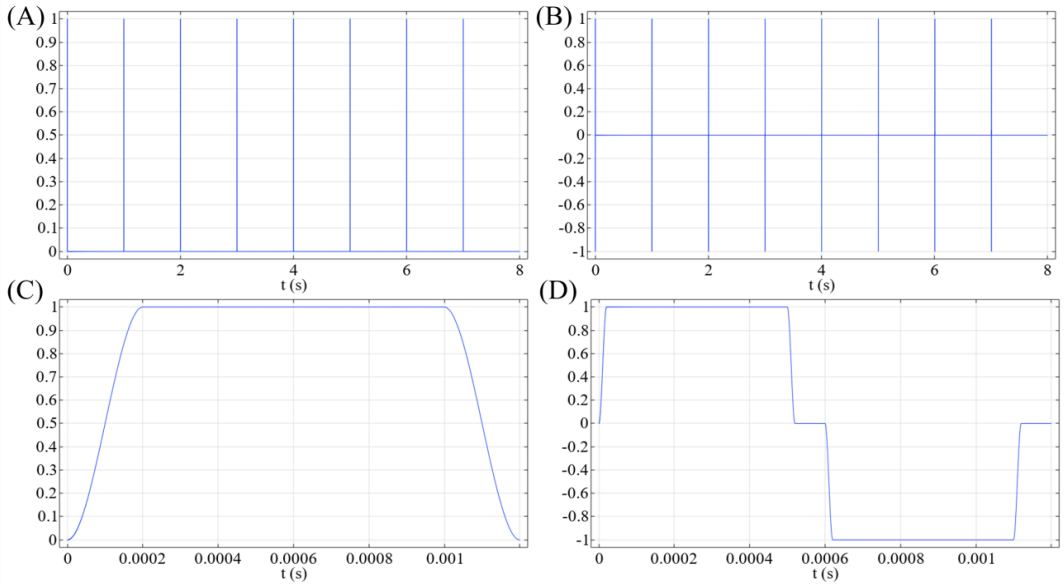

***Figure 3**: Pulse waveform function used in the model. (A) The graph shows the function for monophasic pulses, which was applied to the anode surface and multiplied with the corresponding voltage (40 V – 400 V). (B) The graph shows the function for biphasic pulses, where positive pulse was applied to the anode and negative pulse to the cathode and and multiplied with the corresponding voltage (40 V – 280 V). (C) A single monophasic pulse function. (D) A single biphasic pulse function.*

Pulse waveform function, used in the model was either 8 x 1 ms monophasic pulses with frequency of 1 Hz as shown on Figure 3A or 8 x 1 ms (500 µs positive followed by 500 µs negative) biphasic pulses with frequency of 1 Hz as shown on Figure 3B. Voltage was varied from 40 V to 400 V for monophasic pulses and from 40 V to 280 V for biphasic pulses in the *Electrode surface* node that was set as the boundary condition in the model.

The spatial concentration profiles for Na^+, Cl^-, H^+ and OH^- are presented on Figure 4 and were obtained as a 'cut-line 2D' datasets in COMSOL. For better visualization of the concentration profiles on Figure 4 and Figure 6, the diffusivity

was multiplied by a factor of 10^3 in the *Electrolyte, Diffusion* section of the model. Na^+ ions are depleted close to the positive anode surface, since it repels positive ions. At the cathode, the concentration of Na^+ ions is increasing since the positive ions move towards the negative electrode (Figure 4A). There is no reaction for Na^+ ions in the model; therefore, their concentration remains constant throughout the simulation. Negatively charged chloride ions are moving towards the positive anode and away from negative cathode (Figure 4B). In the simulation Cl^- are reactants in the chlorine evolution reactions, therefore their concentration decreases over time. Positive H^+ is a product of the oxygen evolution reactions at the anode, while negative OH^- is a product of hydrogen evolution reaction at the cathode. H^+ and OH^- concentration at the corresponding electrode surface increases (Figure 4C and D).

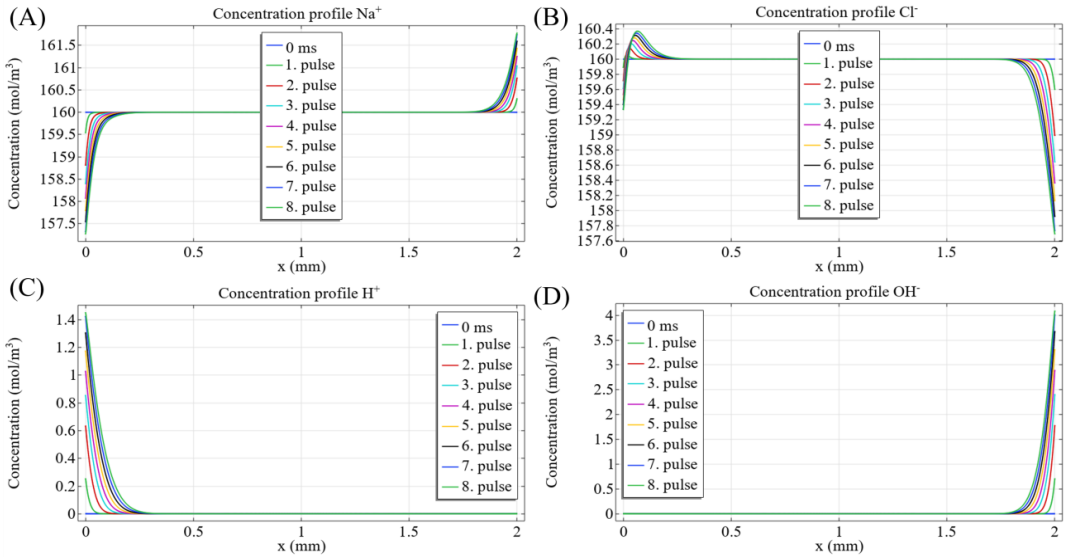

Figure. 4: *Simulated (A) Na^+, (B) Cl^-, (C) H^+ and (D) OH^- concentration (mM) vs. distance (mm) after each monophasic pulse application. Applied voltage = 40 V. Spatial coordinate, x component presents the anode surface at 0 and cathode at 2 mm, respectively. For better visualization of the graphs, the diffusivity was multiplied by a factor of 10^3.*

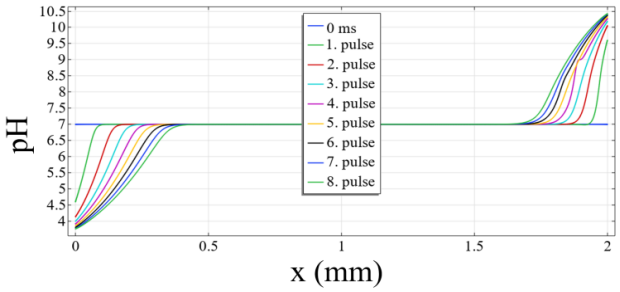

***Figure 5**: Simulation of the pH profile for monophasic pulses. As the H^+ ions are produced at the anode, the pH decreases to a more acidic value. On the other hand, at the cathode, OH^- ions are produced, therefore the pH increases to a more alkaline value. Small oscillations can be seen on the cathode side, which could be due to the pH calculation. Applied voltage = 40 V. For better visualization of the graphs, the diffusivity was multiplied by a factor of 10^3.*

With the known concentration of produced H^+ and OH^- ions, a pH profile of the electrolyte domain can be obtained as a function of distance between the anode and the cathode. The pH profile is shown on Figure 5. Acidic pH develops close to the anode, while alkaline pH develops close to the cathode.

4.1. Dissolution of metal ions

As the voltage applied in the numerical model reaches the threshold value of the reaction potential of electrode material and is higher than the equilibrium potential the dissolution of metal ions starts to occur. With increasing voltage, an increase in concentration of released metal was observed. The highest concentration of released metal ions was observed as expected, for the highest pulse amplitude, i.e., 400 V pulses. Figure 6 presents how the dissolution of (A) aluminium and (B) iron ions takes place after each 40 V pulse application. Figure 6C and D show the release of metal ions when pulses with amplitude of 400 V were applied. The concentration of released aluminium ions is slightly higher than for iron ions because more electrons play a role in the electrochemical reaction of aluminium dissolution and is in line with the experimental results. Since the anode is positively charged, the concentration profiles of positive metal ions moves away from the anode towards the negative cathode due to diffusion in between the pulses and migration in electric field during the pulses.

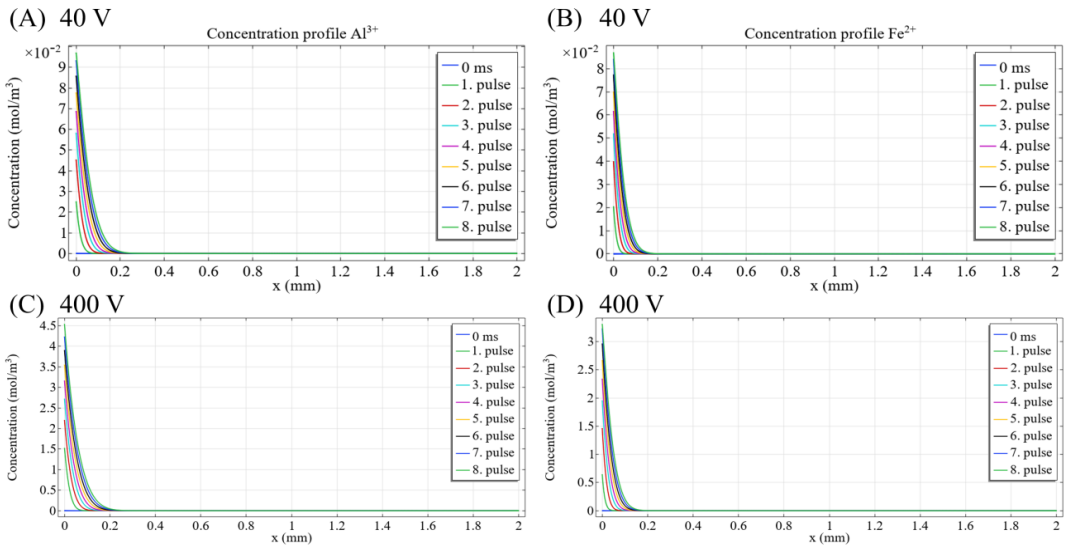

***Figure 6**: Concentration of released metal ions for (A) aluminium and (B) iron as a function of distance from the anode after each monophasic pulse with applied voltage = 40 V.(C) and (D) show the concentration profiles of aluminium and iron ions after application of 400V monophasic pulses. Spatial coordinate, x component presents the anode surface at 0 and cathode at 2 mm, respectively. For better visualization of the graphs, the diffusivity was multiplied by a factor of 10^3.*

In order to compare numerical results with the experimental values the molar concentration (mM) of the released metal ions was calculated. Firstly, a concentration surface integral was calculated over the modelled geometry. The surface integral value was then multiplied by the height (h) of the electrodes to obtain the amount of released metal ions in moles. The obtained values were then divided by the volume (50 µl) used in the experiments by Kotnik *et al.*[34] As the pulse amplitude increases, the energy for the electrochemical reactions is higher leading to a higher concentration of released metal ions.

Biphasic pulses were also employed in the numerical model. As a first approximation, the biphasic pulse function was applied to the anode for the positive polarity (500 µs) and to the cathode for the negative polarity (500 µs). Therefore, the anodic and cathodic chemical reactions were interchanged during the simulation to model biphasic pulse application. Increase in the dissolved metal ions was observed for both aluminium and iron ions, with increasing biphasic pulse amplitude. However, metal release with biphasic pulses compared to monophasic ones was five times lower for aluminium and almost four times lower for iron ions. Interestingly, for higher amplitudes both Al^{3+} and Fe^{2+} dissolution obtained from the model compare well at low amplitudes for both monophasic and biphasic pulses (Figure 7 and 8) but deviates from experimental values for monophasic pulses.

4.2. Model validation

Modelled numerical results were compared to other peer-review numerical studies found in the literature. Specifically, the accuracy of the equations and input parameters for each electrode reaction was verified. The spatial concentration profiles for Na^+, Cl^-, H^+ and OH^- were also compared to the ones found in previous studies, to make sure the physics of our model is indeed correct.[23,51,55]

Model results were then compared with the experimental data published in a study by Kotnik *et al.*[34] Good agreement between experimental and simulated data can be observed for monophasic pulse voltages < 160 V as shown on Figure 7. However, as the voltage of the applied pulses increases our numerical model apparently no longer adequately describes all of the reactions taking place, therefore, the concentration of released metal ions in the model is lower than the one obtained in the experiments. Discrepancies between numerical and experimental results at higher voltages (> 200 V) could be due to additional electrochemical processes taking place at the electrode-electrolyte interface that are not accounted for in the presented numerical model. For example, the roughness of the electrode surface increases with dissolution of metal ions and corrosion, therefore a larger surface is exposed to the electrolyte during the experiments, possibly leading to a higher concentration of released metal ions.[22,32] Furthermore, metal ions can react with other compounds in the electrolyte. Passivation and activation of aluminium electrode occurs depending on the applied potential, the pH and Cl^- concentration. It has been shown that that higher pH and Cl^- concentration increase the anodic reaction rates and consequently concentration of released aluminium.[53,62] Experimental studies suggest that when aluminium electrodes are used, a cathode dissolution of Al^{3+} can also occur due to a chemical attack of OH^- ions.[63]

Finally, the chloride ions present in the solution can also attack the stainless steel electrode and cause continuous dissolution of iron through pitting corrosion.[64] What is more, Fe^{2+} ions undergo iron oxidation to form Fe^{3+} in the presence of chlorine.[65] In the experimental study[34], Fe^{2+} as well as Fe^{3+} ions were detected (due to analytical method used), resulting in higher release iron concentration in the experimental study. Metal ions released from the anode might also increase medium conductivity[54] leading to higher currents in the electrolyte which would increase the concentration of released metals. Furthermore, stainless steel is an iron alloy; therefore, it usually consists of not only iron, but also chromium, copper, nickel, manganese, and other metals, which are not accounted for in our model and could lead to additional electrochemical reactions.

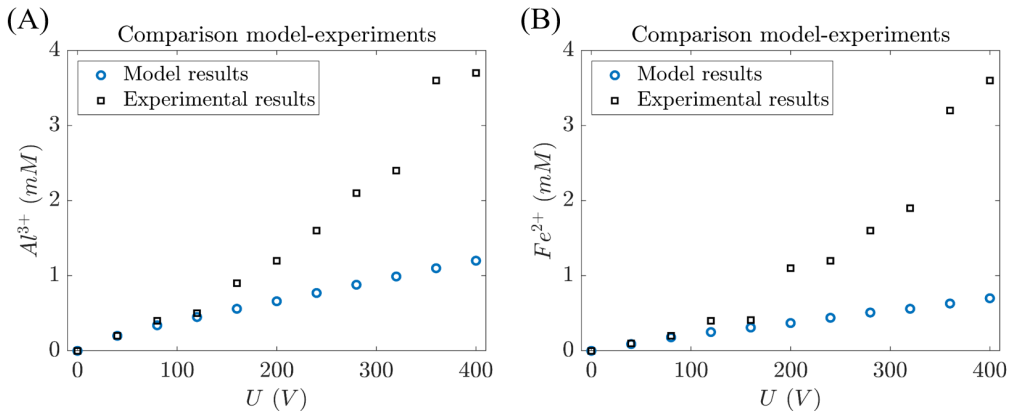

***Figure 7**: Comparison of the simulated metal release data for monophasic pulses with the experimental data from Kotnik et al.* [34] *for (A) aluminium and (B) iron ions.*

Furthermore, apart from water proteolysis reaction, electrolyte reactions are not modelled in our simulation and could explain the differences between the experimental and modelled values. In addition, pH changes have an effect on higher release of metal ions, which is not accounted for in our model. What is more, in the experiments, the cell growth medium was studied, which could result in additional electrochemical reactions, due to complex composition of the medium,

compared to the one used in the model, where NaCl suspension was modelled.

Interestingly, the modelled concentration of dissolved metal fits the experimental results much better, when biphasic pulses are used (Figure 8). The amplitude for biphasic pulses was modelled as peak-to-peak value; therefore, the single electrode-electrolyte voltages were lower, leading to better correlation between numerical and experimental results.

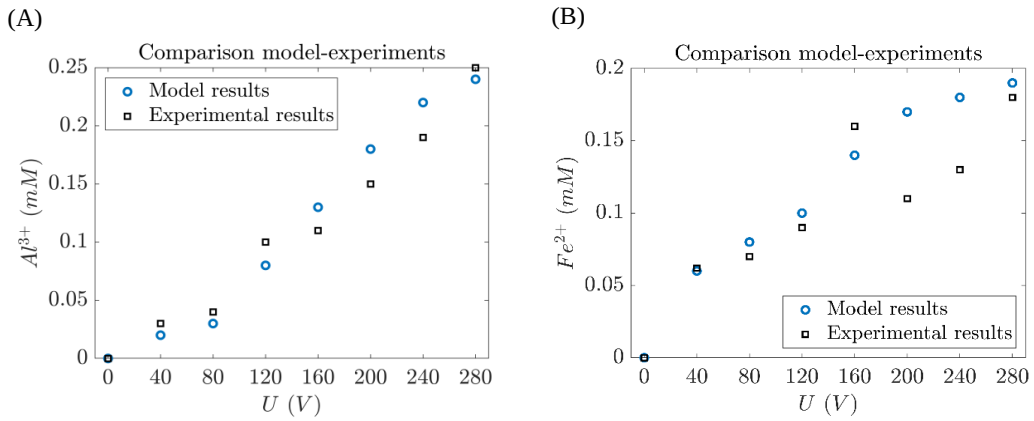

***Figure 8**: Comparison of the simulated metal release data for biphasic pulses with the experimental data from Kotnik et al. [34] for (A) aluminium and (B) iron ions.*

The modelled dissolution of aluminium ions for biphasic pulses (Figure 8A) is in good agreement with the experimental results for pulse amplitudes up to 120 V, followed by a slight overestimation of the modelled results for higher amplitudes. Similarly, the agreement between model and experimental results for iron dissolution with biphasic pulses (Figure 8B) is good for amplitudes up to 160 V, while at higher amplitudes the model shows a higher release of metal ions as the one obtained in experiments.

Overall, we were able to develop a numerical model for description of electrochemical reactions and metal dissolution from aluminium cuvettes and iron electrodes during application of monophasic and biphasic electric pulse waveform. However, the presented model would need further improvements to sufficiently describe the metal dissolution at higher voltages as well. Additional electrochemical reactions at the electrode-electrolyte interface should be identified and modelled, as well as secondary reactions of released aluminium and iron ions in the electrolyte. Nevertheless, with the given model, fewer experimental studies would be required for optimization of electroporation pulses protocols in order to achieve lower metal dissolution.

5. CONCLUSION

Electrochemical numerical models are important for a better understanding of reactions occurring at the electrode-electrolyte interface during the application of electric pulses. In our study, we developed a numerical model to describe dissolution of aluminium and iron ions at the electrode-electrolyte interface during application of 8 x 1 ms monophasic and biphasic pulses. The monophasic and biphasic electric pulse waveforms were introduced in the model as input parameters, thus providing the possibility to study the metal release in dependence of different pulse protocols with varying interphase and interpulse

delays. We were able to validate the model using experimental result published in the paper by Kotnik *et al.*[34] However, the model also showed a lack of complete description of electrochemical reactions at amplitudes higher than 160 V. The numerical model therefore still needs further development, such as the introduction of additional electrochemical reactions at the electrode-electrolyte interface, secondary reactions in the electrolyte and pH induced modifications. Nevertheless, the developed and validated numerical model can be in principle used for optimization of protocols used in electroporation-based technologies and treatments.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influence the work reported in this paper.

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3.3 Paper 3

Title: **Calcium ion effect on phospholipid bilayers as cell membrane analogues**

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Povzetek: Prisotnost kovinskih ionov lahko vpliva na stabilnost in strukturo fosfolipidnih dvoslojev. Zelo pomembna je interakcija fosfolipidnih molekul z dvovalentnimi kationi, kot so kalcijevi ioni, ki igrajo pomembno vlogo v številnih celičnih procesih. Cilj naše raziskave je bil ugotoviti učinke kalcijevih ionov na fosfolipidne membrane z uporabo dveh analogov celične membrane, liposomov in ravninskih lipidnih dvoslojev, ter prvič s kombinacijo dveh instrumentalnih metod: elektroforeze (instrumentacija nES GEMMA) in električnih meritev (kapacitivnosti in upornost). Liposomi in ravninskih lipidni dvosloji so bili pripravljeni iz fosfatidilholina, holesterola in fosfatidiletanolamina. Liposome smo pripravili iz posušenih lipidnih filmov s hidratacijo, medtem ko smo ravninske lipidne dvosloje oblikovali z uporabo metode Mueller-Rudin. Kalcijevi ioni v različnih koncnetracijah so bili dodani na zunanjo oziroma na eno stran lipidne membrane. Pri obeh proučevanih analogih celične membrane smo opazili spremembe v lastnostih fosfolipidnega dvosloja zaradi prisotnosti kalcija. Opazili smo zmanjšanje premera liposomov, kar je lahko povezano s tesnejšim slojem fosfolipidov ali lokalnimi poškodbami membrane. Podobno smo opazili povišanje upornosti in znižanje kapacitivnosti ravninskega lipidnega dvosloja v prisotnosti kalcijevih ionov, kar je lahko posledica povečane togosti in tesnejše zgradbe lipidnih molekul v dvosloju.

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Calcium ion effect on phospholipid bilayers as cell membrane analogues

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ABSTRACT

Ion attachment can modify stability and structure of phospholipid bilayers. Of particular importance is the interaction of phospholipids with divalent cations, such as calcium ions playing an important role in numerous cellular processes.

The aim of our study was to determine effects of calcium ions on phospholipid membranes employing two cell membrane analogues, liposomes and planar lipid bilayers, and for the first time the combination of two instrumental setups: gas-phase electrophoresis (nES GEMMA instrumentation) and electrical (capacitance and resistance) measurements. Liposomes and planar lipid bilayers consisted of phosphatidylcholine, cholesterol and phosphatidylethanolamine. Liposomes were prepared from dried lipid films via hydration while planar lipid bilayers were formed using a Mueller-Rudin method. Calcium ions were added to membranes from higher concentrated stock solutions.

Changes in phospholipid bilayer properties due to calcium presence were observed for both studied cell membrane analogues. Changes in liposome size were observed, which might either be related to tighter packing of phospholipids in the bilayer or local distortions of the membrane. Likewise, a measurable change in planar lipid bilayer resistance and capacitance was observed in the presence of calcium ions, which can be due to an increased rigidity and tighter packing of the lipid molecules in the bilayer.

1. Introduction

Effects of metal cations on biological membranes are of considerable interest since ion attachment can alter the stability and structure of phospholipid bilayers [1,2]. One particular important biophysical question is the interaction of phospholipid membranes with divalent metal cations, such as calcium. Calcium is an important and ubiquitous second messenger involved in the regulation of a variety of different cellular processes. In addition, it plays a crucial role in the maintenance of cell homeostasis [3]. Therefore, the concentration of calcium ions in the intracellular and extracellular environment is highly regulated [4,5]. Changes in calcium concentration can lead to various events such as cell fusion, remodeling of the cell membrane and increased leakage of cellular components [6–8]. Excessive influx and uptake of calcium in cellular storages, signifies cell stress and can lead to a cellular overload, which consequently causes cell death [5,9].

Calcium has recently also been used for medical treatments, such as calcium electroporation. Electroporation is a process of electrically induced increase in permeability of the cell membrane for molecules which otherwise cannot diffuse across the membrane barrier [10]. Calcium electroporation is a novel anti-tumor treatment where large quantities of calcium are internalized by the cell during an electroporation process [11]. Calcium electroporation has been shown to induce tumor necrosis associated with ATP depletion [12–15]. What is more, calcium electroporation also considerably inhibits cell migration capabilities [14]. The anti-tumor effectiveness of calcium electroporation has been demonstrated *in vivo* [12,16,17], *in vitro* [17–19] and in clinical trials [20–23].

Effects of calcium ions on cell membrane stability, structure and cellular processes have been investigated extensively and a large number of different studies have been conducted to better understand the interactions between calcium and phospholipids forming the cell membrane: (i) It was observed that binding of calcium ions causes a conformational change of the phospholipid polar headgroups [24,25] leading to a reduction in phospholipid area per molecule [26,27]. (ii) It was also demonstrated that calcium ions, when adsorbed onto a membrane, reduce the surface charge density of the phospholipids [28] and cause partial dehydration of the bilayer [1,29]. (iii) Significant increase in membrane thickness, and membrane rigidity was observed as well [30,31]. (iv) What is more, several molecular dynamic (MD) studies show a sequential

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binding of calcium ions to the phospholipids leading to structural changes of the lipid membrane [32,33], namely likewise a decrease in area per lipid.

Hoeljolt and colleagues demonstrated that the addition of calcium ions could also influence the transition temperature of the lipids, namely increasing it [34]. Calcium ion effects on phospholipids has been studied on various simplified cell membrane models, such as liposomes and planar lipid bilayers in the past [1,2,35–39]. Liposomes are spherical vesicles surrounded by a phospholipid double layer encapsulating an aqueous lumen. Their resemblance to a biological cell membrane exceeds the planar lipid bilayer, which is considered as a small fraction of a total cell membrane applied for electrophysiological measurements. Planar lipid bilayers consist of two phospholipid sheets with the hydrophobic tails pointing toward the center of the sheet, shielded from the aqueous environment by their hydrophilic phospholipid headgroups. Since planar lipid bilayers are accessible from both sides, their analysis is relatively simple, straightforward [40] and occurs in a well-controlled environment [41,42].

In our study, liposomes and planar lipid bilayers are used as models of biological membranes to investigate the effects of calcium ions on phospholipid bilayers in more detail by comparing two analytical setups. A change in liposome size due to calcium ion addition was observed and quantified via gas-phase electrophoretic measurements. Gas-phase electrophoresis was first described by Kaufman and colleagues in 1996 [43]. Samples are electrosprayed from a volatile electrolyte solution. Subsequently, drying of droplets and charge equilibration in a bipolar atmosphere occurs. Single-charged, surface-dried, polydisperse aerosol is then size-separated in a high laminar-sheath flow and a tunable electric field. By variation of the electric field strength, monomobile aerosol particles are obtained which are then assessed via particle number-based detection as recommended by the European Commission for nanoparticle characterization (2011/696/EU from October 18th, 2011). Relating the electric field strength necessary for the monomobile aerosol to pass the size separator, and hence ultimately the particle size versa particle count values yields the corresponding GEMMA spectrum. Such a setup is known under different names – nano Electrospray Gas-phase Electrophoretic Mobility Molecular Analyzer (nES GEMMA), nES Differential Mobility Analyzer (nES DMA), LiquiScan ES, Scanning Mobility Particle Sizer (SMPS) or MacroIMS – and has already been used in the past for liposome analysis [44–46] or analysis of lipid-containing structures [47–49]. Subsequently, electrical measurements on planar lipid bilayers were carried out, to determine changes in the bilayer properties induced by calcium ion addition. Even small calcium ion concentration related changes in the phospholipid head-group structure could alter the electrical properties of the phospholipid surface and produce measurable changes of the lipid bilayer's properties. We aimed to gain a better insight on interactions between calcium ions and membrane phospholipids comparing two analytical setups and indeed, we observed an effect of calcium ions on liposomes and planar lipid bilayers as cell membrane analogues in both setups.

2. Materials and methods

2.1. Chemicals

Ammonium acetate (NH_4OAc, $\geq$ 99.99 %), ammonium hydroxide (ACS reagent), n-decane (ReagentPlus, $\geq$ 99.00 %), hexane (ACS reagent), calcium chloride ($CaCl_2$, anhydrous, $\geq$ 96.00 %) were purchased from Sigma Aldrich (Steinheim, Germany). Chloroform (Spectronorm quality) was obtained from VWR BDH Chemicals (Roncello, Italy), methanol (LiChrosolv), potassium chloride (KCl) and 2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid (HEPES) from Merck (Darmstadt, Germany). Nitrogen and CO_2 gas was from Messer (Gumpoldskirchen, Austria). The lipids l-α-phosphatidylcholine,hydrogenated (Soy) (HSPC), 1, 2-dioctadecanoyl-*sn*-*glycero*-3-phosphoethanolamine (18:0 PE, DSPE), cholesterol (Chol) and 1-palmitoyl-2-oleoyl-*glycero*-3-phosphocholine (POPC) were from Avanti Polar Lipids (Alabaster, AL, USA obtained via Instruchemie (Delfzyl, The Netherlands)).

2.2. Buffers and electrolytes

NH_4OAc (40 mM, pH 8.4) filtered through a 0.2 μm pore size syringe filter (surfactant free cellulose acetate membrane from Sartorius, Göttingen, Germany) was used for vesicle preparation and as aqueous electrolyte for nES GEMMA.

KCl (0.1 M) and HEPES (0.01 M) were mixed together in a 1:1 volumetric ratio and applied as an aqueous electrolyte in the measurement chamber for planar lipid bilayer measurements. $CaCl_2$ solutions were prepared at indicated concentrations by dissolving $CaCl_2$ in either NH_4OAc solution for liposome measurements or KCl/HEPES solution for planar lipid bilayer measurements.

2.3. Liposome preparation

Liposomes from HSPC:Chol:DSPE (4:3:3 M ratio) or POPC:Chol:DSPE (4:3:3 M ratio) were prepared from dried thin lipid films via hydration [50]. Lipids were dissolved in methanol:chloroform (1:3 mixture [v:v]) prior to formation of a thin, regular film under a constant stream of nitrogen gas. The film was additionally dried for approximately 2 h in a desiccator following its hydration with 1 mL NH_4OAc solution. This yielded a dispersion of 10 mM total lipid concentration. Vortexing and heating in a water bath at about 65°C lead to detachment of the lipid film from the flask surface. Subsequently, small unilamellar vesicles were prepared via extrusion with 21 passes through two pre-wetted 100 nm pore size, polycarbonate membranes (Avanti Polar Lipids) applied in the same membrane orientation. Finally, liposome stock solutions were stored overnight in brown glass vials at 4°C prior to analysis.

2.4. Liposome nES GEMMA measurements

Gas-phase electrophoresis was carried out on a TSI Inc instrument (Shoreview, MN, USA): A nES aerosol generator (model 3480) equipped with either a ^{210}Po α-particle source or an alternating corona discharge unit [51], a nano differential mobility analyzer (nDMA, model 3080) and a n-butanol-based ultrafine condensation particle counter (CPC, either model 3025A or a similar model, namely 3776C) were applied. The samples were introduced to the nES unit via a 25 μm inner diameter, fused silica capillary with a homemade tip [52], generating a stable Taylor cone. A fresh capillary was employed for each day of measurement in order to exclude cross-contaminations. 4.0 lb per square inch differential (psid, approx. 28 kPa) and 0.1 L per minute (Lpm) CO_2 and 1.0 Lpm compressed, particle-free air were employed for transport of particles from the capillary through the neutralization/charge reduction chamber and to the nDMA unit. The applied air was additionally dried (Donaldson Variodry Membrane Dryer Superplus, Leuven, Belgium) to facilitate drying of nES derived nanodroplets. The covered size range of the nDMA was from 4.85 nm to 180.0 nm by application of a sheath flow of 2.5 Lpm. All of the experiments were carried out at room temperature (~21 °C). Every sample was measured four times, for 180 s each, corresponding to 150 s of voltage adjustment and a 30-second window for the instruments to return to idle state of low voltage again.

Prior to gas-phase electrophoresis, six different $CaCl_2$ concentrations in NH_4OAc solution were added (0 mM, 5 mM, 10 mM,

20 mM, 30 mM and 40 mM) to the liposome solutions during spin filtration employing 10 kDa molecular weight cut-off filters (MWCO, polyethersulfone membrane, VWR, Vienna, Austria) leading to a 1:10 [v:v] dilution of the initial stock. The spin filtration of liposomes was carried out in a centrifuge using 9300 g for approximately 7 min. For liposome samples containing $CaCl_2$, the last spin filtration round was always performed in pure NH_4OAc in order to remove unbound, non-volatile calcium ions.

2.5. Planar lipid bilayer formation

Planar lipid bilayers were formed using a Mueller-Rudin method (Mueller et al. 1962). In short, the corresponding amount of POPC or a mixture of HSPC:Chol:DSPE (4:3:3 M ratio) dissolved in chloroform were dried under a N_2 stream and subsequently dissolved in n-decan to obtain a final 20 mM lipid concentration. All of the experiments were carried out at room temperature (~21 °C). A Delrin measurement chamber from Warner Instruments (Hamden, CT, USA) with 150 μm diameter aperture separating the two cuvettes was used to form planar lipid bilayer. The aperture was pretreated with 20 mM lipids dissolved in hexane. After evaporation of hexane, each compartment was filled with 4 mL of KCl/HEPES electrolyte solution. For planar lipid bilayer formation, a small drop of lipids dissolved in n-decane was applied to the aperture using a glass rod. A stable planar lipid bilayer was formed after approximately 30 min and subsequent the capacitance was measured to be around 0.5 $\mu F/cm^2$ for POPC and 0.4 $\mu F/cm^2$ for mixed (HSPC:Chol:DSPE) bilayers.

2.6. Planar lipid bilayer resistance and capacitance measurements

Electrical measurements on planar lipid bilayers were performed using a LCR meter E4980A from Keysight (Santa Rosa, CA, USA) connected to four Ag/AgCl electrodes (In vivo metric, Healdsburg, CA, USA) immersed in the electrolyte solution in the Delrin measurement chamber (Warner Instruments, USA). The LCR meter was set to measure resistance *Rp* and capacitance *Cp* in parallel. The AC voltage was set to 20 mV and the frequency to 2 kHz. Data points were acquired each quarter of a second to obtain measurements of the resistance and capacitance over time. The LCR meter was connected through an Ethernet connection and controlled with MATLAB R2019a (MathWorks, Natick, MA, USA) Toolbox using SCPI protocol. The measured data, capacitance and resistance of each planar lipid bilayer were also processed with MATLAB R2019a.

Formation of a planar lipid bilayer required a 30 min stabilization period, after which a corresponding volume of 9 M $CaCl_2$ solution in KCl/HEPES was stepwise added to one of the compartments in the Delrin measurement chamber to obtain six different $CaCl_2$ concentrations (0 mM, 5 mM, 10 mM, 20 mM, 30 mM and 40 mM).

2.7. Statistical analysis

Datasets for liposome analysis with each calcium ion concentration (raw data obtained from instrument software, MacroIMS manager v2.0.1.0) were combined via their median to yield a corresponding nES GEMMA spectrum. Size precision of the applied nES GEMMA instrument for liposome analysis was ± 0.1 nm. Each spectrum was cut at 40 nm particle diameter in order to blank the low-sized calcium related peaks from the nES GEMMA spectra. Gaussian curve was fitted to each cut spectrum via Matlab R2019a to obtain diameter values and statistical parameters. For comparison of different concentration spectra, a one-way ANOVA on Ranks was used. All pairwise multiple comparisons were made by Tukey's test. We rejected the null hypothesis of analysis if the p-value of the test was less than 0.05 ($p < 0.05$).

Resistance and capacitance measurements of planar lipid bilayers for each calcium ion concentration were combined via their median to obtain the corresponding dataset. For comparison of planar lipid bilayers datasets a one-way ANOVA on Ranks was used. All pairwise, multiple comparisons were made by Tukey's test. We rejected the null hypothesis of analysis if the p-value of the test was less than 0.05 ($p <<< 0.05$).

3. Results and discussion

The effect of calcium ions on phospholipid membranes was studied using two different cell membrane models, namely globular liposomes and planar lipid bilayers. Calcium ions were added to liposomes and planar lipid bilayers in order to observe phospholipid modifications as a function of calcium ion concentration, employing two different measuring methods, nES GEMMA for liposomes and resistance/capacitance measurements on planar lipid bilayers.

3.1. Liposome analysis

Firstly, we focused on liposomes as *in-vitro* cell membrane analogues. In contrast to planar lipid membranes, vesicles exhibit a strong membrane curvature. In addition, the phospholipid surface of vesicles exceeds the section of a planar lipid membrane. Due to the latter, we expected to observe similar effects upon calcium ion addition to liposomes than when employing planar lipid bilayers, yet at higher calcium ion concentrations.

Liposomes encapsulating NH_4OAc solution were applied and calcium ions were added to the vesicles' surrounding medium for incubation during the spin-filtration process. Subsequently, excess small-diameter particles as well as unbound calcium ions were removed via spin-filtration. Other low-sized calcium related peaks were cut out of the spectrum using Matlab R2019a software.

In the absence of calcium ions, the apex of the liposome peak was determined to be at 39.5 ± 2 nm (n = 4 measurements) for POPC:Chol:DSPE liposomes and 71.2 ± 0.7 nm (n = 12 measurements) for HSPC:Chol:DSPE liposomes by fitting the Gaussian curve to the raw data. When calcium ions were added at different concentrations to liposome samples a slight but significant shift in the liposome particle diameter was observed. In a first attempt, liposomes consisting of POPC:Chol:DSPE were analyzed; however, the increased unspecific lipid aggregation rendered them not completely suited for our analysis. As can be seen on Fig. 1A, the liposome peak is hardly distinguishable therefore; the Gaussian curve does not fit well to the data.

Nevertheless, a change in liposome diameter can be observed as seen on Fig. 1B, namely a decrease in particle diameter for 5 mM and 10 mM $CaCl_2$ and an increase in particle diameter for higher calcium concentrations (20 mM, 30 mM and 40 mM $CaCl_2$). An 22% increase in liposome diameter was measured between 0 mM $CaCl_2$ and 40 mM $CaCl_2$ Fig. 2.

In the next set of experiments, HSPC:Chol:DSPE liposomes were used due to their optimal size distribution and ease of analysis using our analytical setup. The liposome peak is easily distinguishable as seen on Fig. 3A and a decrease in liposome size with increasing calcium concentration was observed (Fig. 3B). A 10% decrease in liposome diameter was measured between 0 mM $CaCl_2$ and 40 mM $CaCl_2$. A decrease in the surface-dry liposome volume due to calcium ion binding is also supported by dynamic light scattering (DLS) data [53]. Shrinkage of liposome particles is likely to be caused by a decrease of area per lipid caused by calcium ions as demonstrated by MD simulations [54].

However, this decrease in liposome size is surprising as such, as simple attachment of non-volatile buffer components to analytes

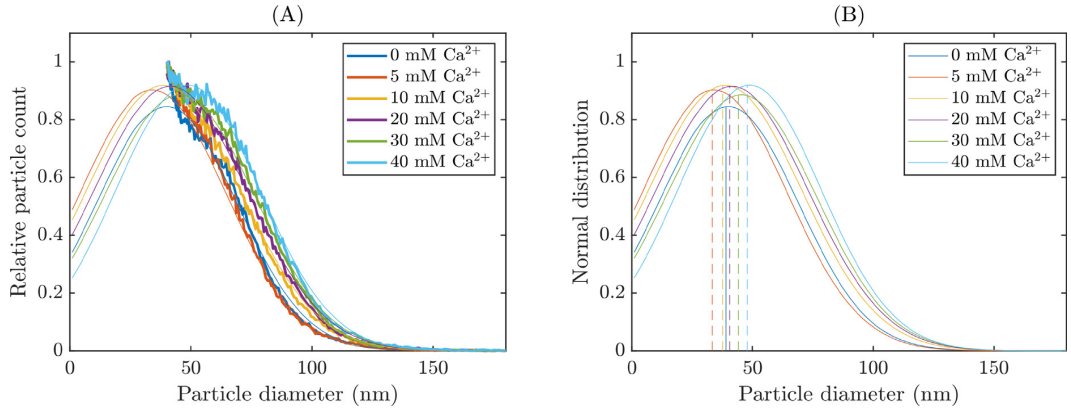

Fig. 1. POPC:Chol:DSPE liposome particle size distribution as assessed via nES GEMMA. (A) Particle size distributions of POPC:Chol:DSPE liposome samples containing 0 mM $CaCl_2$ (N = 4) , 5 mM $CaCl_2$ (N = 4), 10 mM $CaCl_2$ (N = 4), 20 mM $CaCl_2$ (N = 4), 30 mM $CaCl_2$ (N = 4) and 40 mM $CaCl_2$ (N = 4) are depicted. Data is related to the highest peak and is scaled using Matlab rescale function. (B) The fitted Gaussian curve for each specific calcium concentration. Vertical lines represent the liposome diameter for each calcium ion concentration.

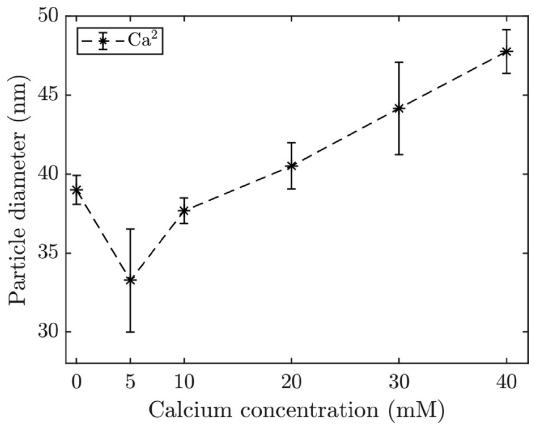

Fig. 2. POPC:Chol:DSPE liposome diameter change as a function of calcium concentration. An increase in POPC:Chol:DSPE liposome size can be observed with addition of $CaCl_2$ to the liposome sample. Raw data was fitted to the Gaussian curve to obtain the diameter points. Represented is the median value of all measurements at specific concentration and standard deviation.

upon nES GEMMA analysis usually results in higher surface-dry particle diameter values [55] as seen for POPC containing liposomes Fig. 4. Hence, a calcium ion dependent decrease in vesicle size might either be related to tighter packing of phospholipid moieties in the bilayer and therefore reduced particle size or local distortions of the lipid bilayer leading to partial loss of encapsulated vesicle components (ammonium acetate, water) upon surface drying during gas-phase electrophoresis. Similar effects (a decrease in vesicle size) was also observed upon addition of a pore-forming peptide (GALA) to liposomes and incubation at acidic pH (unpublished data of a different experimental series).

3.2. Planar lipid bilayer analysis

Subsequently, planar lipid bilayer electrical properties were measured by a LCR meter connected directly to the electrodes in the measurement chamber. As a measure of the influence of calcium ion addition on the lipid bilayer properties, we determined changes in its equivalent parallel capacitance *Cp* and resistance *Rp*. The measured electrical properties can be correlated to the bilayer thickness (d) and permeability using basic equations for resistance (R [Ω]) and capacitance (C[F]) (see equation (1) and (2) below):

$$c = \frac{Cp}{A} = \frac{\varepsilon A}{dA} = \frac{\varepsilon}{d} \tag{1}$$

$$R = \frac{\rho d}{A} \tag{2}$$

A planar lipid bilayer can be represented as a capacitor that is capable of storing charge in an electric field. The bilayer specific capacitance c [μF/cm^2] is calculated as measured capacitance divided by the area of the aperture and is as such used to compare capacitance of different measurements setups. c is directly proportional to its dielectric constant (ε, [F/m]), and is inversely proportional to the thickness of the bilayer (d, [m]). Resistance of the planar lipid bilayer can also be measured from the values of applied voltage and measured current flowing through the formed bilayer. Resistance of the bilayer is directly proportional to the specific electrical resistance of the bilayer (ρ , [Ωm]) and its thickness (d, [m]), and is inversely proportional to the surface area (A, [m^2]) of the bilayer.

For planar lipid bilayer experiments, in a first attempt POPC lipids were chosen due to their simplicity and ease of bilayer formation. Furthermore, due to the absence of a net charge on POPC we were able to focus primarily on specific lipid–ion interactions. However, since POPC is uncharged, the interaction of calcium ions is expected to be relatively weak compared to charged phospholipids. Nevertheless, even small changes in the lipid head group could significantly alter the electrical properties of the bilayer surface. Therefore, measurement of bilayer's electrical properties was applied as a sensitive technique for determination of calcium binding to the lipid structures.

We measured the changes in resistance (Fig. 5A) and capacitance (Fig. 5B) of the POPC planar lipid bilayer in response to calcium ion addition. A measurable increase in bilayer resistance R and a decrease in bilayer specific capacitance c was observed, which we relate to an increase in planar lipid bilayer thickness in the presence of calcium ions. Changes in the bilayer's resistance and capacitance have been reported previously and our results are in accordance with these findings [27,31]. In previous studies,

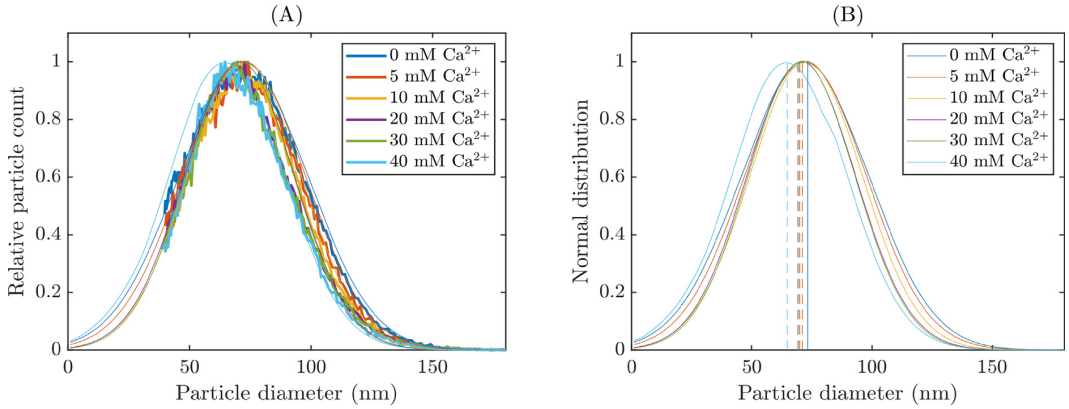

Fig. 3. Liposome particle size distribution as assessed via nES GEMMA. (A) Particle size distributions of HSPC:Chol:DSPE liposome samples containing 0 mM $CaCl_2$ (N = 12) , 5 mM $CaCl_2$ (N = 4), 10 mM $CaCl_2$ (N = 4), 20 mM $CaCl_2$ (N = 8), 30 mM $CaCl_2$ (N = 4) and 40 mM $CaCl_2$ (N = 8) are depicted. Data is related to the highest peak and is scaled using Matlab rescale function. (B) The fitted Gaussian curve for each specific calcium concentration. Vertical lines represent the liposome diameter for each calcium ion concentration.

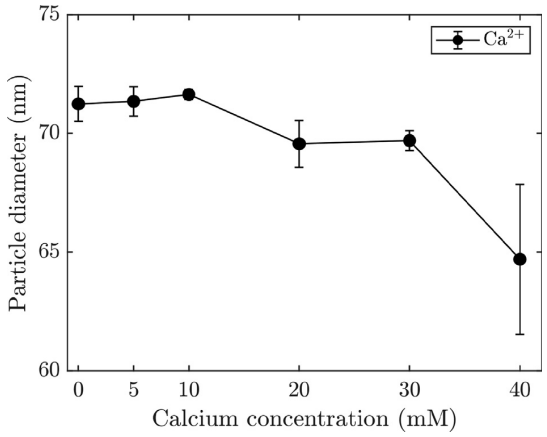

Fig. 4. HSPC:Chol:DSPE liposome diameter change as a function of calcium concentration. A decrease in HSPC:Chol:DSPE liposome size can be observed with addition of $CaCl_2$ to the liposome sample. Raw data was fitted to the Gaussian curve to obtain the diameter points. Represented is the median value of all measurements at specific concentration and standard deviation.

a considerable effect of divalent cations on the packaging order of the lipid molecules was observed as well, and measured as an increase in resistance and a decrease in capacitance. Obtained results can be correlated with a "rigidification" of the planar lipid bilayers in the presence of divalent cations, due to ordering of the lipid molecules and a more tightly packed lateral structure. As mentioned previously, MD simulations [54] show a decrease in area per lipid due to calcium ions, which correlates to an increase in bilayer thickness and is in accordance with our experimental results showing an increase in bilayer resistance and a decrease in capacitance.

In the next step, we measured the changes in resistance (Fig. 5A) and capacitance (Fig. 5B) of the HSPC:Chol:DSPE planar lipid bilayers induced by calcium ion addition. Planar lipid bilayers were formed using a mixture of HSPC:Chol:DSPE lipids for better comparison to nES GEMMA analysis of liposomes. These lipids have a more complex structure in comparison to POPC. Additionally, the surface of PE lipids is distinctively different from that of other phospholipid molecules as DSPE also carries a positive charged head group. It is possible that the chemical structure of the lipids influences the binding properties of calcium ions.

Planar lipid bilayers composed of a complex mixture of lipids, show a different behavior from POPC when calcium is added to the system. Contrary to POPC an increase in resistance as well as a slight increase in capacitance was observed, which proves, that not only the bilayer thickness changes due to calcium, but also other properties of the bilayer as well.

Fig. 5 presents electrical measurements of POPC and HSPC:Chol:DSPE bilayers as a function of calcium ion concentration. Capacitance changes more drastically for POPC lipids, which might be due to no net lipid charge. DSPE carries a positive charge, therefore less calcium comes into proximity of the HSPC:Chol:DSPE bilayers minimizing its effect. For POPC planar lipid bilayer the resistance increased for almost 44% from 0 mM $CaCl_2$ to 40 mM $CaCl_2$. Similarly, the resistance increase was seen for HSPC:Chol:DSPE bilayers where resistance with addition of 40 mM $CaCl_2$ increased for 43%.

Smaller changes were measured for capacitance differences, namely for POPC bilayers the capacitance decreased for almost 8% between 0 mM $CaCl_2$ and 40 mM $CaCl_2$. Whereas for HSPC:Chol:DSPE bilayers capacitance remained almost constant and increased for only about 0,4%.

To sum up, we showed that two different instrumental techniques could be used to detect calcium-induced changes on either liposomes or planar lipid bilayers. However, the choice of the lipid molecules used to build cell membrane models plays an important role in calcium modifications. Differences in measurements between POPC and HSPC:Chol:DSPE bilayers could be due to the fact that the lipid composition greatly influences the membrane's properties, mainly its permeability. What is more, calcium ions increase the phase transition temperature of the lipids and since the permeability of the lipid membrane is highest around the lipid transition phase, the addition of calcium ions will result in higher transition temperatures and planar lipid bilayers will therefore be more tightly packed at room temperature, where our measurements were performed. When calcium ions were added to POPC containing lipid structures, the bilayer's resistance increased while the capacitance decreased which can be correlated to an increased bilayer thickness and decreased area per lipid. Liposome measurements showed an increase in particle diameter, which can be due to the attachment of non-volatile buffer components to analytes upon nES GEMMA analysis or a decrease in area per lipid and consequently increased

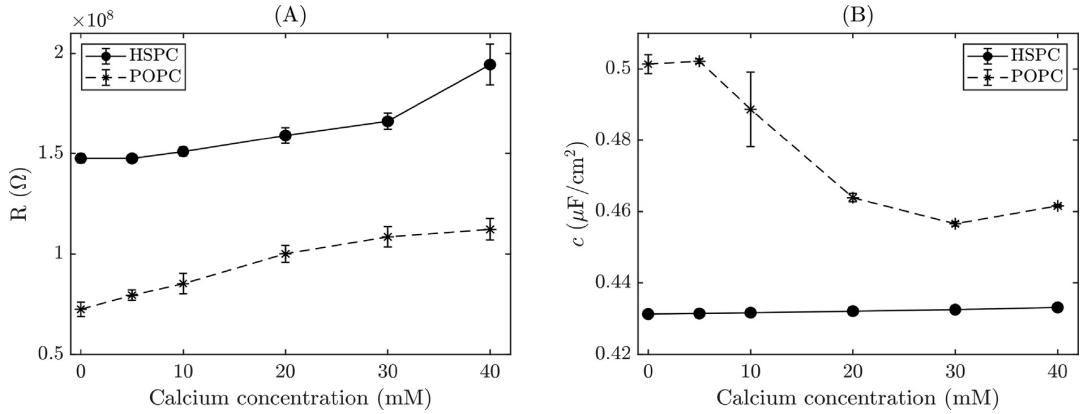

Fig. 5. Difference between POPC and HSPC:Chol:DSPE bilayers. (A) It can be seen that the resistance increases with increasing calcium concentration for POPC (N = 27) and HSPC:Chol:DSPE (N = 23) planar lipid bilayers. (B) Capacitance on the other hand decreases for POPC planar lipid bilayers and slightly increases for HSPC:Chol:DSPE planar lipid bilayer showing a more complex interaction of HSPC and DSPE lipids with calcium ions.

bilayer thickness. A more complex behavior was observed for HSPC and DSPE containing cell membrane analogues. With HSPC:Chol:DSPE planar lipid bilayers, an increase in resistance was observed, which can again be correlated to an increased bilayer thickness and decreased area per lipid. However, the capacitance did not change significantly. HSPC:Chol:DSPE liposomes showed a decrease in particle diameter with increasing calcium ion concentration, which could be due to a decreased area per lipid and consequent tighter packaging of the molecules in the lipid bilayer. This is in line with the results presented by Hoejholt et al [34], where they showed that lipid composition and heat capacity of the membrane can influence the effect of calcium electroporation.

4. Conclusion

Two different cell membrane analogues were used to observe the effect of calcium ions on phospholipids using for the first time the combination of two different measurement techniques i.e. gas-phase electrophoresis of liposomes and electrical measurements of planar lipid bilayers.

We observed an increase in POPC:Chol:DSPE liposomes and a decrease in HSPC:Chol:DSPE liposome diameter due to calcium ion addition upon gas-phase electrophoresis. Therefore, we conclude that calcium ions cause structural changes in the phospholipid membranes. Calcium binds to the phosphate group of the phospholipid molecules thus creating a conformation change in the lipid head group. These small changes can also be observed on planar lipid bilayers via electrical measurements. An increase of bilayer's resistance and a decrease in bilayer's capacitance was observed due to calcium binding to POPC lipids. When a complex mixture of HSPC:Chol:DSPE lipids was employed for bilayer formation, different results were obtained as both resistance and capacitance increased due to calcium binding. This leads us to believe that not only area per lipid and therefore membrane thickness change but also other properties of the phospholipid bilayer are influenced by calcium ion attachment.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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3.4 Paper 4

Title: **The role of lipid oxidation on electrical properties of planar lipid bilayers and its importance for understanding electroporation**

Authors: **Katja Balantič**, Victor U. Weiss, Ernst Pittenauer, Damijan Miklavčič and Peter Kramar

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Povzetek: Lipidna oksidacija je verižna reakcija, ki povzroči degredacijo lipidnih molekul in lahko pojasni dolgotrajno prepustnost celične membrane po elektroporaciji. Namen naše raziskave je bil opazovati razlike v električnih lastnostih ravninskih lipidnih dvoslojev zaradi oksidacije lipidov. Fosfolipidi so bili (i) kemično oksidirani na zraku, (ii) s $KMnO_4$ inducirano oksidacijo ali (iii) s Fentonovo reakcijo. Produkte oksidacije smo nato analizirali z masno spektrometrijo. Ravninski lipidni dvosloji so bili zgrajeni iz neoksidiranih ali oksidiranih lipidov. Električni lastnosti ravninski lipidnih dvoslojev, upornost in kapacitivnost sta bili izmerjeni z merilnikom impedance. Električna prevodnost je bila nato izračunana kot obratna vrednost izmerjene upornosti. Z uporabo predhodno razvite merilne naprave smo na stabilen dvosloj dovedli linearni naraščajoči napetostni signal, ter tako izmerili porušitveno napetost in življenjsko dobo dvosloja. Z oksidacijo se poveča prevodnosti in kapacitivnosti ravninskega lipidnega dvosloja v primerjavi z neoksidiranim dvoslojem. Z naraščajočo oksidacijo lipidov jedro dvosloja postaja bolj polarno in posledično bolj prepustno. Naše ugotovitve lahko pojasnijo dolgotrajno prepustnost celične membrane po elektroporaciji, skladno z rezultati poskusov na celicah.

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The role of lipid oxidation on electrical properties of planar lipid bilayers and its importance for understanding electroporation

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ABSTRACT

Electroporation is a useful tool for the manipulation with the cell membrane permeability. Underlying physicochemical processes taking place at the molecular level during electroporation are relatively well studied. However, various processes remain unknown, one of them is lipid oxidation, a chain reaction that causes degradation of lipids, and might explain the long-lasting membrane permeability after the electric field has ceased. The aim of our study was to observe the differences in the electrical properties of planar lipid bilayers, as *in vitro* cell membrane models, due to lipid oxidation. Phospholipids were chemically oxidized and oxidation products were analysed using mass spectrometry. Electrical properties, resistance R (Ω) and capacitance C (F) were measured using an LCR meter. Using a previously developed measuring device, a linear increasing signal was applied to a stable bilayer in order to measure its breakdown voltage U_{br} (V) and lifetime t_{br} (μs). We observed an increase in conductance and capacitance of the oxidized planar lipid bilayers when compared to their non-oxidized counterparts. With increasing lipid oxidation, the core of the bilayer becomes more polar, and consequently more permeable. Our findings can explain the long-lasting permeability of the cell membrane after electroporation.

1. Introduction

Electroporation or electropermeabilization is a widely used technique in biotechnology [1,2], medicine [3–5], and food processing [6] for enhancing the permeability of the cell membrane. By application of high-voltage electric pulses, pores are transiently formed in the cell membrane leading to the influx and efflux of various molecules, which otherwise lack the mechanism to cross the hydrophobic membrane barrier [7,8]. However, contrary to results from molecular dynamic (MD) simulations, the membrane's permeability remains high even after the electric field has ceased and the pores have resealed, leading researchers to stipulate that lipid oxidation might contribute to the long-lasting membrane permeability [9–11].

Lipid oxidation is a free radical chain reaction causing the degradation of lipid molecules and is initiated by a radical attack on an acyl chain containing a weak allylic or bis-allylic C–H bond [12]. These double bonds in the fatty acyl chains of unsaturated phospholipids are prone to oxidative damage; in particular to non-specific oxidation caused by reactive oxygen species (ROS). The removal of the hydrogen from the C–H group leaves an unpaired electron on the carbon atom. This creates a carbon-centred lipid radical, which can be stabilized by a shift in double bonds to form a conjugated diene or through the reaction with molecular oxygen to form the lipid peroxide radical [13,14]. Further on, this process creates even more radicals propagating the damage to the nearby molecules since lipid peroxide radicals can abstract hydrogen from a nearby unsaturated lipid molecule, forming a primary oxidation product, hydroperoxide [15]. Oxidative processes can result in various products with truncated lipid tails, as primary products decay further to generate aldehydes as well as carboxylic acids as secondary products [16–18].

Application of high voltage electric pulses could be a stressful event for the cell, leading to oxidative damage of its membrane. Several studies were carried out to confirm that lipid oxidation occurs during electropermeabilization [19], which was indeed shown in bacteria [20], vesicles, liposomes [21] and cells [22–25]. By application of electric pulses, ROS can be generated either in the extracellular medium due to electrochemical reactions or intracellularly due to the destabilization of mitochondrial membranes [25–28]. In a recent MD study it was shown that membrane permeability and conductance increase by several orders of magnitude due to lipid oxidation [11]. In the presence of oxidized

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lipids, the thickness of the bilayer decreases, while the area per lipid increases [29–34], suggesting that, oxidized bilayers are considerably more permeable and conductive than their non-oxidized counterparts [11,35].

Secondary oxidation products were studied as well showing that, due to the formation of aldehydes, the conductance increase is even higher and corresponds to observed conductance changes in cells that were exposed to electric fields [9]. Furthermore, an increase in passive diffusion, as well as spontaneous pore formation can be observed due to increased concentration of oxidized lipids in bilayers. In this context, it was shown that a small number of oxidation products (2.5 %) can already cause a dramatic increase in membrane permeability [36]. It was also observed that membrane oxidation leads to alterations in membrane structure and finally to complete bilayer disintegration [29,37,38]. The extent of lipid oxidation is increased with the number of pulses, pulse duration, and pulse amplitude [22,23,26,39]. Therefore, to further optimize the efficiency of electroporation-based technologies and treatments, in addition to optimizing the electric pulse parameters, we also need to study the relation between the physiological state of the cell membrane, such as its oxidation and its susceptibility to electropermeabilization, to better understand the processes occurring on the molecular level during the application of high voltage electric pulses.

In our study, we measured electrical properties of non-oxidized and chemically oxidized planar lipid bilayers to determine differences due to lipid oxidation. This is a challenging task, which requires characterization of the type and amount of lipid oxidation products. The pathways of oxidative processes vary greatly and depend on the oxidant species, the type of linkage between glycerol and the acyl chain, the degree of unsaturation, and the oxidation conditions. If identification of the oxidation products is relatively simple in lipids with only one bis-allylic hydrogen atom, it becomes more complicated if the degree of unsaturation is increased [12]. Non-oxidized and chemically oxidized lipid molecules with varying degree of unsaturation were analysed by matrix-assisted laser desorption and ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS), which is a simple, but powerful method to characterize lipids and their oxidation products [40,41]. We used different lipid molecules since the unsaturation degree plays a major role in the level of lipid oxidation [21]. We used lipid molecules most commonly studied in MD simulations and as model systems in biomembrane research [9,21,37,42–44].

Planar lipid bilayers as *in vitro* cell membrane models were used to study lipid oxidation processes at the molecular level. This artificial lipid bilayer is an elementary, but still satisfactory model of the cell membrane, used as an electrophysiological technique for measuring the properties of phospholipid molecules, in a controlled environment [45,46]. The composition of the lipid bilayer can be arbitrarily changed to mimic the composition of a true cell membrane with varying degree of oxidized species. With this simple cell membrane model, we were able to thoroughly investigate the electrical properties of lipid membranes that underwent oxidation processes. Electrical properties of non-oxidized or oxidized planar lipid bilayers, such as resistance R (Ω) and capacitance C (F) were measured, and membrane conductance was calculated as an inverse of the measured resistance. A linear increasing signal was applied to a stable bilayer to measure its breakdown voltage U_{br} (V) and the lifetime t_{br} (μs) of the planar lipid bilayer [47,48]. Indeed, we were able to detect measurable differences in the electrical properties of planar lipid bilayers, built from either non-oxidized or oxidized lipid molecules.

2. Material and methods

2.1. Chemicals

N-decane (ReagentPlus, ≥ 99.0 %), hexane (ACS reagent), $KMnO_4$, $FeCl_2$ and H_2O_2 were purchased from Sigma Aldrich (Steinheim, Germany). Chloroform (Spectronorm quality) was obtained from VWR BDH Chemicals (Roncello, Italy), methanol (LiChrosolv), potassium chloride (KCl) and 2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid (HEPES) from Merck (Darmstadt, Germany). Nitrogen was from Messer (Gumpoldskirchen, Austria). The lipids 1-palmitoyl-2-oleoyl-*glycero*-3-phosphocholine (POPC), 1,2-dioleoyl-*sn-glycero*-3-phosphocholine (DOPC), 1-palmitoyl-2-linoleoyl-*sn-glycero*-3-phosphocholine (PLPC) and 1,2-dilinoleoyl-*sn-glycero*-3-phosphocholine (DLPC) were from Avanti Polar Lipids (Alabaster, AL, USA obtained via Instruchemie (Delfzyl, The Netherlands)). 2′,4′,6′-Trihydroxyacetophenone monohydrate (THAP) matrix substance for MALDI-MS (≥99.5 %) was from Sigma Aldrich (St. Louis, MO, USA).

2.2. Buffers and electrolytes

KCl (0.1 M) and HEPES (0.01 M) were mixed together in a 1:1 volumetric ratio and applied as an aqueous electrolyte in the measurement chamber for planar lipid bilayer measurements.

2.3. Lipid oxidation

All lipids were chemically oxidized prior to bilayer formation using the protocol described in the literature [49]. Briefly, (i) oxidation was carried out with atmospheric air, where lipids in chloroform were dried under a N_2 stream to obtain a dry thin lipid film at the bottom of the flask. Lipids were then left to oxidize under atmospheric air for 1 day. Chloroform was then added, and lipids were used for further experiments. Oxidation was also initiated using either (ii) 2 M $KMnO_4$ solution or a mixture of (iii) 100 mM $FeCl_2$ and 500 mM H_2O_2 solution. Solutions of chemical oxidants were added to the dry thin lipid film at the bottom of the flask and incubated for 10 min at 37 °C using $KMnO_4$ solution or for 1 h using $FeCl_2$/H_2O_2 solution. Afterwards 1 ml of chloroform: methanol [1:1 vol ratio] was added to stop the oxidation. Vortexing and centrifuging were used to separate the polar and non-polar phase and to extract the oxidized lipids, which were dissolved in chloroform and used for further experiments.

2.4. Mass spectrometry analysis

MALDI MS analysis of non-oxidized and chemically oxidized lipids was carried out using an UltrafleXtreme reflectron TOF-MS (Bruker, Bremen, Germany) in positive ion mode at 200 Hz laser repetition rate and accumulating 5000 laser shots per sample. Lipids were dissolved in methanol and pre-mixed (1:1 (v:v)) with methanolic THAP solution (15 mg/ml). 0.6 μl of the final solution was applied to a stainless steel MALDI MS target.

2.5. Planar lipid bilayer formation

Planar lipid bilayers were formed using a Mueller-Rudin method [50,51]. Briefly, the corresponding amount of lipids was dissolved in chloroform and dried under a N_2 stream and subsequently dissolved in *n*-decan to obtain a final 20 mM lipid concentration. Experiments were carried out at room temperature (~21 °C). A Delrin measurement chamber from Warner Instruments (Hamden, CT, USA) with 150 μm diameter aperture separating the two cuvettes was used to form the planar lipid bilayer. The aperture was pretreated with 20 mM lipids dissolved in hexane. After the evaporation of hexane, each compartment was filled with 4 ml of KCl/HEPES electrolyte solution. For planar lipid bilayer formation, a small drop of lipids dissolved in *n*-decane was applied to the aperture using a glass rod. A stable planar lipid bilayer was formed after approximately 30 min and subsequently, capacitance was measured to confirm the stability of the bilayer. In accordance with earlier studies, each experiment was repeated to a minimum of 10 to 20 times to confirm the trend and to avoid errors [52].

This bilayer formation ensures that our lipid molecules are not exposed to air during the formation process. However, due to deposition

of the lipids dissolved in organic solvent directly into the aqueous electrolyte, the solvent itself can play a role in the stability and thickness of the bilayer.

2.6. Planar lipid bilayer resistance and capacitance measurements

Electrical properties of planar lipid bilayers were measured using a system developed in the Laboratory for Biocybernetics, University of Ljubljana, Faculty of Electrical Engineering [48]. The system (Fig. 1) consists of two units that are used for (i) electrical measurements of intact planar lipid bilayers and (ii) to expose the planar lipid bilayer to a linear rising voltage signal.

Electrical measurements on intact planar lipid bilayers were performed using an LCR meter E4980A from Keysight (Santa Rosa, CA, USA) connected to four Ag/AgCl electrodes (In vivo metric, Healdsburg, CA, USA) immersed in the electrolyte solution in the Delrin measurement chamber (Warner Instruments, USA). The LCR meter was set to measure resistance *Rp* and capacitance *Cp* in parallel. The AC voltage was set to 20 mV and the frequency to 2 kHz. Data points were acquired each quarter of a second to obtain measurements of the resistance and capacitance over time.

The breakdown voltage (U_{br}) and lifetime (t_{br}) of the planar lipid bilayer were determined by applying a linear rising voltage signal to a planar lipid bilayer [47]. The signal generator Agilent 33220A, Keysight (Santa Rosa, CA, USA) was used to apply a voltage ramp at 4.8 kV/s. Breakdown voltage was defined as the voltage at the moment t_{br} when a sudden increase of transmembrane current occurred. To detect the current a custom amperometer was developed. Voltage and current signals were measured using an oscilloscope HDO6104A-MS, Teledyne Lecroy (New York, NY, USA). Time of breakdown t_{br} was defined as the lifetime of the lipid bilayer at a chosen slope of the linear rising voltage signal.

A manual switch enables us to differentiate between the two units of the system. The LCR meter, signal generator and oscilloscope were connected through an Ethernet connection and controlled with MATLAB R2019a (MathWorks, Natick, MA, USA) Toolbox using SCPI protocol. Measured capacitance, resistance, breakdown voltage and lifetime of each planar lipid bilayer were processed using MATLAB R2019a.

The specific capacitance c (μF/cm^2) of the planar lipid bilayer was calculated using Eq. (1).

$$c = C_p/A \tag{1}$$

Where C_p is the measured capacitance and A is the area of the aperture (1.76e^{-4} cm^2) used for planar lipid bilayer formation. The electrical conductivity σ (S/cm^2) of the planar lipid bilayer was calculated using Eq. (2) and is the inverse of the measured resistance R_p.

$$\sigma = 1/(R_p{}^*A) \tag{2}$$

Specific capacitance and conductance were normalized to the area A of the aperture for easier comparison with the data found in the literature.

To study the effect of lipid peroxidation on the conductivity of planar lipid bilayers, either oxidized or non-oxidized lipids were used. 10 measurements for each lipid species were carried out for each oxidation protocol. Differences in conductance σ and specific capacitance c of the planar lipid bilayers build from either non-oxidized or oxidized lipid molecules were studied.

2.7. Statistical analysis

Resistance and capacitance measurements (N = 10) of planar lipid bilayers for each oxidation procedure and each specific lipid molecule were combined via their mean to obtain the corresponding dataset. Likewise, measurements of the breakdown voltage and bilayer's lifetime (N = 10) for each oxidation procedure and each specific lipid molecule were combined via their mean to obtain the corresponding dataset. Fig. 2 presents raw data. For comparison of planar lipid bilayers datasets, one-way analysis of variance (ANOVA) was used, followed by pairwise comparisons using a multiple comparison Tukey's significant difference procedure. We rejected the null hypothesis of analysis if the p-value of the test was <0.05 ($p < 0.05$). All of the test were performed using Matlab R2019a.

3. Results and discussion

In our experiments, we measured electrical properties of planar lipid bilayers to detect differences between non-oxidized and oxidized bilayers due to lipid oxidation. Bilayer's conductivity, calculated as the

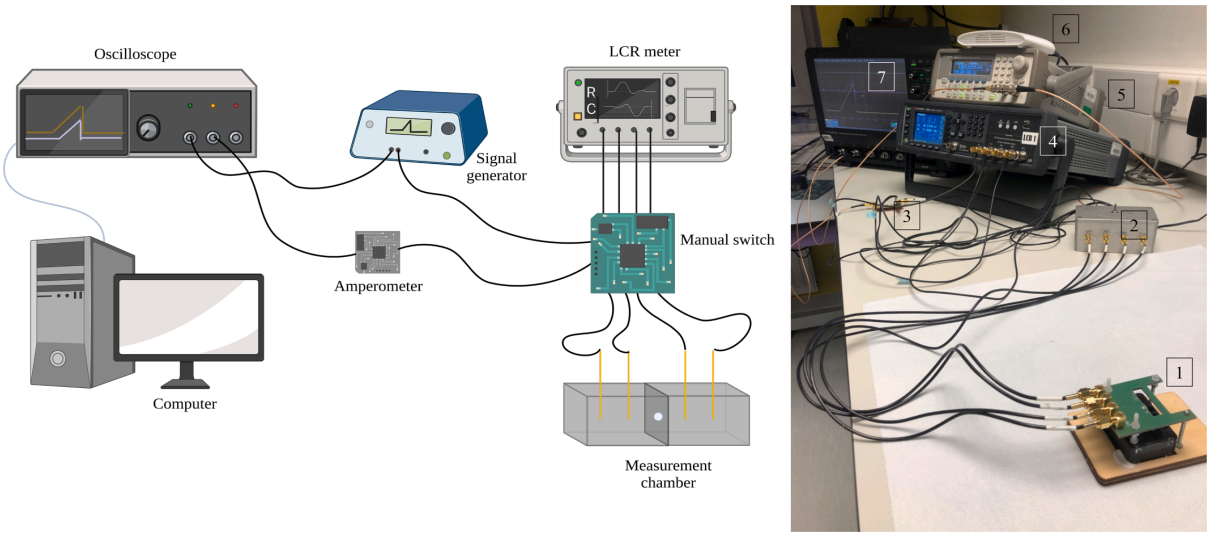

Fig. 1. (Left) A schematics representation of the system developed in the Laboratory of Biocybernetics to measure the electrical properties of planar lipid bilayers. (Right) System in our lab: (1) Measurement chamber, (2) Manual switch, (3) Amperometer, (4) LCR meter, (5) Signal generator, (6) Ethernet Hub, (7) Oscilloscope.

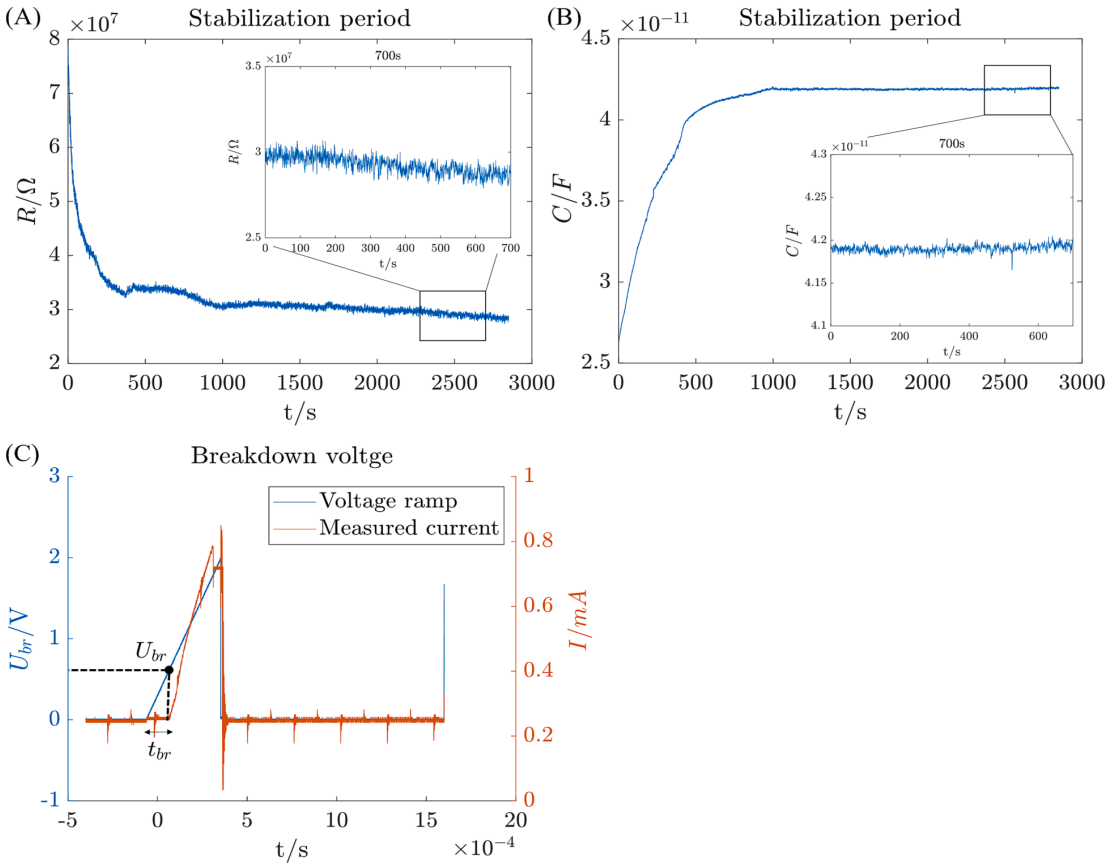

Fig. 2. An example of raw data for a single bilayer measurement. (A) Resistance and (B) capacitance were measured after a minimum of 30 min stabilization period. As the lipids are applied to the aperture in the measurement chamber, the resistance is high and capacitance is low, since a large quantity of lipid molecules is present around the aperture. As the lipids start to self-assemble into a bilayer, the resistance decreases and capacitance increases until both values reach a plateau. When the values of resistance and capacitance were stable, a 700 s measurement of each lipid bilayer was obtained for further analysis. (C) The breakdown voltage U_{br} was measured as the voltage at which the increase in the current was detected. The lifetime t_{br} was measured as the time at which the breakdown of the bilayer occurred.

inverse of the measured resistance, specific capacitance, U_{br} and t_{br} were measured and compared. Oxidation of lipid molecules was carried out using oxidizing agents with varying strength to note different degrees of oxidation. MS analysis was applied to detect produced oxidized species. Below, firstly, the detected oxidation products via MS analysis are described, followed by the analysis of the observed differences in the electrical properties of the planar lipid bilayers due to lipid oxidation.

3.1. Mass spectrometry analysis of lipid molecules

We chemically oxidized the lipids and determined the most common oxidizing products of each specific lipid molecule. The lipid molecules used were: (i) POPC with a single double bond on unsaturated acyl chain at *sn*-2. (ii) DOPC with two double bonds, one each per an acyl chain. (iii) PLPC, which has two double bonds on the unsaturated linoleic acid at *sn*-2. Lastly, (iv) DLPC with four double bonds, 2 each per an acyl chain. The acyl chains differ in the number and the position of double bonds. Since double bonds are highly reactive and play a key role in lipid oxidation reactions, we observed the differences occurring due to variance in double bond number and their position.

3.1.1. Chemistry of lipid oxidation

We used three different oxidation procedures, each one generating radical species with varying strength and, consequently, a different quantity of oxidation products. The most commonly formed oxygen radicals for each oxidation protocol are given below.

The sequential reduction of molecular oxygen (Eq. (3) present in atmospheric air leads to formation of oxygen radicals such as the superoxide anion radical ($O_2^-\bullet$), singlet oxygen ($^1O_2$) and, if H_2O is present, the hydroxyl (HO•) and hydroperoxyl (HOO•) radical [53,54].

$$O_2 + e^- \rightarrow O_2^-\bullet \quad (3)$$

The superoxide radical can further be reduced to form hydrogen peroxide H_2O_2.

$$O_2^-\bullet + e^- + 2H^+ \rightarrow H_2O_2 \quad (4)$$

Furthermore, hydrogen peroxide can further dissociate into the hydroxyl radical (HO•).

$$H_2O_2 + e^- \rightarrow OH^- + HO\bullet \quad (5)$$

Another important ROS is the singlet oxygen, which is formed by the photolysis of the triplet oxygen.

$$^3O_2 \xrightarrow{light} {}^1O_2 \quad (6)$$

Singlet oxygen can react with water vapour in the atmosphere to form hydroxyl radicals. Hydroxyl radicals, being highly reactive can then combine with another O_2 molecule to form a hydroperoxyl radical (HOO•).

$KMnO_4$ is a strong oxidant that does not produce any toxic by-products. Oxidation via $KMnO_4$ was carried out at 37 °C in neutral pH, which leads to the formation of radicals as Mn^{7+} is reduced to form Mn^{4+} (Eq. (7)). Therefore, the permanganate (VII) ion is a strong oxidizing agent.

$$MnO_4^- + 2H_2O + 3e^- \rightarrow MnO_2 + 4OH^- \quad (7)$$

Lastly, oxidation via the Fenton reagents leads to the formation of hydroxyl and hydroperoxyl radicals. Iron Fe^{2+} reacts with H_2O_2 and is oxidized to Fe^{3+} during which a hydroxyl radical (HO•) is formed (Eq. (8)). Fe^{3+} can then further react (Eq. (9) with another molecule of H_2O_2 to form hydroperoxyl radical (HOO•).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + OH^- \quad (8)$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HOO^{\bullet} + H^+ \quad (9)$$

These radicals are powerful, non-selective oxidants. Among the ROS, HO• is the most potent damaging radical which can react with all biological molecules, the most susceptible ones being unsaturated fatty acids.

3.1.2. Produced oxidized species

Non-oxidized lipid molecules used in this study and their most common oxidized counterparts that were also found on MS spectra are presented in Fig. 3. Cleavage of a double bond present on *sn*-2 acyl chain in POPC (Fig. 3A) produced an aldehyde (Fig. 3C) at *m/z* 650.4 and a carboxylic acid (Fig. 3D) at *m/z* 666.4 on the MS spectra (Fig. 4A) resulting from the abstraction of terminal carbon atom at C9. Adducts with H^+, K^+ and Na^+ were detected. For DOPC molecules with two oleic acid chains (Fig. 3E), different oxidation scenarios are possible. It is possible to oxidize the one double bond on *sn*-1 or *sn*-2 acyl chain, which results in an aldehyde (Fig. 3G) found on MS spectra (Fig. 4B) at *m/z* 676.4. Furthermore, with $FeCl_2/H_2O_2$ oxidation, we were able to detect an elimination of the *sn*-2 acyl chain at *m/z* 504.3 (Fig. 3H).

PLPC contains saturated palmitic acid at the *sn*-1 position and unsaturated linoleic acid at the *sn*-2 position (Fig. 3I), where oxidation is expected to take place, so the short-chain products are formed as the result of linoleic acid oxidation. Two double bonds are present on the *sn*-2 chain, the first one at position C9 and the second one at position C12. It

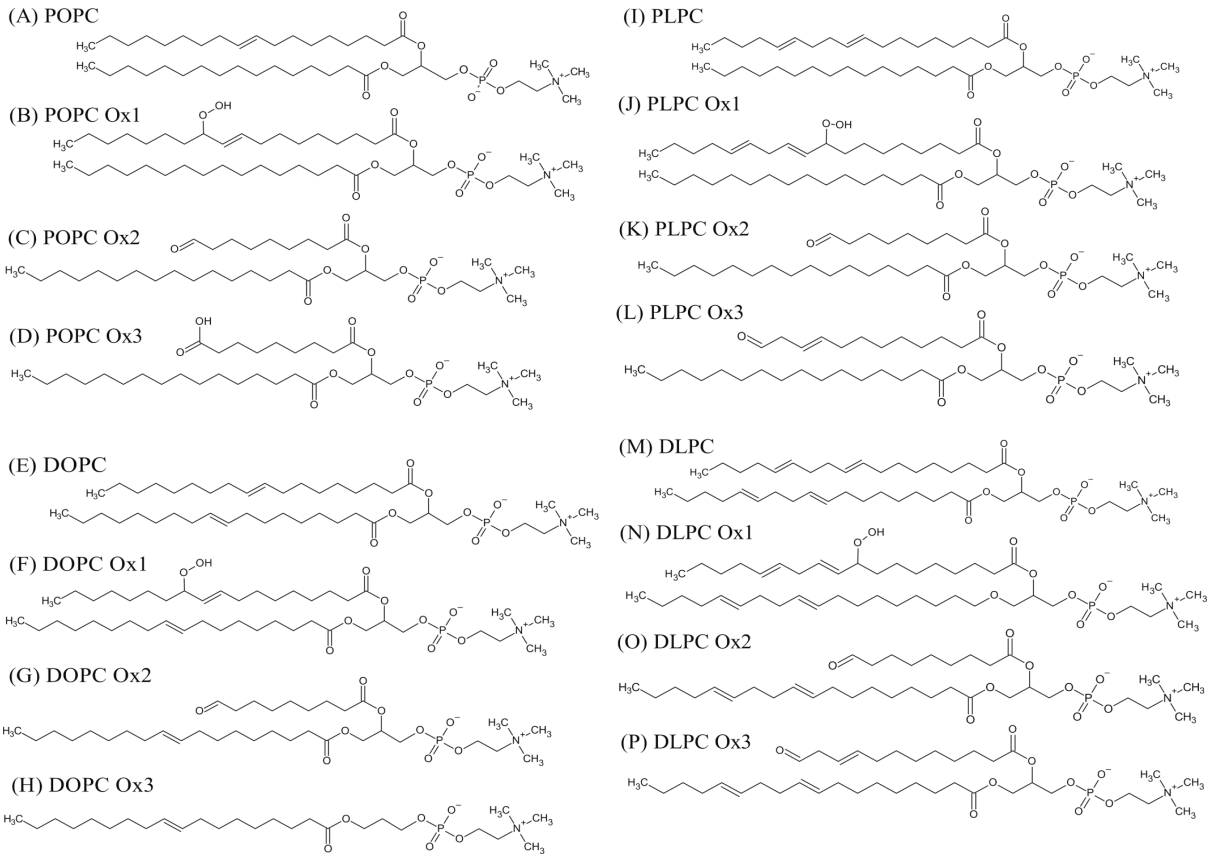

Fig. 3. Non-oxidized lipids used in the study and their oxidized products. (A) Native POPC molecule, (B) hydroperoxide, a primary product of POPC oxidation, (C) oxidation product of POPC with an aldehyde terminal group and (D) oxidation product of POPC with a terminal carboxylic group. (E) Native DOPC molecule, (F) hydroperoxide, a primary product of DOPC oxidation, (G) oxidation product of DOPC with an aldehyde terminal group and (H) elimination of *sn*-2 acyl chain. (I) Native PLPC molecule, (J) hydroperoxide, primary oxidation product of PLPC, (K) oxidation product of PLPC with an aldehyde at position C9 and (L) position C12. (M) Native DLPC molecule, (N) hydroperoxide, primary oxidation product, (O) oxidation product of DLPC with an aldehyde terminal group at C9 and (P) C12.

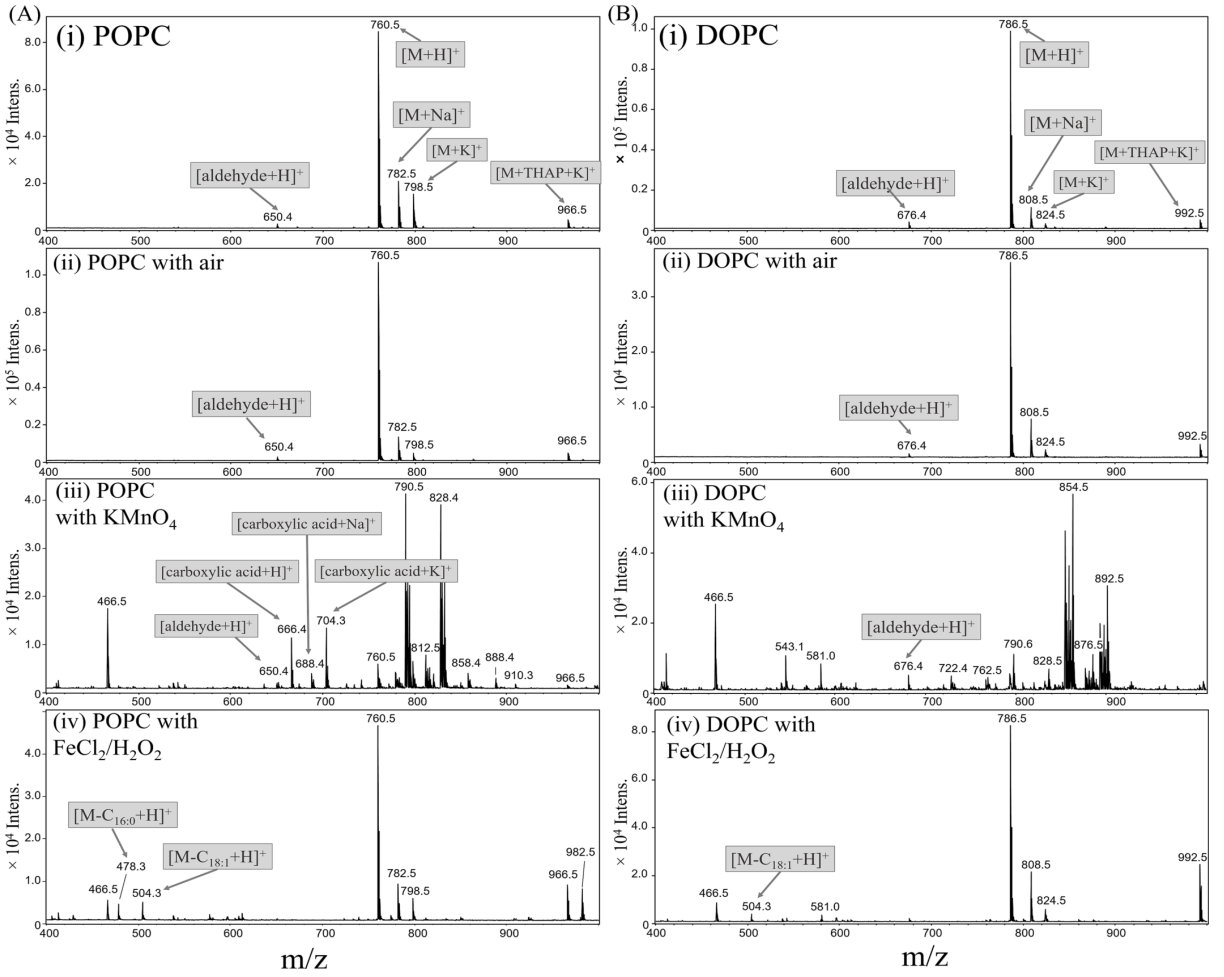

Fig. 4. Mass spectra for (A) POPC, and (B) DOPC lipids. (A) and (B) group on graph include spectra for (i) non-oxidized lipids, (ii) lipids oxidized with air, (iii) $KMnO_4$ and (iv) with Fenton reaction ($FeCl_2/H_2O_2$).

is possible to only oxidize the double bond at position C12 or to shorten the acyl chain even further and oxidize the double bond at position C9. Again mainly aldehydes were found as terminal functional groups due to oxidation, two different aldehydes (Fig. 3K,L) were found, one at C12 with *m/z* 690.4 and one at C9 with *m/z* 650.4 (Fig. 5A). The same is true for DLPC lipid (Fig. 3M) oxidation where the shortenings can occur at two different positions, but this time on each acyl chain. A shorter aldehyde (Fig. 3O) can be detected at *m/z* 674.4 and the longer aldehyde (Fig. 3P) at *m/z* 714.4 (Fig. 5B). Regardless of the position of the double bond, oxidation of an acyl chain usually results in its cleavage and formation of short-chain products of various chain lengths with terminal aldehyde as a functional group (–CHO).

Furthermore, oxidizing agents also influence the formation of oxidation products especially the occurrence of either short or long-chained products. When lipids were oxidized with air, the peaks for oxidation products were low, and only some trace amounts of aldehydes were found for POPC, DOPC and PLPC lipids. In our protocols, air proved to be the weakest oxidizing agent. All of the lipids were dissolved in chloroform and handled in the presence of nitrogen, nevertheless the MS spectra of non-oxidized and air oxidized lipids are not significantly different, leading us to believe that either a trace amount of aldehydes was already present in our native samples, or air does not significantly oxidized the lipids.

$KMnO_4$ oxidation was indeed much stronger than air and resulted in many different products. For POPC lipids, aldehyde as well as carboxylic acid peaks were detected. The peaks for native POPC, DOPC and PLPC molecules are no longer detectable after $KMnO_4$ oxidation since native molecules are cleaved to form the corresponding oxidation products. However, oxidation with $KMnO_4$ produced large peaks even at molecular weights higher than the native lipid molecule, leading us to believe that some of the oxygen remains bound to our lipid molecules even after lipid extraction. This is seen especially on POPC (Fig. 4A) and DOPC spectra (Fig. 4B). The insertion of oxygen atoms without breakdown of the lipid structure generates so-called long-chain products. $FeCl_2$ and H_2O_2 oxidation results in cleavage of a fatty acid from the POPC and DOPC lipid molecule. For PLPC aldehyde was detected, while DLPC is so highly unsaturated that only short-chain products were present, native DLPC molecule was no longer detectable. Several undefined peaks can be found in PLPC (Fig. 5A) and DLPC spectra (Fig. 5B) since they have more possible oxidation scenarios, acyl chains can be cleaved at two

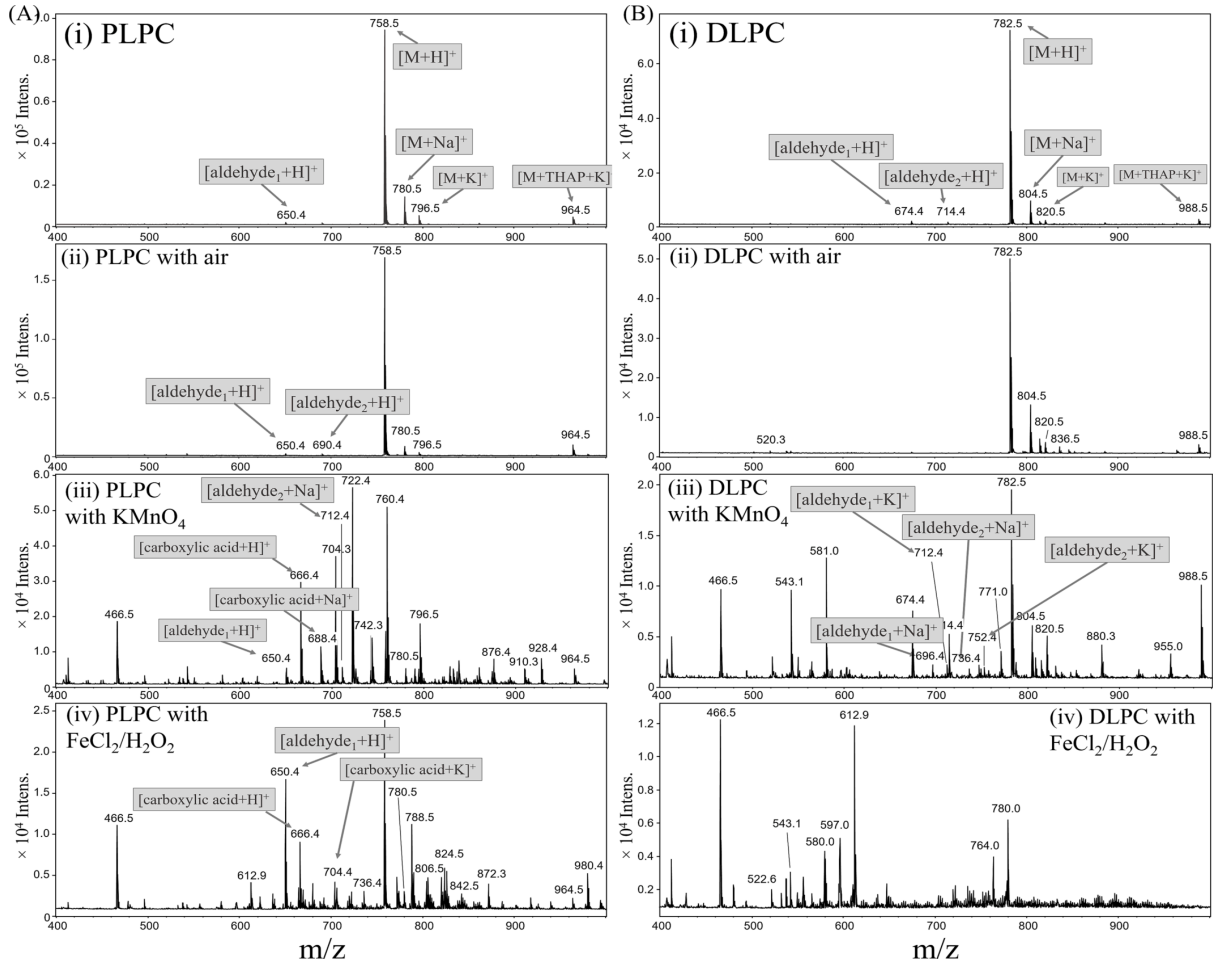

Fig. 5. Mass spectra for (A) PLPC, and (B) DLPC lipids. (A) and (B) group on graph include spectra for (i) non-oxidized lipids, (ii) lipid oxidized with air, (iii) $KMnO_4$ and (iv) with Fenton reaction ($FeCl_2/H_2O_2$).

different positions, and therefore more peaks can be found in the respective MS spectra. Nevertheless, all three oxidation protocols generate similar oxidation products although the yields of the individual compounds are obviously different.

3.2. *Electrical properties of planar lipid bilayers*

Electrical properties of planar lipid bilayers, namely conductivity, specific capacitance, breakdown voltage and lifetime were determined by measuring voltage and current across a planar lipid bilayer. Results show that we are able to detect differences in the electrical properties of non-oxidized and oxidized planar lipid bilayers.

3.2.1. *Conductivity measurements*

A much higher specific conductance of the oxidized planar lipid bilayers, compared to non-oxidized ones, was observed (Fig. 6) indicating that lipid oxidation could be the cause of increased membrane permeability during and after electroporation. The conductivity of the bilayer measures the ability of electrical current and ions to pass the bilayer. With increasing lipid oxidation, the core of the bilayer will become more polar, due to chemical changes and consequently more permeable [55]. Therefore, an increase in conductivity is as expected. Increased conductivity can also affect action potential generation and propagation [56]. What is more, transient pores can occur in the planar lipid bilayer, due to the presence of oxidized species and applied voltage, which could also lead to an increase in specific conductance [57,58]. Our measurement system applies a voltage to the planar lipid bilayer, which could in principle lead to the formation of water fingers that protrude inside the hydrophobic core of the membrane; nevertheless, the bilayer would remain stable. It was shown before, that the presence of oxidized species in the bilayer lowers the activation barrier for pore formation [34]. This gives another possibility for interpretation of an increased specific conductance, not being only due to a decrease in membrane thickness or more hydrophilic bilayer interior, but also because of a lower membrane barrier potential in the presence of oxidized lipid molecules.

Interestingly, as seen in Fig. 6, the conductivity of non-oxidized planar lipid bilayers was the highest for POPC lipids containing a single double bond. This is contrary to expectations since POPC lipids should form the thickest bilayers and therefore have the lowest conductivity. This contradiction can be attributed to the method utilized for bilayer formation. Using the "painting method" for bilayer formation, stable bilayers are readily formed with saturated lipid molecules.

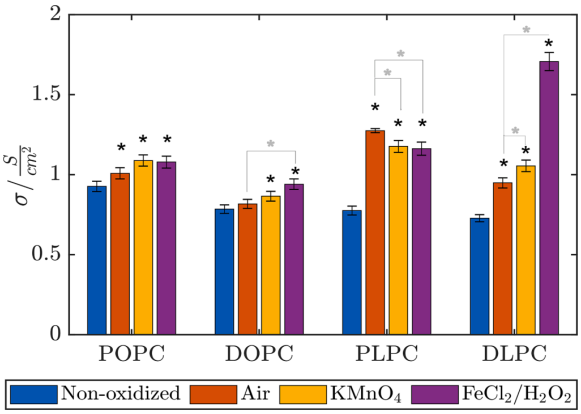

Fig. 6. Specific conductance for each specific lipid bilayer using different oxidation protocols. An increase in membrane conductivity is observed with lipid oxidation. Data are expressed as means ± standard deviations (n = 10 measurements). Black asterisks (*) show statistically significant differences ($p < 0.05$) between non-oxidized and oxidized bilayer, grey asterisks (*) show statistically significant differences ($p < 0.05$) between oxidized groups.

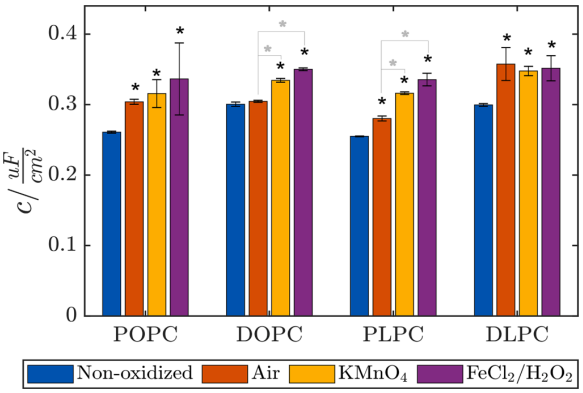

Fig. 7. Specific capacitance for each specific lipid bilayer using different oxidation protocols. Specific capacitance increases due to lipid oxidation. Data are expressed as means ± standard deviations (n = 10 measurements). Black asterisks (*) show statistically significant differences ($p < 0.05$) between non-oxidized and oxidized bilayer, grey asterisks (*) show statistically significant differences ($p < 0.05$) between oxidized groups.

However, when lipids with a higher degree of unsaturation are employed, the bilayers become less stable and their formation requires a larger droplet of lipids to be applied to the aperture in the measurement chamber. Therefore, with higher degree of unsaturation, a larger lipid quantity is needed for bilayer formation, leading to thicker bilayers and possibly to formation of multilayers thus lowering the conductivity for highly unsaturated PLPC and DLPC lipids. The application of multiple droplets to the aperture, could also lead to a higher yield of the solvent within the membrane, consequently thickening the interface and decreasing the conductance. What is more, the nonlinear increase in measured electrical properties for PLPC and DLPC lipids can also be related to a larger degree of lipid unsaturation and presence of various oxidation products. Nevertheless, our aim was to develop a measuring system as a proof of concept to observe differences between non-oxidized and oxidized bilayers for the same lipid species, which was indeed successful for all lipid molecules used.

Conductance of non-oxidized POPC bilayers was 0.92 ± 0.06 S/cm^2. For oxidation with air, the conductance increased for 9 %, while the increase for $KMnO_4$ and $FeCl_2/H_2O_2$ induced oxidation was 16 % and 15 % respectively. DOPC bilayers in their native state had a conductance of 0.79 ± 0.04 S/cm^2. Oxidation with air slightly increased the conductance of the DOPC bilayer for 4 %, while the increase was larger for $KMnO_4$ oxidation with 9 % and a 19 % increase for $FeCl_2/H_2O_2$ oxidation. Non-oxidized PLPC bilayers have a conductance of 0.77 ± 0.05 S/cm^2, their conductance significantly increases with air oxidation, for 65 %. $KMnO_4$ induced oxidation increased the PLPC conductance for 52 % and $FeCl_2/H_2O_2$ for 50 %. DLPC bilayers in the absence of oxidized species have a conductance of 0.73 ± 0.02 S/cm^2. Conductance for oxidation with air and with $KMnO_4$ increased for 28 % and 43 % respectively, while the oxidation via Fenton reagents increased the conductance, more than two-fold, for 134 %, leading us to believe that due to the presence of oxidized species, transient pores form spontaneously and further increase the conductivity of the bilayer.

3.2.2. Capacitance measurements

According to our measurements, specific capacitance increased due to lipid oxidation (Fig. 7), which has been reported previously and our results are in agreement with these findings [9]. Oxidation chemically changes the hydrophobic tail region of the lipid molecules, with insertion of more polar functional groups, which increases the dielectric coefficient of the bilayer and consequently results in measureable increase in the lipid bilayer's specific capacitance.

Capacitance of the hydrophobic region is associated not only with the dielectric coefficient but also with the thickness of the phospholipid acyl chains. Oxidation leads to formation of short-chain lipid products, creating thinner bilayers, which results in an increased capacitance.

Specific capacitance of non-oxidized POPC, DOPC, PLPC and DLPC bilayers was 0.26 ± 0.005 μF/cm^2, 0.30 ± 0.01 μF/cm^2, 0.25 ± 0.001 μF/cm^2 and 0.29 ± 0.008 μF/cm^2 respectively. Oxidation of POPC bilayer with air increased the specific capacitance for 15 %, oxidation via $KMnO_4$ for 19 % and with Fenton reagents for 35 %. Specific capacitance of DOPC bilayers increased for 1 %, 10 % and 17 % when lipids were oxidized with air, with $KMnO_4$ or $FeCl_2/H_2O_2$ respectively. Oxidation of PLPC bilayers with air increased the specific capacitance for 12 %, $KMnO_4$ induced oxidation increased the capacitance for 24 %, and the use of Fenton reagents for 32 %. The highest capacitance increase of DLPC bilayers, for 20 % was achieved with oxidation with air; however, the change was not significantly different between other two oxidation protocols. Oxidation with $KMnO_4$ and with $FeCl_2/H_2O_2$ lead to an increase in specific capacitance for 14 % and 19 % respectively.

The value of measured specific capacitance in our study is low compared to previously published reports, which could be due to the incorporation of the hydrocarbon solvent *n*-decane into the membrane [59,60]. What is more, the torus of the solvent surrounding the bilayer could lead to deviations from the previous results as well [61]. Nevertheless, the specific capacitance of the oxidized bilayers increases as seen on Fig. 7, due to the formation of polar regions in previously non-polar environment, and the presence of short-chain oxidation products, which leads to an increase in the bilayer's dielectric coefficient and smaller thickness of the bilayer. However, the differences in specific capacitance, for PLPC and DLPC lipids using the same oxidation protocols are less pronounced than conductivity changes. The fact is that the modifications of the bilayer structure due to the presence of oxidized species are complex. Oxidation induced changes in the acyl chains can also lead to modifications in the head group region of the lipid molecules, where the area per head is increased and functional groups resulting from oxidation are added to the interface [62]. Furthermore, the occurrence of long-chain oxidation products can increase the bilayer thickness. These effects can all lead to smaller specific capacitance differences between non-oxidized bilayers and their oxidized counterparts.

3.2.3. Breakdown voltage and lifetime of planar lipid bilayers

Breakdown voltage and lifetime of planar lipid bilayer are important values when determining the electroporation properties of planar lipid bilayers. Firstly, when applying the voltage ramp, the charge build up on the planar lipid bilayer occurs. Above the critical voltage (U_{br}), defects are caused in the planar lipid bilayer allowing an increase of the transmembrane current, afterwards the bilayers usually collapse [58].

The measured U_{br} (Fig. 8) and t_{br} (Fig. 9) for different bilayers did not prove to be statistically significant, leading us to believe that using our system to obtain the breakdown voltage and lifetime measurements is not a sufficiently sensitive method to detect the differences between non-oxidized and oxidized lipid bilayers. It is interestingly also possible that lipid oxidation does not play a key role in stability of planar lipid bilayers. It was shown recently, that oxidized lipid bilayers can retain their integrity even under applied electric field [34].

We expected that the measurements of breakdown voltage and the lifetime of the oxidized bilayer would show that the voltage needed for the breakdown of the bilayer decreases with lipid oxidation, the same should be true for the bilayer lifetime. The only significant decrease in breakdown voltage and lifetime of the bilayer was obtained for DLPC lipids oxidized with air. DLPC lipids are highly unsaturated and therefore very prone to oxidation. Even though air did not prove to be a strongest oxidant in our experiments, the protocol does not lead to formation of long-chain products. MS spectra for DLPC lipids shows that $KMnO_4$ oxidation leads to formation of aldehydes as well as long-chain products and Fenton reagents lead to no detectable peak for native DLPC molecule, however no known oxidation products were detected either. Therefore, we can only observe a decrease in stability and faster breakdown for DLPC bilayers oxidized with air.

3.3. Lipid oxidation effect on electrical properties of planar lipid bilayer

The study of oxidation effects on different lipid molecules and the measurement of their electrical properties proved to be a challenging task. The bilayer formation becomes more demanding with increasing degree of lipid unsaturation and even more so with the presence of oxidized species. Oxidized bilayers are less stable and a larger quantity of lipid molecules is needed for bilayer formation. Nevertheless, our measurement system enabled us to show, that oxidative stress, and an increasing number of oxidized species perturbing the planar lipid bilayer, leads to an increase in bilayer's conductivity and specific capacitance as summarized in Table 1.

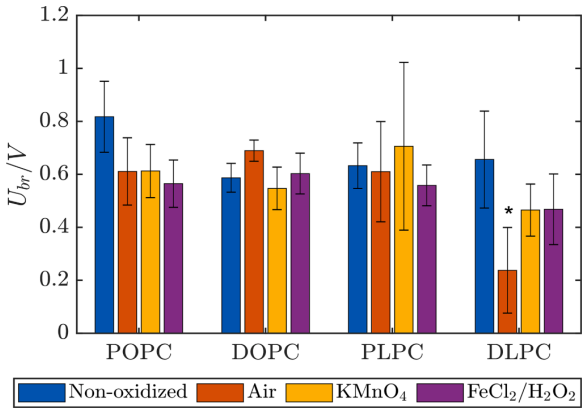

Fig. 8. Breakdown voltage for specific lipid bilayer using different oxidation protocols. A significant decrease in U_{br} was measured for DLPC lipids oxidized in air. Data are expressed as means ± standard deviations (n = 10 measurements). Asterisks (*) show statistically significant differences ($p < 0.05$) between non-oxidized and oxidized bilayer.

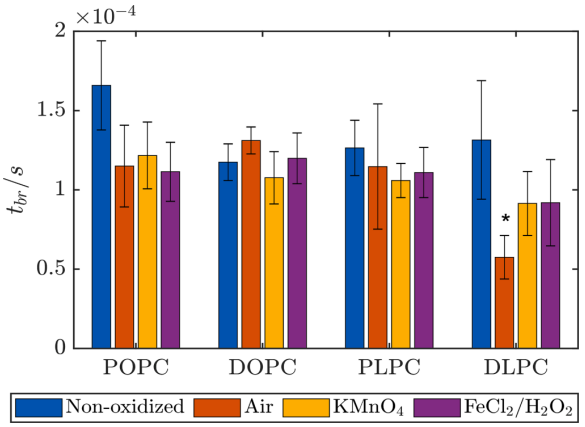

Fig. 9. Lifetime of planar lipid bilayer for specific lipid bilayer and different oxidation protocols. A significant decrease in t_{br} was measured for DLPC lipids oxidized in air. Data are expressed as means ± standard deviations (n = 10 measurements). Asterisks (*) show statistically significant differences ($p < 0.05$) between non-oxidized and oxidized bilayer.

Table 1
Oxidation products and differences in electrical properties for each lipid molecule. –CHO ending corresponds to an aldehyde, –COOH to a carboxylic acid, and M−C16:0, M−C18:1 to fragmentation products. For conductivity and specific capacitance, the mean values with standard deviation are presented. The arrows ↑ indicate an increase in the measured electrical properties for oxidized bilayers compared to their non-oxidized counterparts.

Lipid	Non-oxidized	Air	$KMnO_4$	$FeCl_2/H_2O_2$
POPC	–CHO	–CHO	–CHO, –COOH, less native POPC, long-chain products	Fragmentation: $M-C_{16:0}$, $M-C_{18:1}$
σ (S/cm²)	0.92 ± 0.06	↑ 1.01 ± 0.05	↑ 1.07 ± 0.04	↑ 1.06 ± 0.05↑ 0.35 ± 0.04
c (μF/cm²)	0.26 ± 0.005	↑ 0.30 ± 0.01	↑ 0.31 ± 0.035	↑ 0.35 ± 0.04
DOPC	–CHO	–CHO	–CHO, no native DOPC, long chain products	Fragmentation: $M-C_{18:1}$
σ (S/cm²)	0.79 ± 0.04	↑ 0.82 ± 0.02	↑ 0.86 ± 0.03	↑ 0.94 ± 0.03
c (μF/cm²)	0.30 ± 0.01	↑ 0.303 ± 0.005	↑ 0.33 ± 0.01	↑ 0.35 ± 0.008
PLPC	–CHO	–CHO	–CHO, –COOH, no native PLPC, long-chain products	–CHO, –COOH, long-chain products
σ (S/cm²)	0.77 ± 0.05	↑ 1.27 ± 0.01	↑ 1.17 ± 0.04	↑ 1.16 ± 0.04
c (μF/cm²)	0.25 ± 0.001	↑ 0.28 ± 0.02	↑ 0.31 ± 0.015	↑ 0.33 ± 0.03
DLPC	–CHO	/	–CHO, long-chain products, less native DLPC	Fragmentation, no native DLPC
σ (S/cm²)	0.73 ± 0.02	↑ 0.94 ± 0.03	↑ 1.05 ± 0.04	↑ 1.71 ± 0.05
c (μF/cm²)	0.29 ± 0.008	↑ 0.35 ± 0.035	↑ 0.33 ± 0.025	↑ 0.345 ± 0.03

For POPC lipids, the increase in specific conductance and capacitance is significantly different between non-oxidized and oxidized bilayers (Figs. 6 and 7); however, there is no significant difference between different oxidation protocols. From the POPC MS spectra (Fig. 4A), we were able to observe that air did not produce many oxidation products, while $KMnO_4$ oxidation resulted in high peaks for aldehyde and carboxylic acid products, with H^+, K^+ and Na^+ adducts.

Furthermore, a much smaller peak for native POPC lipid was detectable after $KMnO_4$ oxidation, indicating that the majority of lipids underwent oxidation. Even though high peaks for oxidation products and lower quantity of native POPC were detected on MS spectra, oxidation via $KMnO_4$ did not result in a significant rise in specific conductance and capacitance compared to non-oxidized bilayers. This lack of substantial increase can likely be attributed to the presence of long-chain products as oxygen atoms are incorporated into the acyl chains of the lipid molecules. This long-chain products increase the thickness of the bilayer, minimizing the increase in electrical properties. Oxidation via Fenton reagents led to a decrease in the intensity for native POPC peak and production of two oxidation products due to a removal of either oleic (m/z 478.3) or palmitic acid (m/z 504.3). However, native POPC lipids were still detectable after $FeCl_2/H_2O_2$ oxidation, and since, bilayers would still preferentially form with non-oxidized native molecules, the increase in specific conductance and capacitance with $FeCl_2/H_2O_2$ oxidation was not as pronounced as expected.

MS spectra of DOPC lipids (Fig. 4B) shows similar results as the one obtained for POPC lipids, due to the similarity in the number and position of the double bond on the oleic acid moiety. $KMnO_4$ oxidation produced mainly aldehyde as an oxidation product, native DOPC was no longer detectable. Long-chain products due to addition of O_2 molecules can be found on the MS spectra for $KMnO_4$ oxidation, leading to much thicker bilayers and less significant differences in electrical properties (Figs. 6 and 7). Oxidation with $FeCl_2/H_2O_2$ did result in a lower intensity of native DOPC peak and elimination of *sn*-2 acyl chain from DOPC molecule (m/z 504.3), therefore producing short-chain products, which resulted in the highest increase in specific conductance and capacitance for DOPC bilayers. Oxidation with air did not show statistically significant change between non-oxidized and oxidized bilayers.

When bilayers were built from PLPC lipids, oxidation with air caused a highest increase in membrane conductivity, which is surprising, since MS spectra (Fig. 5A) did not show many oxidation products; what is more, the increase in specific capacitance was not as high as conductivity increase. This led us to believe that not only the bilayer thickness but also the change in the dielectric coefficient due to oxidized lipid tails in the bilayer interior has a significant effect on the specific capacitance for PLPC lipids. With air oxidized PLPC lipids, two aldehydes were detected as well as a high peak for native PLPC lipids. Therefore, a stable bilayer was readily formed with lower lipid quantity, leading to a thinner bilayer with a fraction of integrated aldehydes. Therefore, the conductivity increase was high, while the small presence of oxidized species did cause an increase in the dielectric coefficient, and the difference in specific capacitance between non-oxidized and air oxidized PLPC is not as high. Oxidation with $KMnO_4$ and $FeCl_2/H_2O_2$ produced many oxidation products, mainly aldehydes and carboxylic acids. With $KMnO_4$ oxidation, native PLPC molecule was no longer detectable. However, a less significant increase in PLPC conductance (Fig. 6) was measured for $KMnO_4$ and $FeCl_2/H_2O_2$ oxidation compared to air oxidation. This could be explained by a larger lipid quantity need for formation of a stable PLPC bilayer in the presence of $KMnO_4$ and Fenton reagents, therefore increasing the thickness of the bilayer and lowering the conductivity for $KMnO_4$ and $FeCl_2/H_2O_2$ oxidation. On the other hand, the specific capacitance increase (Fig. 7) was the highest when $FeCl_2/H_2O_2$ was used to oxidized PLPC lipids, probably due to a large increase in dielectric coefficient.

For DLPC the Fenton reagents produced the highest amount of short-chain products, original DLPC was no longer detectable on the MS spectra (Fig. 5B) and consequently the highest increase in membrane conductance (Fig. 6), almost two-fold. The measurements of specific capacitance (Fig. 7) showed that there is no significant change between different oxidation protocols for DLPC lipids. Again, leading us to believe that bilayer thickness as well as dielectric coefficient affects the capacitance measurements. When lipids were oxidized using $KMnO_4$ or $FeCl_2/H_2O_2$, chemicals were introduced to the dry lipid film, therefore an ion or a molecule could be left over from the oxidizing chemicals to bind to the lipid. Particularly the $KMnO_4$ induced oxidation leads to the addition of one or two O_2 molecules. Addition of H_2O_2 may also occur. Consequently, not only short-chain but also long-chain products can be formed, leading to a much thicker bilayer with a lower specific capacitance. What is more, the breakdown voltage and lifetime of planar lipid bilayers decreased for highly unsaturated DLPC lipids oxidized with air, which again suggests that the presence of oxidized lipids renders the bilayer less stable. Among all of the lipid molecules studied, the highest change in the electrical properties, especially conductivity was measured for DLPC lipids, due to their high unsaturation and susceptibility to oxidation.

A drawback of our bilayer formation method is the unknown composition of oxidized bilayers. We cannot determine the degree of oxidized species present in the bilayers, only the detection of oxidation products on MS spectra prior to bilayer formation is possible. However, the results show that we are indeed able to detect differences in the electrical properties of non-oxidized and oxidized planar lipid bilayers. What is more, a so-called "folding method" could be used for bilayer formation, where a lipid droplet is deposited on the surface of the aqueous solution in the measurement chamber and bilayers are formed by rising the levels of the solution. With this method, the solvent effect becomes negligible and stable bilayers are formed faster since lipid monolayers form on the surface of the aqueous solution, prior to the bilayer formation. Furthermore, a smaller quantity of lipids is needed for bilayer formation; therefore, our conductivity results for non-oxidized bilayers might not be contradictory anymore. The down side of this method is that lipid molecules are exposed to air during the formation process, however, since our results show that air is not the strongest oxidant; the presence of oxygen from the atmosphere could be neglected in future experiments.

The results obtained can explain the long-lasting membrane permeability after electroporation. Since application of high voltage electric pulses can lead to oxidation of lipid molecules, the conductivity of the membrane increases, which means that not only hydrophilic pores, but also lipid oxidation plays a role in non-selective transport during and after electroporation. Oxidized lipid molecules have to be enzymatically repaired or replaced in order to maintain membrane structure and function [63], which takes time, and that can explain how the cell membrane permeability can be increased even up to several minutes after the application of electric pulses has ceased [11].

4. Conclusions

In our study, we were able to demonstrate that lipid oxidation leads to an overall increase in the planar lipid bilayer conductivity and specific capacitance, thus showing the possibility of increased membrane permeability due to lipid oxidation. This finding can also explain prolonged membrane permeability after the application of high-voltage electric pulses in the process of electropermeabilization. Once an electric field is applied, lipid membranes are under attack of ROS leading to lipid oxidation. Different functional groups, such as aldehydes, carboxylic acid and other short-chain products can be found in the tail regions of oxidized lipids as shown by mass spectrometry. This may lead to structural and chemical changes of the lipid bilayer, which can alter the membrane properties. We detected increased conductivity and specific capacitance of oxidized lipid bilayers compared to their non-oxidized counterparts which most probably occurs due to formation of polar regions in the previously hydrophobic interior of the bilayer. A significantly higher change in electrical properties was observed for lipids with multiple double bonds, which are more prone to oxidation and result in a variety of different oxidation products. Measurements of breakdown voltage and lifetime of the planar lipid bilayer however did not show significant differences, leading us to believe, that the oxidized bilayers retain their stability even under applied electric field.

CRediT authorship contribution statement

Katja Balantič: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. **Victor U. Weiss:** Methodology, Supervision, Writing – original draft, Writing – review & editing. **Ernst Pittenauer:** Methodology, Supervision, Writing – original draft, Writing – review & editing. **Damijan Miklavčič:** Supervision, Writing – original draft, Writing – review & editing. **Peter Kramar:** Conceptualization, Methodology, Supervision, Investigation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [Peter Kramar reports financial support was provided by University of Ljubljana Faculty of Electrical Engineering. Peter Kramar reports a relationship with University of Ljubljana Faculty of Electrical Engineering that includes: employment.].

Data availability

Data will be made available on request.

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4 Discussion

The work carried out in this thesis is presented in three papers published in international scientific journals. The results of each contribution are presented and discussed in detail in the papers; therefore, this section summarizes the results published in the papers and presents in more detail the results that have not yet been published.

4.1 Electroporation effects on cell membrane

Electroporation is a useful tool to manipulate the cell membrane permeability with different effects on membrane structure and stability. The first paper, titled *"The good and the bad of cell membrane electroporation"* (Balantič *et al.* 2021), presents the principles of electroporation and its applications. The paper begins with a description of the cell membrane structure, being a thin, sheet-like biological structure that surrounds every cell and forms a selective barrier between the intracellular and extracellular environments. Its main function is to keep the cell's components inside while preventing unwanted substances from entering the cell. At the same time, it enables the selective transport of vital nutrients into the cell and of waste products in the opposite direction.

Transport of molecules across the plasma membrane can be both passive and active [1]. Large and charged molecules such as proteins, nucleic acids, and synthetic drugs cannot pass cell membranes at all [125]. Numerous therapeutic molecules are of this type, and to get them into the cells where they function, various techniques have been developed to increase the permeability of the plasma membrane permeability.

Furthermore, the effects of the electric field on various cell membrane components such as phospholipid bilayer, proteins, and the cytoskeleton are described. Electroporation has been reported to induce lipid oxidation in bacteria, plant cells, and mammalian cells [8] as well as in liposomes composed of polyunsaturated phospholipids [22]. All reactive oxygen species (ROS), regardless of their origin, can lead to oxidation of lipids during electroporation [126]; however, because ROS are short-lived, only those that originate in close proximity to the cell membrane and have access to lipid tails will cause lipid damage. It has been shown that ROS oxidize only those parts of the membrane that are electropermeabilized [20]. In addition, electroporation of cell membranes affects membrane proteins to varying degrees, with the worst-case scenario leading to their inactivation by denaturation due to the local temperature increase induced by the electric pulses [127]. It was also shown that exposure of cells to electric pulses increased the conductance of transmembrane Na^+/K^+-ATPase and decreased transmembrane ionic currents through voltage-gated ion channels [8].

What is more, the application of electric pulses can compromise the integrity of the cytoskeleton. When cells are exposed to electric pulses, the network of microfilaments and microtubules can be disrupted [128]. These effects are voltage-dependent and reversible, as the cytoskeleton can recover completely within hours without significant loss of cell viability. Disruption of microfilaments has even been shown to protect the cell from death by external electric pulses [129]. Electroporation of vesicles with actin filaments has been shown to cause membrane rigidification, blocking any major deformation of the vesicles and preventing the formation of large membrane pores [130]. The mechanism of cytoskeletal disruption is thought to involve conformational changes and electromechanical processes, although this is not entirely clear to date [146].

In summary, we conclude that the challenge that remains to be solved in the context of cell membrane electroporation is to decipher the molecular mechanism behind the application of high-voltage pulses. Only through a thorough understanding of the processes at the molecular level will we arrive at reliable electroporation treatment protocols. In particular, the contribution of electrochemical reactions, the effects of released metal ions, and lipid oxidation on the cell membrane are far from being well understood.

4.2 Numerical model describing electrochemical reactions

Numerical models are a useful tool for better understanding of the electrochemical reactions that occur at the electrode-electrolyte interface during the application of high-voltage electric pulses. In the paper (submitted on July 17, 2023) *"In silico numerical model of aluminium and iron dissolution during electric pulse application for electroporation"*, we have developed a numerical model to describe the electrochemical reactions and metal dissolution at the electrode-electrolyte interface during the application of monophasic and bipahsic high-voltage pulses, thus for the first time providing a numerical model for *in silico* optimization of electroporation parameters and protocols with the possibility to use the electroporation pulse waveform as an input parameter to the model.

Experimental data of electrolyte contamination during electroporation was found in the study by Kotnik *et al.* They carried out a mass spectrometry analysis to determine the concentration of released metal ions, namely aluminium and iron ions, in dependence of different electroporation protocols. Both monophasic and biphasic electric pulses were used, with varying voltages from 0 V to 400 V for monophasic pulses and from 0 V to 280 V for biphasic pulses. A lower concentration of released aluminium and iron ions was measured when biphasic pulses where used compared to monophasic pulses.

A finite element method based software COMSOL Multiphysics 6.0 (Comsol Inc., Burlington, MA, USA) was used for all numerical computations. The electrochemical model consists of two electrodes, namely the anode and the cathode surrounded by the electrolyte, in our case NaCl. Using Nernst-Planck equations, we solved the problem for several variables, such as the concentration of individual ions (Na+, Cl-, H+, and OH-) and metal ions according to the material used for the electrode in the model. The solution domain to the problem is the electrolyte phase, where transport equations take place. There are two boundaries; the anode surface and the cathode surface, where reactions take place. In addition to the transport equations, kinetics of the electrode reactions were introduced at the anode and cathode boundary.

Electric potential was applied to the anode and cathode surface as an external

electric potential condition. Monophasic and biphasic electric pulses were used in the model. For eight monophasic pulses the pulse duration was 1 ms, frequency was 1 Hz. The electric pulse was obtained by subtracting two Heaviside functions using COMSOL's built in function. The pulse rise time was set to 0.001 ms. For eight biphasic pulses, each pulse was of 1 ms total duration (500 µs of positive polarity followed by 500 µs of negative polarity). For biphasic pulses the electric pulse was obtained in similar way to monophasic one.

The results show that Na^+ ions are depleted near the positive anode surface since it repels positive ions. At the cathode, the concentration of Na^+ ions increases as the positive ions move toward the negative electrode. In our model, there is no reaction for Na^+ ions; therefore, their concentration remains constant throughout the simulation. Negatively charged chloride ions move toward the positive anode and away from the negative cathode. In the simulation, Cl^- ions are reactants in the chlorine evolution reactions; therefore, their concentration decreases with time. Positive H^+ is a product of oxygen evolution reactions at the anode, while negative OH^- is a product of hydrogen evolution reactions at the cathode. Their concentration at the corresponding electrode surface increases. With the known concentration of H^+ and OH^- ions generated, we are also able to obtain a pH profile in the electrolyte domain as a function of the distance between the anode and cathode.

An increase in the concentration of released metal ions was observed with increasing voltage amplitude, for both electroporation protocols, i.e. monophasic and biphasic pulses. As expected, the highest concentration of released metal ions was observed for monophasic, 400 V pulses. Since the anode is positively charged, the concentration profile of positive metal ions moves from the anode to the negative cathode due to diffusion between pulses and electric migration during pulses. With biphasic pulses, the metal release was lower compared to monophasic pulses. In particular, it is five times lower for aluminium and almost four times lower for iron ions.

Good agreement between experimental data and numerical modelling results was found for voltages up to 160 V for monophasic pulses. However, as the voltage of the applied pulses increases, our numerical model apparently no longer adequately describes all the reactions that take place, which is why the concen-

tration of released metal ions is lower in the model than determined in the experiments. Discrepancies between numerical and experimental results at higher voltages could be due to additional electrochemical processes at the electrode-electrolyte interface that are not accounted for in the current numerical model. For example, the roughness of the electrode surface increases with the dissolution of metal ions, so that a larger surface area is exposed to the electrolyte during the experiments, possibly leading to a higher concentration of released metal ions. Interestingly, the modelled metal concentration agrees much better with the experimental results when biphasic pulses are used. The amplitude for biphasic pulses was modelled as a peak-to-peak value; therefore, the voltages between each electrode and the electrolyte were lower, resulting in a better correlation between numerical and experimental results.

Using the developed numerical model, we demonstrated that the pulse polarity and amplitude strongly affect the dissolution of metal ions. The model can be further used to study the optimal pulse parameters for high-voltage pulse applications to ensure lower electrolyte contamination and higher electrode stability in medicine, biotechnology and food industry. With the developed numerical model the optimization can be achieved in a fast and reliable way without the need for extensive experimental work.

4.3 The effect of metal ions on lipid membranes

In the paper *"Calcium ion effect on phospholipid bilayers as cell membrane analogues"* (Balantič *et al.* 2022), the effect of metal ions on phospholipid bilayers as *in vitro* cell membrane models was studied. Recent research suggests that metal ions influence the structure of the cell membrane, which is pivotal in the context of electroporation treatments, where the alteration of cell membrane structure is the main treatment mechanism. Metal ions can be already physiologically present in biological environment or as shown in Chapter 4.2., dissolved from the electrodes during high-voltage electric pulse application. As a result, investigating the influence of metal ions on the cell membrane becomes imperative for enhancing the safety and efficiency of electroporation treatments.

In order to study the effect of metal ions on phospholipid bilayers in a controlled environment, we decided to use two cell membrane models, liposomes and planar lipid bilayers. First, calcium was selected as the divalent cation for study because it is abundant in the biological environment and plays a key role in cell signalling. Moreover, calcium electroporation was proposed as a novel treatment in electrochemotherapy. Second, the effect of aluminium and iron ions on lipid membranes was also investigated (not yet published), since the electrodes used for electroporation treatments are usually made of aluminium or stainless steel i.e. an iron alloy. Metal ions in different concentrations were added to the external environment of the liposomes or to one side of the planar lipid bilayer. Metal ion concentrations used in our experiments were chosen based on previous studies of metal dissolution [19], [60]. The lower concentration values (10 mM) were obtained from the electrolyte contamination study by Kotnik *et al.* Higher concentrations (50 mM) of dissolved metal ions were measured in the study by Vižintin *et al.*

We measured the changes in phospholipid structure as a function of metal ion concentration, using two different measurement methods: Nano-electrospray gas-phase electrophoretic mobility molecular analyzer (nES GEMMA) for liposomes and measurements of the electrical properties of planar lipid bilayers. A change in liposome size due to metal ion addition was observed and quantified via gas-phase electrophoretic measurements using nES GEMMA. This technique utilizes the electrospray process to create aerosol sample droplets. Subsequently, drying of droplets and charge equilibration in a bipolar atmosphere occurs. Single-charged, surface-dried, polydisperse aerosol is then size-separated in a high laminar-sheath flow and a tunable electric field. By variation of the electric field strength, monomobile aerosol particles are obtained which are then assessed via particle number-based detection.

Furthermore, electrical measurements on planar lipid bilayers were carried out, to determine changes in the bilayer properties induced by metal ion addition. An impedance meter was used to measure the resistance and capacitance of the planar lipid bilayers. Employing a system developed in our laboratory, the breakdown voltage and lifetime of the planar lipid bilayers were measured as well. The electric resistance measures the specific property of the material, in our case the phospholipid bilayer, to resist electric current. Planar lipid bilayers are

good insulators with high electric resistance. Also, planar lipid bilayers have the ability to store electric charge as described by the measured electric capacitance. However, the measured capacitance depends on several factors, including the geometry and thickness of the bilayer and the dielectric coefficient of the bilayer. The addition of positively charged metal ions leads to changes in the structure of the planar lipid bilayers and, consequently, in the measured values of electric resistance and capacitance. Additionally, the breakdown voltage and lifetime of the planar lipid bilayers were measured with a system developed in our laboratory. The breakdown voltage represents the minimum voltage required to rupture the lipid bilayer and allow the passage of molecules or substances through the membrane. The breakdown voltage thus describes the strength of the membrane. The lifetime of a planar lipid bilayer, in turn, represents the time from the application of the voltage ramp to its rupture.

4.3.1 Calcium ions

Initially, we focused on liposomes as *in vitro* cell membrane analogues. In contrast to planar lipid bilayers, vesicles exhibit strong membrane curvature and are more similar to the biological cell. We observed a decrease in measured liposome size with increasing calcium concentration. The shrinkage of liposomes is likely due to a calcium ion-induced decrease in area per lipid. In addition, a calcium ion-dependent decrease in vesicle size could also be related to either tighter packing of the phospholipid moieties in the bilayer, resulting in a smaller vesicle size.

The electric properties of planar lipid bilayers, namely resistance and capacitance, were studied as a function of calcium ion concentration. A measurable increase in the resistance of the bilayer and a decrease in the specific capacitance of the bilayer were observed, which we attribute to an increase in the thickness of the planar lipid bilayer in the presence of calcium ions. A significant effect of divalent cations on the packing order of lipid molecules was also observed, expressed in an increase in resistance and a decrease in capacitance. The obtained results are consistent with a "rigidification" of planar lipid bilayers in the presence of divalent cations, which is due to the ordering of lipid molecules and a more densely packed lateral structure.

4.3.2 Aluminium ions

Aluminium ions are abundant in the environment and can also be dissolved from aluminium cuvettes during high-voltage pulse application. We therefore investigated the effect of Al^{3+} ions on lipid membranes using planar lipid bilayers as *in vitro* cell membrane models. The electric properties of the bilayers, namely resistance, capacitance, breakdown voltage, and bilayer lifetime, were measured as a function of metal ion concentration.

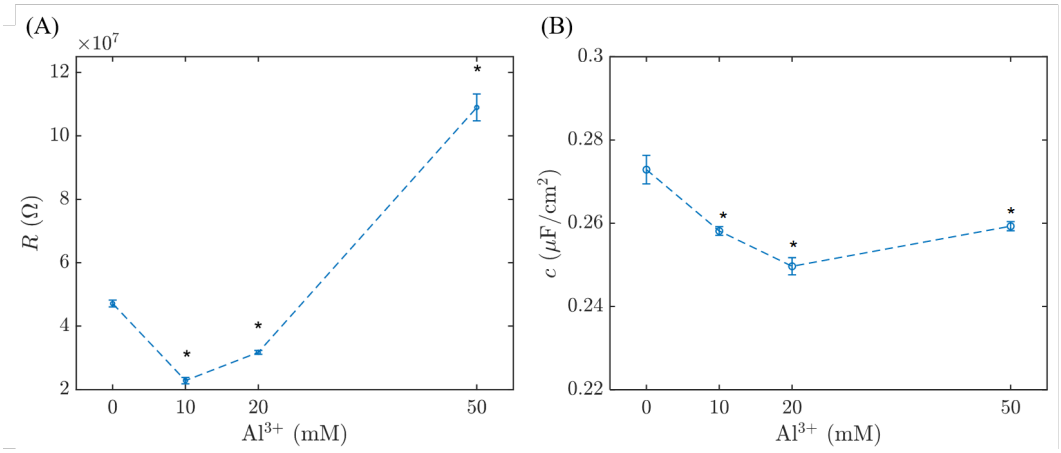

Figure 4.1: Electrical properties of planar lipids bilayers as a function of aluminium concentration. (A) Resistance and (B) specific capacitance of planar lipid bilayers were measured. Data are given as mean ± standard deviations (n = 10 measurements). Black asterisks (*) indicate statistically significant differences ($p \leq 0.05$) between 0 mM metal concentration and higher concentrations (10 mM, 20 mM, 50 mM).

When a low concentration (20 mM) of metal ions was added to the bilayers, the resistance and the specific capacitance decreased as the conductivity of the electrolyte increased due to the higher concentration of ions in the solution. When a higher metal concentration was used (50 mM), the resistance increased significantly due to rigidifcation of the bilayers. A slight decrease in specific capacitance was observed as well, most likely due to a thicker bilayer in the presence of metal ions.

Figure 4.1. shows the (A) resistance and (B) capacitance measurements for lipid bilayers in the presence of aluminium ions. The breakdown voltage and lifetime of planar lipid bilayers were also measured, and the results are presented in Figure 4.2 (A) and (B), respectively. With increasing aluminium concentration,

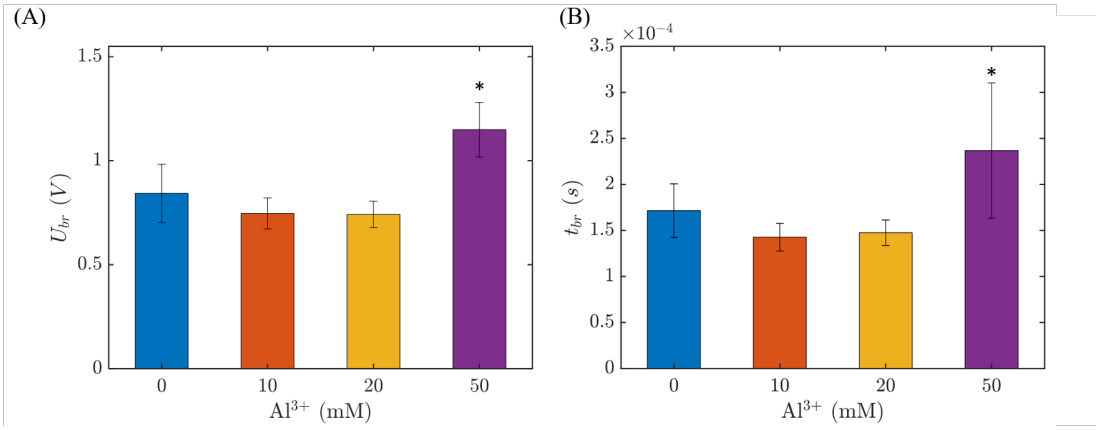

Figure 4.2: Breakdown voltage and lifetime of planar lipids bilayers in dependence of aluminium concentration. (A) U_{br} and (B) t_{br} of planar lipid bilayers were measured. Data are given as mean ± standard deviations (n = 10 measurements). Black asterisks (*) indicate statistically significant differences ($p \leq 0.05$) between 0 mM metal concentration and higher concentrations (10 mM, 20 mM, 50 mM).

the voltage needed for bilayer breakdown increased, and the bilayer lifetime was longer, leading us to believe that the presence of aluminium increases the thickness and rigidity of the bilayers.

4.3.3 Iron ions

The changes in the electrical properties of planar lipid bilayers due to the addition of iron ions are presented in Figure 4.3, resistance (A) and capacitance (B) were measured. An increase in resistance was observed at higher iron concentrations (50 mM), leading us to believe that the presence of iron ions rigidifies the bilayer. On the other hand, the capacitance decreased with a higher concentration of metal ions, since the thickness of the bilayer increased. The breakdown voltage and lifetime of planar lipid bilayers are presented in Figure 4.4 (A) and (B), respectively. As the iron ion concentration increased, the voltage needed for the breakdown of the bilayer increased and the lifetime of the bilayer before breakdown was longer.

Our results show that metal ions in high concentrations have a rigidification effect on lipid membranes. Therefore, the study of metal dissolution during elec-

troporation and the resulting effects that metal ions have on membrane structure is of great importance. Due to rigidification of the bilayer caused by metal ions, it is likely that the membranes will be less susceptible to electroporation. In addition, metal ions may also act as catalysts in the cascade of chemical reactions leading to lipid oxidation.

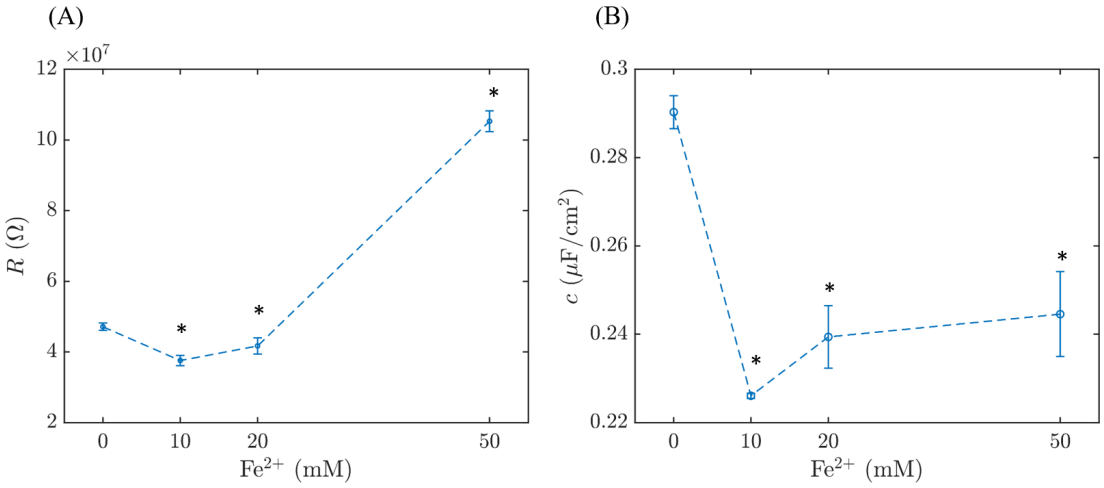

Figure 4.3: Electrical properties of planar lipids bilayers in dependence of iron concentration. (A) Resistance and (B) specific capacitance of planar lipid bilayers were measured. Data are given as mean ± standard deviations (n = 10 measurements). Black asterisks (*) indicate statistically significant differences ($p \leq 0.05$) between 0 mM metal concentration and higher concentrations (10 mM, 20 mM, 50 mM).

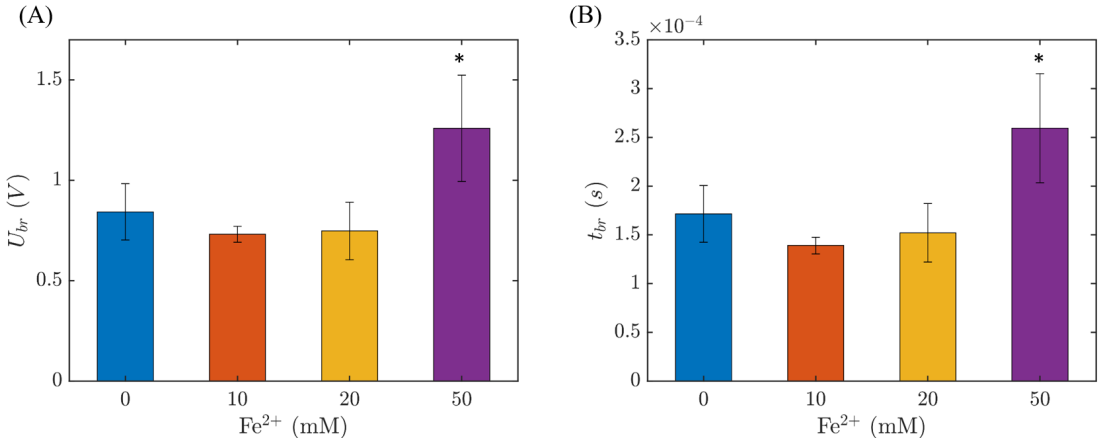

Figure 4.4: Breakdown voltage and lifetime of planar lipids bilayers in dependence of iron concentration. (A) U_{br} and (B) t_{br} of planar lipid bilayers were measured. Data are given as mean ± standard deviations (n = 10 measurements). Black asterisks (*) indicate statistically significant differences ($p \leq 0.05$) between 0 mM metal concentration and higher concentrations (10 mM, 20 mM, 50 mM).

4.4 The role of lipid oxidation on properties of planar lipid bilayers

The last paper, *"The role of lipid oxidation on electrical properties of planar lipid bilayers and its importance for understanding electroporation"* (Balantič *et al.* 2023) presents the study of lipid oxidation effects on the electrical properties of planar lipid bilayers. It has been shown before, that electroporation leads to lipid oxidation due to electrochemical reactions at the electrode-electrolyte interface and consequent formation of reactive oxygen species or through the destabilization of mitochondrial membranes. Nowadays, lipid oxidation is believed to be the cause of the long-lasting cell membrane permeability as observed in cell experiments, even after the application of electric pulse has ceased.

Lipid molecules with varying degrees of unsaturation were chemically oxidized using air, $KMnO_4$ and Fenton reagents ($FeCl_2$ and H_2O_2). Obtained oxidation products were analyzed using mass spectrometry. The most commonly found oxidation products were aldehydes. Planar lipid bilayers of either non-oxidized or oxidized lipid molecules were then prepared and their electrical properties were measured. Using a system developed in our laboratory we were able to measure

the resistance, capacitance, breakdown voltage and lifetime of non-oxidized and oxidized planar lipid bilayer.

We detected an increase in the conductivity and capacitance of lipid bilayers made from oxidized lipids compared to their non-oxidized counterparts, which led us to believe that oxidized bilayers are indeed more permeable than non-oxidized bilayers. A much higher conductivity of the oxidized planar lipid bilayers compared with the non-oxidized ones, suggests that lipid oxidation may be the cause of the increased membrane permeability after electroporation. The conductivity of the bilayer measures the ability of the electric current, i.e., ions, to pass through the bilayer. With increasing lipid oxidation, the core of the bilayer becomes more polar, due to the presence of oxidized functional groups and consequently more permeable. The thickness of the bilayer decreases due to formation of short-chain oxidation products. In addition, transient pores can form in the planar lipid bilayer due to the presence of oxidized species and the applied voltage, which can also increase the conductivity of the bilayer. The presence of oxidized species in the bilayer lowers the activation barrier for pore formation. This provides another possible interpretation for increased conductivity, which is not only due to a decrease in membrane thickness or a more hydrophilic interior of the bilayer, but also due to a lower membrane barrier potential in the presence of oxidized lipid molecules.

The capacitance of oxidized bilayers increases due to the formation of polar regions in a previously non-polar environment and the presence of short-chain oxidation products, resulting in an increase in the dielectric coefficient of the bilayer and a decrease in the thickness of the bilayer. However, for the same lipid molecules and oxidation protocols, the changes in capacitance are less pronounced than the changes in conductivity. It must be emphasized that the changes in bilayer structure due to the presence of oxidized species are complex. Oxidation-induced changes in the acyl chains can also lead to modifications in the head group region of lipid molecules, where the area per head is increased and functional groups resulting from oxidation are added to the interface. Furthermore, the appearance of long-chain oxidation products can increase the thickness of the bilayer. All of these effects can lead to smaller capacitance changes between non-oxidized and oxidized bilayers.

The measured breakdown voltage and lifetime for different bilayers proved not to be statistically significant. It is quite possible that our system for measuring breakdown voltage and lifetime is not sensitive enough to detect the differences between non-oxidized and oxidized lipid bilayers. However, it is surprisingly also possible that lipid oxidation does not play a critical role in the stability of planar lipid bilayers.

Overall, our results can explain the long-lasting membrane permeability after electroporation. It is known that the application of high-voltage electric pulses can lead to the oxidation of lipid molecules, therefore the conductance of the membrane increases, implying that not only hydrophilic pores but also lipid oxidation plays a role in non-selective transport during and after electroporation. Oxidized lipid molecules must be enzymatically repaired or replaced to maintain membrane structure and function, which takes time, and that can explain why cell membrane permeability can remain increased even up to several minutes after the application of electric pulses has ceased.

5 Conclusions

Electroporation is a useful tool for increasing the permeability of the cell membrane. The physicochemical processes involved in the application of high-voltage electric pulses are relatively well studied. However, several underlying processes remain unknown. The aim of this doctoral dissertation was to investigate some of these processes, namely electrochemical reactions at the electrode-electrolyte interface, dissolution of metals from the electrodes, and the effects of metal ions and lipid oxidation on the electrical properties of planar lipid bilayers.

A challenge in developing electroporation protocols is in predicting and optimising the factors that cause electrochemical reactions. One potential tool for optimising electroporation protocols is by modelling the reactions that occur near the electrodes. We have developed a numerical model that describes the dissolution of metal ions during electroporation, using different pulse waveforms as input parameters to the model. The developed numerical model consists of a two-dimensional geometry and was solved using COMSOL Multiphysics. Different pulse amplitudes and polarities were used in the model and the results were validated with experimental results of electrolyte contamination by Kotnik *et al.* [19] With higher voltages and with the use of monophasic pulses, the concentration of released metal ions increases. Overall, the developed and validated model can further be used to optimise parameters for high-voltage electric pulse application in medicine, biotechnology and food industry, without the need for extensive experimental work.

Further on, the effect of metal ions on different cell membrane models was investigated. First, biologically present calcium ions were studied because of their role in cell signaling and their use in calcium electroporation. Subsequently,

the effects of Al^{3+} and Fe^{2+} ions that can be released from the electrodes during electroporation, were investigated as well. Two *in vitro* cell membrane models were employed for the study, liposomes and planar lipid bilayers, and for the first time the combination of two instrumental setups; gas-phase electrophoresis and electrical measurements of planar lipid bilayers. We observed a decrease in liposome size due to the presence of metal ions. What is more, the presence of metal ions resulted in increased resistance and decreased capacitance of planar lipid bilayers. We showed that the addition of metal ions can change the structure of phospholipid bilayers, leading to bilayer rigidification. Metal ions bind to the phosphate group of phospholipid molecules, causing a conformational change in the lipid head group and consequently thicker bilayers. Therefore the study of metal ions effect on the cell membrane becomes imperative for enhancing the safety and efficiency of electroporation treatments.

Finally, the effects of lipid oxidation on the cell membrane permeability were studied. Planar lipid bilayers were used as *in vitro* cell membrane models to study lipid oxidation processes at the molecular level. First, phospholipid molecules were chemically oxidized and the oxidation products were detected by mass spectrometry. Subsequently, planar lipid bilayers were formed from non-oxidized or oxidized lipid molecules and their electrical properties such as conductivity, capacitance, breakdown voltage and lifetime were measured. We found an increase in membrane conductivity and capacitance due to the presence of oxidized species, leading us to believe that lipid bilayers actually become more permeable due to the presence of oxidized species. A much more pronounced change in electrical properties was observed for lipids with multiple double bonds, which are more susceptible to oxidation and lead to a variety of different oxidation products. The finding that the presence of oxidation products increases conductivity of planar lipid bilayers may explain the prolonged membrane permeability after electroporation.

Electroporation of lipid membranes has been studied and developed over the past 40 years. However, there are still challenges that need to be investigated to better understand the underlying mechanisms of high-voltage pulse application and to further improve electroporation-based treatments. A fundamental challenge that remains to be resolved is the description of the molecular mechanisms that occur during and after electroporation. Only a complete understanding of

these processes at the molecular level will unlock the full potential of electroporation and its reliable application. Preclinical and clinical studies have confirmed the great potential for electroporation-based treatments in cancer [147] and gene therapy [148] as well as in tissue ablation [133]. However, it is clear that there is room for further technical improvement to increase the precision and specificity of these treatments and to reduce or eliminate the side effects that sometimes occur. In this context, the processes that take place directly at the electrodes during electroporation need to be better controlled, for example the electrochemical reactions and metal dissolution. The metal ions released from the electrodes cause conformational changes in the lipid membrane and may also act as catalysts in the cascade of chemical reactions that cause lipid oxidation. What is more, the contribution of electric pulses to increased cell membrane permeability due to lipid oxidation and protein modifications is far from clear. In this regard, numerical models and experimental research explaining the processes during the application of high-voltage electric pulses are of great importance for a better understanding and improved safety of electroporation-based technologies and treatments.

6 Original scientific contributions

Development of numerical model to describe electrochemical reactions taking place at the electrode-electrolyte interface during electroporation.

Numerical models can predict the outcome, efficacy, and possible adverse effects of electroporation treatments. To this end, we have formulated a numerical model that describes the electrochemical processes involved in metal release from aluminum cuvettes and stainless steel electrodes using different pulse parameters. A two-dimensional numerical model was developed using COMSOL Multiphysics and validated with experimental results. Monophasic and biphasic pulses with varying amplitudes were used in the model and concentration profiles of dissolved metal ions were obtained showing that monophasic pulses and higher amplitudes increase the concentration of released metal ions. With the model, we are now able to further optimize electroporation protocols without the need for extensive experimental work since different pulse waveforms can be for the first time implemented as input parameters in the developed numerical model.

Experimental evidence that metal ions, which are physiologically present in our body or are released from the electrodes during electroporation affect the properties of lipid bilayers.

Various metal ions have a complex effect on lipid membranes, which we studied employing *in vitro* cell membrane models such as planar lipid bilayers and liposomes. The effects of metal ions were studied using gas-phase electrophoresis and electrical measurements of planar lipid bilayers. We concluded that metal ions added to lipid membranes are responsible for changes in the structure of the bilayer leading to decreased liposome size and higher resistance of planar

lipid bilayers. At high concentrations, metal ions can increase the rigidity of the phospholipid bilayer, due to electrostatic interactions between metal ions and charged phospholipid headgroups, leading to thicker bilayers by allowing them to be packed more densely. Due to the tighter packaging order of the phospholipids the voltage needed for the breakdown of the planar lipid bilayer is higher and lifetime of the bilayers is longer. Metal ions can also act as catalysts in lipid oxidation reactions and cause oxidative damage to lipid membranes. Therefore, investigating the influence of metal ions on the cell membrane becomes imperative for enhancing the safety and efficiency of electroporation treatments.

Experimental evidence that lipid oxidation causes measurable changes in electrical properties of planar lipid bilayers.

Lipid oxidation leads to chemical changes in lipid membranes and may explain the long-lasting membrane permeability after the application of high-voltage electric pulses. It is known that the electric field can accelerate the oxidation process by promoting the production of reactive oxygen species (ROS). The study of lipid oxidation is a challenging task, so we decided to use planar lipid bilayers to observe oxidation induced changes at the molecular level in a controlled environment. Lipid membranes, which are normally electric insulators, become more conductive in the presence of oxidized species and the resulting increased polarity of acyl chains. In addition, the capacitance of oxidized lipid bilayers increases due to thinner bilayers and the increased dielectric coefficient in the presence of oxidation-related functional groups. This finding may explain the prolonged membrane permeability after electroporation, as observed in experiments on cells.

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The role of lipid oxidation on electrical properties of planar lipid bilayers and its importance for understanding electroporation

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