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Ultrasound and electric pulses for transdermal drug delivery enhancement: Ex vivo assessment of methods with in vivo oriented experimental protocols

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ABSTRACT

In our present study we focus on two physical enhancement methods for transdermal drug delivery: ultrasound and electric pulses either alone or in combination. Great emphasis has been given on the design of the experimental system and protocols, so the results and the conclusions drawn from them would have greater relevance for in vivo use and later translation into clinical practice. Our results show a statistically significant enhancement of calcein delivery (after one hour of passive diffusion following treatment) already after 5 minutes of ultrasound application, or only 6 × 100 short high voltage electrical pulses. We also experimented with combinations of the two enhancement methods hoping for synergistic effects, however, the results showed no evident drastic improvement over single method. Looking closer at physics of both methods, this absence of synergy in our in vivo oriented experimental setting is not surprising. The mechanism of action of both methods is the creation of aqueous pathways in the stratum corneum leading to increased skin permeability. However, when used in combination (regardless of the order of methods), the second method was unsuccessful in adding many new aqueous pathways in the stratum corneum, as it acted preferentially near the sites of the existing ones.

1. Introduction

Transdermal drug delivery (TDD) is being studied as a method for noninvasive drug administration. However, the use of this method has been limited because the superficial layer of the skin, the stratum corneum, is not sufficiently permeable to allow effective transfer of drugs into the skin and/or the bloodstream. Skin as the largest organ of human body is covering an area of approximately 2 m^2 and is providing contact between the body and the external environment. The barrier properties of skin are attributed to the outermost layer, the stratum corneum (SC), which is highly lipophilic. Although SC is only 10–20 μm thick, it allows only small, lipophilic drugs to permeate the skin in very small concentrations. Most large hydrophilic macromolecules, such as peptides and proteins, are unable to permeate passively. Therefore, enhancement techniques are required to assist in the transport of such molecules across the SC (Williams, 2003; Yarmush et al., 2014; Zorec et al., 2013b). In this study, we focused on two physical enhancement methods: the use of ultrasound (sonoporation - SP) and electric pulses (electroporation - EP), both known to create aqueous pathways through the SC in order to increase its permeability.

Electroporation causes transitory structural perturbation of lipid bilayer membranes resulting from the application of short duration high voltage pulses. Electric field induces aqueous pore formation in the cell membrane and can provide a local driving force that facilitates the transport of molecules. Electroporation is typically used on the unilamellar phospholipid bilayers of cell membranes of cells in vitro or in the tissue to introduce a variety of molecules, such as drug molecules or DNA (Yarmush et al., 2014). However, when used on skin, electroporation can create aqueous pathways through the SC, which is in fact a stack of lipid bilayer membranes. In this way, various molecules with a wide range of molecular weights and physicochemical properties have been

Abbreviations: TDD, transdermal drug delivery; SC, stratum corneum; EP, electroporation; SP, sonoporation.

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successfully delivered (Denet et al., 2004; Mori et al., 2003; Vanbever and Preat, 1999).

Skin sonoporation is the use ultrasound to temporarily increase SC permeability and deliver therapeutic compounds into and/or through the skin. At this point we would first like to devote a few words to the terminology in the area of TDD with ultrasound. Nearly every study published so far uses the term sonophoresis to describe ultrasound-mediated TDD. In this paper, a distinction between the terms sonophoresis and sonoporation is made. The term sonoporation describes better the creation of aqueous pathways through the SC as a result of ultrasound application. Similar distinction is made in the area of electrically-mediated TDD where both terms are used: electroporation as well as electrophoresis, however, they are used to describe two different mechanisms: (i) electroporation is the creation of aqueous pathways, and (ii) electrophoresis is the migration of charged species in the electric field. In this paper, in order to avoid ambiguity, similar distinction between mechanisms was used for ultrasound-mediated TDD: The term sonoporation is used to describe the creation of aqueous pathways in the SC with ultrasound, while sonophoresis describes the migration of the delivered molecules via acoustic streaming and convection effects.

Ultrasound can perturb mammalian tissue primarily by acoustic cavitation, but also by thermal effects (Lavon and Kost, 2004; Mitragotri and Kost, 2004; Park et al., 2014; Polat et al., 2011b; Smith, 2007). Cavitation is caused by the oscillation and collapse of the cavitation bubble in the ultrasound field, while thermal effects are attributed to the absorption of ultrasound energy. To date, different ranges of ultrasound frequencies were used for sonoporation and can roughly be divided into: (i) low frequency sonoporation (20–100 kHz) and (ii) high frequency sonoporation (1–16 MHz). Based on previous studies, low frequency ultrasound appears to be more effective to increase skin permeability and deliver a wide range of molecules, including large hydrophobic and hydrophilic compounds, than high frequency ultrasound (Park et al., 2014; Polat et al., 2010, 2011b; Smith, 2007).

Most pre-in-vivo electroporation studies reported in the literature were conducted in differently sized glass diffusion cells - Franz cells (Chizmadzhev et al., 1998; Pliquett et al., 1995, 1998, 2002, 2005; Pliquett and Gusbeth, 2004; Pliquett and Weaver, 1996; Prausnitz et al., 1993; Vanbever et al., 1999; Vanbever and Preat, 1995; Zorec et al., 2013a). Those cells consist of two compartments – the donor, containing the molecule to be delivered, and the receiver compartment filled with buffer – separated by a skin sample. In this way the transport of the molecule from one compartment to the other can be easily monitored. However, although diffusion cells represent the gold standard in skin passive diffusion studies, they are not the ideal in vitro setup to be used in TDD studies with electroporation or sonoporation. Due to the geometric constraints of the diffusion cell, delivering the electric pulse to the skin while inside the diffusion cell is challenging. Namely, the electric pulses are delivered through the electrodes placed in the donor and the receiver solution. This in vitro experimental setup differs greatly from the in vivo situation. In an in vivo or clinical application of skin electroporation, variations of electrode configurations are used in which all of the electrodes are placed externally on the skin's surface. The electric field associated with the Franz cell type setup (where one electrode is placed on the external side and one electrode is placed on the internal side of the skin) is unlikely to closely resemble the intended in vivo case. Further, as the buffer represents – from the electrical point of view – a non-negligible part of the whole system, most of the delivered voltage rests in the buffer itself and only a small part on the skin. Thus when considering the published results of such electroporation studies, care must be taken not to interpret the voltage applied to the electrodes as the actual voltage delivered across the skin sample, which depends strongly on the placement of the electrodes in the donor and the receiver solution, as well as the electrical conductivities of the solutions, and is not easily controlled. Also, the delivery of electric pulses into the buffer inevitably causes electrochemical reaction that may change electrical characteristics of the buffer-skin system during, and possibly after the pulse delivery, either reversibly or irreversibly.

Similarly, most pre-in-vivo sonoporation experiments are conducted in some kind of glass diffusion cells where the ultrasound transducer is immersed in the donor solution (Alvarez-Roman et al., 2003; Lopez et al., 2011; Polat et al., 2011a, 2012; Sarheed and Rasool, 2011). Again, we would like to point out a few potential pitfalls of such experiments. First, the sound waves are deflected from the glass walls of the diffusion cell. The deflections are superimposed, creating a non-uniform sound field whose parameters are now not the same anymore as those of the ultrasound delivered by the transducer. Further, most ultrasound devices used in the experiments are laboratory sonicators used in nanoparticle production, cell lysis, mixing, cleaning and many other applications. High ultrasonic vibrations are usually delivered through a cylindrical metal rod whose tip is placed in the donor solution. In order to fit into the donor compartment, this tip is much smaller than the donor orifice and the skin sample positioned between the two compartments, creating a powerful sound field in the vicinity of only small portion of skin sample directly under the sonicator tip. All of these experimental circumstances, as in the case of electroporation experiments, differ greatly from the in vivo or clinical applications. Also, overheating is less problematic in the diffusion cells with the donor compartment exposed to surrounding air, making heat dissipation easier. In contrast, for in vivo applications the skin is covered with usually larger ultrasound horn, creating an occlusion that lessens heat dissipation and makes overheating potentially more problematic.

Quite a few pre-in-vivo studies were conducted with ultrasound and electric pulses as transdermal delivery enhancers. Wide ranges of different pulse and ultrasound parameters were used in those studies, such as ranges of different pulse voltages, durations, number of pulses, even their shape (electroporation), or ranges of different ultrasound frequencies, durations of exposure, output powers and duty cycles (sonoporation), mostly in different types of glass diffusion cells. We conducted ex vivo experiments on full-thickness porcine ear skin and we did not specifically focus on experimenting with ranges of parameters as their effects have to some extent been identified and described by the published work of other researchers. Instead, we put great emphasis on the design of the experimental system, such as the geometry and the placing of the electrodes and the ultrasound transducer, as well as the SP and EP protocols that were designed so they can have greater relevance for in vivo use and can therefore easily be translated into the clinics. The details are described in the following sections.

Further, the efficacy of transdermal molecular delivery can be increased by using two or more enhancement methods in combination. Such coupling of different enhancement methods can prove more successful and can result in more molecular delivery with less provided energy. In other words, the effects can be not only additive but synergistic. Because possible mechanisms by which ultrasound and electric field enhance transdermal transport are different, the combination of these two methods might result in such synergistic effect. As mentioned before, the use of individual techniques have already been investigated, however we have only found two studies dealing with a combination of these two methods (Kost et al., 1996; Petchsangsai et al., 2014). We therefore experimented with the combination of electroporation and sonoporation again focusing on creating the

experimental conditions as close to intended in vivo situation as possible.

2. Materials and methods

2.1. Skin preparation

In present study porcine skin was used because it represents a good model of human skin with regard to its physical properties. Porcine skin was obtained from local slaughterhouse immediately post-mortem (Farme Ihan LLC, Šentjur, Slovenia), before washing with steam and detergent in order not to compromise skin integrity. Skin samples were prepared within three hours post-mortem. Excess subcutaneous fat was removed, and the skin was carefully trimmed to obtain a full thickness skin of approximately 2–3 mm thickness. Prepared skin samples were frozen until use, for no longer than one month.

2.2. Ultrasound application

Ultrasound was applied to skin samples with a commercially available device used in cosmetics and dermatology (CaVite, Iskra Medical LLC, Ljubljana, Slovenia), using ultrasound transducer shown in Fig. 1a. The free field ultrasound pressure was measured using a piezoelectric hydrophone (8103 hydrophone, Brüel & Kjær, Nærum, Denmark) at the distance of 5 mm from the transducer face, which is a comparable distance of the skin to transducer in actual experimental setup. The measured peak to peak amplitude of the ultrasound pressure was 166 kPa, with no difference observed for positive or negative pressure. The walls of the bath were made from Plexiglas® and lined with SA-J35 ultrasound absorber (Hangzhou Applied Acoustics Institute, Hangzhou, China), so only progressive ultrasound wave was present in the measuring setup (Jelenc et al., 2012). The device was operated in a continuous mode at 30 kHz frequency (for full protocols see Table 1). Treated skin sample was placed on a rubber base (SA-J35 ultrasound absorber) in order to avoid sound wave reflections. A 1 cm × 1 cm depression of 4 mm depth was cut into the rubber base in order to create a deepening where skin sample was placed. This concave deepening in the skin sample was filled with distilled water to create a reservoir for the coupling medium. Ultrasound transducer was placed on top. According to the literature, 4 mm distance is in the range of recommended distances between the transducer and the skin sample to cause cavitation leading to the creation of aqueous pores in the SC. In order to avoid overheating of skin, the temperature was measured before, during and after the treatment with infrared thermometer (RS-1327, RS Components Ltd., UK).

Table 1
Ultrasound and electric pulse application protocols.

	Protocol number	Ultrasound duration	Frequency	Duty cycle	Ultrasound pressure
Ultrasound (SP: sonoporation)	SP1 SP2 SP3	2.5 min 5 min 7.5 min	30 kHz	100%	166 kPa
	Protocol number	**Pulse voltage**	**Number of pulses between electrode pairs**		**Pulse duration/ spacing**
Electric pulses (EP: electroporation)	EP1 EP2 EP3 EP4 EP5 EPcontrol	200 V 100 V 200 V 200 V 200 V 0 V	100× Continuously for 30 s Continuously for 30 s Continuously for 15 s Continuously for 5 s electrode applied on skin for 30 s		100 μs/ 100 μs
Combination (EP + SP)	EP1 + SP2 EP4 + SP2 EP5 + SP2				

2.3. Electric pulse application

Electroporation pulses were applied using a square wave pulse generator (Green Skin Pore electroporator, Iskra Medical LLC, Ljubljana, Slovenia), with an array of 7 pin electrodes arranged in a honeycomb configuration (Fig. 1b) applied externally on skin sample fixed on a Styrofoam base. Skin was treated with electric pulses of 100 V and 200 V amplitude and different number of pulses (for all EP and SP delivery protocols see Table 1). Pulse duration and spacing between pulses were both fixed at 100 μs. The pulses were delivered between the middle electrode (anode, numbered electrode #1) and the 6 surrounding electrodes (cathodes, numbered 2–7), pairwise: 1–2, 1–3, . . . 1–7. Actual voltages delivered on skin and electric current were measured with LeCroy Oscilloscope (Wavepro 7300A) using differential voltage probes (ADP305, High Voltage Differential Probe, LeCroy) and current probes (AP015, LeCroy).

2.4. Ex vivo skin permeation studies

Ex vivo permeation studies of calcein (0.1 mM in phosphate buffer) were performed using full thickness skin (2–3 mm thick on

Fig. 1. (a) Ultrasound transducer, 3.5 cm diameter; (b) Electroporation pin electrodes arranged in honeycomb configuration. Electrode separation: 5 mm

average). Calcein is a charged and hydrophilic molecule and does not permeate significantly by passive diffusion through untreated SC. Diffusion area of a skin sample was 2.5 cm^2 and 6 repetitions of each protocol were performed. After the treatment – either with sonoporation or electroporation or both – 100 μl of 0.1 mM calcein was placed on the skin diffusion area and was removed and washed with buffer after 1 hour of passive diffusion.

2.5. Skin lysis protocol

Following the 1 hour of passive diffusion, skin areas exposed to passive diffusion were cut into equally-sized samples. Tissue samples were ground in a laboratory mortar, covered with sufficient volume of cold RIPA buffer (10 ml of RIPA/1 g tissue). The lysate was transferred into the centrifuge tube that was first incubated on ice for 30 minutes then centrifuged at 5400 rpm and 4 °C for 20 min. When finished, the supernatant was collected and the fluorescence (proportional to the concentration of calcein that permeated through the SC into the skin) was measured with a spectrofluorometer (Jasco, FP-6300).

2.6. Chemicals

Calcein was diluted in phosphate buffer solution, which was prepared in bi-distilled water (pH 6.5, 100 mM). Calcein, sodium chloride and RIPA buffer were purchased from Sigma–Aldrich (St. Louis, MO, USA), while potassium chloride, di-sodium hydrogen phosphate and potassium dihydrogen phosphate were purchased from Merck (Darmstadt, Germany).

2.7. Data analysis

The results are presented as the quantity of calcein in the supernatant. SigmaPlot 11.0 (Systat, USA) was used for statistical analysis. As the analysis showed that the data was in general not normally distributed, a nonparametric test (Student–Newman–Keuls nonparametric test) was used to check for statistically significant difference. As this test is not very conservative (it is more likely to show statistical significance), significance level was chosen more conservatively ($p < 0.01$). The data is presented with first and third quartile box plots, median and mean values and whiskers denoting min and max values.

3. Results and discussion

3.1. Ultrasound (sonoporation)

A great emphasis of our study was given to the design of the experiment and the protocols in order to eliminate as many accompanying effects as possible and focus exclusively on the effect of ultrasound-induced cavitation causing increase in SC permeability. With this in mind, as well as to avoid safety issues important for future clinical application, we did not use surfactants as skin penetration enhancers that were used in many of the published studies in combination with sonoporation (Lopez et al., 2011; Mitragotri et al., 2000; Polat et al., 2011a, 2012; Tezel et al., 2002) and were proven to greatly enhance ultrasound-mediated delivery.

Ultrasound can be applied concurrently with drug application (simultaneous protocols) or prior to drug application (pretreatment protocols). While the simultaneous-type protocols seem the most sensible approach, most experimental results shows either conflicting results (Sarheed and Rasool, 2011) or no improvement over pretreatment protocols (Lavon and Kost, 2004; Mitragotri and Kost, 2004; Polat et al., 2010, 2011b). During our preliminary experiments, we compared simultaneous vs. pretreatment approach, postulating that simultaneous protocols should, theoretically, be more efficient, as they would take advantage of two mechanisms instead of just one: sonoporation (creation of SC pathways by cavitation), and sonophoresis (migration of molecules via convection and acoustic streaming). However, simultaneous protocols with calcein present in the ultrasound coupling medium consistently showed no advantage over pretreatment approach (data not shown). Yet these results are not at all as surprising as they seem at first. Namely, as the cavitation depends greatly on the physical properties of the coupling medium (density, viscosity, gas content), the presence of calcein (or a drug for clinical applications) may actually suppress cavitation leading to sonoporation of the SC. Second, as the cavitation and aqueous pathway creation (skin permeability is increased for a prolonged period of time) is the predominant mechanism in low-frequency sonoporation, the contribution of sonophoretic mechanisms (relevant only during ultrasound application) such as acoustic streaming and convection microcurrents is much lesser during short ultrasound application times that are preferred for clinical applications. Furthermore, cavitation may cause degradation of the drug when present in the coupling medium during sonication. Also, to avoid overheating and burns, the coupling medium can be frequently replaced or circulated, which increases the cost of the treatment if the drug is present during sonication. As all of the described reasons make simultaneous protocols less appropriate in the clinical setting, we did not pursue simultaneous protocols further.

Further, we also tried ultrasound on skin pretreated with microneedles (roller with embedded microneedles). The idea was to replace the sonoporation mechanism with microneedles in order to undoubtedly create large enough pathways through the SC, then use ultrasound not to cause further structural perturbation of the SC but to push calcein molecules through the pathways via sonophoresis (calcein was present in the coupling medium during sonication). Much to our surprise, calcein concentration in skin after the microneedle+ultrasound treatment was even lower than after ultrasound treatment alone (data not shown). However, similar results have been reported before by Park et al. (Park et al., 2010). In their study, when different sonication protocols were preceded by skin treatment with microneedle roller, this lowered the efficacy of ultrasound treatment. They hypothesized that the micro-sized channels created by microneedles acted as air pockets instead of as transporting channels in sonophoresis. On the other hand, some studies demonstrated effectiveness of microneedle-pretreatment prior to sonification (Petchsangsai et al., 2014; Yoon et al., 2010). An even more innovative and successful approach was reported by Chen et al. (2010) where hollow microneedle array filled with the molecule to be delivered was used to pierce the upper skin layers, which was followed by ultrasound application before the removal of the microneedles to enhance the delivery.

We tried to keep temperature-induced increase in skin permeability as low as possible. Namely, the literature reports that the contribution of thermal effect towards increased permeability is considerable and that every 10 degrees increase in skin temperature leads to doubling in the skin permeability (Sarheed and Rasool, 2011). Moreover, avoiding excessive heating that may cause burns and skin damage is necessary in order to make the method more clinically acceptable. To this objective, different approaches were used, such as periodic replacement of the coupling medium or the use of lower duty cycle to allow for heat dissipation during OFF periods (Polat et al., 2011a,b). In our study, we opted out of using lower duty cycles because such protocols require longer application time to attain the same efficacy, again lowering their clinical relevance (Sarheed and Rasool, 2011). Also, frequent switching between ON and OFF periods may cause the cavitation bubbles to dissolve back into the coupling medium during the OFF periods, rendering the method

less efficient (Polat et al., 2011b). We decided to cool the coupling medium down to 10 °C before applying it to skin (however, it warmed up by the time the small amount of it was pipetted onto skin sample). During sonication, we measured the skin temperature with infrared thermometer every two minutes, after pausing ultrasound application and removing the transducer. The temperatures never exceeded 30 °C. After the treatment, the skin did not show any damage or visible changes.

As anticipated, the results in Fig. 2 demonstrate that the delivery of calcein increases with increasing time of ultrasound exposure. We linearly increased exposure time, but only up to 7.5 min, as longer protocols are clinically less desired. Statistical test showed that not all the data was normally distributed, therefore, to check for significant differences between protocols, a nonparametric test (Student–Newman–Keuls nonparametric test) was used. Protocols SP2 and SP3 (5 and 7.5 min exposure time) are significantly different from passive diffusion (control-PD), while protocol SP1 (2.5 min exposure time) is not. Further, even though the increase in sonication time visually seems to suggest evident trend in the success in calcein delivery, the statistical test only showed significant difference between protocols SP1 and SP3 (2.5 and 7.5 min), and protocols SP1 and SP2 (2.5 and 5 min) due to the scatter of the data. The data is presented with first and third quartile box plots, median and mean values and whiskers denoting min and max values.

The scatter of data for sonication protocols 1–3 in Fig. 2 is considerably higher than the scatter of the control (passive diffusion), suggesting that the reason for this dispersion is the treatment protocol itself, and not sample variation. Park et al. (2010) report that standard deviation was greatly reduced when ultrasound contrast agent (UCA) was used as a cavitation enhancer in the coupling medium. Namely, the uniform size and the stability of microbubbles in the ultrasound contrast agent assure a more controllable cavitation leading to better treatment repeatability. However, the above-mentioned study used high-frequency ultrasound (1 MHz), with commercially available UCA containing microbubbles whose resonance frequencies are in the MHz range, intended for widely used diagnostic purposes (Sboros, 2008). Aiming at scatter reduction or even higher efficacy of the method for low-frequency sonoporation, a coupling medium containing larger microbubbles with kHz resonance frequencies should be used. Unfortunately, such media are not commercially available, so deionized water was used in our study, which does not offer any control over gas content that may vary from one experiment to the next.

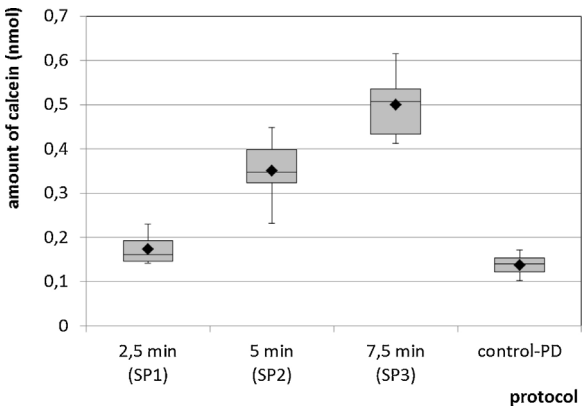

Fig. 2. Amount of calcein in skin after different ultrasound exposure times (protocols SP1-SP3). Passive diffusion only without any skin treatment was used as a control (control-PD). The data is presented with first and third quartile box plots, median (horizontal line between quartiles), mean (black diamonds) and whiskers denoting min and max values.

3.2. Electric pulses (electroporation)

A number of studies have been performed in the area of the use of electroporation for TDD, using different pulse protocols and employing theoretical and experimental approach to explain the mechanisms involved and to find an optimal protocol. Mainly, two types of pulses have been used in skin electroporation: (i) exponentially decaying (Pliquett et al., 2005; Pliquett and Gusbeth, 2004; Vanbever et al., 1999), and (ii) square-wave pulses (Denet and Preat, 2003; Dujardin et al., 2002; Pavselj and Miklavcic, 2011, 2008 Zorec et al., 2013a) of different lengths and amplitudes. When comparing different pulse protocols, pulse length and amplitude are the most influential factors. Namely, to achieve creation of micrometer-sized aqueous pathways, a high enough pulse amplitude is essential and is achieved with short duration-high voltage square-wave pulses or the first part of high-voltage exponentially decaying pulses. On the other hand, long duration low voltage square-wave pulses or the long low voltage tail of exponentially decaying pulses result in higher cumulative pulse energy and create regions of increased permeability within the SC that are relatively large (up to hundreds of μm) and long lasting. Also, long low voltage pulse regime may provide substantial electrophoretic drive of charged molecules through the skin.

However, long low voltage pulses, while undoubtedly improving the efficacy of the treatment, may cause slight burns, muscle contractions and unpleasant sensations when applied in vivo. With clinical applications – safety and patient compliance – in mind, we therefore limited our pulse protocols to short, high-voltage pulse regimes. The pulses were applied with the device Green Skin Pore through an array of spring-loaded pin electrodes arranged in a honeycomb configuration (Fig. 1b) that were developed in collaboration with Iskra Medical LLC, aimed at transdermal delivery applications. An important feature of the device is the electric current limitation at 20 mA. Namely, the delivery of high-voltage pulses to skin surface causes electroporation and increased permeability of the SC, accompanied by the increased electrical conductivity of the tissue leading to sudden rise in the electric current through the tissue. The electric current measurements and the closed-loop system of the device reduces the voltage of the delivered pulses when current limitation is reached – i.e. when the SC is electroporated – to ensure the safety of the treatment and increase patient compliance without compromising the efficiency of the treatment.

In our study, pulse protocols differed in the number of delivered pulses of two different voltage amplitudes: 100 V and 200 V (Table 1). Pulse duration and spacing between pulses were both fixed at 100 μs for all protocols. The pulses were delivered between the middle electrode (anode, numbered electrode #1) and the 6 surrounding electrodes (cathodes, numbered 2–7), pairwise: first between electrodes 1–2, then 1–3, . . . 1–7. The shortest protocol – protocol EP1 – only took 120 ms to deliver so the electrode was not moved during pulse delivery. Conversely, for the rest of the electroporation protocols – protocols EP2 to EP5 – the electrode was moved in circular motion during pulse delivery (5 pulses between each pair continuously for 5–30 s), to ensure more homogeneous SC electroporation of the treated area. Namely, during pulse delivery between the anode and the cathode, only a small part of the area between the two pin electrodes is covered by a sufficiently high electric field to cause SC electroporation, as the electric field is very inhomogeneous (Pavšelj et al., 2010). By moving the electrode in circular motion during pulse delivery, larger portion of the skin surface under the electrode is exposed to a high enough electric field, leading to increased efficiency of the treatment.

Actual voltages delivered on skin and electric current through tissue were measured with an oscilloscope and some of them are

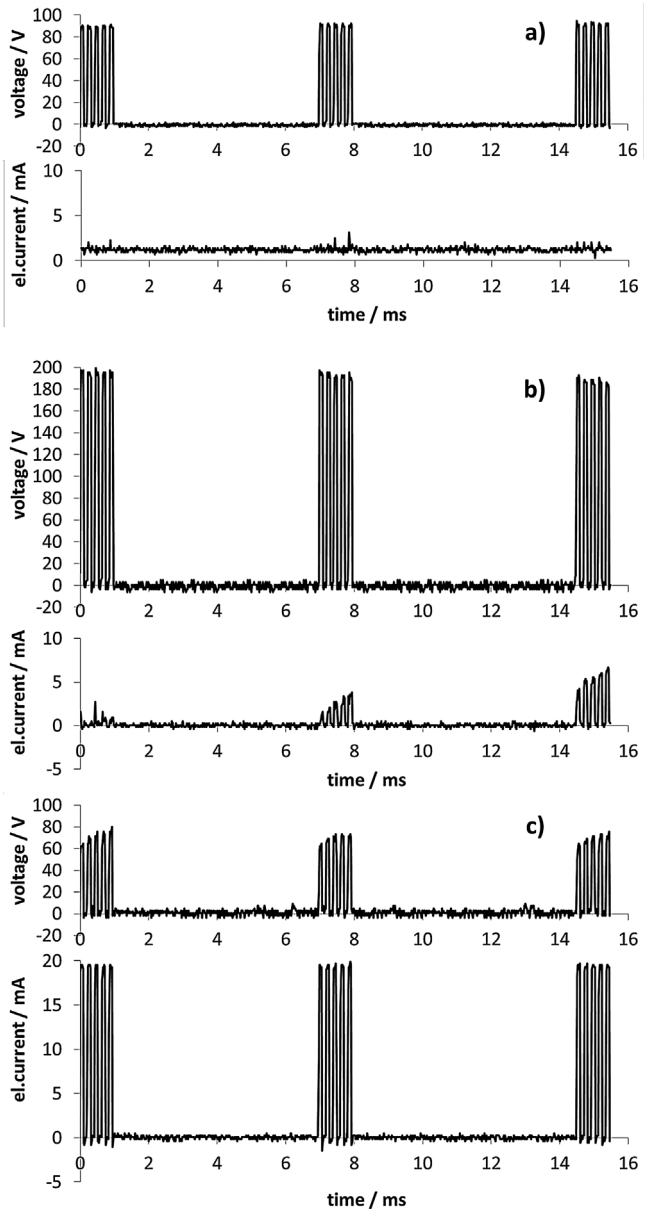

Fig. 3. The voltage delivered on the electrodes (top panels) and the electric current through the tissue (bottom panels). (a) protocol EP2: 100 V, 30 s continuous delivery measured at t = 29 s, (b) protocol EP3: 200 V, 30 s continuous delivery measured at $t = 0$, (c) protocol EP3: 200 V, 30 s continuous delivery measured at $t = 29$ s. For all protocols 5 pulses were delivered between each electrode pair, which was continuously repeated for 30 s (for the protocols EP2 and EP3 presented in the figure). The signals were measured between one electrode pair; hence zero values between periods of 5 pulses during which time pulses are delivered between other 5 electrode pairs.

shown in Fig. 3. The voltage delivered on the electrodes is shown on top panels, while the electric current through the tissue is presented on bottom panels of Fig. 3, for protocols: (a) EP2 (100 V, 30 s continuous delivery) at $t = 29$ s, (b) EP3 (200 V, 30 s continuous delivery) at $t = 0$, (c) EP3 (200 V, 30 s continuous delivery) at $t = 29$ s. Looking at Fig. 3a, 100 V pulse amplitude seems to be too low to cause SC electroporation, as the non-permeabilized, highly resistant stratum corneum represents a barrier to the flow of electric current (shown in bottom panel) even after 30 seconds of pulse delivery. However, 200 V pulse amplitude successfully permeabilizes the SC, as evidenced by the increase of the electric current already after the first 5 delivered pulses (Fig. 3b bottom panel, measured at $t = 0$). Further, the result of the electric current limitation and the closed-loop system of the device is evident from Fig. 3c, measured just before the end of protocol EP3 delivery: the electric current depicted in the bottom panel has reached the maximum 20 mA and did not drop despite the reduced delivered voltage (top panel). Interestingly, even though this reduced voltage is significantly below 100 V (the voltage amplitude of protocol EP2 that in itself was not able to cause SC permeabilization), the electric current stays at 20 mA, indicating high level of SC electroporation.

The amount of calcein in skin after one hour of passive diffusion following treatment with different electroporation protocols is shown in Fig. 4. In line with signals measured during pulse delivery, 100 V pulse amplitude does not increase skin permeability for calcein, even when the protocol is applied for 30 s (protocol EP2 showed no statistically significant improvement over passive diffusion: control PD). But when voltage is increased to 200 V, even with a much lower number of pulses, calcein delivery shows statistically significant enhancement when compared to passive diffusion (protocol EP1 vs. control PD), although there is no statistically significant difference between protocols EP1 and EP2.

Furthermore, as 200 V seems to be above the threshold voltage causing stratum corneum electroporation and increase in permeability to molecular transport, we assumed that calcein delivery would increase by increasing the number of pulses. Indeed 200 V pulses delivered for 30 s (protocol EP3) showed significantly higher calcein delivery over protocol EP1 (only 100 pulses between each electrode pair, the duration of the whole protocol was 120 ms). However, when we reduced the pulse application time from 30 s to 15 s, the calcein delivery was not reduced but enhanced greatly (EP3 vs. EP4). Since this was not an anticipated outcome, we repeated the experiments, but the result was the same. As surprising as this seemed at first glance, this decreased efficacy of longer pulse application times was probably due to skin damage with repeated application of high-voltage pulses after SC has been electroporated. Namely, we noticed some changes on skin samples after protocol EP3 (200 V, 30 s): the formation of a thin crust, probably due to a slight burn, which was most likely the reason for the reduced calcein delivery during one hour of passive diffusion following treatment. We reduced pulse application time further, from 15 s (protocol EP4) down to 5 s (protocol EP5). Even with only 5 s of electroporation, the calcein delivery enhancement was still very high. On the other hand, the scatter of data increased substantially with pulse application time reduction (due to large

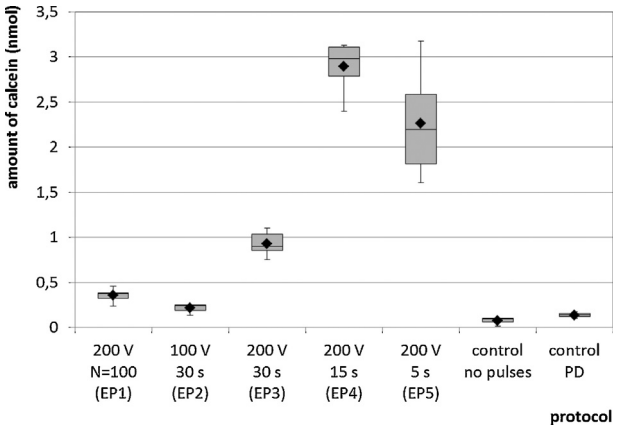

Fig. 4. Amount of calcein in skin after different electroporation protocols (see Table 1). Passive diffusion only without any skin treatment was used as a control (control-PD). Also, we assessed possible contribution to increased SC permeability because of electrode array movements on skin (control no pulses: circular movements of electrode array on skin for 30 s). The data is presented with first and third quartile box plots, median (horizontal line between quartiles), mean (black diamonds) and whiskers denoting min and max values.

scatter there is no statistical difference between protocols EP4 and EP5). This was not entirely unexpected, as the electrode was moved on skin during pulse delivery to achieve more homogeneous permeabilization of the SC, which lessened the repeatability of the treatment. Namely, moving the electrode array around skin surface, it is difficult to maintain the same circular motion and the same pressure on the electrodes from one treatment to the next. These differences cancel out at longer pulse application times, but are amplified at shorter times, hence larger scatter of data. Lastly, we assessed how much, if any effect the electrode array rubbing on the skin surface had on increased SC permeability. To this end, one more control group was added: no pulses were applied to skin, only the electrode array was moved in the same circular movements on skin surface for 30 s. The results revealed no contribution of skin rubbing towards skin permeability enhancement (Fig. 4).

3.3. Ultrasound and electric pulses combined

In many cases, transdermal molecular delivery can be enhanced further if two or more treatment methods are used in combination. The aim of such approaches is not only achieving an additive effect, where the effect of two methods is equal to the sum of the effect of the two methods used separately. Such coupling of methods can result in synergistic effect, where the efficacy of the combination is greater than the sum of their separate effects. Because mechanisms by which ultrasound and electric pulses enhance transdermal transport are different, the combination of these two methods might result in such synergistic effect. Namely, different mechanisms may contribute to TDD enhancement: (a) the creation of aqueous pathways in the SC with electric pulses (electroporation), (b) further creation of aqueous pathways with ultrasound (sonoporation) and (c) if the delivered molecule is present in the coupling medium during sonication: the migration of the molecules via acoustic streaming and convection effects of ultrasound (sonophoresis).

The results of calcein delivery using combination methods compared against single methods (EP or SP) are summarized in Fig. 5. We chose 5 min of sonication to be used in combination with electroporation (protocol SP2), as the calcein delivery enhancement was already significantly different from passive diffusion, while the duration of the protocol was not too long. Further, SP2 was combined with three electroporation protocols: EP1, EP4 and EP5. We excluded the other two electroporation protocols – EP2 and EP3 – as the former was not significantly different from passive diffusion and the latter caused some unwanted changes on skin, reducing calcein delivery enhancement.

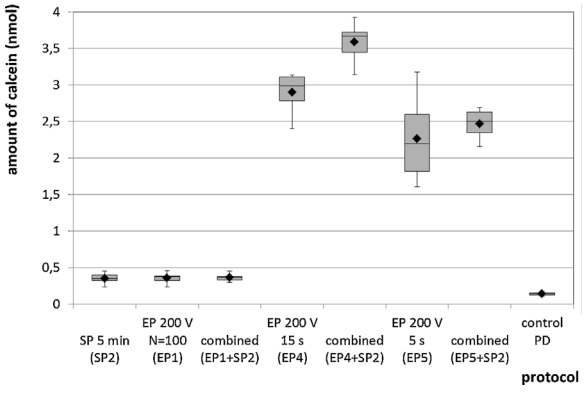

Fig. 5. Amount of calcein in skin after combination methods, compared against single methods. Passive diffusion only without any skin treatment was used as a control (control-PD). The data is presented with first and third quartile box plots, median (horizontal line between quartiles), mean (black diamonds) and whiskers denoting min and max values.

Contrary to our expectations, only the combination of protocols EP4 + SP2 showed statistically significant advantage over EP4 alone, but even this combination does not seem to result in drastic improvement over single method use. The combination EP1 + SP2 does not show any improvement from EP1 alone, not even a visual trend. The last combination of the two methods we experimented with, EP5 + SP2 seems to visually suggest a slight increase in the calcein delivery enhancement when compared to EP5 alone. However, this difference is not statistically significant, which may be due to the high scatter of the EP5 protocol.

As the electroporation + sonoporation sequence did not result in any dramatic improvement over single method use, we reversed the order of the methods, treating skin samples first with ultrasound, followed by electroporation. However, the ordering of the methods did not have any influence on the outcome of the treatment, that is to say that transdermal delivery of calcein was the same (no statistical difference or visual trend) for EP + SP or SP + EP combination (data not shown). Looking closer at physics of both methods, this absence of synergy between sonoporation and electroporation for transdermal drug delivery enhancement is not entirely surprising. Namely, when electric pulses are applied to skin, aqueous pathways are created through the stratum corneum as a result of electroporation. However, when this is followed by application of ultrasound, the cavitation in the coupling medium fails to add (many) new aqueous pathways, most probably because the collapse of cavitation microjets at the skin surface responsible for SC sonoporation preferentially occurs at sites of uneven skin surface, i.e. at electroporation-created pathways. Similarly, when the sequence of methods is reversed, the second method – in this case electroporation – is unsuccessful in adding many new aqueous pathways in the SC to the existing, sonoporation-created ones. In this situation, the preexisting pathways inhibit the formation of new ones as they provide paths of low resistance in the SC for the electric current, which renders the electric field across the SC too weak to cause electroporation of the intact regions of the SC.

Interestingly, the study published by Kost et al. (1996), using the combination of ultrasound and electric pulses to enhance TDD, reports a significant increase in molecular delivery when the two methods are used in combination, compared to single method use. However, there are some significant differences between their work and ours, summarized in three main points: (i) they used MHz-range ultrasound (causing less structural changes on skin than kHz ultrasound), (ii) the experimental system was different (diffusion cells with electrodes for electroporation one on each side of the skin) and (iii) different experimental protocols (1 h long application of ultrasound and electric pulses with calcein or sulforhodamine present). The described dissimilarities probably pronounced the effects of electrophoresis and sonophoresis, which is also evidenced by their results. On the other hand electroporation and sonoporation (causing longer-lasting aqueous pathways through the SC with no phoresis) were predominant mechanisms in our study.

A more recent study by Petchsangsai et al. (2014) explored the use of different combinations of microneedles, electric pulses and ultrasound to enhance TDD of a large molecule (FITC-dextran 4.4 kDa molecular weight). Focusing exclusively on the use of electric pulses and the ultrasound alone or in combination, the results of this study seem to confirm our findings, although ultrasound application was different to ours, the experimental molecule was present during sonication and was much larger than in our study. Nevertheless, when ultrasound (20 kHz) was applied for 2 min after the skin has been treated with electric pulses

(99×300 V, 1 ms duration, similar electrode array to ours), molecular delivery was comparable to single method use.

4. Conclusions

Our study deals with transdermal drug delivery using two physical methods of enhancement: the use of ultrasound (sonoporation – SP) and electric pulses (electroporation – EP) alone or in combination. As the effects of different parameters of both ultrasound and electric pulses on skin have – to a reasonable extent – been described in the studies published so far, we focused on the design of the experimental system and the experimental protocols, so the results and the conclusions drawn from them would have greater relevance for in vivo use and later translation into clinical practice. Therefore, full thickness porcine skin was used and was not treated in the Franz diffusion cells in order to create experimental circumstances closer to in vivo situation. In this way, we avoided ultrasound reflections from glass walls while entirely covering skin sample by the ultrasound horn. For the electroporation part of the study, the pulses were delivered directly on skin with an array of external pin electrodes and not into the solution, which created experimental circumstances much closer to in vivo situation. Also, the ultrasound and the electric pulse protocols were chosen with clinical applications in mind. For that reason, the parameters were chosen conservatively enough to be noninvasive and easily tolerated by the patient: only short, high-voltage electroporation pulses were used (longer pulses may cause burns, muscle contractions and unpleasant sensations), delivered with a closed loop system device that limits the electric current to 20 mA and drops the pulse amplitude when current limit is exceeded. Further, the acoustic pressure of the ultrasound used was measured at 166 kPa, corresponding to about 1.8 W/cm^2 of temporal average at skin distance, which is in the lower end of the ranges of ultrasound powers reported in the literature. Also, the SP and EP protocols were kept as short as possible, again to increase their clinical relevance, as lengthy protocols are much less practical. For that reason we used 100% duty cycle during ultrasound application as well as decided not to pursue the optimization of convection effects that require longer ultrasound application to become evident and comparable to sonoporation mechanism, as well as carry along some concerns over drug degradation.

Our results show a statistically significant enhancement of calcein delivery (after one hour of passive diffusion following treatment) already after 5 min of ultrasound application, or only 6×100 short high voltage electrical pulses. We also experimented with combinations of the two enhancement methods hoping for synergistic effects, however, the results showed no evident dramatic improvement over single method use. The mechanism of action of both methods was the creation of aqueous pathways in the stratum corneum leading to increased skin permeability. Taking advantage of convection and acoustic streaming effects of ultrasound (sonophoresis) requires a longer sonication protocols that would be more appropriate for miniaturized, wearable devices and sustained drug delivery applications. For such applications it would be advisable to use them in combination with one of the methods acting on the stratum corneum to increase its permeability: electroporation, microneedles, laser, sonoporation; however, further studies are needed to maximize the efficacy of such combinations.

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