Skin Electroporation: Rapid Measurements of the Transdermal Voltage and Flux of Four Fluorescent Molecules Show a Transition to Large Fluxes Near 50 V

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Abstract ■ Large molecular fluxes and tight control are highly desired for transdermal drug delivery, which motivated this study of molecular transport due to high voltage pulsing. We used a flow-through sampling system (time resolution of ~14 s) to measure the response of human skin in vitro to a series of exponential pulses (time constant of 1 ms; peak transdermal voltages $[U_{\text{skin,o}}]$ of 0 V to ~300 V, one pulse every 5.6 s). Four negatively charged, hydrophilic fluorescent tracer molecules were employed: sulforhodamine, lucifer yellow, cascade blue, and calcein (molecular weights of 450 to 625 Da). Although differences in their molecular transport profiles were observed, all four molecules exhibited a transition from small to large fluxes at $U_{\text{kin,o}} \approx 50$ V. This behavior may reflect a transition from electroporation of the skin’s appendages to electroporation of the multimembrane bilayer membranes within the stratum corneum.

Introduction

Many studies have shown that applying a series of short (~1 ms), high voltage (~100 V skin) electrical pulses to human skin can dramatically increase the rate of transdermal molecular transport$^{1-20}$ hypothesized to involve electroporation.$^{1}$ This method may provide a useful technique for the transdermal delivery of drugs. Although many experiments have been conducted, using a wide range of molecules,$^{1-12}$ electrical conditions,$^{1,3,7,9,13-18}$ and types of skin,$^{3,5,15,17,18,20}$ few studies have been conducted to determine the quantitative relationship between the electrical conditions and molecular transport.

While many authors have described the average transdermal flux across the skin in terms of the peak applied voltage,$^{1,3,4,9,14,16,18}$ others have shown that it is actually the peak transdermal voltage ($U_{\text{skin,o}}$) that governs transport.$^{7}$ For a given experimental system, the applied voltage may be correlated with the transdermal voltage, but with widely varying experimental apparatuses in use,$^{1,3,17,20-22}$ comparison of the applied voltages used by different authors is not easily accomplished. Hence, it appears to be more useful to report the average molecular flux versus $U_{\text{skin,o}}$. Theoretical calculations also predict a relationship between the molecular flux and $U_{\text{kin,o}}$. In addition, a recent theory has predicted a transition from electroporation of the skin’s appendages at 5 V < $U_{\text{kin,o}}$ < 30 V, to electroporation of the multilamellar bilayer membranes within the stratum corneum at $U_{\text{kin,o}}$ > 30 V.$^{25}$

Many studies have used 1 h aliquot measurements of the receptor compartment to study the molecular flux across the skin.$^{1-6,9-12,14-18}$ However, other studies used a “flow-through” system, which pumps the contents of the receptor compartment to a detector.$^{7,21}$ The first use of this method improved the time resolution to approximately 1 min$^{18}$ and allowed for the measurement of the molecular flux of various fluorescent tracers through the skin, with repeated pulsing.$^{7,18}$ These changes in molecular flux were detectable with 1 h aliquot measurement techniques. Since then, the use of various flow-through systems has been carried out in conjunction with high voltage pulsing of human skin in several studies.$^{7,18,21,23,26,27}$ Thus, to obtain quantitative information during electroporation, the simultaneous measurement of the peak transdermal voltages, on a per-pulse basis, and the molecular transport across the skin in real time were sought. The goal of this study was to determine the relationship between the peak transdermal voltage and the resulting molecular flux.

Materials and Methods

Phosphate-buffered saline solution (PBS) was prepared by adding 138 mM NaCl, 8.1 mM Na2HPO4, 2.7 mM KCl, and 1.1 mM KH2PO4 (150 mM total of dissolved salts) to deionized water and stirring under vacuum for ~30 min until the solution was clear. Donor solutions were prepared by adding 1 mM of fluorescent tracer to degassed PBS and stirring for ~30 min until the tracer dissolved. Four different fluorescent donors were used, containing one of either sulforhodamine, lucifer yellow, cascade blue, or calcein (Molecular Probes, Eugene, OR) (see Table 1 for their physical properties). These molecules were chosen since they were all hydrophilic and had roughly the same molecular weight (450 Da to 625 Da). The nominal electronic charges on these molecules varied from −1 to −4 (Table 1).

Table 1—Physical Properties of the Fluorescent Tracers

<table>
<thead>
<tr>
<th>name</th>
<th>MW (Da)</th>
<th>nominal charge</th>
<th>excitation wavelength (nm)</th>
<th>emission wavelength (nm)</th>
<th>source</th>
</tr>
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<tbody>
<tr>
<td>sulforhodamine</td>
<td>607</td>
<td>−1</td>
<td>586</td>
<td>607</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>lucifer yellow</td>
<td>457</td>
<td>−2</td>
<td>428</td>
<td>535</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>cascade blue</td>
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<td>−3</td>
<td>399</td>
<td>421</td>
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<tr>
<td>calcein</td>
<td>623</td>
<td>−4</td>
<td>496</td>
<td>517</td>
<td>Molecular Probes</td>
</tr>
</tbody>
</table>

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Stored cadaver skin at $-80^\circ C$ was thawed to room temperature and then submerged for 2 min in deionized water at 60 °C ("heat-stripping"). The epidermis (including the stratum corneum) was scraped off of the dermis, cut into 3/4 in. diameter circles, and stored at 4 °C, 95% humidity, for up to 10 days. The epidermis was used in all of the experiments described here.

The permeation chambers were specially designed, side-by-side chambers, with a skin exposure area of 0.64 cm$^2$ (circular opening, 0.9 cm in diameter) (Crown Bio Scientific, Somerville, NJ). Each chamber half had one large port near the skin (for the measuring electrodes and the flow-through system) and two small ports away from the skin (for the pulsing electrodes and the electrode flow-protection system). Water heated by a thermostated water bath to 37 °C was continually pumped through both outer jackets, to maintain the skin and the donor and receptor solutions at 37 °C. And stored at 4 °C, 95% humidity, for up to 10 days. The epidermis was used in all of the experiments described here.
The polyacrylamide solution was composed of 0.75 mL for the donor compartment and sealed in place with Parafilm. Both compartments running through the polyacrylamide gel in Figure 1. The outflow from the receptor compartment electrode went into a holding compartment (a centrifuge tube), which fed into the pump and the spectrofluorimeter.

Before each experiment, the permeation chamber was prepared as follows. Plastic tubes were inserted through both compartments and sealed in place with Parafilm (these are the hollowed-out sections running through the polyacrylamide gel in Figure 1). The measuring flow-through electrode was inserted into the receptor compartment and sealed in place with Parafilm. Both compartments were turned sideways, and polyacrylamide solution was poured in, completely immersing everything, to a volume of ~2.1 mL for the donor compartment and ~200 μL for the receptor compartment. The polyacrylamide solution was composed of 0.75 g of 19:1 acrylamide:N′-methylenebis(acrylamide) powder, 43.75 μL of 440 mM (NH₄)₂S₂O₈ solution, and 3 μL of N,N,N′,N′-tetramethylmethylenediamine in 5 mL of PBS. The polyacrylamide solution was allowed to harden (~10 min). After the solution solidified, the outer plastic tubing and the Parafilm were removed from the outer ports, leaving behind an empty channel through the gel. The flow-protected electrodes were inserted into this channel. The receptor compartment measuring electrode was not removed.

Fluorescence measurements were taken with a spectrofluorimeter (Fluorescence Master Series Spectrometer, Photon Technology International, Brunswick, NJ). The cuvette was specially constructed by drilling two holes into a 1 mL polystyrene microcuvette, to allow for inflow and outflow from the receptor compartment. The final volume of the cuvette was ~200 μL and was the minimum amount needed by the spectrofluorimeter for accurate detection and quantification. Mixing within the cuvette was accomplished by the dripping inflow of fluid down into the cuvette bottom (the drops entering the cuvette were a sizable fraction of the total volume of the cuvette). The performance of this system was very close to an idealized continuous-stirred tank reactor, and the residence time for the combined chamber-tubing-pump-cuvette system (volume of ~400 μL) was measured to be 14 s. This was taken to be the time resolution for flux measurements in these experiments.

High voltage exponential pulses were delivered by a specially modified pulser (Electroporation System 600, BTX Industries, San Diego, CA). The time constant was set to 1 ms. Pulses with peak transdermal voltages between 0 V and ~150 V across the skin were applied for 1 h at the rate of one pulse every 5.56 ± 0.01 s (640 pulses total). This rate was used to facilitate comparison with other experiments in the literature where pulses were given every ~5 s. The negative terminal of the pulser was connected to the pulsing electrode in the donor compartment, and the positive terminal to the receptor compartment, to provide a favorable driving force for the negatively charged tracers.

Current delivered across the skin during pulsing was measured by using the voltage drop across a 1.2 Ω, 50 W noninductive resistor, in series with the side-by-side channel (see Figure 2). The voltage drop across the resistor was measured by a 10x probe connected to a digital oscilloscope (Hewlett-Packard 54601). The oscilloscope was connected through an RS-232 interface card to a computer.

The voltage drop from the receptor to the donor compartment was measured by connecting a differential high voltage probe (Active Differential Probe B9017RT, Yokogawa Corp., Newman, GA) to the measuring electrodes in each compartment. This probe was also connected to the oscilloscope. The computer was programmed to count and save both voltage waveforms recorded by the oscilloscope for every pulse.

In a typical experiment, prepared, stored human skin was floated in PBS for ~1 min to remove the wax paper backing. The skin was loaded into the side-by-side chambers (prepared as described above), with the stratum corneum facing the donor compartment. The condition of the skin was checked by measuring its electrical impedance using an LCR meter (Model 5R715 LCR Meter, Stanford Research Systems, Sunnyvale, CA). The skin was discarded if the electrical resistance was >20 kΩ/cm² (ref 8). The resistances of the PBS and the polyacrylamide gel were negligible (total of 250 Ω) compared to the skin’s resistance (typically ~40 000 Ω in this system).

The apparatus was then allowed to sit for 1 h to fully hydrate the skin and check for leaks (i.e., holes or tears in the skin). Leaky skin (passive flux above the detection limit, ~10⁻¹⁰ mol/m² s) was discarded.

PBS was pumped across the pulsing electrodes at ~6 mL/min. PBS was also pumped through the receptor compartment and the spectrofluorimeter at the rate of 0.0322 mL/s. Both the spectrofluorimeter computer and the oscilloscope computer were programmed to record data during pulsing.

After the experiment, the data from the two computers were downloaded onto a Sun Sparc 10 workstation (Sun Microsystems, Palo Alto, CA) for analysis. To analyze the fluorescence, voltage, and calibration data, a program was written in MatLab S (Mathworks, Natick, MA) that would calculate the voltage drop appearing across the skin for each pulse, and the instantaneous molecular flux across the skin, using appropriate deconvolution routines. The two data streams were then synchronized to give the molecular flux across the skin on a per-pulse basis.

Results and Discussion

Measurements Yielding Average Values Can Give Significant Errors—To simulate the 1 h aliquot measurements commonly used in the literature, the transdermal molecular fluxes measured during each experiment were averaged over 1 h. Plotting these data versus either the applied voltage (Figure 3A) or U̇skin,0 averaged over 1 h (Figure 3B) resulted in a lot of scatter in the data.

Since it was known that the applied voltage does not correctly predict the actual transdermal voltage, the scatter in Figure 3A was to be expected. More evidence of this can be seen in Figure 4, where the average U̇skin,0 values were plotted versus the applied voltages, showing a poor relationship. However, the average fluxes plotted...
against the averaged $U_{\text{skin,0}}$ values in Figure 3B also show a poor relationship. Thus, by using average measurements of either the molecular flux or $U_{\text{skin,0}}$, significant errors can be introduced into the measurements, motivating the use of a flow-through system.

Others have reported molecular flux versus either the peak applied voltage, $1,3,4,9,13,16,18$ or the average peak transdermal voltage, $6,7,12,14-16,18$ Many authors have reported only average fluxes after 1 h of pulsing. $1-6,9-12,14-16,18$ Those methods, while easier to apply, do not properly account for the time-varying nature of the flux and voltage, and can be misleading, as was demonstrated here.

**Molecular Flux and Transdermal Voltage Vary with Repeated Pulsing**—Figure 5 shows the molecular flux, plotted for each pulse during a typical experiment, for each of the four fluorescent donor molecules (5A, sulforhodamine; 5B, lucifer yellow; 5C, cascade blue; and 5D, calcein). The molecular flux reported is the deconvoluted average flux through the skin, measured during the first 14 s after each pulse, the time resolution of this experimental apparatus.

Even though the four donor molecules had roughly similar molecular weights (450 Da to 625 Da) and were all hydrophilic and negatively charged, several different profiles of flux versus time were observed. The flux of

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**Figure 5**—The molecular flux as a function of pulse number, plotted for four donor molecules: sulforhodamine (A), lucifer yellow (B), cascade blue (C), and calcein (D); representative experiments shown. Error bars have been removed for clarity. In all cases, 640 pulses were applied to the skin, 1 pulse every 5.56 s. A flow-through system was used to measure the flux through the skin in real time, with a time resolution of 14 s. Even though these molecules had similar properties (all were hydrophilic, 450 Da to 625 Da, and negatively charged), very different behaviors were observed. (A) Sulforhodamine flux increased throughout the course of the experiment. (B) The flux of lucifer yellow increased to a steady state rate. (C) The flux of cascade blue also went to steady state. (D) The flux of calcein quickly reached a maximum and then began steadily decreasing with repeated pulsing.

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**Figure 6**—The transdermal voltage across the skin for each pulse in a typical experiment. Error bars have been removed for clarity. The peak transdermal voltage quickly drops (from $\sim 180$ V to $\sim 140$ V) with repeated pulsing and then continues decreasing at a slower rate afterward (from $\sim 140$ V to $\sim 130$ V). Note that the peak transdermal voltage does not reach steady state, even after 1 h, and hence reporting an average peak transdermal voltage can be misleading.
sulforhodamine (Figure 5A) increased steadily, without reaching a steady state plateau. Longer experiments (data not shown) indicated that the flux does not reach a steady state plateau, even after 7 h of pulsing. However, the fluxes of lucifer yellow (Figure 5B) and cascade blue (Figure 5C) did reach steady state. Also, the flux of calcein (Figure 5D) first increased quickly during pulsing, and then decreased with additional pulsing. This decrease is not an artifact, since the flow protection of the pulsing electrodes was used to prevent contamination of the donor and receptor compartments by electrochemical byproducts or pH changes from the electrodes. Other controls (iontophoresis and passive exposure of the skin to calcein, data not shown) have confirmed that calcein does not react with the skin. This decrease in calcein flux has also previously been reported.7,18,21,23,26

Figure 6 shows a typical voltage drop across the skin during each pulse. $U_{\text{skin}}$ drops quickly with the first few pulses, continually decreases more slowly with repeated pulsing, and does not go to a steady state. This behavior was observed regardless of the tracer used.

Thus, both the molecular flux and the transdermal voltage vary with repeated pulsing. More experiments will be needed to better understand why different behaviors in the transdermal fluxes of similar molecular compounds was observed.

Onset of Molecular Transport at $\sim 50$ V—Combining Figures 5 and 6 yields a plot of the molecular flux versus

Figure 7—The molecular flux on a per-pulse basis through the skin, plotted versus the peak transdermal voltage for every pulse, for each of the four donor molecules: sulforhodamine (A), lucifer yellow (B), cascade blue (C), and calcein (D). Error bars have been removed for clarity. In all four cases an almost linear relationship can be seen between the flux and the peak transdermal voltage, with a transition around $\sim 50$ V. This is indicated by the dotted line (this is not a quantitative determination; it is simply for illustrative purposes only). The trend observed here is consistent with theoretical predictions of a transition to electroporation of the stratum corneum around $\sim 30$ V.25 (A) Sulforhodamine flux. Data from seven experiments are shown here. Since the flux of sulforhodamine continually rises throughout the experiment (see Figure 5A), the flux on a per-pulse basis starts toward the bottom of the graph and continues to rise throughout the course of the experiment. The peak transdermal voltage slowly decreases with repeated pulsing, causing the “track” of data points to lean slightly to the left. (B) Lucifer yellow flux. Data from 13 experiments are shown here. The flux of lucifer yellow reaches a steady state profile (Figure 5B), resulting in a “cloud” of points at the top of each track. Each track leans slightly to the left, due to the decrease in peak transdermal voltages with continued pulsing. (C) Cascade blue flux. Data from 13 experiments are shown here. The flux of cascade blue reaches steady state with repeated pulsing (Figure 5C), causing a “cloud” of points at the top of each track. Each track leans slightly to the left, since the peak transdermal voltage decreases with repeated pulsing. (D) Calcein flux. Data from 14 experiments are shown here. The flux of calcein quickly increases during the start of pulsing and then begins to slowly decline with repeated pulsing (see Figure 5D). Hence, the tracks of calcein fluxes on this figure actually start toward the top of the figure and then head downward with repeated pulsing. Since the peak transdermal voltage also decreases with repeated pulsing, each track leans toward the right. At $<50$ V, the calcein flux tails off; at these voltages, most of the electroporation is appendageal.26
the peak transdermal voltage, as can be seen in Figure 7 (7A, sulfadiazine; 7B, lucifer yellow; 7C, cascade blue, and 7D, calcin). These data were plotted as the molecular flux versus $U_{\text{skin},0}$ for each pulse, irrespective of the pulse number, for all of the experiments. These data are from the same experiments shown in Figures 3 and 4.

For each molecule, a nearly linear relationship can be observed between the molecular flux and $U_{\text{skin},0}$ for voltages $\geq 50$ V (indicated by dotted lines in Figure 7). However, the molecular flux tapers off for voltages $\leq 50$ V. Although the molecular flux varied both in time, and from molecule to molecule, a transdermal voltage of at least $50$ V seems to be necessary to cause significant molecular transport across the skin, regardless of the fluorescent tracer used.

Theoretical calculations and supporting experimental evidence in the literature have predicted electroporation predominantly at the appendages at $U_{\text{skin},0} < 30$ V, and electroporation of the lipid–cornocyte matrix at $U_{\text{skin},0} > 30$ V. Although those calculations were based on a different system than the one used in the current study (for example, full-thickness instead of heat-stripped skin, 25 °C instead of 37 °C, rectangular pulses instead of exponential pulses), the value of 30 V for a transition to lipid–cornocyte electroporation (based primarily on electrical measurements) is reasonably close to the value determined in this study of $\leq 50$ V for the onset of significant molecular transport (based primarily on fluorescence measurements). On the basis of those calculations, the transcellular pathway through the skin, accessible primarily at higher transdermal voltages, allows significantly more molecular transport than the appendageal pathways that predominate at lower transdermal voltages. Most of the molecular transport takes place through the stratum corneum, not through the appendages, possibly through the localized transport regions described in the literature.6,8,10,20,22

**Conclusion**

This study showed that during high voltage pulsing of the skin, both the molecular flux and the peak transdermal voltage need to be measured with high time resolutions, on the order of seconds, not hours, due to the changing nature of both the transdermal voltage and the resultant molecular flux. Molecules which are very similar (similar molecular weights, similar charges, etc) can pass through the skin at significantly different rates and profiles. Careful measurements during repeated pulsing (exponential pulses, 1 ms time constant, 1 pulse every 5.6 s) have shown that the transdermal molecular flux increases markedly at transdermal voltages $> 50$ V.

**References and Notes**

27. Kost, J.; Pliquett, U.; Mitragotri, S.; Yamamoto, A.; Langer, R.; Weaver, J. Synergistic Effect of Electric Field and
Acknowledgments

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