



Nanosecond electrochemotherapy using bleomycin or doxorubicin: Influence of pulse amplitude, duration and burst frequency

Eivina Radzevičiūtė^{a,b}, Veronika Malyško-Ptašinskė^b, Julita Kulbacka^c, Nina Rembiałkowska^c,
Jurij Novickij^b, Irutė Girkontaitė^a, Vitalij Novickij^{b,*}

^a State Research Institute Centre for Innovative Medicine, Department of Immunology, Vilnius, Lithuania

^b Faculty of Electronics, Vilnius Gediminas Technical University, Vilnius, Lithuania

^c Department of Molecular and Cellular Biology, Faculty of Pharmacy, Wrocław Medical University, Wrocław, Poland

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ABSTRACT

Electroporation is a pulsed electric field (PEF) induced phenomenon, which effectiveness varies dependent on pulse parameters. This work focuses on nano-electrochemotherapy with bleomycin and doxorubicin to derive protocols as effective as European Standard Operating Procedures on Electrochemotherapy (ESOPE), which employ conventional microsecond range pulses. As a model, murine Lewis lung carcinoma (LLC1) cell line was used. The effects of pulse duration (100–500 ns), PEF amplitude (6–10 kV/cm) and pulse repetition frequency (10 kHz, 100 kHz, 1 MHz) were studied. A total of 75 ns protocol variations have been used. For detection of cell permeabilization, Yo-Pro-1 and flow cytometry were employed. Cell viability was evaluated 24-, 48-, or 72-hours post-electroporation. Nanosecond parametric protocols resulting in comparable treatment efficiency as ESOPE (1.3 kV/cm × 100 μs × 8) have been proposed. It was shown that high-frequency nanosecond electrochemotherapy with bleomycin or doxorubicin could be an alternative for established ESOPE protocols.

1. Introduction

Nanosecond electric field pulses (nsPEF) when used for electroporation offer better energy control of the pulse bursts and enable precise management of the Joule heating [1], also are less associated with oxidation due to electrolysis [2] and depending on the PEF parameters are less likely to induce severe muscle contractions [3] compared to conventional microsecond range (100 μs) pulses. In the last decades, nsPEF were typically employed as a tissue ablation modality based on electroporation [4,5] and were hardly used in the context of electrochemotherapy presumably due to higher voltages required to achieve successful permeabilization [6], low electrophoretic component associated with ultrashort pulses and the limited availability of nsPEF generators [7]. Additionally, the kinetics and the size of the pores forming when induced by nsPEF electric fields are different compared to μsPEF and msPEF procedures [8,9]. The pore size is a function of the duration of the electric field pulse [10], thus it is established that the size of the pores formed by nsPEF is smaller than with μsPEF [11–13]. At the same time, the increased number of nanosecond pulses permeabilizes cells to a greater extent by increasing the pore number and not the pore

diameter [8], which also can have an influence on molecular uptake [14]. Considering, that the most of electro-diffusive molecular transport occurs after the nanosecond pulse application [15,16], specific nsPEF parametric protocols should be derived to compensate the smaller pores and lack of electrophoretic component for nsPEF to be applicable in electrochemotherapy (i.e. ensure sufficient mass transfer) on par with ESOPE procedures.

The concept of successful bleomycin entry when the cells are permeabilized by nsPEF was successfully shown [17]. Since then works focusing nano-electrochemotherapy started to appear indicating high applicability of nsPEF for controlled delivery of cytotoxic drugs [2,18–21]. Usually, electrochemotherapy protocols are characterized by the pulse parameters of the burst, where the pulse amplitude, duration and the number of the pulses are heavily focused. Nevertheless, the effects of repetition frequency also have a role, which is minor for practical applications in kHz or Hz range (1 Hz and kHz protocols can be used interchangeably [22]). Sensitization and de-sensitization phenomena occur in sub-Hz range [23–25] with the total time of the treatment exceeding several minutes. However, 4 years ago, a new nsPEF phenomenon leading to higher electroporation (higher number of

* Corresponding author at: Electronics Faculty, Naugarduko g. 41, LT-03227 Vilnius, Lithuania.

E-mail address: vitalij.novickij@vilniustech.lt (V. Novickij).

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permeabilized cells and molecular uptake) during ultrafast repetition frequency pulses (500 + kHz) was detected [26–28]. For example, the number of electroporated cells can be doubled solely by the decrease of the delay between the pulses without any changes in the total energy of the burst [29]. Based on *in silico*, *in vitro* [27] and *in vivo* data [19], we have shown that it is possible to achieve a threshold repetition frequency when the depolarization time of the membrane is longer than the time delay between the pulses, and thus, the cell transmembrane voltage starts to accumulate during the nsPEF burst leading to higher molecular uptake. This phenomenon can be employed in nano-electrochemotherapy to compensate weaker molecular diffusion (due to smaller pores and lack of electrophoretic component) when compared to microsecond pulses.

Electrochemotherapy also involves application of cytotoxic drugs and currently the dominating one is bleomycin [30,31]. Efforts to use other drugs such as cisplatin, doxorubicin, mitomycin C, paclitaxel, carboplatin or even calcium ions are performed [32–35]. Bleomycin (BLM) was introduced in preclinical trials more than three decades ago and yet it is still one of the most popular drugs [36]. Bleomycin is a hydrophilic, highly toxic drug, which under normal circumstances, passes the membrane barrier in a limited amount. Once inside the cancer cells BLM induct DNA strand breaks and causes free radicals, inducing cell death. *In vitro* BLM kills cells by two different mechanisms, depending on the amount of drug in the permeabilized cell: mitotic cell death or pseudoapoptosis [37]. However, cytotoxicity of bleomycin can be increased with electroporation to introduce drug directly into the cell cytosol [30,38,39]. In our study, also doxorubicin was involved, which belongs to anthracycline group agents. This antineoplastic drug works by intercalating between the DNA base pairs that causes inhibition of DNA synthesis and blocks the activity of DNA topoisomerase II [40]. As well as with BLM or other chemotherapeutics, DOX cytotoxicity to target cell can be increased using electric field exposure [41].

We have limited our study to these two drugs because both drugs enter the cell via passive diffusion, therefore, having molecules of different molecular weight is beneficial for comparison purposes, especially in nsPEF context (due to smaller pores and limited electrophoresis). Bleomycin is a large molecule (~1.5 kDa), therefore, molecular transport (in this context via electroporation) is critical to the success of chemotherapy [42], while doxorubicin is smaller (~0.54 kDa) enabling better passive diffusion. Importantly, both drugs are of comparable or higher molecular weight than the typical electroporation detection fluorescent markers [43] (i.e., YO-PRO-1 (~0.64 kDa)), thus the permeabilization data in this work can be adequately interpreted and compared with other works. It's not the case with cisplatin (~0.3 kDa), where more complex, but higher resolution membrane permeabilization detection assays have to be employed to assess permeabilization, e.g., Ca^{2+} or Ba^{2+} ions [44], and thus it's not used in our study.

Previously, both these drugs were mainly studied in the microsecond electroporation context [30,45]. The nanosecond electrochemotherapy range is poorly covered. Recently, a dataset on *in vitro* nsPEF electrochemotherapy with bleomycin and cisplatin was published by Vizintin et al. [2]. It was shown that nano pulses cause lower metal release from electrodes than $8 \times 100 \mu\text{s}$ pulses and are comparably effective for cell killing as ESOPE pulses. The study mainly focused the effects of 200 ns, 400 ns and 550 ns pulses delivered at 10 Hz [2]. In our work, we aim to significantly expand the parametric range and the flexibility of protocols, therefore, the feasibility of 100–500 ns pulses with a step of 100 ns, delivered in a burst of 10 is investigated. We have covered the 10 kHz, 100 kHz and 1 MHz pulse repetition frequencies in a 6–10 kV/cm PEF intensity range (a total of 75 ns protocols employed in the study).

The results of our work are applicable for further *in vivo* and clinical research, enabling derivation of equivalent nsPEF pulse parameters depending on available infrastructure.

2. Materials and methods

2.1. Pulsed power setup and protocols

The experimental setup consisted of up to 3 kV, 100 ns – 1 ms square-wave high-voltage pulse generator (VilniusTECH, Vilnius, Lithuania) and a commercially available electroporation cuvette with a 1 mm gap between electrodes (Biorad, Hercules, USA). The voltage applied to the cuvette was 0.6–1 kV, corresponding to a 6–10 kV/cm electric field, respectively. The 100–500 ns pulses were delivered in bursts of 10 (using 10, 100 kHz or 1 MHz) for nsPEF protocols. A total of 75 different protocols were used. As a reference, $1.3 \text{ kV/cm} \times 100 \mu\text{s} \times 8$ pulse sequence was introduced to compare the efficiency of nsPEF treatments with established and clinically used protocol (ESOPE) [46,47].

The nsPEF experiments have been limited to 10 kV/cm, which was a limitation of our infrastructure due to high load of the generator (high conductivity cell buffer (RPMI 1640), $\sigma_m = 1.3 \text{ S/m}$). We also limited the duration in the 100–500 ns range to ensure non-thermal supra-electroporation. Lastly, we did not perform experiments in low-conductivity sucrose-based buffers, since the polarization/de-polarization time of cell membrane would be significantly altered and thus, would be less applicable for comparison with *in vivo* or clinical works.

The typical waveform of the pulse is shown in Fig. 1.

The waveform was acquired using DPO4034 oscilloscope (Tektronix, Beaverton, USA) and P4250 probe (Cleqee, Shenzhen, China). The applied protocols are summarized in Table 1.

The temperature rise of $2.8 \pm 0.4 \text{ }^\circ\text{C}$ was detected when the $1.3 \text{ kV/cm} \times 100 \mu\text{s} \times 8$ (ESOPE) protocol was used (Pt1000 sensor, Innovative Sensor Technology, Wattwil, Switzerland). Such deviations in temperature are typical for ESOPE protocols [49]. The influence of Joule heating during nsPEF was not assessed because the highest intensity sequence ($10 \text{ kV/cm} \times 500 \text{ ns} \times 10$) has 2.7-fold lower energy input than the ESOPE protocol. The energy input of nsPEF sequences varied between 0.02 and 0.22 J to ensure a non-thermal procedure.

2.2. Cells

LLC1 carcinoma cells were maintained in RPMI 1640 supplemented with 2 mM glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin and 10 % of fetal calf serum (FCS). All cell culture reagents were obtained from Gibco (Thermo Fisher Scientific, Grand Island, NY, USA). The experimental day LLC1 cells were detached by aspirating several times with a culture medium, afterward centrifugated and resuspended in the RPMI 1640 growth medium at a concentration of 0.5×10^6 /ml for electrochemotherapy viability assay and 2×10^6 /ml for permeabilization measurements.

2.3. Fluorescence detection assay

Cell permeabilization was evaluated using YO-PRO-1 (YP) (Sigma-Aldrich, Burlington, MA, USA) and flow cytometry. The cells (45 μl) were mixed with YP (5 μl) for the final YP concentration of 1 μM . The 50 μl samples were placed inside the cuvette between the electrodes and treated by PEF. After 3 min incubation at room temperature 150 μl of phosphate buffered saline (PBS) was added to the sample. The samples were analyzed by flow cytometry (10,000 cells) using BD Accuri C6 flow cytometer (Becton, Dickinson and Company, New Jersey, United States). The experimental scheme is shown in Scheme 1.

YP fluorescence was evaluated using a bandpass filter of 530/30 nm. A shift of fluorescence spectra and the cells in the defined gate (which was defined based on the untreated control) have been interpreted as fluorescence positive (permeabilized), while the cells outside the gate have been interpreted as non-fluorescent (non-permeabilized), which is a standard procedure in electroporation studies.

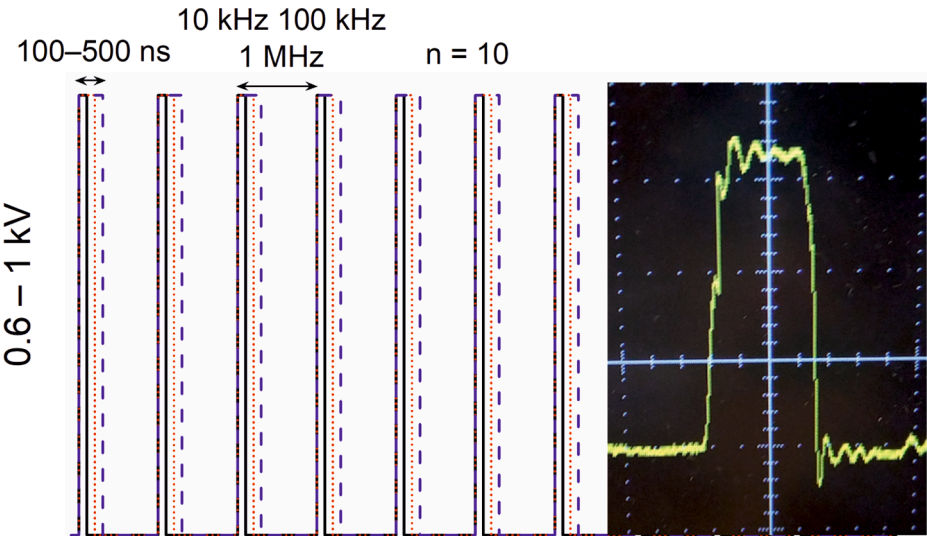
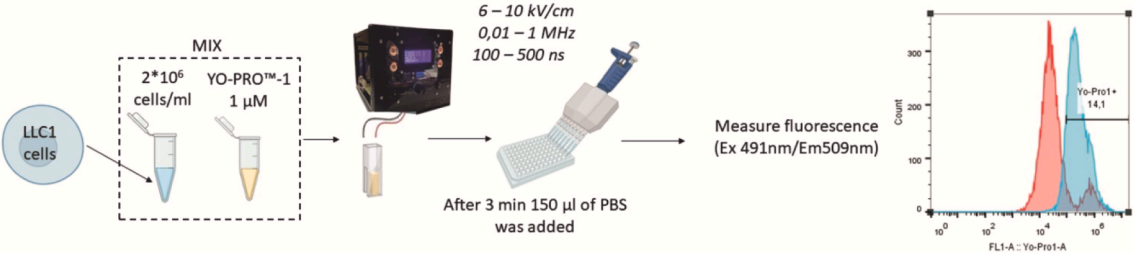


Fig. 1. The waveform of the applied electrical pulses (200 ns pulse snapshot is shown).

Table 1
Pulse parameters of applied protocols, where V – applied voltage, I – current, t – pulse on-time per treatment, R – cuvette impedance. Dose calculated according to [48].

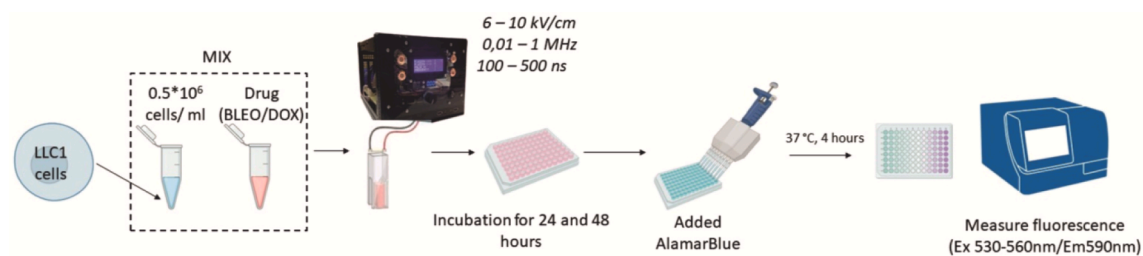
Electric field [kV/cm]	Duration	Number of pulses, n	Repetition frequency	Dose ($V^2 \times t$) [$V^2 \times s$]	Energy ($I^2 \times R \times t$) [J]	Energy density [kJ/L]
6	100 ns100 ns100 ns100 ns	1010101010	10 kHz, 100 kHz, 1 MHz	0.36	0.02	0.31
7				0.49	0.02	0.43
8				0.64	0.03	0.56
9				0.81	0.04	0.70
10				1.00	0.04	0.87
6	200 ns200 ns200 ns200 ns	1010101010	10 kHz, 100 kHz, 1 MHz	0.72	0.03	0.63
7				0.98	0.04	0.85
8				1.28	0.06	1.11
9				1.62	0.07	1.41
10				2.00	0.09	1.74
6	300 ns300 ns300 ns300 ns	1010101010	10 kHz, 100 kHz, 1 MHz	1.08	0.05	0.94
7				1.47	0.06	1.28
8				1.92	0.08	1.67
9				2.43	0.11	2.11
10				3.00	0.13	2.61
6	400 ns400 ns400 ns400 ns	1010101010	10 kHz, 100 kHz, 1 MHz	1.44	0.06	1.25
7				1.96	0.09	1.70
8				2.56	0.11	2.23
9				3.24	0.14	2.82
10				4.00	0.17	3.48
6	500 ns500 ns500 ns500 ns	1010101010	10 kHz, 100 kHz, 1 MHz	1.80	0.08	1.57
7				2.45	0.11	2.13
8				3.20	0.14	2.78
9				4.05	0.18	3.52
10				5.00	0.22	4.35
1.3	100 μs	8	1 Hz	13.52	0.59	11.76



Scheme 1. Experimental scheme for evaluation of permeabilization of cell membrane using YO-PRO-1 as a fluorescent marker and flow cytometry.

2.4. Electrochemotherapy and viability assay

Experimental design is shown in [Scheme 2](#). The 25 μl of LLC1 cells suspension in growth medium (0.5×10^6 cells/ml) were mixed with 25 μl of respective chemotherapeutic drug. To evaluate electrochemotherapy effects, different concentrations of the drugs were used,



Scheme 2. Experimental scheme for evaluation of cell viability after nanosecond electrochemotherapy.

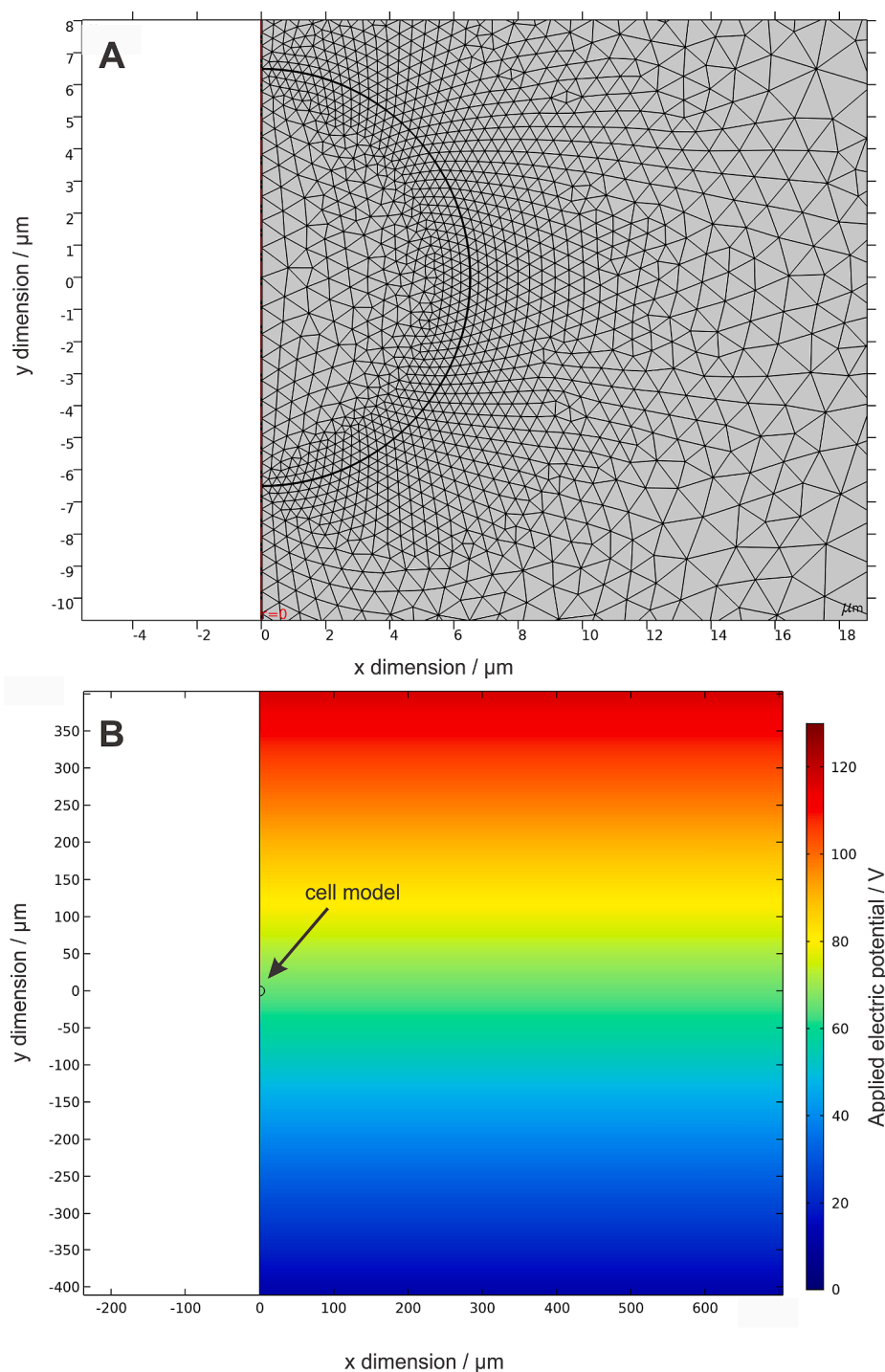


Fig. 2. The COMSOL model of a biological cell, where (A) mesh structure; (B) extracellular medium when a voltage potential is applied.

bleomycin (BLM) 0.08–6.7 $\mu\text{g}/\text{ml}$ (Medac, Germany) and 0.2–5.8 $\mu\text{g}/\text{ml}$ of doxorubicin (DOX) (Ebewe Pharma, Austria).

Mixed suspension was placed inside the cuvette between the electrodes and treated by different conditions of nsPEF, transferred to 96-well plate. Afterward, 150 μl of growth media was added to each well and the sample left for the incubation period. Subsequently, the LLC1 cells viability was evaluated after 24, 48, and 72 h. After needed incubation, 10 μl of AlamarBlue cell viability reagent (Thermo Fisher Scientific, USA) was added in to each well. The cells were kept in an incubator for the next 4 h and the fluorescence was measured using Synergy 2 microplate reader and Gen5™ software (BioTek, USA). The excitation wavelength was 540/20 nm and the emission was evaluated at 620/40 nm.

2.5. Modelling transmembrane voltage

A simple cell polarization model based on finite element method (FEM) was developed in COMSOL (COMSOL Multiphysics, Sweden) using an axisymmetric geometry. The cell was approximated as a conductive sphere ($\sigma_c = 0.32 \text{ S}/\text{m}$ [50], radius of 6.5 μm [51]) with a thin layer (5 nm) of low electrical conductivity ($1.1 \times 10^{-7} \text{ S}/\text{m}$ [52]) representing a lipid membrane similar to the previous study [27]. Briefly, the extracellular medium (1.3 S/m) was approximated as a cube with 1 mm edge. The cathode and anode were defined as aluminum

electrodes (1 mm gap). A free triangular mesh with 15,559 domain elements and 583 boundary elements was generated. The 0.6 and 1 kV high frequency (1 MHz) pulse bursts (100, 300 and 500 ns, $n = 10$) were defined as an input (corresponding to 6 kV/cm and 10 kV/cm PEF). As a reference, 130 V and 100 μs pulses were used to model ESOPE protocol. The mesh structure and the cell model when 130 V voltage was applied are shown in Fig. 2.

This model allows to approximate the capacitive charging/discharging of the membrane during and after electrical pulse. It should be noted, that the pores and their influence on the conductivity of the membrane are not taken into account.

2.6. Statistical analysis

One-way analysis of variance (ANOVA; $p < 0.05$) was used to compare different treatments. Tukey HSD multiple comparison test for the evaluation of the difference was used when ANOVA indicated a statistically significant result ($p < 0.05$ was considered as statistically significant). The data was post-processed in OriginPro software (OriginLab, Northampton, MA, USA). All experiments were performed at least in three repetitions, and the treatment efficiency was expressed as mean $\pm$ standard deviation.

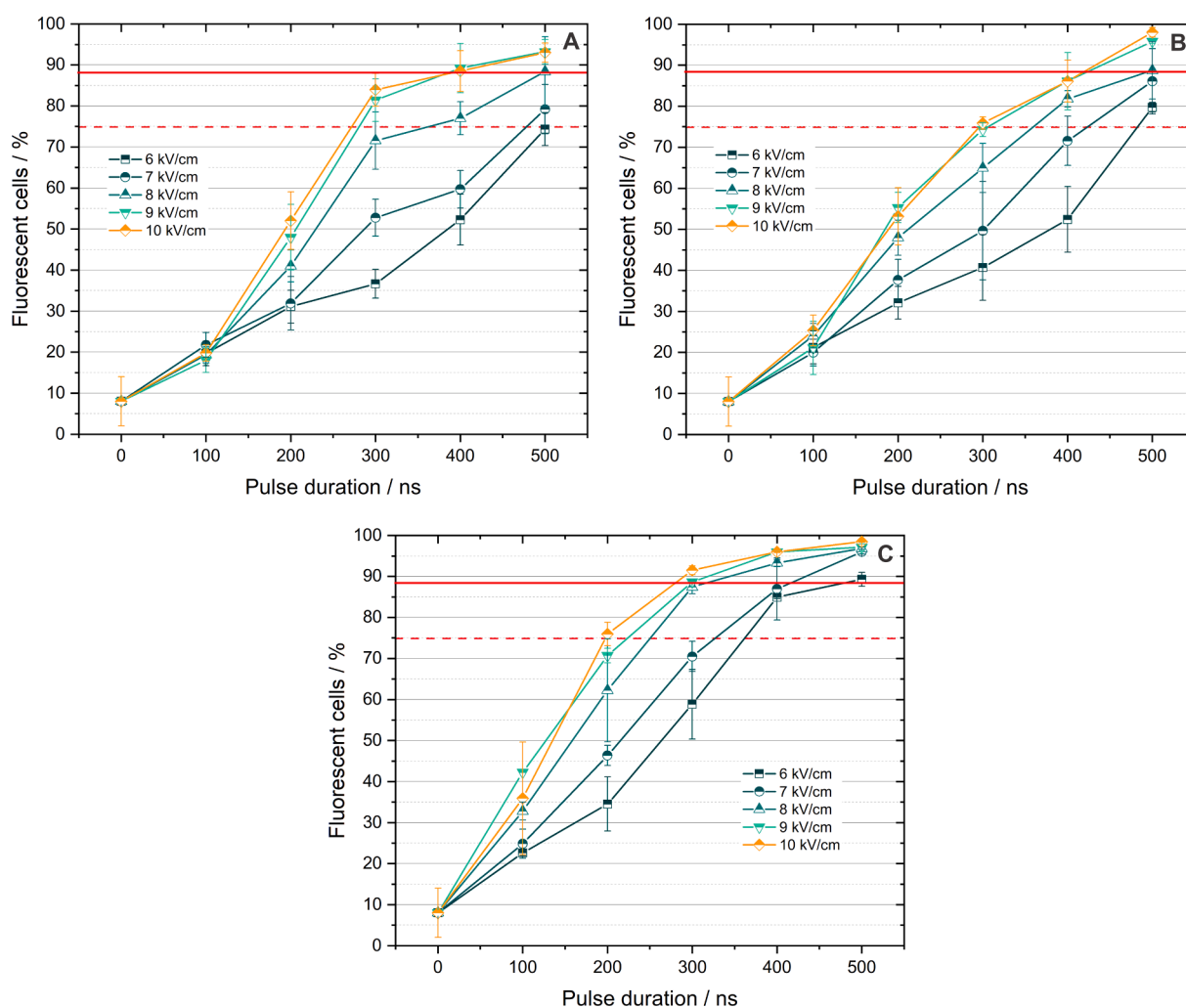


Fig. 3. Dependence of cell membrane permeabilization on electric field intensity, pulse duration and applied burst frequency, where A) 10 kHz bursts; B) 100 kHz bursts and C) 1 MHz bursts of 10 pulses. Red line shows permeabilization acquired by the reference ESOPE protocol (1.3 kV/cm $\times$ 100 μs $\times$ 8). Red dashed line indicates a 75 % permeabilization level to highlight parameters triggering high permeabilization, which are of particular interest for electrochemotherapy.

3. Results

3.1. Cell membrane permeabilization

The effects of nanosecond pulse bursts on cell membrane permeabilization have been evaluated. Bursts of 10 pulses (100 – 500 ns) have been supplied with varied pulse repetition frequency (10, 100 kHz and 1 MHz). Cell membrane permeabilization was confirmed using a fluorescent marker (YP) and the results are summarized in Fig. 3.

It can be seen that electroporation in the nanosecond range (supra-electroporation) can be controlled by all the varied parameters (PEF amplitude, pulse duration and burst frequency). With increase of the pulse duration the electric field required to trigger same level of electroporation is reduced. For example, 10 kV/cm $\times$ 200 ns pulses delivered at 10 kHz, result in $\sim$ 50 % cells being permeabilized (Fig. 3A), while for 300 ns pulses the same level of permeabilization is reached already with 7 kV/cm electric field. The 9–10 kV/cm $\times$ 500 ns protocols are the most effective. The permeabilization triggered by 9 kV/cm electric field in the whole range of pulse durations is on average lower than 10 kV/cm, but the difference is not statistically significant.

Frequency-wise the responses triggered by 10 kHz and 100 kHz are comparable in the 6–10 kV/cm region (Fig. 3A and B). The differences between same protocols are sub-10 % or not statistically significant. Significant improvement of electroporation is triggered in the 1 MHz region (Fig. 3C). In most of the cases the same pulsing protocols return higher permeabilization due to higher burst frequency. As a result, the number of protocols resulting in $>$ 75 % permeabilization is higher compared to the 10 and 100 kHz parametric sequences. As a reference, the cells were also electroporated with 1.3 kV/cm $\times$ 100 μ s $\times$ 8 protocol (ESOPE) returning 88 ± 5 % of cells being electroporated (red line, Fig. 3).

3.2. Microsecond electrochemotherapy

For adequate comparison of electrochemotherapy efficiency and interpretation of nanosecond electroporation results, firstly, the electrochemotherapy was performed with 1.3 kV/cm $\times$ 100 μ s $\times$ 8 (ESOPE) protocol with various concentrations of drugs (BLM and DOX).

In Fig. 4(A) it can be seen that a threshold concentration of drug exists when BLM electrochemotherapy starts to be effective.

For the LLC1 carcinoma cell line the threshold BLM concentration is 0.7 μ g/ml (after 48 h cell killing efficiency $>$ 50 % (LD50 dose)). Independently on the evaluation time (48 or 72 h) the efficiency of BLM electrochemotherapy is the same ($\sim$ 60 % viability reduction). Without electroporation BLM does not affect cell viability in the whole range of tested drug concentrations.

The DOX itself inhibits cell viability already when the concentration exceeds 1.25 μ g/ml (presumably due to different molecular size and passive diffusion, with DOX being a smaller molecule), while electroporation significantly improves the treatment efficiency. When used with the same (as BLM) concentration of 0.7 μ g/ml, electrochemotherapy with DOX returns similar efficiency ($\sim$ 60 % viability reduction). Different to BLM, DOX is effective already after 24 h. Based on the results, the 0.7 μ g/ml concentration has been selected to be used in nanosecond electroporation experiments for both drugs. Also, the evaluation times have been limited to 24 and 48 h since for both drugs the evaluation time (48 h or 72 h) does not affect the quantitative efficiency of electrochemotherapy.

3.3. Nanosecond electrochemotherapy

Electrochemotherapy employs reversible electroporation, therefore, the viability of the cells after the highest intensity (10 kV/cm) pulses without drugs was checked and compared with ESOPE protocol. The results are summarized in Table 2.

It can be seen that the PEF protocols didn't trigger any significant

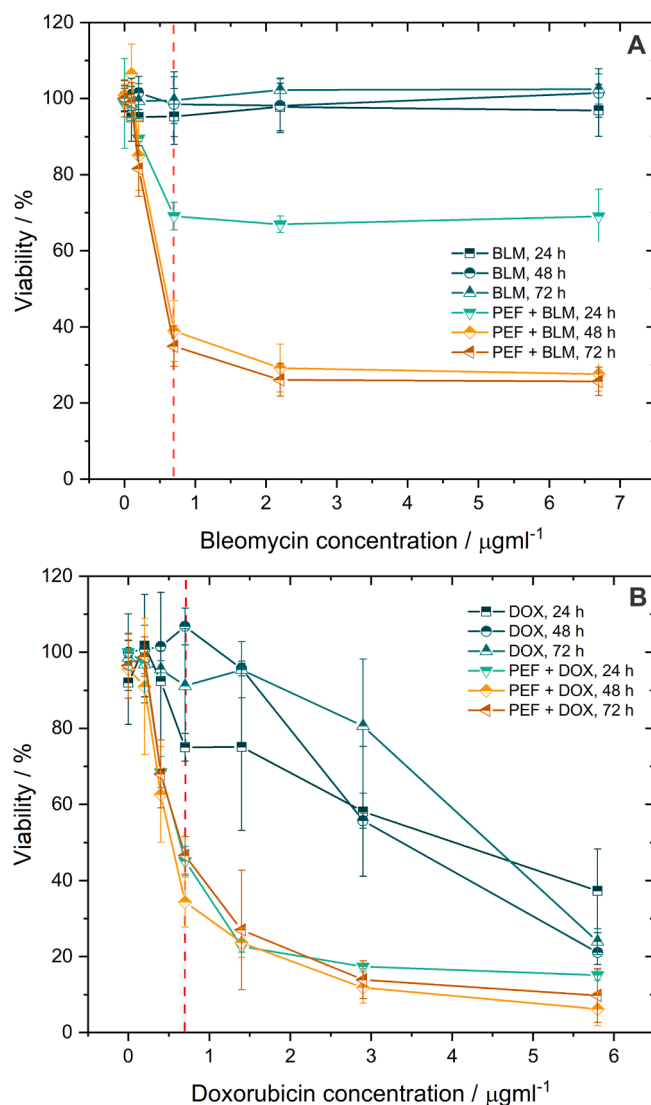


Fig. 4. Dependence of electrochemotherapy efficiency on drug concentration evaluated 24, 48 and 72 h post-treatment, where A) BLM electrochemotherapy; B) DOX electrochemotherapy. Red dashed lines highlight the drug concentration resulting in $>$ 50 % cell killing efficiency (LD50) after 48 and 72 h.

Table 2

Dependence of cell viability (48 h post-treatment) on pulse repetition frequency using highest intensity 10 kV/cm nanosecond pulse bursts. The data are normalized to untreated control.

Protocol	Viability (%)		
	10 kHz	100 kHz	1 MHz
10 kV/cm $\times$ 100 ns $\times$ 10	96.6 $\pm$ 2.9	101.5 $\pm$ 3.8	97.5 $\pm$ 5.2
10 kV/cm $\times$ 200 ns $\times$ 10	97.4 $\pm$ 0.7	99.1 $\pm$ 2.5	94.2 $\pm$ 1.6
10 kV/cm $\times$ 300 ns $\times$ 10	95.1 $\pm$ 0.6	99.5 $\pm$ 2.1	101.8 $\pm$ 0.7
10 kV/cm $\times$ 400 ns $\times$ 10	93.9 $\pm$ 3.7	99.9 $\pm$ 3.6	98.9 $\pm$ 2.4
10 kV/cm $\times$ 500 ns $\times$ 10	93.1 $\pm$ 3.9	95.8 $\pm$ 0.1	97.5 $\pm$ 0.8
ESOPE			
1.3 kV/cm $\times$ 100 μ s $\times$ 8	99.2 $\pm$ 2.2		

reduction of cells viability, therefore, the PEF effects presented in this study are reversible. Further, the nanosecond BLM electrochemotherapy in all three frequency ranges (10, 100 kHz and 1 MHz) has been studied. The results are summarized in Fig. 5. BLM electrochemotherapy is not effective after 24 h resulting in $\sim$ 30 % cell death when used with ESOPE protocol (red line, Fig. 5A, C, E). Significant improvement ($>$ 20 %, $P <$

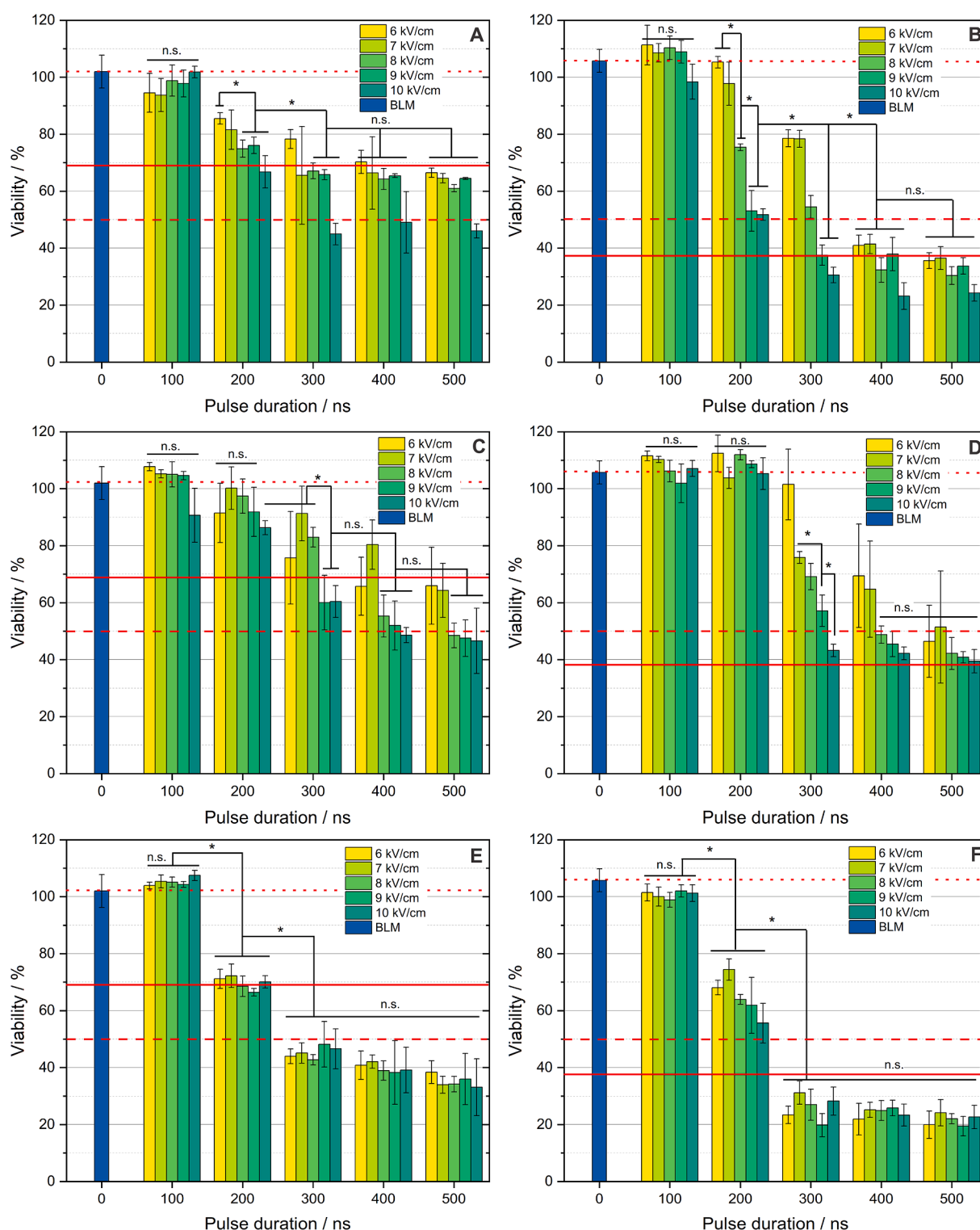


Fig. 5. Dependence of nanosecond BLM electrochemotherapy efficiency on electric field parameters, where A, B) 10 kHz protocols for 24 and 48 h evaluation times post-treatment; C, D) 100 kHz protocols for 24 and 48 h evaluation times post-treatment; E, F) 1 MHz protocols for 24 and 48 h evaluation times post-treatment. Asterisk (*) corresponds to statistically significant ($P < 0.05$) difference between samples. Red line indicates average electrochemotherapy efficiency using ESOP protocol ($1.3 \text{ kV/cm} \times 100 \mu\text{s} \times 8$). Dashed red line used to highlight the 50 % inactivation dose (LD50). Dot red line shows treatment efficiency without nsPEF treatment.

0.05) of cell killing efficiency was detected with 1 MHz pulses. In the whole amplitude range (6–10 kV/cm) for 300–500 ns pulses a saturated response was observed (Fig. 5E). The 200 ns pulses at 1 MHz resulted in a same efficiency as ESOP without any dependence on pulse amplitude. It was not the case for 10 kHz and 100 kHz pulses, where a tendency of

higher PEF amplitude (i.e., 10 kV/cm) being more effective than other protocols was easily detectable. Nevertheless, the 48-h data was of up-most interest when most of the cells had enough time to die by mitosis due to BLM electrochemotherapy. The results are summarized in Fig. 5 (B, D, F). It can be seen that only 300–500 ns protocols trigger cell death

comparable with ESOPE protocol. Similar to 24 h response, the MHz pulses were the best for nanosecond electrochemotherapy offering higher efficiency than the microsecond electrochemotherapy (on average > 10 %).

Nevertheless, again no dependence on the pulse amplitude was

observed (300–500 ns range), while a threshold pulse duration of 300 ns to trigger saturated electrochemotherapy efficiency was required (Fig. 5F).

In case of 10 kHz and 100 kHz pulses the expected dependence on pulse amplitude was present (Fig. 5B, D). On average the 100 kHz

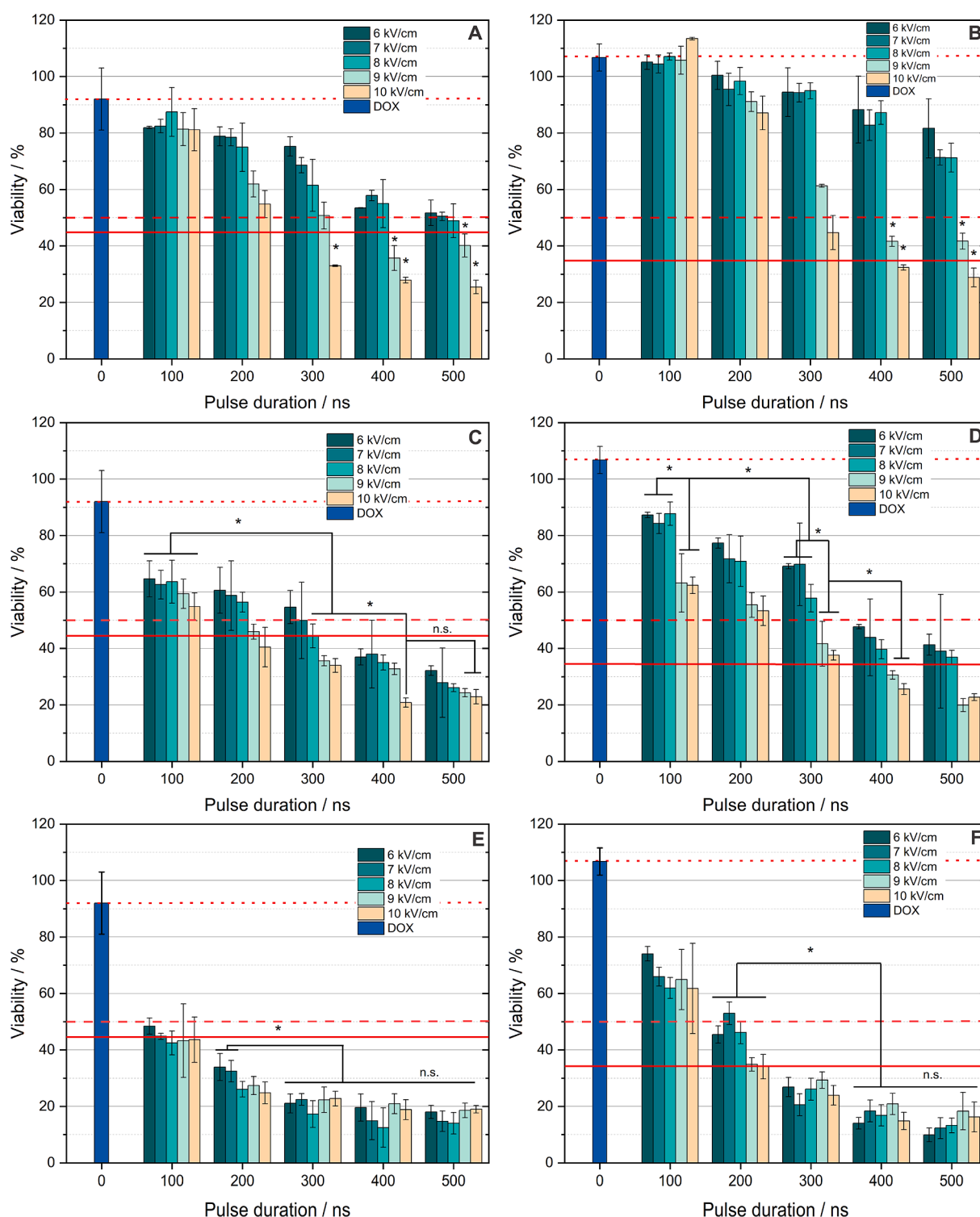


Fig. 6. Dependence of nanosecond DOX electrochemotherapy efficiency on electric field parameters, where A, B) 10 kHz protocols for 24 and 48 h evaluation times post-treatment; C, D) 100 kHz protocols for 24 and 48 h evaluation times post-treatment; E, F) 1 MHz protocols for 24 and 48 h evaluation times post-treatment. Asterisk (*) corresponds to statistically significant ($P < 0.05$) difference between samples. Red line indicates average electrochemotherapy efficiency using ESOPE protocol ($1.3 \text{ kV/cm} \times 100 \mu\text{s} \times 8$). Dashed red line used to highlight the 50 % inactivation dose (LD50). Dot red line shows treatment efficiency without nsPEF treatment.

treatment was weaker than 10 kHz, however, in most of the cases the differences were not statistically significant. The 400 and 500 ns nsPEF protocols (9, 10 kV/cm) were comparable with ESOPE (efficiency-wise), which is in agreement with permeabilization data (Fig. 3).

Similarly, the DOX nano-electrochemotherapy was analyzed. The results are summarized in Fig. 6.

Successful DOX electrochemotherapy (efficiency better than LD50) was detectable already after 24 h. For 10 kHz and 100 kHz pulses a clear dependence both on pulse amplitude and duration was observed (Fig. 6A, C). In case of 10 kHz protocols, the 400 and 500 ns pulses (9, 10 kV/cm) were superior to ESOPE. With increase of frequency (100 kHz) the efficiency of nsPEF improved even further – the 400–500 ns pulses were superior to microsecond procedure in the whole range of studied amplitudes. The MHz pulses were the best, however, different to BLM therapy, the 100 and 200 ns pulses were also effective, which could be attributed to the smaller size of DOX molecule. Yet again, no statistically significant dependence on pulse amplitude was observed (Fig. 6E).

The 48-h response was not as good, since all the protocols, which triggered non-saturated permeabilization (Fig. 3) showed an increase of viability when compared to 24 h results. Nevertheless, the 400, 500 ns protocols (9, 10 kV/cm) were still comparable or better than ESOPE cell killing efficiency-wise. In case of MHz protocols (Fig. 6F), the whole 300–500 ns range of protocols was resulting in better than ESOPE treatment independently on pulse amplitude.

The different rate of dependence of the protocols on PEF amplitude in 10 kHz – 1 MHz frequency range was peculiar. Therefore, in order to evaluate the extent of this dependence, the treatments in the 100–500 ns range were averaged PEF amplitude-wise and the results are presented in Fig. 7.

It can be seen the standard deviation (i.e., dependence on PEF amplitude) is the highest for 10 kHz procedures and the lowest is for 1 MHz protocols. Independently on the drug, MHz pulses are the best for nsPEF electrochemotherapy. In the 300–500 ns range for all 6–10 kV/cm protocols MHz protocols are significantly ($P < 0.05$) better than ESOPE procedure. Interestingly, different response to 10 kHz and 100 kHz protocols was triggered between the drugs. In case of BLM 10 kHz on average was superior than 100 kHz protocols, which was not the case for DOX. Also, in case of DOX electrochemotherapy the treatment dependence on pulse duration can be approximated as a linear function for all the frequency ranges. In case of BLM electrochemotherapy it is true only for 10 kHz (in the 100–500 ns range).

It was concluded that it is possible to derive nsPEF protocols, which are efficiency-wise equivalent to ESOPE procedure.

4. Discussion

The aim of this work was to show that nanosecond pulses can ensure sufficient drug delivery for a cytotoxic therapy comparable (efficiency-wise) with ESOPE both for bleomycin and doxorubicin-based therapies. Currently, there is no systemic coverage of nsPEF protocols for electrochemotherapy or derivation of equivalent pulse parameters in a similar manner as it was performed for μ sPEF [53,54]. The motivation to use nsPEF in electrochemotherapy lies in the unique features nsPEF offer, e.g., use of electric field amplitudes far above the electroporation threshold, while still ensure predominantly reversible electroporation. It is beneficial in terms of electric field distribution in order to ensure that the whole tumor volume is covered by above-threshold electric field. It is especially true for non-invasive parallel plate electrodes, where the tumor can't be fully electroporated due to field inhomogeneity, resulting in tumor regrowth [19]. Also, ultrashort pulse bursts feature a higher frequency component, which potentially allows mitigation of impedance changes [55] and better treatment of heterogeneous tumors when compared to microsecond procedures [4]. Additionally, nsPEF are reported to affect the intracellular organelles, however, it's mostly true for sub-100 ns pulses [56,57], which is not the case in our study.

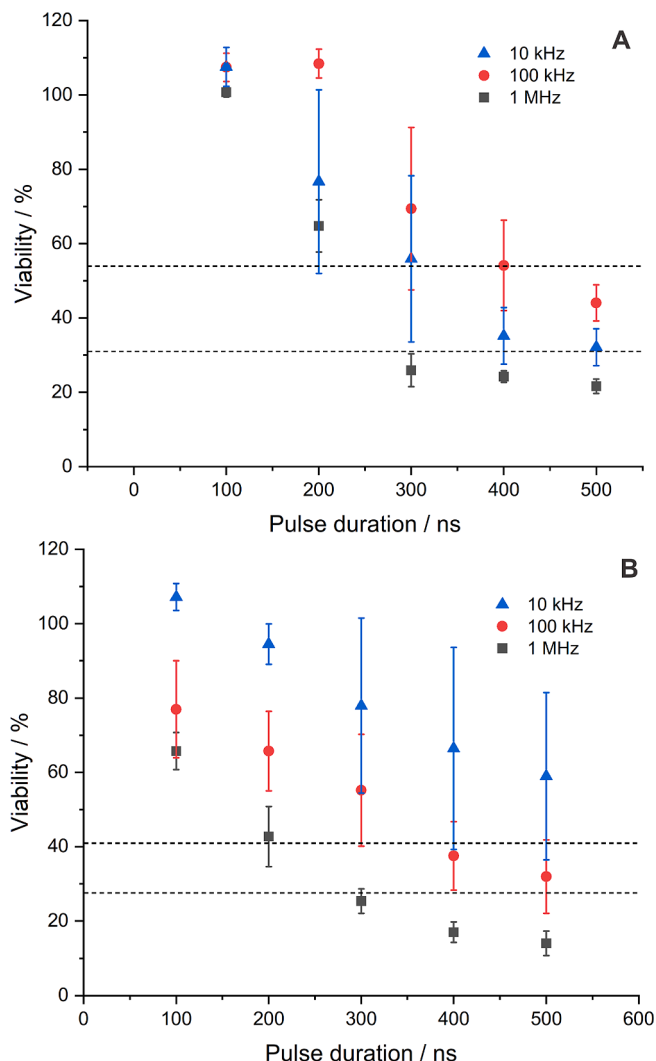


Fig. 7. The dependence of nano-electrochemotherapy efficiency 48-h post treatment on pulse duration, where (A) BLM electrochemotherapy; (B) DOX electrochemotherapy. The data points are represented as mean efficiency of the treatment in the 6–10 kV/cm range and whiskers refer to standard deviation of data. Dashed lines define the range of treatment efficiencies triggered by ESOPE protocol (1.3 kV/cm $\times$ 100 μ s $\times$ 8).

Nevertheless, the lack of electrophoretic component and the smaller pores induced by nsPEF could be highlighted as main concern for application in electro-diffusive drugs delivery context. In order to compensate this, either a higher drug dose should be used to ensure a higher concentration gradient for a more rapid diffusion or tuning of the nsPEF pulse parameters is required, which was performed in this study.

We have found out that for low frequency (10 kHz) pulses (within the studied parameter range) both the pulse amplitude and duration play a significant role. With increase of frequency the influence of pulse amplitude is diminished (refer to Fig. 7), while for 1 MHz protocols it is almost non-existent in the 6–10 kV/cm PEF range. A clear some sort of threshold pulse duration (200–300 ns) was detected in the MHz range determining the saturated on non-saturated electrochemotherapy. It is not straightforward to explain, therefore, we speculate that the effect is influenced by the residual transmembrane potential (TMP), which occurs when the pulses are compressed into a MHz burst. To support the hypothesis, we have performed a FEM simulation of the TMP accumulation during the high frequency burst. The results are presented in Fig. 8.

It can be seen that with increase of pulse duration and/or electric

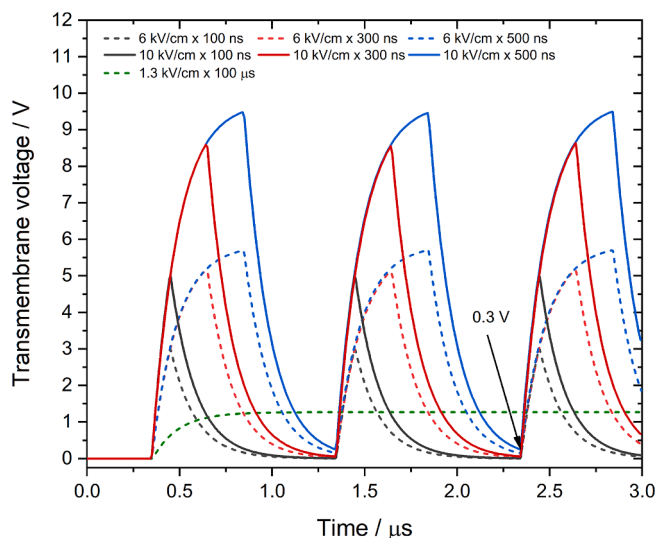


Fig. 8. The dependence of transmembrane voltage on the applied electric field parameters (rested potential not taken into account). An arrow indicates residual transmembrane voltage, which is formed due to insufficient time for membrane depolarization.

field amplitude, the TMP increases due to capacitive charging of the membrane. However, the residual voltage is formed only with the longer pulses with a threshold around 300 ns (with the defined medium conductivity of $\sigma_m = 1.3$ S/m). It might at least partly explain why there is some sort of threshold duration required for successful nsPEF electrochemotherapy in the MHz range. We speculate that the residual voltage improves the stability and/or the size of the pores and thus, positively affects the diffusion of drugs. The proposed hypothesis is in agreement with the data presented in Figs. 5F and 6F. It doesn't mean that nsPEF are not applicable in other frequency ranges (we showed that it is possible) or with shorter pulses, it's just a convenient polarization mechanism to potentiate passive mass transfer.

As it was mentioned previously, nanosecond pulse compression is also advantageous for treatment of superficial tumors with non-invasive electrodes. Without switching to irreversible electroporation (since the TMP amplitude and input energy remains the same, refer to Fig. 8) pulse compression offers unprecedented flexibility for tolerance of field non-homogeneity within the tumor. For superficial tumors the field inhomogeneity could be >50 % within the tumor [19], therefore, ensuring equivalent electrochemotherapy efficiency within a broad range of PEF (in this study 6–10 kV/cm) is very promising for homogenic treatment and full recovery. This is unachievable with ESOPE because increase of the PEF amplitude 30–50 % would result in irreversible electroporation and Joule heating. The results acquired in this study explain why the fully recovered mice were present only in nsPEF group during bleomycin electrochemotherapy and not the ESOPE group [19].

In case of doxorubicin electrochemotherapy, the number of works is significantly lower even for ESOPE protocols. In the Maillet and colleges study biodegradable micelles in a mouse hepatocellular carcinoma xenograft model were loaded with DOX. For drug loading two electroporation protocols (reversible and irreversible) were used. It was shown that electroporation threefold increased DOX uptake [35]. Rembińska et al. [36], used human breast adenocarcinoma (MCF-7) cell line and DOX ECT, which also indicated a synergistic effect. Other *in vitro* results reveal very different DOX ECT efficiencies depending on the treated cell line [37]. However, in our work we could see that after nsPEF protocols the cellular response is already detectable even after 100 ns pulses, which is not the case for BLM therapy. We believe that the phenomenon is influenced by the difference in molecular size of the drugs, therefore, it's indicative that nsPEF should be even more compatible with cisplatin (almost 2-fold smaller than DOX). According

to Vizintin et al. [2] cisplatin nano-electrochemotherapy results in a comparable efficiency as ESOPE treatment, which supports our speculation. It was not the case for BLM-electrochemotherapy [2], however, in the present work we have provided nsPEF protocols to solve the problem.

Finally, moving towards shorter pulse duration range is advantageous in terms of electrolysis. Shorter pulses reduce the oxidation as a product of electrolysis, which enhance generation of reactive oxygen species (ROS) during electroporation [58,59], consequently, reduce the possibility of damage to unsaturated lipids.

It should be noted that in this work we have been limited by the 10 kV/cm electric field due to possibilities of available infrastructure. However, with the PEF amplitude increased, the duration of the pulses can be further reduced, which will prevent residual TMP accumulation at 1 MHz (refer to Fig. 8). Therefore, even higher frequency 2–5 MHz electric field will have to be utilized to make the time delay between the pulses shorter.

5. Conclusions

In this work, the effects of bleomycin and doxorubicin-based nano-electrochemotherapies were studied. It was shown that nano pulses can be as effective as standard ESOPE procedures, however, require a lot smaller energy input, which is advantageous in terms of Joule heating. We have also shown a clear frequency dependence of the nano-electrochemotherapy efficiency. Compressing the nano pulses into MHz range bursts is advantageous for improvement of the treatment efficiency. The results are useful for development of new nano-protocols and derivation of equivalent pulse parameters for logical evolution of conventional electrochemotherapy towards shorter pulse duration range.

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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