10 Electroporation

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CONTENTS

10.1 INTRODUCTION

Controlled drug delivery is generally achieved by manipulating the properties of drugs and/or drug delivery devices or carriers. However, once a drug leaves its carrier (whether it is a syringe, transdermal patch, or microsphere), direct control of drug transport through the body's tissues is generally not possible. If one were able to control the transport properties of biological barriers within the body, then drug delivery could be more completely controlled, including both the release of the drug from a carrier as well as drug transport through tissues to the therapeutic target. Electroporation of biological barriers makes this type of control a possibility.

Electroporation has been applied to drug delivery primarily in three areas. The first application involves loading drug into cells which then act as biocompatible carriers. Second, approaches to enhancing cancer chemotherapy have been explored,

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where locally increased permeability of tumors caused by electroporation can lead to greatly enhanced therapy with fewer side effects, as seen *in vivo* and in the clinic. Finally, electroporation of skin has been shown to occur and increase transdermal transport of molecules by orders of magnitude, as demonstrated through electrical, molecular flux, and microscopy studies *in vitro*.

10.2 BACKGROUND OF ELECTROPORATION

Electroporation involves the creation of transient aqueous pathways in lipid bilayer membranes by the application of a short electric field pulse.[1-5] Permeability and electrical conductance of lipid bilayers are rapidly increased by many orders of magnitude, where membrane changes can be reversible or irreversible. Electroporation occurs when the transmembrane voltage reaches on the order of 1 V for electric field pulses typically of 10 μs to 10 ms in duration. Electroporation is known to occur in metabolically inactive systems, such as black lipid membranes[6] and red blood cell ghosts,[7,8] as well as in living cells[1,3] and tissues.[4,9] While other terms, such as electropermeabilization,[4] are also used in the literature, we have followed the most common practice and refer to this phenomenon as electroporation.[1-3,5]

Electrical studies have shown that membrane resistance can drop orders of magnitude on a timescale of microseconds or faster due to electroporation.[10-12] Typically, upon applying a pulse, the membrane charges and initially remains stable (Figure 10.1). Then, the membrane becomes unstable and electroporation occurs, resulting in dramatically reduced membrane resistance. These changes in membrane electrical properties can be reversible or irreversible, depending largely on pulse parameters and membrane geometry.

Molecular transport across membranes also increases during electroporation. As shown in Figure 10.2, large numbers of macromolecules, such as bovine serum albumin (BSA), can be introduced into cells. Gene transfection can be accomplished when DNA is transported across cell membranes and incorporated into the genetic material of a cell, a technique routinely used in molecular biology.[1,3]

The mechanism of transport caused by electroporation is expected to involve diffusion and/or electrically driven transport (Figure 10.3). During a pulse, transport has been shown to occur by electrophoresis and/or electroosmosis (Figure 10.3A and B), depending on the experimental system.[7,13,14] For small compounds (e.g., $M_r <$ 1000 Da), significant transport can also occur by diffusion after a pulse due to long-lived changes in membrane permeability (Figure 10.3C and D). Postpulse transport of macromolecules is generally much slower.

Although studied mostly in planar bilayer and isolated cell systems, electroporation has also been demonstrated in cells part of monolayers[15,16] and in tissues, including retinal explants,[17] islets of Langerhans,[18,19] rice,[20] and maize[21] tissues, in skeletal muscle,[22] in a number of different tumors,[23-27] and in the dermis[28] and stratum corneum[29-33] of the skin.

The dramatic and often-reversible changes in membrane properties associated with electroporation have been explained with models involving transient creation of aqueous pathways, or "pores," across the lipid bilayer.[34-37] However, direct evidence

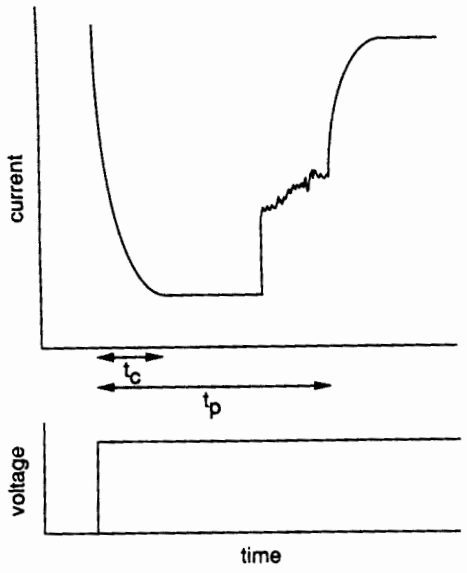


FIGURE 10.1 General features of the events occurring during electroporation of a planar bilayer membrane. During the application of a square-wave voltage pulse (lower graph), the current (upper graph) is initially large due to charging of the membrane. After a characteristic charging time, t_c, the current is approximately constant until it rapidly surges and then fluctuates, believed to be caused by membrane instability. Upon electroporation of the membrane, after a characteristic poration time, t_p, the current rapidly rises due to the creation of aqueous pathways across the membrane. In isolated planar bilayer membranes, electroporation often results in permanent membrane rupture. However, in spherical membranes, cells, and tissues, electroporation is usually partially or fully reversible. (From Abidor, I. G. et al., *Bioelectrochem. Bioenerget.*, 104, 37, 1979. With permission.)

(i.e., visualization) for these pores has not been reported, primarily because electropores are believed to be small (<10 nm), sparse (<0.1% of surface area), and generally short-lived (microseconds to seconds), making their capture by any form of microscopy extremely difficult.[38]

10.3 APPLICATIONS IN DRUG DELIVERY

10.3.1 Loading of Cells As Drug Carriers

For controlled release of drug from an environment protected from the degradative enzymes of the body, drugs have been encapsulated in carrier systems, such as microspheres and liposomes.[39-41] Problems associated with these approaches can include inactivation of drug during the encapsulation process and poor biocompatibility of the carrier.

Electroporation has been used to load a variety of different molecules into red blood cell ghosts, which can act as drug carriers. Ghost-encapsulated compounds

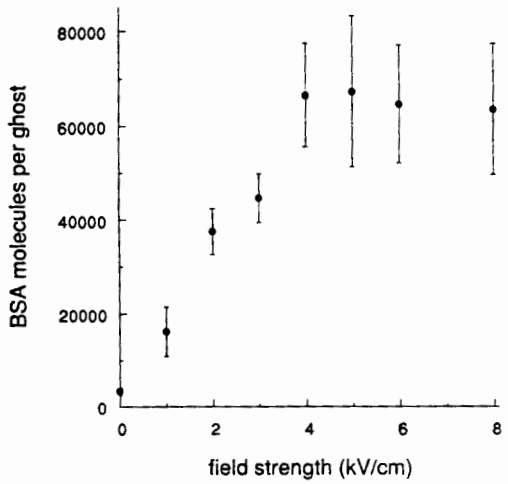


FIGURE 10.2 Uptake of fluorescein-labeled BSA molecules by erythrocyte ghosts as a function of pulse magnitude. Spherical ghosts were suspended in a solution containing BSA and exposed to a single exponential-decay pulse (decay time constant, τ = 1 to 2 ms). Initially, uptake of exogenous BSA increased with field strength, while above approximately 4 kV/cm a plateau in uptake is observed. The BSA concentration inside the ghosts in the high-voltage plateau region is estimated to be only 7% of the extracellular concentration (10^{-5} M). Models of electroporation which account for the dynamic, voltage-dependent behavior of the pore population can explain this subequilibrium plateau.[102] This figure includes data from on the order of 10^6 individual ghosts measured by flow cytometry. Standard error bars are shown. (From Prausnitz, M. R. et al., *Biophys. J.*, 65, 414, 1993. With permission.)

include proteins and enzymes which retain their biological activity.[42] To test the *in vivo* application of this approach, intact murine red blood cells were loaded by electroporation with a model drug, (^{14}C)-sucrose, which was later released *in vivo* in a mouse (Figure 10.4).[43] This carrier system was biologically inert and maintained a constant (^{14}C)-sucrose plasma concentration over the 30-day period of the experiment. While encapsulation by electroporation is a gentle process which can yield biologically active drug inside biocompatible carriers, limitations of the approach include biohazards associated with the likely use of human blood, lack of control over release rates, and very slow release rates for encapsulated macromolecules.

Another application of electroporation for controlled drug delivery involves targeting drug delivery to leukocytes. Because the voltage drop across a cell is a function of the cell diameter, electroporation of large cells occurs at lower bulk field strengths than that of small cells.[1,3] By taking advantage of this targeting mechanism, whole blood was exposed to electroporation pulses which permeabilized leukocytes (which are generally larger than erythrocytes), but left erythrocytes unaffected (i.e., they did not undergo hemolysis).[44] This approach could be used to deliver drugs selectively to leukocytes, which could act as drug carriers targeted to the immune system.

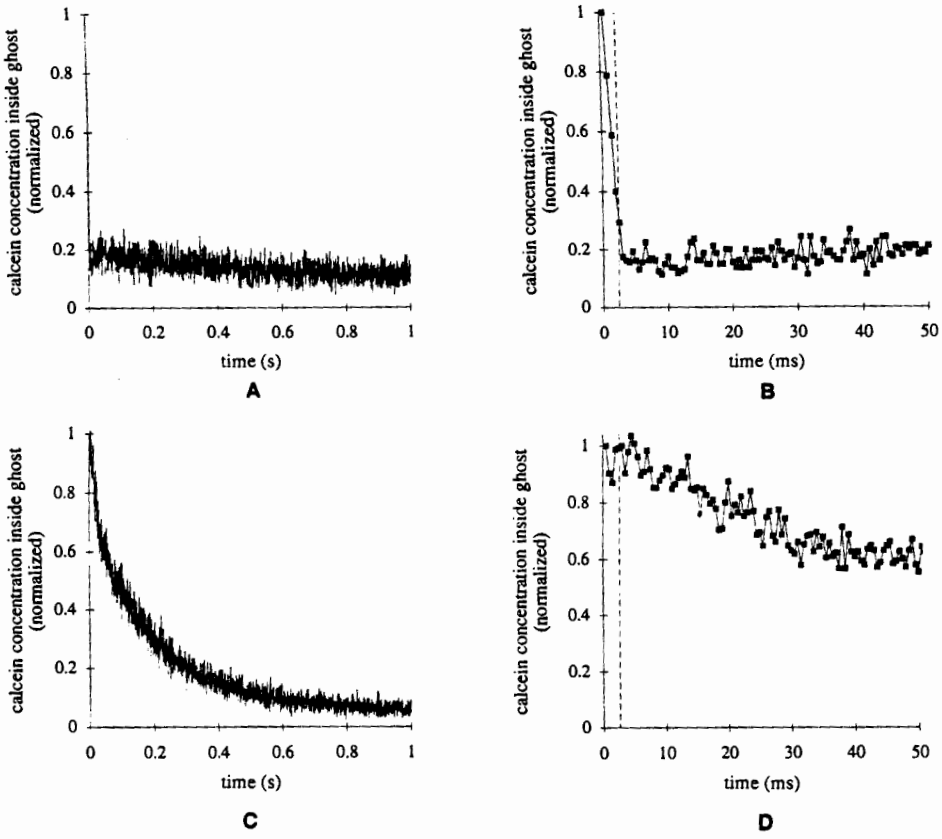


FIGURE 10.3 Normalized calcein concentration inside individual calcein-loaded erythrocyte ghosts during and after single exponential decay electric field pulses ($E = 2.5$ kV/cm; $\tau = 2.5 \pm 1$ ms), measured with a fluorescence microscope photometer with millisecond time resolution. In each case, the ghost was completely emptied of calcein, but over different timescales. (A and B) Most efflux occurred during the pulse, suggesting transport primarily by electrophoresis and/or electro-osmosis. (C and D) Most efflux occurred after the pulse, suggesting transport primarily by diffusion across a permeabilized membrane. Each pair of graphs (e.g., A and B) contains the same data shown on different timescales. The dashed lines indicate the time constant, τ, of the pulse. (From Prausnitz, M. R. et al., *Biophys. J.*, 68, 1864, 1995. With permission.)

10.3.2 Permeabilization of Tumors for Enhanced Chemotherapy

The success of cancer chemotherapy is often limited by the inability of therapeutic agents to reach their targets inside tumors.[45] Moreover, side effects from drugs reaching unintended targets elsewhere in the body are also a significant problem.

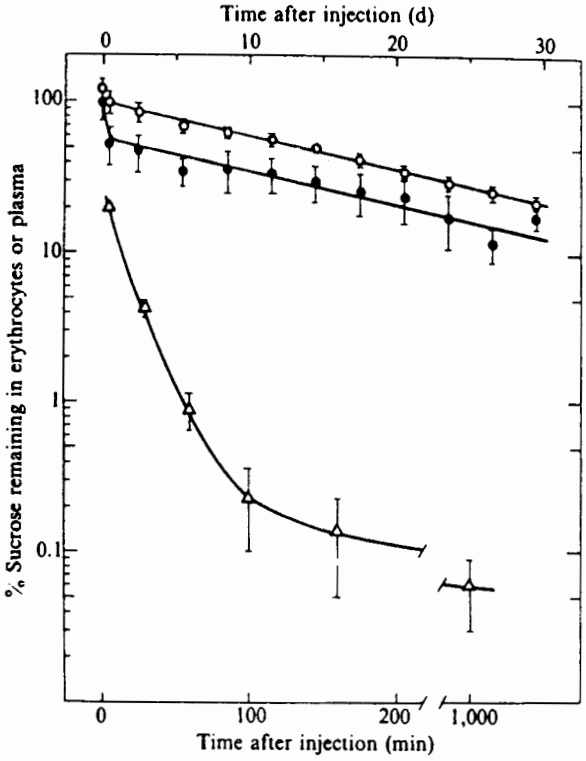


FIGURE 10.4 Elimination of sucrose from the circulation of mice. Murine erythrocytes were loaded with (^{14}C)-sucrose by electroporation and subsequently allowed to reseal. Sucrose entrapped in fully resealed erythrocytes (○), in partially resealed erythrocytes (●), or free in solution (△) was injected into AKR/J female mice. While free sucrose was eliminated within hours, entrapped sucrose was eliminated over a half-life of about 2 weeks, corresponding to the known half-life of mouse erythrocytes. Over the 30-day period of the experiment, the sucrose plasma concentration in the presence of loaded erythrocytes was approximately constant and equal to about 0.1% of the concentration inside the loaded erythrocytes. Standard deviation bars are shown. (From Kinosita, K. and Tsong, T. Y., *Nature*, 272, 258, 1978. With permission.)

To address both of these issues, electroporation of tumors has been used to deliver drug selectively to tumors, thereby increasing drug effectiveness and reducing side effects.[23-27] Work has been performed *in vitro*, *in vivo*, and clinically using the drug bleomycin.

10.3.2.1 *In Vitro*

Electroporation *in vitro* of DC-3F cells, a spontaneously transformed Chinese hamster lung fibroblast line, resulted in increased uptake of bleomycin and consequent drug-induced cell death.[46,47] On average, 400 bleomycin molecules needed to be transported inside each cell to kill it.[47] Because electroporation enhanced transport

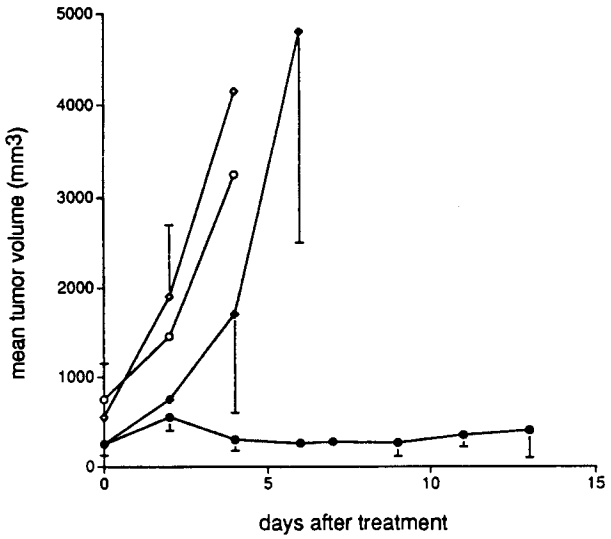


FIGURE 10.5 Tumor growth in mice following different electrical and pharmacological treatments. B16 melanomas in the flanks of C57B1/6 mice received no treatment (◊), or were treated with drug only (○), electric pulses only (♦), or both drug and electric pulses (●). Tumors treated with both drug (500 μg bleomycin, intramuscular injection) and electric pulses (8 pulses applied across the tumors at 1000 V, 100 μs, 1 pulse/s) showed 4 complete regressions and 1 cure out of 11 treated mice. Other treatment protocols resulted in no regression or cures. Because bleomycin crosses cell membranes very poorly, permeabilization of tumors by electroporation pulses is expected to increase bleomycin uptake and thereby increase its therapeutic effect. (From Mir, L. M. et al., *Eur. J. Cancer*, 27, 68, 1991. With permission.)

of drug across cell membranes, the externally applied bleomycin dose required for cell death was up to 10,000 times smaller with electroporation than without.[47] In contrast, electroporation enhanced drug effectiveness only three- to fivefold for lipophilic drugs which could more easily cross cell membranes, suggesting that the mechanism of bleomycin enhancement was related to increased transmembrane transport.[46] Moreover, the effects of melphalan on cells is normally controlled by leucine concentration, since melphalan enters cells via leucine transporters. However, leucine concentration did not influence the effects of melphalan on electroporated cells, suggesting that leucine transporters did not control transport because electroporation created new transmembrane pathways.[46]

10.3.2.2 *In Vivo*

Treatment *in vivo* has been performed on a number of different types of tumors in mouse, rat, and rabbit models.[4,9] In each of the studies, treatment with either bleomycin (by intramuscular or intravenous injection) or electric pulses (applied at the site of the tumor) alone did not have significant effects on tumor size. However, the combination of bleomycin and electroporation resulted in significant reduction in tumor size and prolongation of animal life (Figure 10.5).[23-25,27,48-52] For example, in

one study involving spontaneous mammary tumors in mice, all 38 of the animals treated with electrochemotherapy showed at least partial regression, while 23 showed complete regression, 3 of which were cures.[49] In electrochemotherapy studies, typical electrical protocols have involved application of 1 to 8 square-wave or exponential-decay pulses of 600 to 10,000 V in strength and 0.1 to 7 ms in duration across electrodes of 2 to 25 mm spacing.[53] Local transient edema, superficial skin scabs, and local tissue necrosis can be associated with the protocol.[25,48]

To establish whether or not the mechanism of this therapy involved increased drug uptake by tumors due to electroporation, bleomycin concentration was measured throughout the body 3 days after treatment of mice *in vivo*.[52] Drug concentration in the treated tumors was approximately four times greater in mice that had received electric pulses than those that had not. In all other tissues, drug concentration was the same independent of whether or not tumors had received electrical treatment. This indicates that drug was targeted by electroporation to the site of the tumors. Further mechanistic insight may also come from experiments which have shown that the combination of bleomycin electrochemotherapy with treatment with interleukin-2 further increases the number of animals cured.[54-56] This suggests that the immune system plays a role in tumor cell death associated with electrochemotherapy.

As expected for electroporation, the success of electrochemotherapy treatment depends strongly on pulse voltage and duration. In general, tumoricidal effects are seen above a minimum threshold voltage and are functions of pulse voltage at higher voltages.[25,52] One investigator has found that reduction of tumor size varies directly with pulse length and the square of pulse voltage, indicating that the effects of electrochemotherapy may be a function of pulse energy.[51]

10.3.2.3 Clinical Studies

In a Phase I–II clinical trial including eight patients, head and neck squamous cell carcinomas were treated with four or eight square-wave pulses of 780 V in strength and 100 μs in duration across electrodes of 6 mm spacing.[26,57] Out of 40 treated nodules, 23 showed clinical complete response and 6 showed only partial response. Side effects were limited to short, painless muscle contractions at the time of the pulses. Electrochemotherapy has also been performed on patients with melanomas.[58]

10.3.3 Electroporation of Skin for Transdermal Drug Delivery

Although transdermal drug delivery has the potential to be a noninvasive, user-friendly method of delivering drugs, its clinical use has found limited application due to the remarkable barrier properties of the outermost layer of the skin, the stratum corneum.[59,60] As a result, chemical, iontophoretic, ultrasonic, and other methods of enhancement have been studied as approaches to increase rates of transport. First reported in 1992,[61] application of low-duty-cycle, high-voltage pulses has been shown to have dramatic effects on skin properties, including large increases in transdermal transport of a variety of different compounds, including macromolecules.[30-33,53,62-74] These effects are believed to be caused by skin electroporation,

involving transient structural changes in the intercellular lipid bilayers of the stratum corneum. Although both electroporation and iontophoresis involve electric fields, the two phenomena are fundamentally different. While iontophoresis acts primarily on the drug, involving skin structural changes as a secondary effect,[59,60] electroporation is expected to act directly on the skin, making transient changes in tissue permeability.

10.3.3.1 Increases in Molecular Transport across Skin

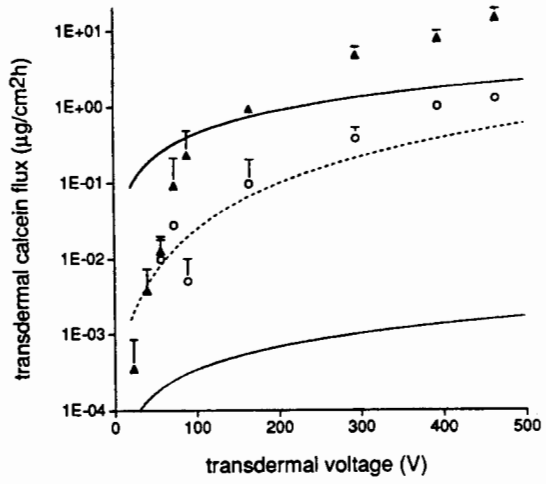
10.3.3.1.1 Transdermal Flux of Calcein

While electroporation of unilamellar, phospholipid cell membranes is well known, electroporation of the multilamellar, nonphospholipid bilayers found in the intercellular spaces of stratum corneum has only recently been investigated. To determine if electroporation of the stratum corneum occurs, human cadaver epidermis under physiological conditions was subjected to electric pulses which cause electroporation in other systems. Most work focused on the transdermal transport of calcein caused by low-voltage constant electric fields (iontophoresis) and high-voltage pulsed electric fields (hypothesized to involve electroporation). Because of the overall hydrophobic character and net negative charge of the stratum corneum, transdermal transport of negatively charged hydrophilic drugs is especially challenging. Therefore, calcein, a moderate-sized (M_r = 623 Da), highly polar ($z = -4$ net charge) molecule,[75] was selected as a model drug because its transport across skin is particularly difficult.

The effects of exposing human epidermis *in vitro* to exponential-decay electric field pulses (decay time constant, τ = 1 ms) at a rate of 12 pulses/min is shown in Figure 10.6A as a function of voltage.[30] Fluxes before pulsing were below the detection limit (of order 10^{-4} mg/cm^{2}h, imposed by background fluorescence), while fluxes during pulsing were up to four orders of magnitude above this limit. Flux increased nonlinearly with increasing pulse voltage; that is, flux increased strongly with increasing voltage below approximately 100 V and increased weakly with increasing voltage at higher voltages.

Transdermal calcein transport due to electric field pulses of both forward polarity and alternating polarity is shown (Figure 10.6A).[30] Here, forward-polarity pulses correspond to the positive electrode in the receptor compartment and the negative electrode in the donor compartment. For this configuration, electric field pulses could both cause structural changes in the skin, possibly due to electroporation, as well as move calcein across the skin by electrophoresis through both previously existing and newly created transport pathways. In contrast, alternating-polarity pulses were applied such that the electrode polarity alternated with each pulse. Although each pulse was either completely positive or completely negative, the total time integral of voltage over all pulses was zero. Both forward- and alternating-polarity pulses resulted in significant increases in transdermal flux.

Theoretical analysis of skin electroporation has predicted that the multilamellar lipid bilayers of human stratum corneum could electroporate at voltages on the order of 100 V.[76,77] The resulting predictions of one model[77,78] are shown in Figure 10.6A. This model, which accounts for the geometric and physiochemical properties of the skin and the compounds being transported, was developed from first principles and



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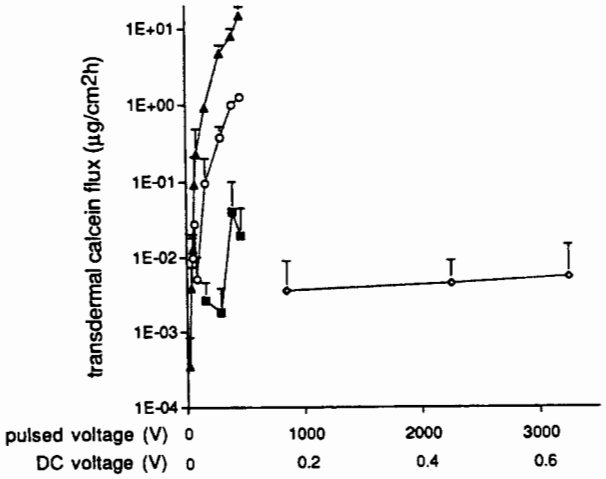


B

FIGURE 10.6

requires no fitted parameters. It has previously been shown[78] to predict passive and low-voltage iontophoretic transport across skin. The lower, dotted curve represents the prediction of the model for calcein transport during forward-polarity pulses through unaltered skin; the prediction of the model is very poor. In contrast, the upper, solid line represents the prediction for transport across skin containing transient pores, having characteristics consistent with what is known about single-bilayer electroporation; the prediction is much better. Finally, the middle, dashed line

represents the prediction for transport during alternating-polarity pulses through porated skin, which is also in good agreement with the data. This analysis both shows that a theoretical basis exists for changes in skin structure and establishes that these changes are capable of explaining some of the characteristic effects of high-voltage pulsing.

Enhancement of transdermal transport at low voltage (e.g., <1 V) can generally be explained by electrophoresis and/or electro-osmosis without changes in skin structure.[79,80] Therefore, if protocols having the same electrophoretic and electro-osmotic driving force provide different degrees of enhancement, it suggests that changes in skin properties occurred. Fluxes caused by low-duty-cycle, high-voltage pulsing were therefore compared with fluxes caused by continuous low voltages which would provide the same total electrophoretic and electro-osmotic driving force. Transport enhancement by electrophoresis or electro-osmosis is proportional to the time integral of voltage.[81] Therefore, continuous application of 0.1 V should provide the same driving force as 500 V applied at a 1:5000 duty cycle (1-ms pulse applied once every 5 s). As seen in Figure 10.6B, application of continuous DC voltages caused fluxes three orders of magnitude smaller than pulsing under "equivalent" forward-polarity conditions. Moreover, while alternating-polarity pulses also dramatically increased transport, no enhancement resulted from equivalent low-voltage AC current which provided the same time-averaged driving force for transport (data not shown).[70] Finally, increases in transport of two orders of magnitude have been seen when the polarity is such that electrophoresis and electro-osmosis oppose transdermal transport (Figure 10.6B). These comparisons suggest that electrophoresis and/or electro-osmosis alone cannot explain the large flux increases observed during high-voltage pulsing, which indicates that changes in skin properties occurred.

One of the key features of electroporation seen in single-bilayer membranes is dramatically increased transport by a mechanism involving structural changes in the

FIGURE 10.6 Experimental data and theoretical predictions for the transdermal flux of calcein under different electrical conditions. (A) Calcein flux during forward-polarity (▲) and alternating-polarity (○) pulses (see text for definitions). Flux was increased by up to four orders of magnitude. Theoretical predictions are also shown for transport across unaltered skin during forward-polarity pulsing (dotted line), transport across skin containing electropores during forward-polarity pulsing (solid line), and transport across skin with electropores during alternating-polarity pulsing (dashed line). (B) Calcein flux during forward-polarity (▲), alternating-polarity (○), and reverse-polarity (■) pulsing and during DC iontophoresis (◊). The upper axis indicates pulsing voltage electrically "equivalent" to continuous DC voltages on the lower axis (see text). Dotted lines connecting the points in each data set are shown to aid the reader, but have no physical interpretation. For both graphs, fluxes represent average values during 1 h of electrical exposure. Either a continuous DC voltage or a series of intermittent (12 pulses/min) exponential-decay pulses (τ = 1 to 1.3 ms) was applied to human epidermis *in vitro*. Standard deviation bars are shown. (Compiled from Prausnitz, M. R. et al., *Proc. Natl. Acad. Sci. U.S.A.*, 90, 10504, 1993, Prausnitz, M. R. et al., *J. Controlled Release*, 38, 205, 1996, and Edwards, D. A. et al., *J. Controlled Release*, 34, 211, 1995. With permission.)

membrane barrier.[1,3] Figure 10.6 suggests that this is seen in skin, too. A second feature of electroporation is that over a range of conditions, these changes are reversible.[1,3] This is also seen in skin. In experiments that identified long-lived changes in skin permeability, pulses at or below approximately 100 V were shown to cause no detectable long-lived changes in skin permeability, while higher voltage pulses appeared to cause lasting changes,[30] which did not go away, even after 18 to 24 h.

Experiments performed to assess the combined effects of electroporation and ultrasound (1 MHz, 1.4 W/cm^2, continuous application) on skin found a synergistic effect which increased transdermal flux two- to threefold and reduced transport lag time compared with electroporation alone.[65] Both ultrasound-induced lipid bilayer disordering and cavitation-related convection may have contributed to the increased effects of electroporation. Ultrasound also lowered the minimum voltage required for electroporation-induced flux increases by 13%,[65] possibly due to partial disordering of lipid bilayer structure by ultrasound.

Finally, limited work on electroporation *in vivo* has been performed on hairless rats,[30,53] assessed by measuring serum concentrations of calcein delivered transdermally. At voltages between 30 and 300 V, fluxes in excess of 10 μg/cm^{2}h were observed, which is at least two orders of magnitude greater than controls. *In vivo* fluxes did not increase with voltage, suggesting that a rate-limiting step other than transport across the stratum corneum existed, perhaps uptake of calcein from a skin depot into the bloodstream. No visible skin damage was observed after pulsing at voltages below 150 V; erythema and edema were evident at higher voltages. Long-term biochemical and pathological studies are needed.

10.3.3.1.2 Transport Number Analysis

Transport numbers represent the fraction of total current carried by a given ionic species, which can be regarded as the efficiency of electrophoretic transport of that species.[81,82] By calculating the transport number ($t_{calcein}$), or transport efficiency, associated with different electrical conditions, the pathways available to ion transport can be partially characterized. Here, $t_{calcein}$ is a measure of the efficiency with which calcein can be transported through pathways across skin relative to transport of small ions, such as sodium or chloride. Thus, transport numbers can give information about the effective average size of transport pathways.

During both iontophoresis and high-voltage pulsing, $t_{calcein}$ has been shown to increase with increasing current and voltage (Figure 10.7).[70] This means that the changes in skin properties caused by increased current or voltage increase transport of calcein more than small ions, perhaps due to creation of larger transport pathways. At the lowest voltages and currents, small ion transport was favored over calcein transport by a factor of 1000 ($t_{calcein} \approx 10^{-5}$, compared with the predicted maximum value, corresponding to pathways much larger than calcein, $t_{calcein,\ max} \approx 10^{-2}$)[70] (Figure 10.7). Under these conditions, transport pathways may have dimensions similar to that of calcein (Stokes–Einstein radius, $r_{calcein} = 0.6$ nm).[77] In this case, transport of small ions such as sodium or chloride (crystal ionic radius, $r_{Na^+} = 0.1$ nm and $r_{Cl^-} = 0.2$ nm)[83] would be less hindered, while calcein would experience considerable steric hindrance. Pathways that might show this selectivity could include

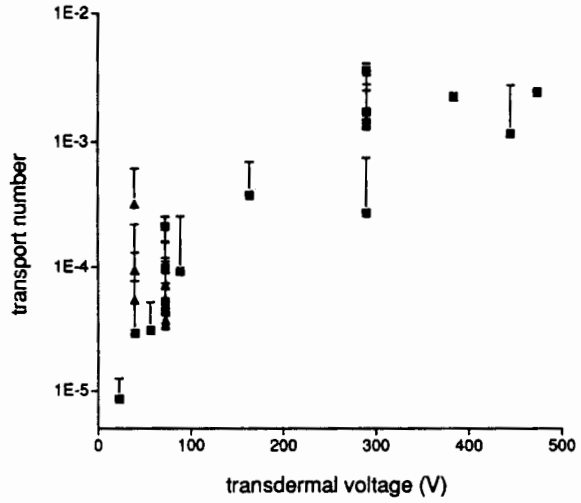


FIGURE 10.7 Transport number for calcein transport across human epidermis *in vitro* during exponential-decay pulses (■) and square-wave pulses (▲). For each point, pulses of a constant duration between 30 μs and 1 ms were applied for 1 h at a constant rate between 10^{-1} and 10^{4} pulses/min. Transport number, which is a measure of the efficiency with which the electric field transported calcein, increased with increasing voltage. Standard deviation bars are shown. (From Prausnitz, M. R. et al., *J. Controlled Release,* 38, 205, 1996. With permission.)

transport between intercellular lipid bilayers of the stratum corneum ($r = 0.7$ nm)[84] or through intercellular junctions in the lining of shunt pathways. Another possibility is that many pathways exist that only allow small-ion transport, such as angstrom-size "leaks" in lipid bilayers created by random thermal motion,[85] along with a few much larger routes which readily permit passage of calcein, perhaps associated with appendages.

At the highest voltages and currents, where $t_{calcein}$ approached its predicted maximum value ($t_{calcein,\ max} \approx 10^{-2}$)[70] (Figure 10.7), larger pathways may exist, with dimensions much larger than calcein. In this case, small-ion transport would remain unhindered while calcein transport would become less hindered. This would increase $t_{calcein}$ by increasing calcein transport relative to small ions. However, with this data alone, the absolute size of these pathways or whether they were newly created or enlargements of preexisting pathways cannot be assessed.

Finally, $t_{calcein}$ has been shown to have no clear dependence on pulse length, rate, energy, waveform, or total charge transferred.[70] This is consistent with known mechanisms of single-bilayer electroporation, where pore characteristics are believed to be determined largely by voltage.[1,3]

10.3.3.1.3 Transdermal Flux of Other Compounds

While many skin electroporation studies have been performed with calcein as a model drug, electroporation-enhanced transport of a number of other molecules has also been reported. By using a protocol similar to that described for calcein, enhanced

transport of three other moderate-sized, polar molecules across skin has been achieved by electroporation: Lucifer Yellow (M_r = 457 Da, z = −1 net charge),[30] sulforhodamine (M_r = 607 Da, z = −1 net charge),[33] and an erythrosin derivative (M_r = 1025 Da, z = −2 net charge).[30] By using a different protocol, involving fewer but longer and somewhat lower-voltage pulses, electroporation has been shown to increase transport of metoprolol (M_r = 267 Da, z = +1 net charge)[32,69] and fentanyl (M_r = 336 Da, z = +1 net charge).[86]

In studies of metoprolol flux across electroporated hairless rat skin *in vitro*,[32,69] the mechanism of transport was found to be diffusion through permeablized skin during the pulsing protocol. Collection of drug in reservoirs within the skin was also found to be significant. Transdermal transport was determined to increase directly with pulse length (above a threshold of 80 ms) and with the square of pulse voltage for a series of low-voltage (less than ~65 V across the skin), long-duration (τ > 80 ms) exponential-decay pulses. This may suggest that transport was a function of applied energy.[69] However, this dependence on energy did not apply when other protocols were used (e.g., square-wave or higher-voltage pulses).[32,69]

More recently, transport of macromolecules has been shown to be enhanced by electroporation. By using a protocol similar to that used in the calcein studies, oligonucleotides (M_r = 4.8 and 7 kDa)[64] and heparin (see discussion below)[71] were transported across skin *in vitro* at therapeutically useful rates. Moreover, using a protocol which applied a single initial electroporation pulse which was followed by iontophoresis, the transport of luteinizing hormone–releasing hormone (LHRH; M_r = 1182 Da, z = +1 net charge),[31] (arg^8)-vasopressin (M_r = 1084 Da, z = +2 net charge),[87] and neurotensin (M_r = 1693 Da, z = +1 net charge)[87] were also enhanced by electroporation.

LHRH flux is shown in Figure 10.8 as a function of current density of iontophoresis following a single exponential-decay electroporation pulse (300 to 400 V, τ = 5 to 9 ms) or iontophoresis alone.[31] The application of a single pulse caused changes in skin permeability such that flux during subsequent iontophoresis was an order of magnitude greater. Calculation of transport numbers from those data show a tenfold increase following electroporation,[31] indicating that the high-voltage pulse resulted in larger transdermal transport pathways.

Heparin is a macromolecule (M_r = 5 to 30 kDa) in widespread clinical use which is often administered by continuous infusion, due to the poor oral bioavailability of heparin, its short half-life, and the risk of bleeding complications.[88] Transdermal delivery of heparin would be a desirable alternative, if heparin could be transported across skin at therapeutic rates. Under passive conditions (no electric fields) transdermal heparin flux is negligible.[71] However, while applying short (τ = 1.9 ms), high voltage (150 to 350 V across skin) pulses to the skin at a rate of 12 pulses/min, rates of transdermal heparin transport were between 100 and 500 mg/cm^{2}h (Figure 10.9A). This level of transport is therapeutically relevant (see below). Moreover, heparin transported across the skin was biologically active (Figure 10.9A).

Constant current iontophoresis (0.1 to 1 mA/cm^2) also enhanced heparin transport, but to a much lesser extent (Figure 10.9A). The currents used during low-voltage iontophoresis were selected for two reasons. First, they bracketed the maximum current density (~0.5 mA/cm^2) which patients tolerate during clinical iontophoresis.[89] Second, the time-averaged current passed during high-voltage pulsing

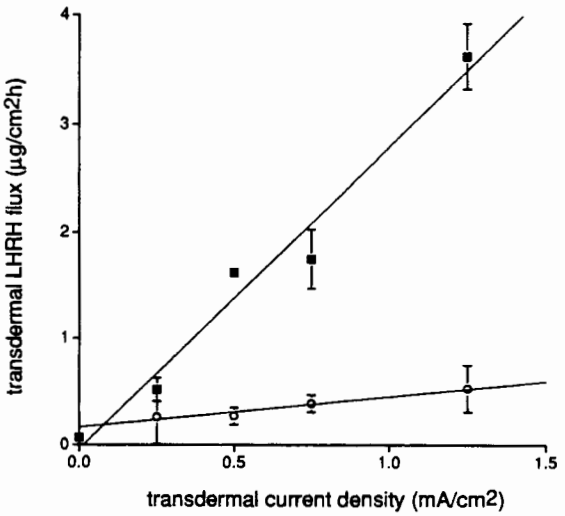


FIGURE 10.8 Transdermal transport of LHRH as a function of current density due to iontophoresis following a single electroporation pulse (■) and due to iontophoresis alone (○). This figure suggests that the application of a single electroporation pulse before iontophoresis can significantly increase the flux of LHRH relative to iontophoresis alone. Pre-iontophoresis pulses were exponential decay (τ = 5 ms) and applied 1000 V across the electrodes. Linear regressions are shown. (From Bommannan, D. B. et al., *Pharm. Res.*, 11, 1809, 1994. With permission.)

was in the range of 0.1 to 1 mA/cm^2.[71] Therefore, both low- and high-voltage protocols had the same time-averaged current and therefore passed the same number of ionic charges across the skin, making comparisons of transport efficiency (i.e., transport number analysis) more direct.

Heparin transport numbers during high-voltage pulsing ($t_{heparin}$ = 0.054 ± 0.006) were calculated to be about an order of magnitude greater than during iontophoresis ($t_{heparin}$ = 0.007 ± 0.002) (Figure 10.9B) . These values indicate that approximately 5% of the current was carried by heparin during pulsing, compared with only 0.7% during iontophoresis.[71] This significant difference in transport numbers implies that heparin transport was significantly less hindered during high-voltage pulsing than during iontophoresis, perhaps because high-voltage pulses caused transient changes in skin microstructure by creating new and/or enlarged aqueous pathways for transport across the skin, and thereby increasing $t_{heparin}$. Low-voltage iontophoresis does not cause these changes in skin structure[59,60] and, therefore, has less effect on $t_{heparin}$. Nevertheless, all measured transport numbers were significantly less than the maximum value ($t_{heparin}$ = 1),[71] indicating that although heparin transport was less hindered during high-voltage pulsing, in all cases transport was still significantly hindered. Unless transport pathways are extremely large (i.e., much larger than a heparin molecule), this is expected.[71]

The heparin fluxes reported during high-voltage pulsing (100 to 500 μg/cm^{2}h or 2 to 10 U/cm^{2}h) are therapeutically relevant. For example, administration from a

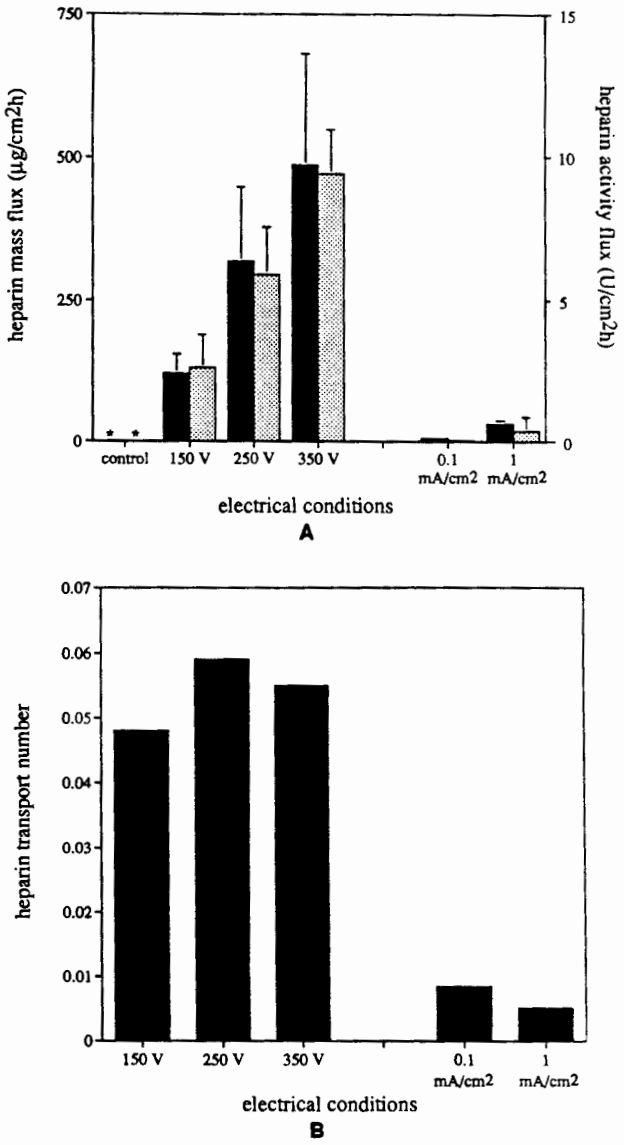


FIGURE 10.9

100-cm^2 patch would result in a heparin delivery rate of 10 to 50 mg/h or 200 to 1000 U/h, based on mass flux and activity flux measurements, respectively (Figure 10.9A). This is in the range sufficient for low-dose prophylaxis of thromboembolism (~500 U/h)[88] and full-dose anticoagulation therapy (700 to 2000 U/h).[88]

10.3.3.1.4 *Rapid Temporal Control by Electroporation*

To better understand the kinetics of transdermal transport by electroporation, a flow-through system has been designed which can give temporal resolution approaching 10 s.[90] By using this system, transdermal transport by electroporation of calcein and sulforhodamine across human epidermis was continuously measured (Figure 10.10).[33] For calcein, the flux reached a steady state within minutes and then decreased below background levels within seconds after pulsing stopped. In contrast, sulforhodamine flux increased continuously during pulsing and did not return to background levels after pulsing.[33]

At first, the curve for calcein flux may appear to contain a lot of noise. However, closer examination shows that the flux oscillated with a regular period of 1 peak/min.[62,67] This is the same rate at which pulses were applied, suggesting that these variations show the effects on transport of individual pulses. This is supported by results seen while pulsing at other rates, where oscillations in flux also occurred at the same rate as pulsing.[33,62,67] As a result of each pulse, the flux initially increased and then decayed as the effects of the pulse decreased. From this and other data, the steady-state lag time for calcein was determined to be the time it took to apply approximately 10 pulses (i.e., in Figure 10.10, where 1 pulse was applied each minute, the lag time was about 10 min), independent of voltage.[62] Moreover, the onset time for transport was the time it took to give 3 pulses, independent of voltage: in Figure 10.10, the first detectable transdermal transport was measured after 3 min.[62]

The pulse-dependent oscillation in calcein flux suggests that calcein was transported primarily during individual pulses.[90] The smooth curve for sulforhodamine flux indicates that sulforhodamine was continuously transported both during and between pulses. Mechanistically, this suggests that calcein transport occurs primarily by electrophoresis during pulses, and not by diffusion between pulses.[33,65] Given the moderate size of calcein (M_r = 623 Da) and its great charge ($z = -4$), this is reasonable. In contrast, sulforhodamine transport could occur largely by diffusion between pulses. Given its much weaker charge ($z = -1$), this is also reasonable.

Because the transport of calcein has been shown to be rapidly responsive to the electric field, electroporation protocols have been designed to achieve desired delivery profiles.[62] For example, continuous low-level delivery of a drug with intermittent

FIGURE 10.9 Flux and transport number of transdermal heparin transport during different electrical protocols. (A) Transdermal heparin flux determined by different assays. Heparin mass flux (■) was determined by radioactivity measurements, while biological activity flux (□) was determined by the whole blood recalcification time assay.[103] In all cases, active heparin was transported across the skin. Standard deviation bars are shown. Asterisk indicates a flux below the detection limit (of order 1 μg/cm²h for radioactivity measurements and 0.1 U/cm²h for biological activity measurements). (B) Heparin transport number during different electrical protocols. During high-voltage pulsing, transport numbers were approximately 0.05, while during low-voltage iontophoresis they were about an order of magnitude smaller. The larger transport numbers seen during high-voltage pulsing suggest the creation of larger aqueous pathways by the electric field. (From Prausnitz, M. R. et al., *Bio/Technology*, 13, 1205, 1995. With permission.)

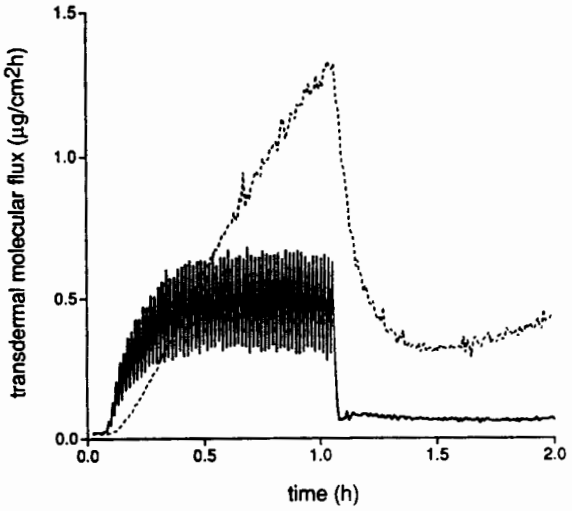


FIGURE 10.10 Simultaneous measurement of the transdermal flux of calcein (M_r = 623 Da, z = −4 net charge; solid line) and sulforhodamine (M_r = 607 Da, z = −1 net charge; dashed line) using a continuous flow-through apparatus. Calcein flux rapidly reached a quasi-steady state and oscillated at a frequency equal to the pulse rate. In contrast, sulforhodamine flux neither oscillated nor reached a steady state. These differences can be explained by calcein transport primarily by electrophoresis during pulses and sulforhodamine transport primarily by diffusion between pulses (see text). Exponential-decay pulses (decay time constant, τ = 1.1 ms) causing a peak transdermal voltage of 200 V were applied to human epidermis *in vitro* at a rate of 1 pulse/min for 1 h. (From Pliquett, U. and Weaver, J. C., *Bioelectrochem. Bioenerget.*, 39, 1, 1996. With permission.)

boli may be a desirable delivery schedule for some drugs. To achieve this type of delivery, iontophoresis was applied to supply baseline delivery, while electroporation pulses provided rapid boli (Figure 10.11A). A more complex delivery profile is shown in Figure 10.11B. In these figures, changes in delivery rates were achieved by changing pulse voltage. However, changes in pulse rate can also achieve similar results.[33,67] Finally, by using an initial series of pulses applied more rapidly to "prime the pump," followed by less rapid pulsing to provide the desired steady-state flux, steady state was achieved within approximately 1 min.[62]

10.3.3.2 Changes in Skin Electrical Properties

Measuring changes in skin electrical properties can give insight into the mechanism of changes in transport properties and the nature of transport pathways. During the application of high-voltage pulses to human epidermis *in vitro*, skin dynamic resistance has been determined as a function of voltage (Figure 10.12).[33,66] Below 40 V, millisecond-long pulses had little effect on resistance. However, at higher voltages the skin dynamic resistance dropped two to three orders of magnitude within microseconds during each pulse. This corresponds to extensive creation or enlargement of pathways for ionic current across the skin.

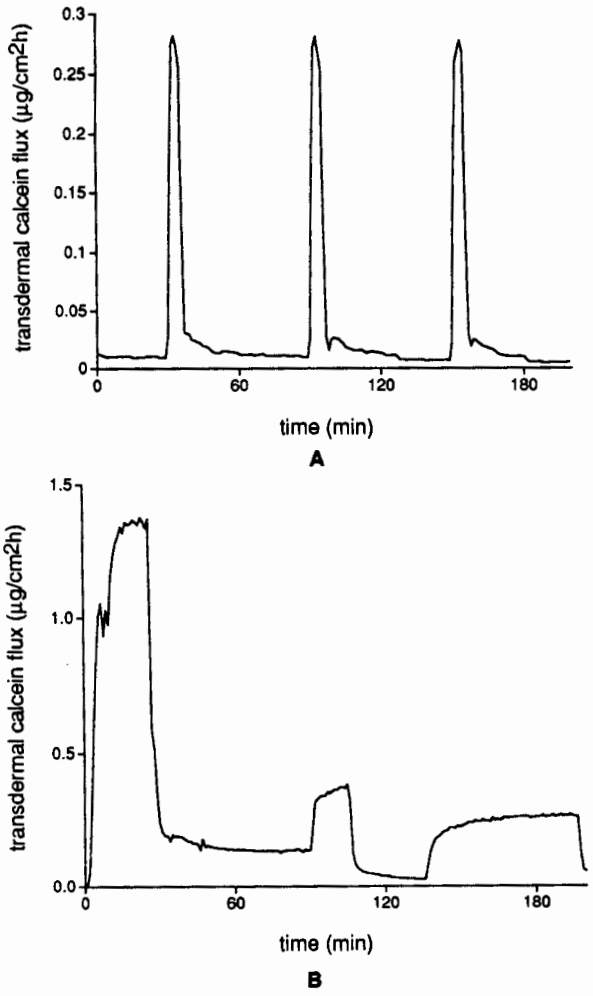


FIGURE 10.11 Complex delivery profiles using transdermal delivery by electroporation. (A) Continuous low-level delivery with intermittent boluses. Low-level delivery corresponded to continuous DC iontophoresis at 14 mA/cm^2. Boli corresponded to pulsing at 115 V and 12 pulses/min for 5 min, each separated by 55 min of iontophoresis. (B) A complex delivery schedule achieved by changing pulse voltage. Pulse rate was held constant at 1 pulse/min, while pulse voltage was changed in the following sequence: 270 V for 30 min, 115 V for 60 min, 165 V for 15 min, 0 V for 30 min, 135 V for 60 min, 0 V for 5 min. Exponential-decay pulses (decay time constant, $\tau = 1$ ms) were applied to human epidermis *in vitro*. (From Prausnitz, M. R. et al., *Pharm. Res.*, 11, 1834, 1994. With permission.)

Measurements of human skin resistivity made milliseconds after application of a single pulse show recovery from about 100 Ω-cm^2 to on the order of 10,000 Ω-cm^2.[33,66,74] Then, within 1 s further recovery occurs, usually bringing skin resistance to within 50% of its prepulse value. This recovery has been observed to occur independent

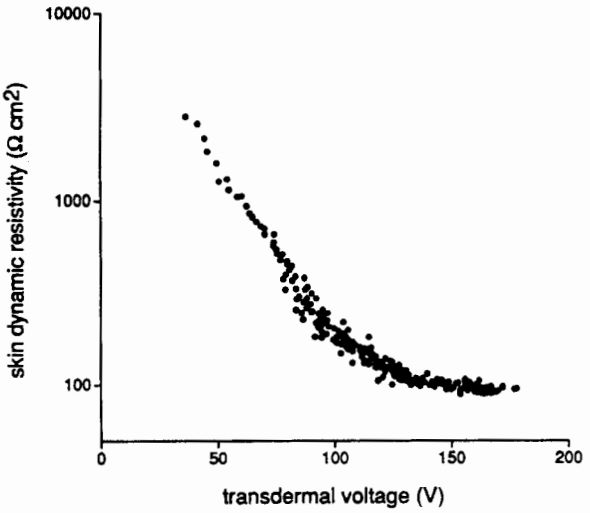


FIGURE 10.12 Skin dynamic resistivity during the application of a high-voltage pulse shown as a function of peak transdermal voltage. On a time scale of microseconds or less, skin dynamic resistivity dropped by two to three orders of magnitude (prepulse skin resistivity was on the order of 100,000 kΩ cm^2). Resistivity changes were generally reversible for pulses between 40 and 100 V. Pulses greater than 100 V were only partially reversible, while pulses less than 40 V caused insignificant changes in skin resistivity. Dynamic resistance was determined 20 μs after the onset of an exponential-decay pulse (τ = 1 ms) applied to human epidermis *in vitro*. (From Pliquett, U. et al., *Biochim. Biophys. Acta*, 1239, 111, 1995. With permission.)

of voltage after single pulses.[66] After 1 s, over a time scale of minutes, skin resistance can recover further, exhibiting either complete or partial recovery. At the highest voltages, recovery after ~1 s occurs to a much less extent. The time scales and degrees of both onset and recovery are characteristic of known electrical properties of single-bilayer electroporation.[1,3]

These dramatic but largely reversible effects of electroporation can be compared with the effects of a well-accepted medical procedure: a needle stick. Puncturing and removing from the skin a small needle (28 gauge) caused skin resistance drops which were not reversible and were at least as great as those caused by skin electroporation at the highest voltages.[74] This suggests that although electroporation is not fully reversible under all conditions, the irreversible changes it causes may be less damaging than the effects of a small needle.

When multiple pulses are applied to the skin, resistance continues to drop with each successive pulse until 15 to 30 pulses have been applied, after which additional pulses do not decrease resistance further.[33,66] As the number of pulses and the rate at which they are applied are increased, skin dynamic resistance during pulsing and the degree of recovery after pulsing both decrease. When many (e.g., 100) multiple pulses are applied at high voltage, skin resistance may return to only 10% of its prepulse value, indicating significant irreversibility.

Measurements made immediately after high-voltage pulsing have also shown up to sixfold increases in skin capacitance which later recover to prepulse values.[33,66,74] Capacitance has been found to increase with the square of voltage,[66,74] suggesting an energy-dependent mechanism. Increased capacitance may indicate changes in skin lipids[91] since skin capacitance is generally attributed to stratum corneum lipid bilayers.[92,93] In contrast, low-voltage electric fields have been shown to cause no or much smaller changes in skin capacitance.[79,93,94]

The combination of calcein transport and skin electrical property measurements allows estimation of what fraction of the skin is available to ion transport and over what characteristic times transport pathways become accessible. By measuring skin resistance and assuming that ion transport pathways are filled with saline, the area fraction of skin made up of these pathways, F_{ion}, has been estimated.[70] After minutes to hours of conventional iontophoresis (up to a few volts), human skin resistivity can drop to between 1000 and 10,000 Ω-cm^2).[79,80,95,96] This corresponds to ion transport pathways occupying an estimated 0.01 to 0.001% of skin surface area ($F_{ion} \approx 10^{-5}$ to 10^{-4}).[70] This is the same area occupied by hair follicles and sweat ducts in human skin ,[97] consistent with these shunt routes being the sites of transport during iontophoresis, as shown previously.[98-100]

Making the same calculation for high-voltage pulses, during which skin resistance drops to approximately 100 Ω-cm^2,[33,66,70,74] indicates that about 0.1% of skin surface area becomes available to ion transport ($F_{ion} \approx 10^{-3}$).[33,70] Electroporation of single bilayers is also believed to cause poration of up to 0.1% of membrane area.[101,102] These estimates suggest that 10 to 100 times more skin area is available for ion transport during high-voltage pulsing than conventional iontophoresis. This may correspond to a shift from iontophoretic transport largely through shunt routes to transport predominantly through electropores within the bulk of stratum corneum.

Differences between pathways available to small ions and those available to calcein can also be considered. Because F_{ion} gives the fraction of skin area containing ion pathways and the ratio $t_{calcein}/t_{calcein,\,max}$ gives the fraction of ion pathways available to calcein transport, then the fraction of skin area available to calcein transport ($F_{calcein}$) can be estimated as the product of these two quantities.[70] By using this relationship, $F_{calcein}$ was estimated to range from 10^{-6} to 10^{-3} during high-voltage pulsing and from 10^{-8} to 10^{-4} during iontophoresis.[33,70]

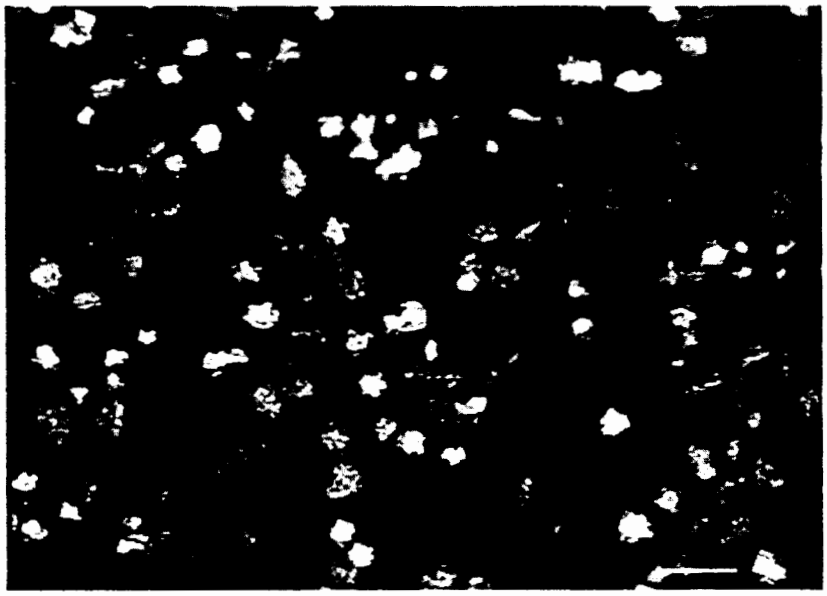
Finally, the timescale over which these transport pathways become accessible has been estimated. Changes in skin resistance due to conventional iontophoretic exposures generally occur over a characteristic time of at least minutes.[79,80,95,96] In contrast, ion transport pathways created by high-voltage pulses become accessible at least eight orders of magnitude more quickly, within a characteristic time of microseconds.[33,66,70,74] Electroporation of single bilayers is also known to occur on a timescale of microseconds or faster.[1,3] Given that high-voltage pulsing causes a one to two order of magnitude greater reduction of resistance that occurs at least eight orders of magnitude more quickly than is typical for iontophoresis, it seems unlikely that the mechanistic bases for changes associated with the two protocols are the same.

10.3.3.3 Microscopic Imaging of Transdermal Transport

Fluorescence microscopy has been used to understand more fully the nature, size, and location of transport pathways in skin exposed to high-voltage pulses. In these studies, human skin was exposed to fluorescent probes (calcein and/or sulforhodamine) and electrical protocols *in vitro* using either (1) a side-by-side permeation chamber, from which skin was later removed and examined by scanning confocal fluorescence microscopy[73] or (2) a chamber placed on a conventional fluorescence microscope stage, allowing real-time microscopic evaluation.[68] After iontophoresis at currents up to 1 mA/cm^2, calcein was seen to be heterogeneously transported into the stratum corneum, as revealed by occasional large areas (measuring on the order of 100 μm across) which were more brightly labeled than the surrounding tissue.[73] While fluorescence generally appeared to be intercellular, a signal was sometimes seen to originate from within the interior of keratinocytes. The typical hexagonal outlines of cells were always evident within the brightly labeled regions, suggesting that no gross morphological changes had occurred. Cross-sectional images showed that calcein was present throughout the stratum corneum.[73]

High-voltage pulses applied to the skin resulted in bright regions of similar dimensions to those found after iontophoresis (Figure 10.13A).[68,73] Both intercellular and intracellular fluorescence was observed. Outlines of cellular structures were also evident.[73] Additional experiments showed that the sites of skin fluorescence corresponded to both sites of molecular transport across (as opposed to just into) the skin and paths of ionic current.[68] Moreover, these sites did not correspond to sweat ducts or hair follicles. Transport regions covered between 0.02 and 8% of skin area. Closer examination[68] by digital image analysis revealed that each fluorescent region contained several subpeaks of fluorescence, suggesting additional microstructure within these transport regions.

FIGURE 10.13 Micrographs of human stratum corneum showing fluorescence of calcein transported by high-voltage pulsing *in vitro*. Sites of fluorescence can be interpreted as sites of transdermal calcein transport. (A) Localized transport regions can be seen, having dimensions of 50 to 100 μm. Ten exponential-decay pulses (τ = 1 ms) causing a peak transdermal voltage of 157 V were applied to human epidermis at a rate of 2 pulses/min. This signal-averaged image was collected immediately after the application of pulses using a fluorescence microscope with a digital video-imaging system. Scale bar equals 200 μm. (From Pliquett, U. F. et al., *Biophys. Chem.*, 58, 185, 1996. With permission). (B) A close-up view of a localized transport region, showing a brightly fluorescent area with a nonfluorescent interior, or fluorescent "ring." Exponential-decay pulses (decay time constant, τ = 1.1 ms) causing a peak transdermal voltage of 300 V were applied to human epidermis *in vitro* at a rate of 12 pulses/min for 1 h. Skin samples were flash-frozen and later imaged using scanning confocal fluorescence microscopy. Scale bar equals 50 μm. (From Prausnitz, M. R. et al., *J. Pharm. Sci.*, 85, 1363, 1996. With permission.)



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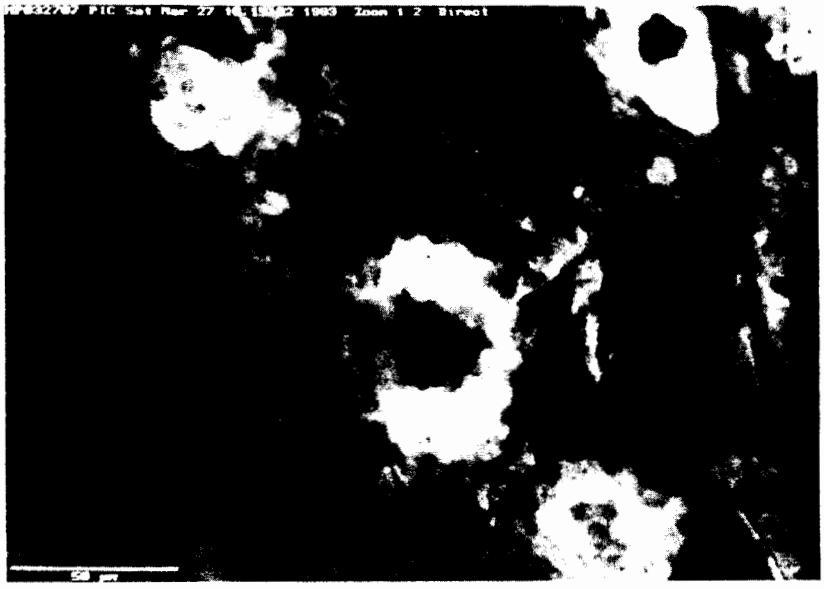

B

FIGURE 10.13

Unlike iontophoresis or pulsing at lower voltages, pulsing at higher voltage (e.g., 300 V across the skin) led to the appearance of bright regions containing dark interiors, i.e., fluorescent "rings" (Figure 10.13B).[73] These rings were never observed in iontophoretic or lower-voltage pulsed samples and were always found in samples pulsed at 300 V. A series of additional experiments[73] involving lipophilic stains and fluorescent nanospheres suggested that while the fluorescent outer rings represent sites of transport which retained calcein, the dark ring interiors represent sites of transport which did not retain calcein at the time of imaging, possibly because of long-lasting local structural changes of nanometer dimensions.

10.4 CONCLUSIONS

Electroporation involves the creation of transient aqueous pathways in lipid bilayer membranes by the application of a short electric field pulse and has been observed in isolated cells and in tissues, including tumors and the skin stratum corneum. For drug delivery, electroporation has been shown to be capable of loading red blood cells with a model drug that can later be released *in vivo*. Moreover, electroporation can selectively permeabilize larger cells (e.g., leukocytes) among a population of smaller cells (e.g., erythrocytes).

In applications that enhance cancer chemotherapy with bleomycin, electroporation of tumors results in increased drug uptake due to membrane permeabilization. *In vitro*, *in vivo*, and clinical studies all show that the combination of bleomycin and electroporation kills tumor cells and often results in complete regression.

Different from isolated cells and cells part of a tissue (e.g., tumors), electroporation of skin for transdermal drug delivery involves permeabilization of the multilamellar, intercellular lipid bilayers in the stratum corneum. Flux increases up to four orders of magnitude have been observed with human skin *in vitro* for a range of hydrophilic molecules up to thousands of daltons in molecular mass (e.g., heparin, oligonucleotides). Sites of transport have been observed to be heterogeneously distributed across the skin. Electroporation-mediated transport is rapidly responsive to changes in electrical conditions, where (1) skin transport properties change over a time scale of microseconds or faster and (2) steady-state transdermal flux can be achieved on a time scale of minutes. Skin electroporation has also been theoretically characterized, indicating that changes in transport due to electroporation of lipid bilayers within the stratum corneum are consistent with experimental results.

Comparison of transdermal transport during low-voltage, constant electric fields (iontophoresis) and high-voltage pulsed electric fields (electroporation) indicated that the transport enhancement seen during electroporation could not be explained by electrophoresis and/or electro-osmosis alone, but suggested the occurrence of skin structural changes. Moreover, the estimated area fraction of skin available to ion transport during electroporation was determined to be up to 0.1%, which is one to two orders of magnitude greater than during iontophoresis, also suggesting the creation (or enlargement) of transport pathways. While a variety of mechanisms could be proposed to explain these results, transient changes in skin structure created by a mechanism related to electroporation is offered as the most-promising hypothesis.

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