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Electroporation with nanosecond pulses and bleomycin or cisplatin results in efficient cell kill and low metal release from electrodes

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ABSTRACT

Nanosecond electric pulses have several potential advantages in electroporation-based procedures over the conventional micro- and millisecond pulses including low level of heating, reduced electrochemical reactions and reduced muscle contractions making them alluring for use in biomedicine and food industry. The aim of this study was to evaluate if nanosecond pulses can enhance the cytotoxicity of chemotherapeutics bleomycin and cisplatin *in vitro* and to quantify metal release from electrodes in comparison to 100 μs pulses commonly used in electrochemotherapy. The effects of nanosecond pulse parameters (voltage, pulse duration, number of pulses) on cell membrane permeabilization, resealing and on cell survival after electroporation only and after electrochemotherapy with bleomycin and cisplatin were evaluated on Chinese hamster ovary cells. Application of permeabilizing nanosecond pulses in combination with chemotherapeutics resulted in successful cell kill. Higher extracellular concentrations of bleomycin – but not cisplatin – were needed to achieve the same decrease in cell survival with nanosecond pulses as with eight 100 μs pulses, however, the tested bleomycin concentrations were still considerably lower compared to doses used in clinical practice. Decreasing the pulse duration from microseconds to nanoseconds and concomitantly increasing the amplitude to achieve the same biological effect resulted in reduced release of aluminum ions from electroporation cuvettes.

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1. Introduction

Exposure of cells/tissue to pulsed electric fields allows transmembrane transport of otherwise impermeant molecules. This phenomenon of increased cell membrane permeabilization due to exposure to short electric pulses is called electroporation, sometimes termed also electropermeabilization or pulsed electric field treatment. The underlying mechanisms of electroporation have been recently reviewed by Kotnik et al. [1]. Electroporation with nanosecond pulses alleviates multiple limitations existing in conventional micro- and millisecond range electroporation. Nanosecond pulses penetrate the cell interior, having more profound effects on the organelles [2–7]; however, as was first theoretically predicted and later confirmed experimentally, the plasma membrane is also affected [8–10]. Permeabilization of the cell membrane to medium sized molecules like bleomycin [11] and siRNA [12], and more recently even the delivery of plasmids [13] was reported using nanosecond pulses. Nanosecond pulses electroporate cells of different sizes and shapes at similar thresholds [14,15]. Electroporation with nanosecond pulses showed induction of apoptosis and antitumor activity [16–23]. The use of pulses of high electric field strength but very short duration means that the energy transmitted by the pulses to the treated volume is very low and leads to a low level of heating, thus minimizing the possibility of thermal damage to tissue [2,24].

Electroporation with longer (i.e. in the micro- and millisecond range) pulses is used in various applications including transfection of cells/transformation of bacteria, extraction of biomolecules from cells, inactivation of microorganisms in water and liquid foods, tissue ablation and electrochemotherapy (ECT) [25–28]. ECT is a local treatment of cancer which combines the use of electric pulses delivered on the tumor area and some standard chemotherapeutic agents for which plasma membrane represents a barrier for reaching their intracellular target. ECT is being introduced into clinical practice based on extensive preclinical data, on its effectiveness on different tumors and on solid evidence of its mechanisms of action [29]. Several mechanisms of action have already been identified: increased membrane permeability and intracellular drug accumulation, vascular disruption, vascular lock and involvement of immune response; the dominant mechanisms being increased cellular uptake of non-permeant or low permeant anticancer drug with

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a very high intrinsic cytotoxicity due to exposure of cells or tumors to cell membrane-permeabilizing electric pulses [30]. Increased cytotoxicity of bleomycin and *cis*-diaminedichloroplatinum (II) (cisplatin), two most often used chemotherapeutics in ECT, was demonstrated *in vitro*, with several-fold potentiation [31,32] and confirmed *in vivo* on different animal tumor models and patients [33–37]. Despite the success of the therapy, some side effects were reported. According to the patients, the most unpleasant or even painful side effects were the sensations during the pulse delivery, which were mainly attributed to peripheral nerve stimulation and muscle contractions [38–43]. The applied electric pulses in ECT are most commonly delivered in trains of eight monophasic pulses of 100 μs duration with 1 Hz or 5 kHz pulse repetition rate. It seems that muscle contractions are significantly reduced when using nanosecond pulses compared to microsecond-long pulses [44,45], although more studies would be needed to further support this point, thus making the use of nanosecond pulses in electroporation-based applications in medicine like ECT compelling. The increased cytotoxicity of bleomycin after electroporation and consecutive cell death *in vitro* was already shown with 10 ns pulses [11] and Tunikowska et al. [46] have reported a case study of successful ECT in feline oral malignant melanoma using bleomycin and 15 ns pulses after CO_2 laser surgery. Novickij et al. [47] showed a comparable delay of tumor growth in mice treated with a combination of doxorubicin and eight 100 μs pulses or 250 800 ns pulses. However, until now, there are no reports of comprehensive exploration of the effects of various nanosecond pulse parameters on effectiveness of reversible electroporation combined with bleomycin or cisplatin which could aid in developing effective ECT protocols for use in the clinic.

In electroporation procedures, the electric field in cell suspension or tissue is usually established by delivering electric pulses through metallic electrodes in contact with the treated medium/tissue. During delivery of high-voltage electric pulses to cells in suspension or in tissue, electrochemical processes occur at the electrode–electrolyte interface, such as electrolysis, generation of radicals and release of metal ions from the electrodes which results in corrosion and fouling of the electrodes, release of the electrode material or/and chemical modification of the medium [48,49]. For research purposes, the use of aluminum cuvettes is common, where delivery of electroporation pulses causes also release of aluminum ions from the electrodes [50–53]. The release of aluminum ions has been associated with a change of the solution pH [52,53]. The released aluminum ions can precipitate nucleic acids and proteins [54,55] and affect the biochemistry of the electroporated cells [51]. It was confirmed experimentally that shortening of the pulse and/or use of biphasic pulses reduces electrochemical reactions [50,56–58]. However, shorter pulses necessitate the use of higher electric field strengths and/or higher number of pulses to achieve the same biological effect as with longer pulses [59]. It thus remains unclear whether shortening the pulse duration to nanoseconds with concomitantly increasing the voltage (and/or number of pulses used) still results in reduced electrochemical reactions.

The aim of our study was to evaluate *in vitro* if nanosecond electric pulses can enhance the cytotoxicity of bleomycin and cisplatin to a similar extent as eight 100 μs pulses commonly used in electrochemotherapy and to quantify the release of aluminum ions from electroporation cuvettes in comparison to eight 100 μs pulses.

2. Materials and methods

2.1. Electroporation set-up and pulse delivery

In cell experiments, a laboratory prototype pulse generator (University of Ljubljana), based on H-bridge digital amplifier with 1 kV MOSFETs (DE275-102N06A, IXYS, USA) [60] was used to deliver eight standard ECT monophasic rectangular pulses of 100 μs duration with 1 Hz repetition rate. In metal release experiments, the Cliniporator Vitae (IGEA, Italy) was used to deliver the eight 100 μs monophasic rectangular pulses with 1 Hz repetition rate. The CellFX System (Pulse Biosciences, California, USA) was used to deliver monophasic nanosecond rectangular pulses of 200, 400 or 550 ns duration (set duration on the pulse generator which fits well also with the full width at half maximum). We delivered 1, 25 or 100 nanopulses at repetition rate of 10 Hz. The voltage and the electrical current were monitored in all experiments. For microsecond pulses, the oscilloscope WaveSurfer 422, 200 MHz, high-voltage differential voltage probe ADP305 and current probe CP030, CP031A or CP150 (all from Teledyne LeCroy, New York, USA) was used. For nanosecond pulses, the current was measured by Pearson current monitor model 2878 (1 V/10 A, 70 MHz) and the voltage was measured by 1 kΩ resistor and Pearson current monitor model 2877 (1 V/1 A, 200 MHz) (all from Pearson Electronics, California, USA); the signals were monitored by the oscilloscope WaveSurfer 3024Z, 200 MHz (Teledyne LeCroy). The electric field strength (E) was calculated by dividing the measured voltage with the distance between the electrodes (i.e. the size of the gap of the electroporation cuvette). The waveforms of a 200 ns and 100 μs pulse are shown in Fig. 1. The energy delivered by the electric pulses was estimated by multiplying the pulse duration and number of pulses with the measured amplitude of the voltage and measured amplitude of the electric current. The cell suspension in the electroporation cuvettes was at room temperature during pulse delivery. Temperature rise was measured by the fiber optic temperature sensor OTG-M170 and ProSens signal conditioner (both OpSens, Canada), The sensor was placed inside a 2 mm or 4 mm gap electroporation cuvette (VWR, Pennsylvania, USA) filled with

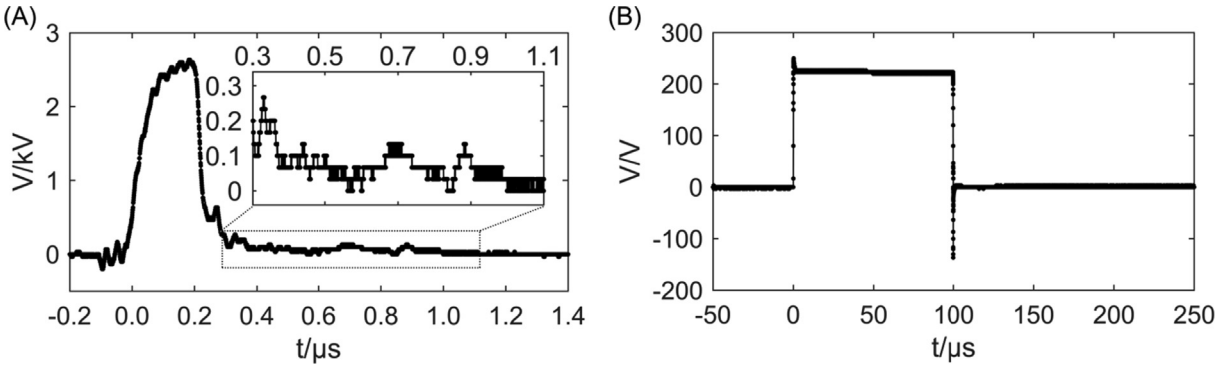

Fig. 1. Waveforms of pulses used in the study: (A) 200 ns, electric field strength of 6.1 kV/cm, and (B) 100 μs, electric field strength of 1.1 kV/cm. (A) the amplitude of the 200 ns pulse from 0.3 to 1.1 μs is zoomed in to show the reflected waves.

cell suspension and temperature was measured before, during and after delivery of electric pulses.

2.2. Cell culture

CHO-K1 Chinese hamster ovary cell line, obtained directly from the European Collection of Authenticated Cell Cultures (ECACC, cat. no. 85051005, mycoplasma free), was grown in Nutrient Mixture F-12 Ham (cat. no. N6658, Sigma-Aldrich, Missouri, United States) supplemented with 10% fetal bovine serum (FBS, cat. no. F9665, Sigma-Aldrich), 1.0 mM L-glutamine (cat. no. G7513, Sigma-Aldrich), 1 U/ml penicillin/streptomycin (cat. no. P0781, Sigma-Aldrich) and 50 μg/ml gentamycin (cat. no. G1397, Sigma-Aldrich) for 2–4 days at 37 °C in a humidified, 5% CO_2 atmosphere. On the day of the experiment, cells were detached with 1 × trypsin-EDTA (cat. no T4174, Sigma-Aldrich) diluted in 1 × Hank's basal salt solution (cat. no. H4641, Sigma-Aldrich). Trypsin was inactivated by Dulbecco's Modified Eagle Medium (DMEM, cat. no. D5671, Sigma-Aldrich) supplemented with 10% FBS (cat. no. F9665, Sigma-Aldrich), 2.0 mM L-glutamine, 1 U/ml penicillin/streptomycin and 50 μg/ml gentamycin (used in this composition through all experiments). Cells were transferred to a 50 ml centrifuge tube and centrifuged 5 min at 180*g* and 23 °C. The supernatant was aspirated, and cells were re-suspended at a cell density of 4×10^6 (for cell survival and cell membrane permeabilization experiments after electroporation) or 4.2×10^6 (for ECT experiments) cells/ml in the complete growth medium DMEM which was used as electroporation medium. Conductivity of complete growth media F-12 Ham and DMEM were measured at room temperature with conductometer MA 5950 (Metrel, Slovenia).

2.3. Cell membrane permeabilization and resealing

To determine the percentage of permeabilized cells at different electric field strengths, the suspension of CHO cells was mixed with YO-PRO-1 iodide (cat. no. Y3603, Thermo Fisher Scientific, Massachusetts, USA) to final concentration of 1 μM right before application of electric pulses. 600 μl (for treatment with nanopulses) or 150 μl (for treatment with 8 × 100 μs pulses) of the cells–YO-PRO-1 mixture was transferred in an electroporation cuvette with 4 mm (nanopulses) or 2 mm (8 × 100 μs pulses) gap, followed by pulse application. 20 μl of the treated cell suspension was transferred to a new 1.5 ml microcentrifuge tube. Three minutes after the last pulse, 150 μl of complete growth medium DMEM was added. To determine the time needed for cell membrane resealing, 600 μl (for treatment with nanopulses) or 150 μl (for treatment with 8 × 100 μs pulses) of suspension of CHO cells was transferred in an electroporation cuvette with 4 mm (nanopulses) or 2 mm (8 × 100 μs pulses) gap, followed by pulse application. 10 μl of the treated cell suspension was transferred to 13 new 1.5 ml microcentrifuge tubes. Every 2 min after the last pulse (until 26 min after the last pulse), 1 μl of 0.01 mM YO-PRO-1 in complete growth medium DMEM was added to the cell suspension in one of the tubes and 3 min after addition of YO-PRO-1, 150 μl of complete growth medium DMEM was added to the cell suspension. Cell suspensions from both cell membrane permeabilization and resealing experiments were gently vortexed before analysis on the flow cytometer Attune NxT (Thermo Fisher Scientific). Cells were excited with a blue laser at 488 nm, and the emitted fluorescence was detected through a 530/30 nm band-pass filter. The measurement was stopped when 10,000 events were acquired. The obtained data were analyzed using the Attune NxT software (Thermo Fisher Scientific). Single cells were separated from all events by gating. The percentage of cells with permeabilized cell membrane and median YO-PRO-1 fluorescence was determined from the histogram of YO-PRO-1 fluorescence. The experiments were repeated 3–4 times per each pulse protocol. The sham control was handled in the same way as the samples with the exception that no pulses were delivered.

2.4. Cell survival after electroporation

Just before pulse delivery, 600 μl (for treatment with nanopulses) or 150 μl (for treatment with 8 × 100 μs pulses) of suspension of CHO cells in complete growth medium DMEM was pipetted in an electroporation cuvette with 4 mm (nanopulses) or 2 mm (8 × 100 μs pulses) gap. The electroporation cuvettes were placed in the cuvette holder and electric pulses were applied (for the sham control no pulses were applied). Then, the cell suspension (20 μl) was transferred to a new 1.5 ml microcentrifuge tube and 25 min after the last pulse, complete growth medium F-12 Ham (380 μl) was added. The cell suspension was gently vortexed and plated (100 μl) in a well of a flat bottom 96-well plate in three technical replicates. Cells were kept at room temperature outside the incubator for the duration of the whole experiment (approximately one hour). The plate was incubated at 37 °C in a humidified, 5% CO_2 atmosphere. Cell survival was assessed by MTS assay. After 24 h of incubation, 20 μl of The CellTiter 96 AQueous One Solution Cell Proliferation Assay (cat. no. G3580, Promega, Wisconsin, USA) was added per well of the 96-well plate. After 2 h 35 min of incubation at 37 °C in a humidified, 5% CO_2 atmosphere, absorbance at 490 nm was measured with the spectrofluorometer Infinite 200 (Tecan, Austria). The survival was calculated by first subtracting the absorbance of the blank (5 μl of complete growth medium DMEM and 95 μl of complete growth medium F-12 Ham) and then normalizing the average absorbance of the three technical replicates of the sample to the absorbance of the sham controls. The experiments were repeated 3–5 times per each pulse protocol.

2.5. Cell survival after electrochemotherapy

Bleomycin sulphate (Medac, Germany) was diluted in water to concentration of 2 mM, aliquoted and stored at −20 °C. On the day of experiment, an aliquot was thawed and diluted in saline solution to prepare working solutions that after addition to the cell suspension resulted in final concentrations of 1 nM, 5 nM, 10 nM, 20 nM, 40 nM, 60 nM, 80 nM 100 nM, 140 nM, 200 nM, 300 nM, 400 nM or 500 nM bleomycin. Cisplatin (Cisplatin Kabi, 1 mg/mL, Fresenius Kabi, Germany) was diluted in saline solution on the day of the experiment to prepare working solutions that after addition to the cell suspension resulted in final concentrations of 10 μM, 30 μM or 50 μM cisplatin. 600 μl (nanopulses) or 165 μl (8 × 100 μs pulses) of the cell suspension was pipetted in 1.5 ml microcentrifuge tubes. Just before electroporation, 32 μl (nanopulses) or 8.8 μl (8 × 100 μs pulses) of appropriate dilution of the drug in saline solution (for control without drug: saline solution only) was added so that the final cell concentration was 4×10^6 cell/ml. Then, 600 μl (nanopulses) or 150 μl (8 × 100 μs pulses) of the cells–drug mixture was transferred in an electroporation cuvette with 4 mm (nanopulses) or 2 mm (8 × 100 μs pulses) gap. 7.5 μl (for MTS assay) or 5 μl (for clonogenic assay) from the remaining cells–drug mixture was transferred to a new 1.5 ml microcentrifuge tube for non-electroporated control. For clonogenic assay, the rest of the cells–drug mixture was mixed with trypan blue and counted with Countess Automated Cell Counter (Invitrogen, Thermo Fisher Scientific) following manufacturer's instructions. Electric pulses were applied to the electroporation cuvettes and 7.5 μl (for MTS assay) or 5 μl (for clonogenic assay) of the electroporated cells was pipetted into a new 1.5 ml microcentrifuge tube. Complete growth medium F-12 Ham (600 μl for MTS assay or 495 μl for clonogenic assay) was added to the electroporated cells and non-electroporated controls 25 min after pulse delivery. Cells were kept at room temperature

outside the incubator for the duration of the whole experiment (approximately one hour). For MTS assay, the cell suspension (110 μl) was plated in a well of a flat bottom 96-well plate in three technical replicates. The plate was incubated at 37 °C in a humidified, 5% CO_2 atmosphere. After 72 h, CellTiter 96 AQueous One Solution Cell Proliferation Assay (22 μl) was added per well of the 96-well plate. The plate was incubated at 37 °C in a humidified, 5% CO_2 atmosphere and after 2 h 35 min, absorbance at 490 nm was measured with the spectrofluorometer Infinite 200. The survival was calculated by first subtracting the absorbance of the blank (110 μl of complete growth medium F-12 Ham) and then normalizing the average absorbance of the three technical replicates of the sample to the absorbance of the non-electroporated controls without the drug. For clonogenic assay, the cell suspension was additionally diluted in growth medium Ham F12 to achieve the desired cell concentration and 2.5 ml was plated in a well of a 6-well plate in three technical replicates. The plates were incubated at 37 °C in a humidified, 5% CO_2 atmosphere. After 7 days of incubation, the growth medium was aspirated and 1 ml of 0.2% crystal violet in 80% methanol (both from Sigma-Aldrich, Merck, Germany) was added to fix and stain the colonies. After 10 min, the crystal violet–methanol mixture was removed and the plates were rinsed in water. Average plating efficiency from the 3 technical replicates was calculated by dividing the number of counted colonies (colonies containing < 50 cells were disregarded) with the number of plated cells. Surviving fraction was calculated by dividing the plating efficiency with the plating efficiency of the untreated control (non-electroporated cells without the drug). The experiments were repeated 3–6 times per pulse protocol. Integrated modulation contrast (IMC) micrographs of cells were taken 72 h after exposure to the electric pulses and/or drug with DM IL LED (Leica, Germany) inverted microscope using 10 × objective.

2.6. Metal release

In contrast to cell experiments where DMEM growth medium was used as electroporation medium, pure NaCl solution was used in metal release experiments because it allowed us to detect very low concentrations of aluminum ions. Electroporation cuvettes with 4 mm gap were filled with 600 μl of 0.9% (w/v) NaCl in water solution, prepared from water for ultratrace analysis (cat. no. 14211, Sigma-Aldrich) and 99.999% pure NaCl (cat. no. 204439, Sigma-Aldrich). After application of electric pulses (no pulses were applied for the sham control), the treated 0.9% NaCl solution (0.5 ml) was transferred to a new 15 ml centrifuge tube and 1.25 μl of 65% HNO_3 (Merck) was added. Experiments were performed in four replicates. For reagent blanks, 65% HNO_3 (5 μl) was added to 2 ml of 0.9% NaCl solution in a 15 ml centrifuge tube. Samples were kept at 4 °C until analysis. Al content in samples was determined by inductively coupled plasma mass spectrometry (ICP-MS). Prior to ICP-MS analysis, the samples were diluted with MilliQ water (18.2 MΩ cm obtained from a Direct-Q 5 Ultrapure water system, Merck Millipore, Massachusetts, USA). Quantification of Al by ICP-MS was performed based on external calibration by measuring Al standards in the concentration range of 0.1 – 1000 μg/l with online internal standardization (25 μg/l solution of Sc, Ge, Y, Rh, In, Ir and Bi). Calibration standard solutions were prepared from Al stock solution (1000 mg/l in 5% HNO_3 obtained from Merck). Surface (SPS-SW1) water reference materials, supplied by Spectrapure Standards AS (Norway), were used to assess the accuracy of determinations. For ICP-MS analysis, an Agilent 7900 ICP-MS (Agilent Technologies, California, USA) equipped with an auto sampler (SPS4, Agilent Technologies) was used. Optimization of instrumental parameters (summarized in Table S1 in Supplementary Material) was performed on daily basis in order to achieve satisfactory sensitivity and low levels of oxides and doubly charged ions.

2.7. Statistical analysis

Membrane resealing data were analyzed with the nonparametric Kruskal–Wallis test because the assumption of equal variances (tested by Levene's median test at $\alpha = 0.05$) was not met. P-values were adjusted with the post-hoc Holm method test ($\alpha = 0.05$). The normalized survival after ECT measured by MTS was compared to the untreated control with one sample *t*-test ($\alpha = 0.05$), survival after ECT measured by the clonogenic assay was compared to the untreated control with Welch's *t*-test ($\alpha = 0.05$). The concentration of released Al after delivery of each pulse protocol was compared to the sham control or to the measured concentration after delivery of 8 × 100 μs pulses with Welch's *t*-test because of unequal variances (tested by F-test). The relative standard deviation was obtained by multiplying the standard deviation by 100% and dividing this product by the average. Data were processed and visualized using Microsoft Excel (Microsoft, Washington, USA), MATLAB R2020a (MathWorks, Massachusetts, USA) and R 3.5.2 [61].

3. Results and discussion

3.1. Cell membrane permeabilization and cell survival after electroporation

To determine the optimal parameters for electrochemotherapy (i.e. highest cell survival and highest cell membrane permeabilization in terms of fraction of permeabilized cells), cell membrane permeabilization and cell survival of CHO cells was measured after exposure to classical 8 × 100 μs pulses and nanopulses at different electric field strengths (Fig. 2 and Fig. S1 in Supplementary Material). Growth medium was not added to cells right after the electroporation but after an incubation period of 25 min at room temperature to allow cell membrane resealing before plating/analyzing the electroporated cells. For delivery of nanopulses, electroporation cuvettes with 4 mm gap filled with 600 μl of sample at room temperature were used to match the CellFX generator and the load in terms of reflections [23]. Delivery of 8 × 100 μs pulses to electroporation cuvettes with 4 mm gap filled with 600 μl of sample would have resulted in an electric current out of the safe operation area of the microsecond pulse generator at amplitudes required for electroporation. Therefore electroporation cuvettes with 2 mm gap filled with 150 μl of sample were used with the microsecond pulse generator.

CHO cells where cultivated in the Ham F-12 growth medium (which is the recommended culture medium for this cell line), however, delivering pulses of higher amplitudes to electroporation cuvettes filled with Ham F-12 medium resulted in arcing. Arcing was not detected when DMEM growth medium was used to deliver pulses of same amplitudes and was thus selected as electroporation medium for cell experiments. Although both media are highly conductive (the conductivity at room temperature was 13.8 mS/cm for Ham F-12 and 14.2 mS/cm for DMEM), resulting in electric currents in the range of tens and even hundreds of amperes, the measured temperature increase after pulse delivery was very small, < 3 °C. Cell death due to thermal damage or effect of temperature increase on membrane permeabilization is thus highly unlikely.

The proportion of cells with permeabilized cell membrane increased with increasing the electric field strength and for shorter pulses, higher electric field strengths were needed to reach the same level of cell membrane permeabilization as with longer pulses; and with increasing the number of pulses, lower electric

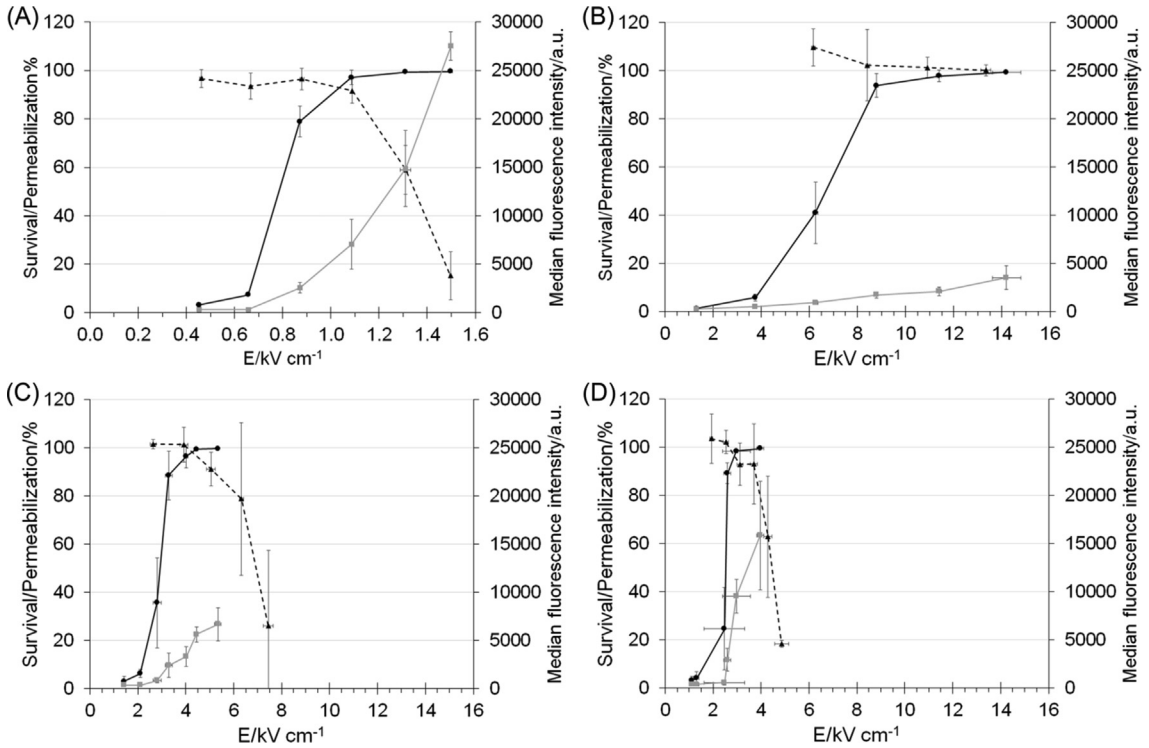

Fig. 2. Cell survival (triangles, dashed black line), cell membrane permeabilization rate (circles, solid black line) and median fluorescence intensity of YO-PRO-1 (squares, solid grey line) at different electric field strengths (E) after delivery of (A) 8 × 100 μs pulses, 1 Hz repetition rate, (B) 1 × 400 ns pulse, (C) 25 × 400 ns pulses, 10 Hz repetition rate, (D) 100 × 400 ns pulses, 10 Hz repetition rate. Note the different scale for (A) 8 × 100 μs pulses. Bars represent standard deviation. Survival and permeabilization curves for other pulse parameters are on Fig. S1 in Supplementary Material.

field strengths were required to achieve the same effect – in accordance with expectations [59].

The optimal values of the electric field strength for the 8 × 100 μs pulses, 1 Hz repetition rate, was determined to be 1.1 kV/cm, which is comparable to previous studies [62,63]. The optimal electric field strengths for the nanopulse treatments were higher and are given in Table 1. We noticed a sample to sample variation in the measured amplitude and electric current on the load when delivering nanopulses with the same amplitude set – the variations were larger at higher voltages. When setting the voltage, which was determined to be the maximal voltage in the safe operation of the CellFX generator, the average calculated electric field strength resulted to be 12.6 kV/cm for 200 ns pulses but 13.2 kV/cm for 400 and 500 ns pulses (Table 1). These differences in the voltage on the load, along with some other possible variations e.g. in the geometry of the electroporation cuvettes, are a source of variation between samples because when a certain voltage was set, the cells were not always exposed to exactly the same electric field.

Table 1
Experimentally determined optimal values of the electric field strength (E) for electroporation.

Pulse parameters	Optimal value of E (kV/cm)
8 pulses of 100 μs, 1 Hz repetition rate	1.1
1 pulse of 200 ns	12.6
1 pulse of 400 ns	13.2
1 pulse of 550 ns	13.2
25 pulses of 200 ns, 10 Hz repetition rate	6.1
25 pulses of 400 ns, 10 Hz repetition rate	3.9
25 pulses of 550 ns, 10 Hz repetition rate	3.1
100 pulses of 200 ns, 10 Hz repetition rate	4.3
100 pulses of 400 ns, 10 Hz repetition rate	2.5
100 pulses of 550 ns, 10 Hz repetition rate	2.5

With increasing the electric field strength, high (i.e. > 99%) cell membrane permeabilization was achieved for all pulse treatments except for 1 pulse of 200 ns; this pulse treatment resulted in 85% cell membrane permeabilization at 12.6 kV/cm (which was previously determined to be the highest electric field strength within the safe operation range of the CellFX pulse generator). For 1 pulse of 200, 400 or 500 ns we did not observe a decrease in cell survival compared to the sham control even at the highest electric field strength, but when using 25 or 100 pulses, the survival decreased at higher electric field strengths. When increasing the number of pulses, the survival started decreasing before the cell membrane permeabilization reached its maximum. For applications based on irreversible electroporation (e.g. tissue ablation), using more pulses of lower electric field strengths might thus be more adequate. However, if the goal is to achieve reversible electroporation (like in ECT), the use of fewer pulses at higher electric fields strengths gives a wider window in which cells are reversibly electroporated.

Increasing the electric field strength above the value that permeabilized > 99% of cell resulted in increased median fluorescence of YO-PRO-1, indicating that at higher electric field strengths, more molecules of the dye entered the cells. As can be seen from Fig. 2 and Fig. S1 in Supplementary Material, the lowest fluorescence intensity was measured with nanosecond pulse protocols with only one pulse – the median fluorescence intensity did not exceed 5 000 a.u. even when applying the maximum voltage; in case of one 200 ns pulse, the maximum measured fluorescence intensity was even bellow 1 500 a.u. At the electric field strength determined to be optimal for ECT, the highest YO-PRO-1 fluorescence intensity was measured for the 8 × 100 μs pulse protocol.

3.2. Cell membrane resealing

Cell membrane resealing was determined by measuring the percentage of permeabilized cells every 2 min from 2 min to

26 min after electroporation with pulses at the optimal electric field strength for ECT, as determined in previous cell survival and cell membrane permeabilization experiments (Table 1). For nanosecond pulse treatments, the time needed for membrane resealing was similar or longer as for the 8 × 100 μs pulses (for which the membrane reseals in < 5 min). After electroporation with twenty-five 200 ns, 400 ns or 550 ns pulses, one 400 ns or 550 ns pulse or one hundred 550 ns pulses, the membrane needed a significantly longer time (> 10 min) to reseal (Fig. 3). Electroporation with nanosecond pulses has been shown to degrade the microtubule network structure and attenuate lysosome movement in calcium-containing and calcium-free solution [64,65]. Since lysosomes are involved in some of the key cell membrane repair mechanism (membrane patching and endocytosis-mediated pore removal) [66,67], modulation of lysosome transport or even direct damage to the lysosomes caused by nanosecond pulses could interfere with the cell membrane repair resulting in longer membrane resealing time.

3.3. Electrochemotherapy with bleomycin and cisplatin

Cytotoxicity of bleomycin and cisplatin was assessed using the MTS assay and for selected parameters also using clonogenic assay. Our results demonstrate that pulses in the nanosecond range are suitable for use in ECT because they increase the bleomycin and cisplatin cytotoxicity severalfold (Figs. 4 and 5). No decrease in cell survival was detected for non-electroporated cells incubated with bleomycin in the range of concentrations used, however, a decrease in survival was observed for non-electroporated cells incubated for 25 min with 50 μM of cisplatin. Higher extracellular concentrations of bleomycin were needed to reduce the cell survival to the same extent as with the standard 8 × 100 μs pulses. The tested extracellular bleomycin concentrations are, however, still considerably lower compared to the therapeutic doses used in clinical practice. Kosjek et al. [68] measured the concentration of bleomycin in the serum of patients with a head and neck cancer who were treated with electrochemotherapy. The determined concentration of bleomycin in the serum was around 2000 nM at 5 min and 900 nM at 40 min after systemic injection of the therapeutic dose (15,000 IU m^{-2}). On the other hand, pulse duration did not have a significant effect on the potentiation of cytotoxicity of cisplatin. The differences between the measured cell survival after electrochemotherapy with bleomycin and cisplatin might be a consequence differences in size, cellular uptake and mechanisms of action of the two drugs. In previous reports, the cytotoxicity of bleomycin is potentiated several hundred-fold or even thousand-fold by ECT, while that of cisplatin up to ten-fold [30]. The plasma membrane dramatically limits the number of bleomycin molecules reaching the cell interior. In the absence of electric pulses, bleomycin molecules penetrate the cells by a receptor-mediated endocytosis [69], while cisplatin enters mainly by passive diffusion through the cell membrane [70]. Bleomycin (ca. 1500 Da) is bigger than cisplatin (ca. 300 Da) and pores produced by nanosecond pulses are believed to be smaller compared to pores produced by longer electroporation pulses [9,71,72] – thus at the same extracellular bleomycin concentration, more bleomycin molecules might have entered into cells through the pores produced by the 8 × 100 μs pulses than through nanopulse-induced pores because the microsecond pulses produced more pores through which bleomycin could enter.

The survival of cells exposed to 8 × 100 μs pulses, 1 Hz repetition rate at 1.1 kV/cm and 25 × 400 ns pulses, 10 Hz at 3.9 kV/cm at different bleomycin concentrations was also determined with the clonogenic assay. For cells electroporated with the micro- or nanosecond pulse protocol in the presence of bleomycin or cisplatin, lower survival was determined by the clonogenic assay as with MTS assay which is in agreement with Jakštys et al. [73] who used among others the MTT assay (a colorimetric assay in principle similar to the MTS assay) and the clonogenic assay. In our study, the survival determined by the clonogenic assay decreased to around 1% when cells where

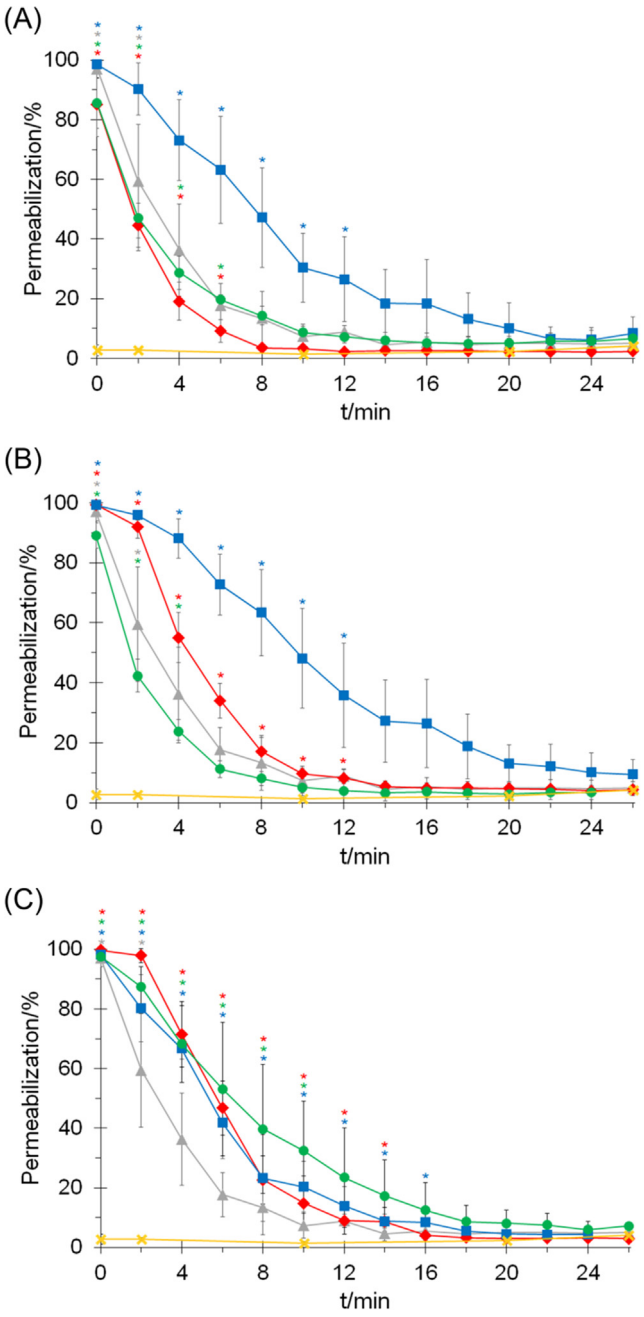

Fig. 3. Cell membrane permeabilization after different time of YO-PRO-1 addition after electroporation with (A) 200 ns pulses, (B) 400 ns pulses, (C) 550 ns pulses, (A, B, C) 8 × 100 μs pulses delivered at 1.1 kV/cm and 1 Hz repetition rate (triangle, grey) and sham control (cross, amber). (A): 1 × 200 ns pulse at 12.9 kV/cm (diamond, red), 25 × 200 ns pulses at 6.3 kV/cm, 10 Hz repetition rate (square, blue), 100 × 200 ns pulses at 4.3 kV/cm, 10 Hz repetition rate (circle, green). (B): 1 × 400 ns pulse at 13.2 kV/cm (diamond, red), 25 × 400 ns pulses at 3.9 kV/cm, 10 Hz repetition rate (square, blue), 100 × 400 ns pulses at 3.0 kV/cm, 10 Hz repetition rate (circle, green). (C): 1 × 550 ns pulse at 13.2 kV/cm (diamond, red), 25 × 550 ns pulses at 3.2 kV/cm, 10 Hz repetition rate (square, blue), 100 × 550 ns pulses at 2.5 kV/cm, 10 Hz repetition rate (circle, green). Bars represent standard deviation, asterisks (*) represent statistically significant ($P < 0.05$) difference from baseline (i.e. the lowest measured permeabilization) determined by the Kruskal–Wallis test with post-hoc Holm method.

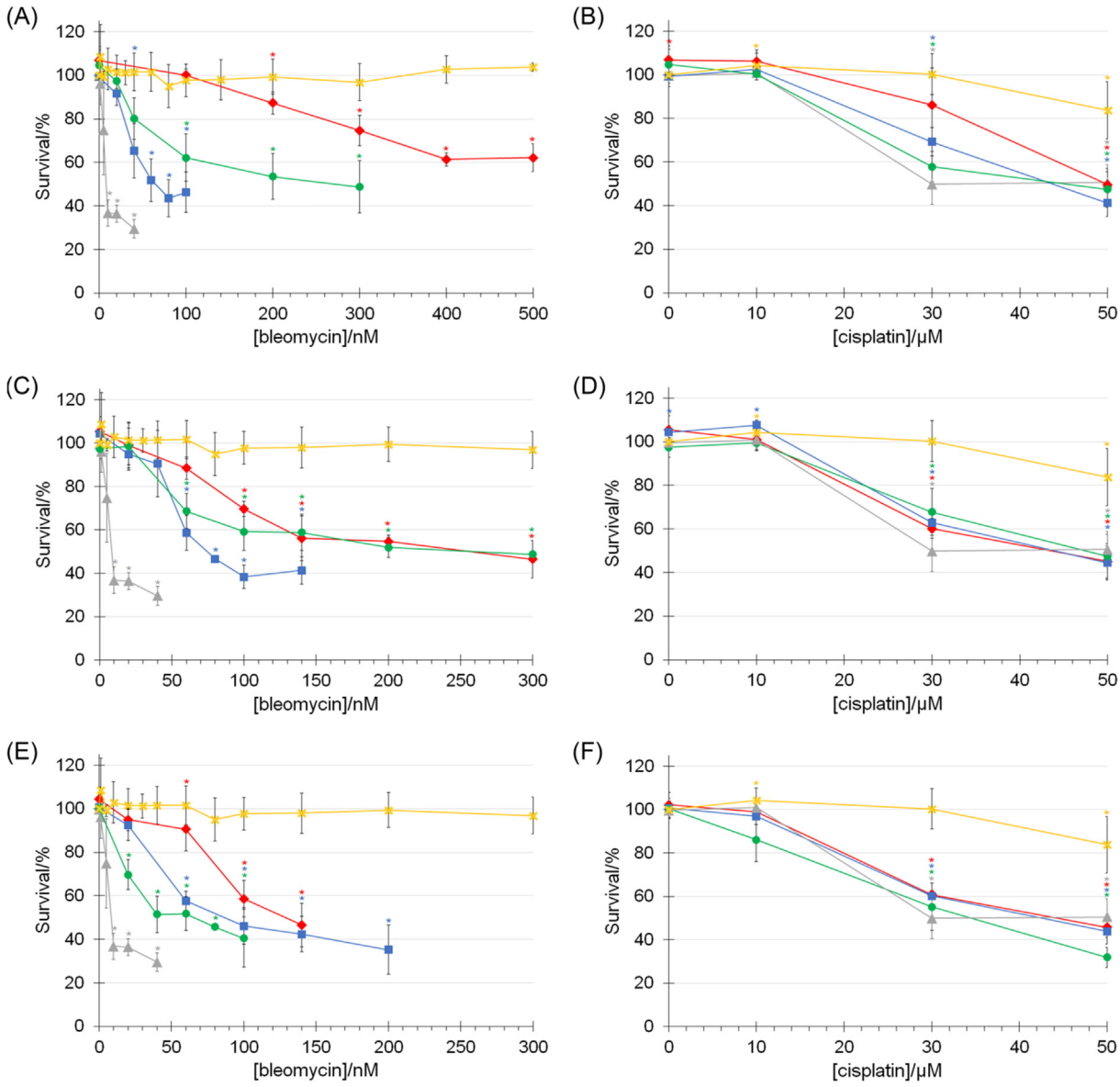

Fig. 4. Cell survival determined by the MTS assay at different (A, C, E) bleomycin and (B, D, F) cisplatin concentrations for non-electroporated CHO cells (cross, amber) and cells electroporated with different nanosecond pulses and 8 × 100 μs pulses delivered at 1.1 kV/cm and 1 Hz repetition rate (triangle, grey). (A, B): 1 × 200 ns pulse at 12.9 kV/cm (diamond, red), 25 × 200 ns pulses at 6.3 kV/cm, 10 Hz repetition rate (square, blue), 100 × 200 ns pulses at 4.3 kV/cm, 10 Hz repetition rate (circle, green). (C, D): 1 × 400 ns pulse at 13.2 kV/cm (diamond, red), 25 × 400 ns pulses at 3.9 kV/cm, 10 Hz repetition rate (square, blue), 100 × 400 ns pulses at 3.0 kV/cm, 10 Hz repetition rate (circle, green). (C): 1 × 550 ns pulse at 8.5 kV/cm (diamond, red), 25 × 550 ns pulses at 3.2 kV/cm, 10 Hz repetition rate (square, blue), 100 × 550 ns pulses at 2.5 kV/cm, 10 Hz repetition rate (circle, green). Bars represent standard deviation, asterisks (*) represent statistically significant ($P < 0.05$) difference from untreated control (*t*-test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

exposed to 40 nM bleomycin and 8 × 100 μs pulses, to 140 nM bleomycin and 25 × 400 ns pulses or to 50 μM cisplatin and 8 × 100 μs or 400 ns pulses, while with the MTS assay, the survival was determined to be around 30 – 50% at these conditions. Since the two assays used to determine cytotoxicity differ in their principles, they give different results. The MTS assay, a colorimetric method for determining the number of viable cells, is based on the bioreduction of the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)–2H-tetrazolium (MTS). It is easy to use and rapid and thus it allows screening many different treatments [74]. The disadvantage of the method is its dependence on the metabolic state of the cell population that can significantly differ from the actual percentage of viable cells – since cells are being stressed by electroporation, their metabolic activity might increase [75]. Clonogenic assay is based on the assumption that each remaining viable cell after exposure will form a colony after sufficient time. For cell lines that do form colonies, clonogenic assay is the best option for assessment of exact number of viable, proliferating cells after treatment [76]. However, the assay is time consuming. The type of cell death caused by bleomycin depends on the number of molecules internalized into the cells. It was reported that bleomycin caused an arrest in the G2-M phase of the cell cycle in electroporated cells at extracellular concentrations in the nanomolar range similar to the present study [77]. The toxic effects of cisplatin are believed to be primarily a consequence of covalent adducts formation between cisplatin and DNA which inhibits DNA replication and cell division [70]. The cells treated with electric pulses and bleomycin

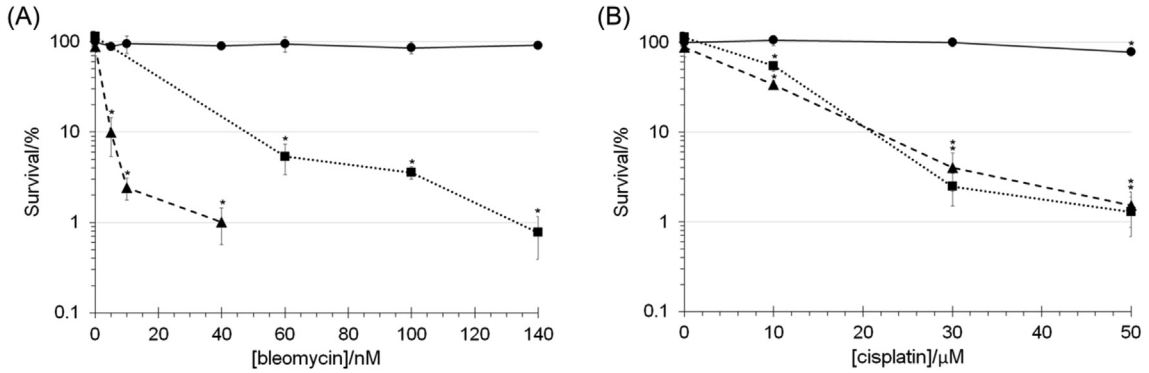

Fig. 5. Cell survival at different (A) bleomycin and (B) cisplatin concentrations determined by the clonogenic assay for non-electroporated cells (circles, solid line), cells electroporated with 8 × 100 μs pulses delivered at 1.1 kV/cm and 1 Hz repetition rate (triangles, dashed line) and cells electroporated with 25 × 400 ns pulses at 3.9 kV/cm, 10 Hz repetition rate (squares, dotted line). Bars represent standard deviation, asterisks (*) represent statistically significant (P < 0.05) difference from untreated control (Welch's *t*-test).

or cisplatin could thus still be metabolically active and produce a signal in the MTS assay, however, they are unable to form colonies in the clonogenic assay.

The lowest decrease in cell metabolic activity was observed for both bleomycin and cisplatin when cells were electroporated with one 200 pulses at 12.6 kV/cm, which is not surprising since this pulse protocol permeabilized the cell membrane of only 85% of the cells. When MTS assay was used to asses cytotoxicity, we observed that the combination of 8 × 100 μs pulses, 1 Hz repetition rate at 1.1 kV/cm and bleomycin resulted in a significant loss of metabolic activity of the cells (to around 40% of the untreated control) when increasing the extracellular concentration of bleomycin up to 10 nM. Higher bleomycin concentration did not further decrease the cells' metabolic activity measured by the MTS assay. The measured cell activity after ECT reached a plateau, i.e. with further increasing the bleomycin concentration, the metabolic activity decreased only slightly. Such a plateau was observed also for cells electroporated with nanosecond pulses, only at higher concentrations of bleomycin compared to the 8 × 100 μs pulses. When using the clonogenic assay, however, this plateau was not observed – cell survival was decreasing with increasing the bleomycin extracellular concentration. For cisplatin, concentrations above 50 μM were not tested because a decrease of survival was observed also for non-electroporated cells incubated for 25 min with 50 μM cisplatin.

Interestingly, when using specific nanopulse electroporation protocols, lower concentrations of bleomycin were needed to achieve a cytotoxic effect – these pulse protocols (25 × 200 ns pulses, 10 Hz at 6.1 kV/cm; 25 × 400 ns pulses, 10 Hz at 3.9 kV/cm; 100 × 550 ns pulses, 10 Hz at 3.1 kV/cm) are also the ones with the longest cell membrane resealing time. The time required for membrane resealing might be affected also by the size of pores that form on the membrane because different repair mechanisms may be activated depending on the pore size. For laser-induced pores it was shown that for smaller pores, longer time is required for membrane resealing than for larger pores [78]. The cell is probably more tolerant to small wounds, but larger pores in the membrane need to be repaired faster for the cell to survive the injury [67]. Since the transmembrane transport is an integral of flux over time, a longer resealing time results in higher intracellular accumulation and consequently higher cytotoxicity of bleomycin. In future studies, measurements of the intracellular bleomycin concentration is required to determine how many bleomycin molecules are being internalized after electroporation with different pulse protocols.

As can be seen from micrographs taken 72 h after ECT experiments (Fig. 6), the combination of electric pulses and bleomycin or cisplatin at concentrations that decreased the cell survival to around 1% according to the clonogenic assay, caused significant morphological changes – the cells were enlarged, of irregular morphologies and also blebbing occurred. On the other hand, no differences were observed in cells that were exposed only to the electric pulses or only to the drug.

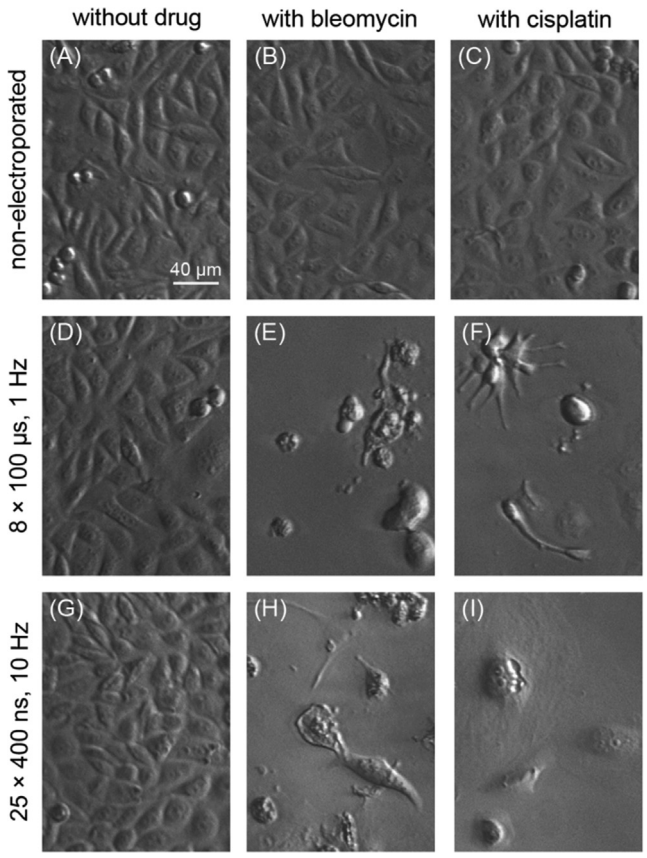

Fig. 6. IMC images of CHO cells 72 h after ECT experiments: (A) control that was exposed neither to electric pulses nor to any drug, (B) non-electroporated cells exposed to 140 nM bleomycin, (C) non-electroporated cells exposed to 50 μM cisplatin, (D) cells exposed to eight 100 μs pulses, 1 Hz, 1.1 kV/cm, (E) cells exposed to eight 100 μs pulses, 1 Hz, 1.1 kV/cm and 40 nM bleomycin, (F) cells exposed to eight 100 μs pulses, 1 Hz, 1.1 kV/cm and 50 μM cisplatin, (G) cells exposed to 25 400 ns pulses, 10 Hz, 3.9 kV/cm, (H) cells exposed to 25 400 ns pulses, 10 Hz, 3.9 kV/cm and 140 nM bleomycin, (I) cells exposed to 25 400 ns pulses, 10 Hz, 3.9 kV/cm and 50 μM cisplatin.

In our study we show that combination of nanosecond pulses and bleomycin or cisplatin, two most commonly used chemother-

apeutic drugs in ECT, causes successful cell kill. In case of contraindications for one drug (e.g. due to allergic reaction or exceeding the cumulative dose), the other could be used. Antitumor effects of ECT with nanosecond pulses and bleomycin/-doxorubicin have already been reported *in vivo* on a feline oral malignant melanoma [46] and on myeloma tumor models in mice [47], however, it is not clear from these reports how the nanosecond pulses, bleomycin/doxorubicin and/or CO_2 laser surgery contributed to the overall success of the therapy because of lack of controls treated with electric pulses, the drugs and CO_2 laser only. Our study is, therefore, providing evidence of effectiveness for ECT with nanosecond pulses and contributes to the optimization of such procedures by elucidating the most effective pulse protocols. Since bleomycin is a medium-sized molecule, our findings can be extended also to other nanosecond electroporation-based applications with the aim to introduce medium sized molecules (like siRNA) into cells.

3.4. Metal release

The concentration of released aluminum ions from electroporation cuvettes was measured after delivery of 8 × 100 μs pulses and nanopulses at previously determined optimal voltages in 0.9% (w/v) NaCl solution. We are aware that the NaCl solution is not usually used as electroporation medium, however, we chose pure NaCl solution for this set of experiments because the growth medium contains aluminum in trace amounts; the pure NaCl solution allowed us to detect even very small amounts of aluminum ions. A new electroporation cuvette from the same manufacturer was used for each sample. Significantly higher concentration of released aluminum from electroporation cuvettes was detected after delivery of 8 × 100 μs pulses than any of the nanosecond pulses (Table 2). Since aluminum alloy and not pure aluminum is used for manufacturing electroporation cuvettes, also other metal ions might be released.

The results of metal release from electrodes after application of electroporation pulses in the nanosecond range thus confirm the theoretical predictions that shorter pulse duration results in less electrochemical reactions [56]. We proved that shorter pulse duration decreases the amount of metal release from electrodes – even if the voltage and/or number of pulses is increased (compared to longer electroporation pulses) to achieve the same biological effect.

Table 2
Concentration of released Al ions from electroporation cuvettes (N = 4) after delivery of different pulses and estimated energies for the 8 × 100 μs and nanosecond pulses, the relative standard deviation (RSD) and estimated energy delivered by the electric pulses. Asterisk (*) represents statistically significant difference (P < 0.05) to the concentration of released Al after delivery of 8 × 100 μs pulses, hash (#) represents statistically significant difference (P < 0.05) to sham control (no pulses delivered) determined by Welch's *t*-test.

Pulse protocol	Energy (J)	[Al] (ng/ml)	
		Average	RSD
blank		6.3*#	3.0
sham control		56.5*	52
8 pulses of 100 μs, 1 Hz frequency 0.9 kV/cm	4.7	2660#	5.2
1 pulse of 200 ns 14.7 kV/cm	0.3	519*	69
1 pulse of 400 ns 15.3 kV/cm	0.7	184*	54
1 pulse of 550 ns 15.3 kV/cm	1.0	427*	75
25 pulses of 200 ns, 10 Hz frequency 6.9 kV/cm	1.8	148*#	12
25 pulses of 400 ns, 10 Hz frequency 4.2 kV/cm	1.4	153*#	7.1
25 pulses of 550 ns, 10 Hz frequency 3.4 kV/cm	1.3	170*#	6.3
100 pulses of 200 ns, 10 Hz frequency 4.2 kV/cm	2.6	414*#	49
100 pulses of 400 ns, 10 Hz frequency 2.8 kV/cm	2.4	411*#	5.9
100 pulses of 550 ns, 10 Hz frequency 2.8 kV/cm	3.3	521*#	13

Electrode material release, corrosion and fouling of the electrodes, products of electrolysis and chemical changes to the treated medium (all consequences of electrochemical reactions at the electrode–electrolyte interface) are limiting factors for application of electroporation-based technologies in the food industry [48]. Uptake of electrolysis species in permeabilized cells induces cell death [79] and metal ions, released from the electrodes during electroporation, are cytotoxic and affect the biochemistry of electroporated cells [50,51,80]. Development of methods for reducing these unwanted electrochemical effects is thus of interest for a wider spread of electroporation-based applications in research, food processing, biotechnology and medical applications of electroporation such as ECT, tissue ablation and gene electrotransfer.

When comparing different nanopulse protocols, the concentration of released metal ions does not seem to correlate with the estimated energy: the lowest energy was estimated for 1 pulse of 200 ns at 14.7 kV/cm, however, for this pulse protocol, we measured one of the highest concentrations of released aluminum ions. This indicates that the amount of released metal ions from electrodes does not solely depend on the applied energy but is affected also by other pulse parameters including pulse duration, voltage, and number of pulses. Perhaps the most unexpected were the concentrations of released aluminum measured after delivery of single nanopulses at the same voltage (which, however, resulted in a slightly different electric field strength: 14.7 kV/cm for 200 ns and 15.3 for 400 and 500 ns). Contrary to expectations that at the same voltage and number of pulses the lowest concentration of released aluminum will be measured for the shortest pulse duration, the highest average concentration of aluminum ion was measured after delivery of one 200 ns (i.e. the shortest) nanopulse. If excluding an outlying measurement (which is probably an experimental error because the measured aluminum concentration was even lower than in sham controls), the mean value for one 200 ns pulse at 14.7 kV/cm increases even more, to 680 ng/ml of aluminum.

Electroporation cuvettes with 4 mm gap filled with 600 μl of the NaCl solution were used in metal release experiments for delivery of all pulses to ensure the same geometry/contact surface of the electrode and electrolyte. This is contrary to cell experiments in which we used electroporation cuvettes with 2 mm gap for delivering the 8 × 100 μs pulses because of the limitations of the microsecond pulse generator, while 4 mm cuvettes were used to deliver nanopulses. To be able to deliver 8 × 100 μs pulses to electroporation cuvettes with 4 mm gap filled with 600 μl of NaCl solution, we needed to use a different pulse generator: the Cliniporator Vitae. In cell experiments, the microsecond pulse generator was set to deliver 8 × 100 μs pulses at 250 V which resulted in an electric field strength of 1.1 kV/cm considering the distance between electrodes to be 2 mm. For metal release experiments, the distance between electrodes was 4 mm, thus, to achieve the same electric field strength, we set the voltage at 500 V on the Cliniporator Vitae. However, 500 V is the minimum voltage for this device and for every successive 100 μs pulse in the train, we observed a drop in the voltage and electric current, resulting in the measured electric field strength of 0.9 kV/cm. Nonetheless, the measured concentration of released aluminum ions after the delivery of 8 × 100 μs pulses was significantly (at least five times) higher than after any of the nanopulse protocols, thus proving our point that metal release from electrodes is significantly reduced if using nanopulses compared to the classical 8 × 100 μs. If the electric field strength of 1.1 kV had been achieved for the 8 × 100 μs, the concentration of the released aluminum ion would most probably be even higher.

Even though the same pulse generator was used to deliver nanopulses, discrepancies in the calculated electric field strength from cell and metal release experiments were observed for some of the pulse protocols (Tables 1 and 2). The source of the

discrepancies might be the use of different medium (DMEM growth medium in cell experiments vs. NaCl solution in metal release experiments) resulting in a different voltage on the load, possible variations in the cuvette's geometry since a new electroporation cuvette was used for every sample, and also the already mentioned differences in the delivered voltage that were observed from sample to sample, especially at very high voltages.

Compared to the reagent blanks (0.9% NaCl solution with addition of HNO_3), significant increase in the concentration of aluminum ions was detected also in sham control samples for which the 0.9% NaCl solution was added to the electroporation cuvette and no pulses were applied. The addition of the NaCl solution caused the development of the double layer, a layer of charged particles and/or orientated dipoles at each electrode–electrolyte interface. Even if no external voltage is applied, chemical reactions occur immediately, and electrons are transferred between the electrode and the electrolyte. The electron transfer results in an electric field between the electrode and the layer of ions/dipoles which influences further chemical reactions, accelerating the oxidation reaction (and consequently metal release from electrodes), while inhibiting the reverse reduction reaction; if no voltage is applied, the competing oxidation and reduction reactions reach an equilibrium [56].

The use of nanosecond pulses has several advantages over conventional micro- and millisecond electroporation pulses: low level of heating, reduced muscle contractions [2,24,44,45] and, as we show in this study, reduced contamination with metal ions released from the electrodes, making nanosecond pulses alluring for use in clinical practice and electroporation-based applications in which electrochemical reactions should be kept at minimum, e.g. food technology.

4. Conclusions

The aim of this *in vitro* study on CHO cells was to evaluate the effects of nanosecond pulse parameters (voltage, pulse duration, number of pulses) on cell membrane permeabilization and resealing and on cell survival after electroporation only and after electrochemotherapy with bleomycin and cisplatin, and compare them to the 8 × 100 μs pulses commonly used in ECT. Cell survival after electroporation decreased and cell membrane permeabilization increased with increasing electric field strength and number of nanosecond pulses. As expected, for shorter pulses, higher electric field strengths were needed to reach the same effect as with longer pulses. We show that the application of permeabilizing nanosecond pulses in combination with bleomycin or cisplatin results in successful cell kill. The pulse duration did not considerably affect the decrease of survival caused by ECT with cisplatin. On the other hand, higher bleomycin concentrations were needed to achieve the same effect with nanosecond pulses compared to the standard 8 × 100 μs pulses, however, the bleomycin concentration was still considerably lower than concentrations used in clinical practice. Nanosecond pulses with parameters that caused the longest cell membrane resealing times were also the ones that decreased cell survival most effectively in ECT experiments with bleomycin. We also show that decreasing the pulse duration from microseconds to nanoseconds with concomitantly increasing the amplitude to achieve the same biological effect results in reduced electrochemical reactions, which in our study was monitored by quantifying the release of aluminum ions from electrodes.

Declaration of Competing Interest

Damijan Miklavčič holds patents on electrochemotherapy that have been licensed to IGEA S.p.a (Carpi, Italy) and is also a consultant to various companies with an interest in electroporation-based technologies and treatments. The other authors have no competing interests.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioelechem.2021.107798.

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