Spatially constrained skin electroporation with sodium thiosulfate and urea creates transdermal microconduits

Ljubomir Ilic\textsuperscript{a}, T.R. Gowrishankar\textsuperscript{a}, Timothy E. Vaughan\textsuperscript{a}, Terry O. Herndon\textsuperscript{b}, James C. Weaver\textsuperscript{a,*}

\textsuperscript{a}Harvard-MIT Health Science and Technology, Massachusetts Institute of Technology, Cambridge, MA, USA
\textsuperscript{b}MIT Lincoln Laboratories, Lexington, MA, USA

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Abstract

Controlled transport of molecules through the skin’s main barrier, the stratum corneum (SC), is a long standing goal of transdermal drug delivery. Traditional, needle-based injection provides delivery of almost any water soluble compound, by creating a single large aqueous pathway in the form of the hollow core of a needle, through which drug is delivered by pressure-driven flow. We extend previous work to show that SC-spanning microconduits (here with diameters of about 200 \textmu m) can be created in vivo by skin electroporation and low-toxicity, keratolytic molecules (here sodium thiosulfate and urea). A single microconduit in isolated SC can support volumetric flow of the order of 0.01 ml s\textsuperscript{-1} by a pressure difference of only 0.01 atm (about 10\textsuperscript{2} Pa), demonstrating that the SC barrier has been essentially eliminated within this microscopic area. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Drug delivery is of great and growing interest \cite{1}, for which the challenge is to introduce both traditional pharmaceuticals (typically less than about 1000 g mol\textsuperscript{-1}) and the newer products of biotechnology, viz. peptides, proteins and nucleic acids (generally larger than 1000 g mol\textsuperscript{-1}). Transdermal drug delivery is of particular interest \cite{2–5} because it involves man-made devices located outside the body, avoids first-pass effects associated with liver metabolism, and has the possibility of providing temporal control of the amount delivered.

Previous studies of skin electroporation \cite{6,7} have shown that systemic transdermal delivery of pharmaceutical compounds should be possible, but may be limited to drugs smaller than about 1000 g mol\textsuperscript{-1}. Thus, for example, drugs such as metoprolol \cite{8} and fentanyl \cite{9} can be transported across skin at relatively large rates. Other studies show that some peptides (e.g. LHRH; \~{}1000 g mol\textsuperscript{-1}) \cite{10}, antisense oligonucleotides (\~{}7000 g mol\textsuperscript{-1}) \cite{11} or the linear, highly charged anticoagulant, heparin (5000 to 20,000 g mol\textsuperscript{-1}) \cite{12} can be transported by electroporation across human skin at potentially...
useful rates. However, delivery of larger molecules, or even small molecules at very high rates, appears difficult.

The hypothesis of skin electroporation is an extension of lipid bilayer membrane electroporation [13], in which elevation of the transmembrane voltage results in creation of new aqueous pathways (Fig. 1) across the multilamellar bilayer membranes of the stratum corneum (SC) [14]. But examination of the SC’s structure [3] shows that the keratin matrix of the corneocytes also present barriers to transport of large molecules.

An unexpected finding from earlier studies was the creation of local transport regions (LTRs) by electroporation [11,15–18]. LTRs typically have a minimum size of a corneocyte (~50 μm characteristic length parallel to the SC surface). Although a detailed understanding of how LTRs form is lacking, a key feature is that LTRs form away from the skin’s appendages (sweat ducts and hair follicles). Only the SC appears to be involved, with a large number (more than 10^3) of new, primary aqueous pathways within a minimum size LTR [19].

A recent study demonstrated that LTRs can be constrained to occur at microholes (40 to 100 μm diameters) in a thin, electrically insulating sheet placed near the skin, even if there is a small gap between the sheet and the skin [20].

An extended hypothesis has been introduced recently, based on the idea that electroporation could introduce low-toxicity, keratolytic molecules into the corneocytes within LTRs [21,22]. The combination of new aqueous pathways through the multilamellar lipid bilayer membranes and subsequent disruption of the keratin matrix could result in dislodgement of entire stacks of corneocytes, creating ~50-μm diameter microconduits (MCs) through the SC (Fig. 1).

Such an opening would present negligible steric hindrance to macromolecule transport. By using temporally controlled pressure-driven flow, large rates of molecular transport should be achieved, for both conventional pharmaceuticals and larger peptides, proteins and nucleotides.

These initial experiments [21] showed that a macromolecule (lactalbumin, ~15,000 g mol^-1) and a smaller, charged molecule (sulforhodamine, ~600 g mol^-1) were transported at almost the same rate across heat-stripped human skin. A fluorescence-labeled antibody (~150,000 g mol^-1) was also transported, after pulsing and in the absence of the pathway-enlarging molecule. Finally, ~1-μm charged beads were transported through microconduits to the other side of the skin specimen. A single pathway-enlarging molecule (sodium thiosulfate) was used throughout. However, a large variability in microconduit creation was observed in these previous experiments. This motivated the present study, which employed both sodium thiosulfate and urea together to achieve much more controlled microconduit creation.

The keratin matrix within corneocytes presents significant steric hindrance to macromolecule transport [23,24]. Therefore we set out to enlarge the primary aqueous pathways (‘pores’) created by electroporation with a controlled, electrochemical process that: (1) disrupts the keratin matrix within the corneocytes of the SC, and (2) concentrates the intervention to a predetermined, microscopic area of skin.

The size of corneocytes provides a naturally occurring characteristic length parallel to the SC surface, viz. ~50 μm. Removal of single stack of corneocytes should therefore provide SC-spanning aqueous pathways (‘microconduits’) that are two to
three orders of magnitude larger than typical macromolecules \cite{21,22}. Creation of microconduits is motivated by the prospect of providing both transdermal drug delivery or interstitial fluid sampling. The present in vitro study focuses on a significantly improved microconduit protocol, and a demonstration that a single microconduit can support useful levels of pressure-driven flow through a specimen of isolated SC. We report a relatively fast (\(\sim 5\) min) electrochemical protocol for the microfabrication of a microconduit that:

(a) is created at a predetermined site in full-thickness human skin;
(b) extends through the stratum corneum; and
(c) involves an area of a few corneocytes (larger than \(\sim 50\) \(\mu\)m, the minimum size microconduit).

2. Materials and methods

2.1. General approach

The present in vitro study involves a partial optimization of an electrochemical process for microconduit creation. The following two steps are carried out simultaneously in the process.

1. Introduction of keratolytic chemicals into corneocytes at a predetermined microscopic area site by high-voltage (HV) pulsing that electroporates the multilamellar bilayer lipid membranes in this region. This creates a spatially constrained LTR with a diameter of \(\sim 200\) \(\mu\)m by using an electrically insulating mask with a single \(\sim 200\) \(\mu\)m microhole that spatially limits the current.

2. Rapid disruption of the keratin matrix within the electroporated corneocytes by a keratolytic solution which has been partially optimized with respect to choice, precedent medical use, and concentration. The goal is rapid, controlled microconduit creation with minimal side effects, both local and systemic.

2.2. Skin preparation

Adult human skin was secured from the abdomen, arm, or back of cadavers (NDRI, Philadelphia, PA; Ohio Valley Tissue and Skin Center, Cincinatti, OH). The skin, stored at \(-70\)\(^\circ\)C, was cut into 1 inch square pieces and thawed to room temperature prior to the start of the experiment. The microconduit creation step was carried out using full-thickness skin. The flow test was carried out on thinned skin, prepared by heat-stripping and/or trypsination.

Heat stripping yields a specimen of thickness \(100\pm 50\) \(\mu\)m. This procedure involves a well established method of immersing the skin in \(\sim 60\)\(^\circ\)C water for 2 min \cite{25}. Overnight removal of the remaining epithelium was achieved by enzymatic trypsin digestion \cite{29}. Specifically, the heat stripped skin was digested in 1\% trypsin (Sigma Chemical Co., T-0134) for 12 h at \(4\)\(^\circ\)C.

2.3. Electrical pulsing

In the present study, the total time of the experiment, \(t\)\(_{\text{total}}\), the interval between pulses, \(t\)\(_{\text{int}}\), the HV pulse decay constant, \(\tau\)\(_{\text{pulse}}\), and the peak current, \(I\), were selected to deliver a total charge of about 40 \(\mu\)C during the pulsing protocol. The voltage across the skin, \(U\)\(_{\text{skin}}\), and the total charge transferred, \(q\), were calculated according to Eq. (1), and Eq. (2), respectively.

\[
U_{\text{skin}} = U_{\text{inner}} - R_{\text{PBS}} \cdot I = U_{\text{inner}} - R_{\text{PBS}} \cdot U_{\text{res}} / 2\Omega,
\]

\[
q = \int_{0}^{t\text{total}} I e^{-t/t\text{pulse}} dt = \int_{0}^{t\text{total}} I t\text{pulse}.
\]

High-voltage (HV) pulses were applied using an exponential pulser (Electroporation System 600, BTX Industries, San Diego, CA). A non-inductive, high power rating (25 W) 2-\(\Omega\) resistor was placed in series with the chamber in order to measure the current through the skin. Measuring electrodes con-
phase and electrophoresis thereafter were carried out in the top-bottom chamber shown in Fig. 2. The chamber is a modified version of a side-by-side chamber where the donor compartment is masked by a non-conducting polyimide sheet with an ~200 μm microhole. The full-thickness skin was mounted between the two compartments with the SC facing the donor and contacting the polyimide sheet. The flow test following the creation of a microconduit (after the skin was thinned) was performed in the side-by-side chamber shown in Fig. 3. A hydrostatic pressure difference between the two chambers was established by a vertical column of aqueous solution on the donor side.

2.6. Positive control

To assess the creation of a microconduit, full-thickness skin specimens were first evaluated by four indicators that were established in positive control experiments, in which an ~200 μm diameter shallow microhole was drilled through the SC of a full-thickness skin specimen by a cobalt microprecision drill bit (Gühring, Germany). The skin specimen was then subjected to electrophoresis with PBS containing fluorescent markers in the donor side (contacting the SC). The rationale was that a small, constant current preferentially drives these negatively charged markers into the low resistance microhole by

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**Fig. 2.** Chamber and circuitry used for electrochemical intervention. Both pulsing and measuring electrodes were made of stainless steel and were 3 cm long. The full-thickness skin was mounted with the SC contacting the 50-μm-thick polyimide sheet containing a 200-μm diameter microhole. The current through the skin was determined from the voltage across the 2-Ω resistor. In all the experiments, the receptor compartment contained PBS (pH 7.4).

**Fig. 3.** Chamber used in flow experiments. The electrochemically treated skin, after thinning by either heat stripping and/or trypsinization, was mounted in the chamber with the SC facing the donor compartment. A column of aqueous solution was added to the donor compartment in order to establish a hydrostatic pressure difference across the skin. The presence of a microconduit in the skin resulted in a continuous decrease in the level of the aqueous column. The column height, $h$, was recorded at a number of time points and used to determine volumetric flow as $dV/dt = A_{\text{rec}}(dh/dt)$.

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sisted of stainless steel wires (0.7 mm diameter, 3 cm long) placed in the inner ports of the chamber parallel to skin surface, at a distance of 1.5 cm between them (Fig. 2). The voltages across the inner electrodes ($U_{\text{inner}}$) and the 2-Ω series resistor ($U_{\text{res}} = I \cdot 2\Omega$) during pulses were stored in a digital oscilloscope (Hewlett Packard 54601) for determining $U_{\text{skin}}$ according to Eq. (1).

2.4. Keratolytic molecules

In the present experiments, we used skin electroporation with a topically applied solution of pathway-enlarging molecules: sodium thiosulfate (STS) and urea. These molecules were chosen by considering established keratin-degrading procedures, and differ from the initial microconduit experiments in which only STS was used [21,22]. Different concentrations of STS (158.1 g mol$^{-1}$; $z = -2$, Sigma Chemical Co., S-1648) and urea (60 g mol$^{-1}$; $z = 0$, GibcoBRL, 15505-035) in phosphate buffered saline (PBS; pH 7.4) were used in the electrochemical process.

2.5. Experimental chambers

The HV pulsing during the microconduit creation
electrophoresis, thereby making it identifiable by 
optical microscopy.

2.7. Negative control

Negative control experiments were performed on 
unperturbed full-thickness skin. The skin was 
mounted in the top-bottom chamber (Fig. 2) with the 
donor compartment containing only PBS. The skin 
was subjected to HV pulsing and electrophoresis with 
the same electrical parameters as in an actual 
experiment involving electrochemical treatment.

2.8. Pulsing control

Pulsing control experiments relate to the electrical 
treatment of the skin without the use of keratolytic 
molecules. The skin was mounted in the top-bottom chamber (Fig. 2) with the donor compartment containing fluorescent markers in PBS. The skin was subjected to HV pulsing and electrophoresis with the same electrical parameters as in an actual experiment involving electrochemical treatment with keratolytic molecules.

2.9. Tests for microconduit presence

The positive control experiments (mechanically 
created skin microhole) were used to establish the 
necessary conditions, viz. indicators for microconduit 
presence. The results associated with an electro-
chemical protocol were compared to these indicators. 
However, the degree of keratin disruption within the 
electrochemically treated region was not easily ascer-
tained using these indicators only. For this reason, a 
combination of visualization and pressure-driven flow was designed as the definite confirmation for the presence of a microconduit, i.e. for complete keratin disruption. The creation of a microconduit was tested using three independent tests: the resistance ratio, the visualization, and the pressure-driven volumetric flow tests. The first test was applied to full-thickness skin, the last test was applied after the skin was thinned by heat-stripping [25] or heat-
stripping and enzymatic digestion of the epidermis, 
the combination yielding isolated SC. The second test was applied to both full-thickness and thinned skin.

2.9.1. Resistance ratio test

High voltage pulsing and the use of keratolytic 
chemicals create SC-spanning aqueous pathways in 
the skin. This change in skin structure is reflected by 
a change in skin resistance. An LCR meter (SR715, 
Stanford Research Systems, Sunnyvale, CA) was 
used to measure the real part of the low-frequency 
skin impedance (100 mV, 100 Hz sinusoidal input). 
A parallel RC model was assumed for the skin. 
Because the capacitance of the skin changes very 
little following HV pulsing, the skin impedance is 
dominated by the skin resistance, \( R_{\text{skin}} \). The decrease in \( R_{\text{skin}} \) following treatment was represented by the 
resistance ratio \( f_R \), defined as

\[
f_R = \frac{R^f_{\text{skin}}}{R^i_{\text{skin}}} \tag{3}
\]

where \( R^i_{\text{skin}} \) and \( R^f_{\text{skin}} \) are the initial and final skin 
resistances, measured before and after the treatment, 
respectively. The resistances were corrected for a 
baseline resistance that accounted for the resistance 
of the PBS between the measuring electrodes and the 
resistance of the electrodes. The baseline resistance 
was measured at the end of each experiment with no 
skin present in the chamber. A large, persisting 
change in skin resistance was used as an indicator for 
the creation of a microconduit.

2.9.2. Visualization test

To confirm the creation of a microconduit, full-
thickness skin specimens were first evaluated by 
visualization using incandescent and fluorescence 
microscopy. The visualization test was repeated on 
the skin following heat stripping and again following 
trypsination. Following the brief electrochemical 
procedure, the skin specimen was subjected to electrophoresis in the presence of PBS containing 
egatively charged, different-size, optical markers (a 
small fluorescent dye and micrometre-sized charged 
beads; as described below). The rationale is that a 
small, constant current preferentially drives these 
markers into the low resistance microhole, thereby 
marking for identification by optical microscopy.

Sulforhodamine (Sigma; 607 g mol\(^{-1}\); \( z = -1 \); red 
fluorescence; 0.5 mM in donor compartment) was 
used as the fluorescent dye, because it is both water 
soluble and sufficiently lipophilic that it stains cell 
membranes [26], allowing tissue accessed by the
microconduit to be stained. The sulforhodamine molecule has a diameter of about 1.5 nm, which allows it to be readily transported through the primary aqueous pathways (about 2 nm diameter) created by HV pulsing, and to pass with negligible steric hindrance through the intact keratin matrix within corneocytes. A 2.5% volume suspension of fluorescent latex beads (Fluoresbrite™, green fluorescence; Polysciences, Inc.; 1.5 μm diameter) with a large negative charge \( \approx -8 \times 10^{-12} \) C \( \approx 5 \times 10^7 \) elementary charges) was used. These microbeads are much larger than typical macromolecules, and can often be detected individually by fluorescence microscopy. Ordinarily such beads do not penetrate the skin [27], owing to both their large charge and size.

On completion of HV pulsing and electrophoresis, the skin was removed from the chamber and rinsed with de-ionized water. The skin was mounted on a microscope slide and examined under a fluorescence microscope (Olympus BH-2, Olympus Co., Woodbury, NY). The fluorescent image of the skin was acquired using a CCD camera (Pixera Corp., Los Gatos, CA). Visualization test was carried out further at two stages: after heat-stripping and after further treatment by enzymatic trypsin digestion. An incandescent (white) light source was placed below the specimen, with observation from above. As light passes through the microconduit, the opening in the skin appears as a single, bright spot surrounded by dark, intact region.

2.9.3. Pressure-driven volumetric flow test

The most direct evidence of the presence of a microconduit is a large solute transport across the epidermis. Thus, a pressure-driven flow across the electrochemically treated skin was used as a test for the presence of a microconduit. Either the heat-stripped skin or the isolated SC was mounted in a side-by-side chamber (Fig. 3). Pressure-driven flow was then measured by establishing a hydrostatic pressure difference between the two compartments of the chamber, using a vertical column of up to 12 cm aqueous solution on the donor side. Volumetric flow was measured by recording the height of the solution column meniscus as a function of time, \( t \), and multiplying by the cross-sectional area of the column to obtain the volume change with time.

A well established fluid dynamics equation [28] was used to relate the diameter, \( D_{MC} \), of the microconduit with the coefficients obtained from the least square fit of a polynomial \( a_1 t^2 + a_2 t \) to the experimental data points for the fluid volume change with time, \( V(t) \). The diameter \( D_{MC} \) was calculated for fluid flow through an orifice as

\[
D_{MC} = \left( \frac{32a_1 A_{col} \cdot 0.6}{g \pi^2} \right)^{1/4}.
\]

Here \( A_{col} \) is the cross-sectional area of the column of aqueous solution, \( g = 9.8 \text{ m s}^{-2} \) is the local value of the earth’s gravitational acceleration, and 0.6 is the aqueous solution coefficient of velocity for a short tube approximation [28], which is appropriate for an \( \sim 200 \mu \text{m} \) diameter microconduit with a thickness ranging from \( \sim 20 \mu \text{m} \) (isolated SC) to \( \sim 100 \pm 50 \mu \text{m} \) (heat-stripped skin).

2.10. Experimental procedure

The experimental procedure involved in the electrochemical creation of a microconduit and in the tests to confirm its presence is described below.

1. A previously thawed full-thickness human cadaver skin specimen was mounted in the top-bottom chamber (Fig. 2), avoiding significant skin deformation by excessive pressure.
2. The integrity of the skin was evaluated by measuring the initial skin electrical resistance between the pulsing electrodes using an LCR meter using a 100 Hz, 100 mV sinusoidal input.
3. In positive control experiments, a shallow 200 μm-microhole was drilled in the skin. In negative control experiments, the skin was left unperturbed. In microconduit creation experiments, the donor compartment was filled with STS-urea solution in PBS and the HV pulsing protocol followed.
4. In order to electrically drive negatively charged fluorescent markers, an hour of 1 mA cm\(^{-2}\) electrophoresis was applied with the donor containing the two fluorescent markers in PBS. The final skin resistance was measured following electrophoresis.
5. The skin was removed from the chamber and
washed in deionized water for 1 h. The skin was then examined under a microscope using both incandescent and fluorescent illumination.

6. Further visualization and flow tests were performed after the skin was heat-stripped, and in some cases, following overnight trypsination. The skin was mounted in the side-by-side chamber (Fig. 3) with a column of aqueous solution on the donor side. The flow was recorded at various time intervals as the decrease in the column level.

7. The chamber was reassembled without the skin to determine the baseline resistance of the electrodes and the PBS in the chamber. This value was used to correct the measured initial and final skin resistances.

3. Results and discussion

The present in vitro study involves a partial optimization of an electrochemical process for microconduit creation. This was achieved by introducing keratolytic chemicals into corneocytes at a predetermined microscopic-area site electroporated by HV pulsing. The goal was rapid, controlled microconduit creation with minimal side-effects, both local and systemic. The presence of a microconduit was tested using three independent tests listed in Section 2.

The results of these tests are summarized in Tables 1 and 2 and Figs. 4–9. Based on positive control results, four features were identified as indicators of the presence of a microconduit. These indicators are:

- \( f_R \), the resistance ratio, which represents the change in skin resistance from the unperturbed state to that following the electrochemical treatment. The presence of a microconduit causes a large increase in the resistance ratio;
- Macro Focus, macroscopic focusing, is the presence of a focused spot on the skin at the site where the microhole of the chamber contacted the skin, as seen in the macroscopic view of the skin. When a microconduit is created using the electrochemical treatment, sulforhodamine present in the donor solution enters the microconduit to form a red fluorescent spot equal in dimension to the microhole in the insulating sheet;
- Micro Focus, microscopic focusing, is the presence of a focused cluster of negatively charged fluorescent microbeads at the site of the microconduit. If a microconduit is created following electrochemical treatment, electrophoresis of skin with highly charged fluorescent beads in the donor compartment causes the beads to be clustered in the microconduit;
- Vert Pool, vertical pooling, denotes the pooling of the beads throughout the depth of the SC (~15–20 \( \mu \)m) when a microconduit is created in the skin. The vertical pooling of fluorescent beads is verified by acquiring fluorescent images of the skin at various depths.

A large resistance ratio accompanied by the presence of the remaining three indicators strongly indicates the presence of a microconduit following the electrochemical procedure.

3.1. Negative control

Five negative control experiments were performed on unperturbed full thickness skin. The skin was subjected to electrophoresis in the presence of the fluorescent markers for the same duration as in an experiment with electrochemical treatment. The resistance ratio was 4±1 following electrophoresis and all three optical indicators were negative (Table 1). Electrophoresis of unperturbed skin did not cause a focused clustering of beads at the site of the microhole (Fig. 4). The macroscopic view of the skin did not show a focused spot where the skin contacted the microhole in the polyimide sheet.

3.2. Positive control

In five positive control experiments, an ~200 \( \mu \)m diameter hole through the SC and about 40±20 \( \mu \)m deep was created by a cobalt microprecision drill bit in full-thickness human cadaver skin. The skin specimen was then subjected to electrophoresis with PBS containing fluorescent markers in the donor compartment (contacting the SC). The resistance ratio following the creation of a hole in the skin was 16±2, indicating the presence of a large, new pathway in the skin. In addition, all three optical indicators were positive (Table 1). A well-defined
Table 1
Results of resistance ratio and optical tests

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a Note that in all experiments, following either control exposure, mechanical microhole creation, or electrochemical intervention, the skin was subjected to 1 h electrophoresis at 1 mA cm$^{-2}$ (over the area of the microhole in the polyimide sheet) before indicators were ascertained. Four indicators were derived from resistance ratio and optical tests. These are denoted by $f_R$ (resistance ratio), Macro Focus (macroscopic focusing), Micro Focus (microscopic focusing), Vert Pool (vertical pooling) and are described in the text. Some of the results are accompanied by images indexed in the rightmost column. The presence of optical indicators are denoted by 1 and their absence by 2, whereas a ± denotes that the particular indicator was not conclusive. Data corresponding to electrochemical treatment experiments are categorized into three groups. The first group corresponds to those experiments in which all three optical indicators were negative and the resistance ratio was less than 10. In the second group, the resistance ratio was between 10 and 20 and some of the optical indicators were positive. In the last group, all the indicators were positive and the resistance ratio was at least 20.

b Indicates the pulsing only control ($n=5$).

Table 2
Results of further visualization and flow tests

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<th>STS (M)</th>
<th>Urea (M)</th>
<th>$U_{\text{skin}}$ (V)</th>
<th>$r_{\text{puls}}$ (ms)</th>
<th>$t_{\text{int}}$ (s)</th>
<th>$t_{\text{total}}$ (s)</th>
<th>I (mA)</th>
<th>q (mC)</th>
<th>$f_R$</th>
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<th>Fluid Flow</th>
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</table>

a The skin in these experiments was heat-stripped and/or trypsinated overnight following the electrochemical process of microconduit creation. The entries in this table are similar to those in Table 1. Two definite confirmations of a microconduit are listed in this table for each experiment. MC Visualized relates to whether the microconduit (MC) was visualized by optical microscopy, either after heat-stripping or trypsinization. Fluid Flow indicates whether a fluid flow was observed in the pressure-driven fluid flow test after trypsinization. The presence of the confirmation is denoted by + and their absence by −.
Fig. 4. Negative control. Full-thickness human skin was subjected only to electrophoresis for 1 h with sulforhodamine and fluorescent beads suspended in saline in the donor compartment. Top left image is the macroscopic view of the skin. The black ellipse encompasses the site of the microhole in the polyimide that contacted the skin. No extensive staining of the skin by sulforhodamine is evident. The top center and top right images are fluorescent images of the skin taken at different depths (30 μm apart). The bright spots which represent fluorescent beads indicate that only discrete binding of beads to the skin. There is no cluster of fluorescent beads present in the skin. The dark region represents the staining of the skin by sulforhodamine. The bottom images are fluorescent images of the skin at a higher magnification. The images are taken at different depths, 5 μm apart. The disjoint presence of beads is clearly visible. Also, the beads do not persist over a large depth, suggesting that there is no pathway spanning the SC in the skin.
spot was visible in the skin, stained red (fluorescence) by sulforhodamine in the donor solution (Fig. 5). The fluorescence image of the skin also revealed a dense cluster of fluorescent beads in the spot. The cluster of beads persisted as the plane of view was moved deeper than 15 μm from the SC surface. The small, constant electrophoresis current preferentially had driven the beads into the low resistance microhole.

3.3. Pulsing control

In five pulsing control experiments, the skin was subjected to HV pulsing and electrophoresis, but without any keratolytic molecules in the donor solution. The HV pulsing of the skin, with about 40 mC of charge delivered, increased the resistance ratio to 6±2 compared with 4±1 in the negative control experiments (Table 1). As in negative control experiments, all three optical indicators were negative. The fluorescence image of the skin showed no areas with clustered beads, only scattered regions with individual beads visible (Fig. 6). These indicators suggest that HV pulsing alone causes no disruption to the keratin matrix and therefore does not create a microconduit in the SC. These observations are consistent with ‘pulsing only’ experiments reported by Zewert et al. [11].

3.4. Electrochemical creation of a microconduit

The present study was designed to optimize the disruption of the keratin matrix within corneocytes following HV pulsing by introducing keratolytic molecules into the corneocytes. A combination of two molecules, sodium thiosulfate (STS), and urea was used at different concentrations. In addition, the parameters of HV pulsing were also varied to improve the process of microconduit creation. The results of these experiments are summarized in Table 1.

Pulsing control experiments demonstrated that HV pulsing alone does not disrupt the keratin matrix. HV pulsing with only urea also caused no disruption of the keratin matrix. Even 10 M of urea increased the resistance ratio only to 3 (Table 1), which is close to that of negative control experiments. All three optical indicators were negative when only urea was used during HV pulsing. This is expected as urea is not known to have any keratolytic properties.

Though urea alone does not cause disruption of the keratin matrix, its presence is important for the creation of a microconduit. Urea, in concentrations less than 1.4 M in our pulsing protocol, did not cause complete disruption of the keratin matrix, irrespective of the amount of accompanying STS. This is evident from the resistance ratio, which ranged between 2 and 13, when the concentration of urea was between 0.1 and 1 M (Table 1). In addition, the optical indicators were not all positive in these cases. Specifically, the clustering of fluorescent beads spanning the SC was not observed (i.e. Vert Pool was negative). These observations suggest that urea in low concentrations does not stabilize the intermediate products of STS-induced keratin degradation. In some cases, the clustering of beads (i.e. Micro Focus) as observed in fluorescence microscopy was inconclusive. In these cases (indicated by ± in Table 1), the fluorescence image showed small clusters of beads in the skin with most of the area occupied by scattered individual beads. In these intermediate range experiments, a focused spot was observed only in the macroscopic view of the skin. This suggests that sulforhodamine, a lipophilic dye, bound to the lipid bilayers exposed by high-voltage pulsing. However, the concentrations of STS and urea were not high enough to cause the disruption of the keratin matrix to enable the much larger 2-μm fluorescent beads to penetrate the corneocyte envelope. A low concentration of urea may also not be sufficient to prevent the oxidation of STS-reduced disulfide bridges.

Previous experiments without an electrically insulating mask [21] showed that electrochemical treatment of SC with 1–3 M STS disrupted the keratin matrix creating ~50-μm diameter microconduit. Here, we show that a similar result could be obtained with a far less concentration of STS when accompanied by urea. STS, in concentrations as low as 70 mM, causes a complete disruption of the keratin matrix in the SC. However, this result was obtained consistently only when STS was used with at least 1.4 M urea. In such an electrochemical treatment, the outcome was similar to that observed in the positive control experiments. The resistance ratio ranged from 20 to 51 when STS and urea were
Fig. 5. Positive control. A shallow 200-μm hole was drilled into the full-thickness skin using a cobalt microprecision drill. The skin was then subjected to electrophoresis for 1 h in the presence of sulforhodamine and latex beads suspended in PBS in the donor compartment. The top left image shows the macroscopic view of the image following electrophoresis. A focused red (here dark) spot (denoted Macro Focus in Table 1) is seen (arrow added to aid identification) at the site where the skin contacted the microhole in the chamber. Top center and top right images are fluorescent images of the skin, imaged 20 μm apart in depth. The images show a dense cluster of fluorescent beads at the site of the microhole (denoted Micro Focus in Table 1). Images in the bottom panel show the fluorescent images at a higher magnification, 15 μm apart in depth. The cluster of fluorescent beads persist over more than 15 μm in depth (denoted Vert Pool in Table 1). Contrast to the negative control experiments, the beads are not dispersed as individual beads, they are instead clustered within the microhole.
Fig. 6. Pulsing control. A full-thickness skin was subjected to HV pulsing, and electrophoresis (Table 1, third row) with only PBS and fluorescent markers in the donor compartment during HV pulsing and electrophoresis, respectively. Top left image (macroscopic view) shows no focused red spot. The low-magnification fluorescent images (top center and top right; 30 μm apart in depth) show no areas with large density of fluorescent beads. Similarly, the magnified fluorescent images (bottom panel, 5 μm apart in depth) show only scattered, granular presence of fluorescent beads. The images suggest that there was no extensive keratin disruption following HV pulsing when keratolytic agents were absent during HV pulsing.
used in concentrations ranging between 0.07 and 0.5 M, and 1.4 and 10 M, respectively (Table 1). In these experiments, all three optical indicators were positive. All these features are equivalent to those observed in positive control experiments suggesting the presence of a large aqueous pathway in the skin spanning the SC.

A 5-min spatially localized electrochemical treatment of full-thickness skin with 0.25 M STS and 5 M urea created a microconduit that was supported by the visual tests. Fig. 7 shows the incandescent and fluorescent images of the full-thickness skin following the electrochemical treatment and 1 h of electrophoresis in the presence of sulforhodamine and beads. HV pulsing in the presence of keratolytic molecules caused a focused red fluorescent spot corresponding to the staining of sulforhodamine in the keratin-disrupted region of the skin. As in the positive control experiments, a focused cluster of fluorescent beads was seen in the microhole (Micro Focus present). The cluster of beads persisted over at least 15 µm deep from the surface of the SC (i.e. Vert Pool was positive). These results, equivalent to those of positive control experiments, suggest the creation of a large aqueous pathway in the skin spanning the SC.

The visualization and resistance tests were used to obtain indicators as evidence for the creation of a microconduit in full-thickness skin. However, the degree of keratin disruption was not easily ascertained using these indicators. Hence, the full-thickness skin was thinned after electrochemical treatment either by heat stripping alone or by trypsinating the heat-stripped skin. Optical and flow tests on thinned

Fig. 7. Electrochemical creation of a microconduit. A full-thickness skin specimen was subjected to HV pulsing in the presence of 0.25 M STS and 5 M urea in PBS (Table 2). The electrochemical protocol is followed by electrophoresis for 1 h at 1 mA cm⁻² (within the microhole) in the presence of large latex beads and sulforhodamine. The macroscopic view of the skin (top left) shows a focused red spot at the site of microhole contact. An arrow has been added to aid in identification. The fluorescent images (top center and top right; 20 µm apart in depth) show a dense cluster of fluorescent beads. The fluorescent images at higher magnification (bottom panel; 5 µm apart in depth) also show a cluster of beads that extends over a depth of 15 µm. These images relate to positive indicators, labeled Macro Focus, Micro Focus, and Vert Pool in Table 1, respectively. The features in these images are essentially the same as those obtained in the positive control experiments. The results suggest that an SC-spanning microconduit has been created in the full-thickness skin following electrochemical intervention.
skin provided confirmatory evidence of the presence of a microconduit (Figs. 8 and 9).

The heat-stripped skin also showed a red fluorescent spot at the site of the microconduit. When heat-stripped skin was exposed to incandescent light from below, in some cases, a bright region was seen at the site of the microhole, as shown in Fig. 8. This provides strong evidence that a microconduit spanning the SC has been created which allows light to pass through. As seen in Fig. 8, the microconduit created by the electrochemical treatment is clearly outlined in the incandescent image of the heat-stripped skin.

Still stronger evidence of a microconduit was provided by the flow tests on skin trypsinated following the electrochemical treatment. Trypsination leaves mostly the SC, therefore, a microconduit spanning the SC should support a measurable flow. Pressure-driven flow was measured by establishing a small hydrostatic pressure difference between the

![Fig. 8. Visualization of a microconduit. A full-thickness skin specimen was subjected to a 5-min electrochemical treatment (Table 2). Top left image shows the macroscopic view of the skin, with a focused red (here dark) spot formed by sulforhodamine staining of the microconduit (arrow). Top right is the corresponding fluorescent image which shows the presence of sulforhodamine in the microconduit (seen as dark regions in the grayscale image). The skin was then heat-stripped to obtain a thinned specimen. Bottom left shows the fluorescent image of the heat-stripped skin. The central bright region is the region of the skin which contacted the microhole in the polyimide sheet. The bottom right is the incandescent image of the heat-stripped skin, illuminated from below using a bright light source. The microconduit in the skin is brightly illuminated by light passing through the microconduit in the skin. This image provides the most striking optical evidence of the presence of a microconduit in the skin.](image_url)

two compartments of the chamber, using a vertical column of up to 12 cm aqueous solution on the donor side. Fig. 9 shows the volume of solute transported through the microconduit as a function of time. The measured flow was compared with fit to theoretical model (Eq. (4)) to estimate the size of the microconduit. The best fit estimate for the microconduit diameter of the specimen shown was 190±20 μm. A pressure difference of 0.01 atm (10 Pa) caused volumetric flow of the order of 0.01 ml s⁻¹ through such a microconduit. This agrees with the resistance ratio values and other indicators which were similar to that observed in positive control experiments.

Fluid flow was consistently observed for different electrochemical treatments (Table 2), even in cases where a microconduit could not be directly visualized. The heat-stripping procedure left a variable number of epithelial layers attached to the SC, which prevented light from passing through a microconduit spanning only the SC. Trypsination removed the epithelium, leaving only the SC; however, the resulting preparation was too thin to provide significant optical contrast between the microconduit and the rest of the intact SC. In such conditions, the direct visualization of a microconduit was not possible. A pressure-driven flow alone provided reliable evidence for the presence of a microconduit.

3.5. Keratolytic molecules

Previous experiments using STS [21] showed that a macromolecule (lactalbumin, ~15,000 g mol⁻¹; ~150,000 g mol⁻¹) and ~1 μm charged beads were transported through microconduits in heat-stripped skin. However, a large variability in microconduit creation was observed in these experiments. This motivated the present study, which employed both STS and urea together to achieve much more controlled microconduit creation. Use of STS alone does not prevent the reduced disulfide bridges to reform by oxidation, reversing any disruption to the keratin matrix. The use of urea as an alkylating agent prevents the oxidation of reduced disulfide bridges [30].

During HV pulsing, negatively charged thiosulfate ions are electrically driven into corneocytes within an LTR, while the electrically neutral urea enters a corneocyte interior passively, or by electroosmosis. The two chemicals disrupt the keratin within electroporated corneocytes, thereby greatly decreasing the steric hindrance over the localized area of the skin exposed to the chemicals and HV pulses (Fig. 1). This results in a greatly enlarged aqueous pathway, viz., a microconduit spanning the SC. In the present study, a partially optimized protocol lasting about 5 min created a microconduit at a predetermined skin site.

Both STS and urea are routinely used to degrade and extract keratin in a two-step chemical process: (1) the reduction of keratin disulfide bridges followed by (2) the stabilization of the resulting kerateines [24]. Both chemicals are relatively nontoxic and have a well-established medical use [30,31].

STS dissociates into two Na⁺ ions and a single thiosulfate ion (112 g mol⁻¹) with two negative charges. The thiol group is a well-known, mild reducing agent able to break the disulfide bridges [21]. Moreover, the thiosulfate ion is smaller in size, and intermediate in negative charge and magnitude, compared to calcein (623 g mol⁻¹; z = −4), and
sulforhodamine (607 g mol⁻¹; z = -1), two fluorescent tracer molecules used extensively in previous skin electroporation studies, and which are believed to readily cross electroporated lipid bilayer membranes and to pass through the keratin matrix within corneocytes in LTRs [15–17,19].

STS is applied systemically (i.v. injection) as an antidote for cyanide poisoning [32,33], and is also used to alleviate the side effects of certain chemotherapeutic agents [34,35]. In the case of cyanide poisoning, STS is given intravenously in a concentration of 1.6 M for a total of 12.5 g administered as a bolus over 1 to 2 min. Previous in vivo experiments using surface electrodes on pleated rat skin without keratolytic agents show that the damage from HV pulsing alone is comparable to (or less than) that from the accepted use of iontophoresis [36]. Thus, lower concentrations of STS within a small region of the viable epidermis for short times, established by the present study as requirements for reliable creation of a microconduit, is expected to have acceptable side effects [22].

Urea (60 g mol⁻¹; z = 0), in concentrations of up to 1.45 M, is an active ingredient in some dermatological preparations [37]. One established medical use involves application to epithelial tissue of the cervix; an example is Amino-Cerv® (8.6% urea) [30]. Urea aids in debridement, dissolves the coagulum, promotes epitelization, and increases the water-binding capacity of the stratum corneum [30]. As a moisturizing ingredient, urea is applied topically to skin in a suitable base in concentrations up to and including 20% or 3.3 M [30]. Finally, urea naturally occurs in blood, with a normal concentration range of 3.6 to 7.1 mM [31]. Thus, urea in topical concentrations of 70 mM is expected to cause no significant side-effects when used in an electrochemical protocol lasting for 5 min.

3.6. Localization of SC disruption

An insulating sheet with a microhole was used in contact with the SC to limit the electrochemical intervention to a very small area of the skin. As shown previously, even without a perfect seal, such a masking causes localized electroporation at microholes 40 to 100 µm in diameter [20]. Electrochemical treatment through a 200-µm microhole in the polyimide sheet was shown to create a 150 to 200-µm diameter microconduit spanning the SC. A straightforward extension of this result suggests that a reduction in the size of the masking microhole would result in a smaller microconduit.

3.7. Rapid creation of microconduits

The protocol used in the present study ensures a reliable creation of microconduit with a 5-min electrochemical intervention. However, the duration of electrical exposure ($t_{pulse} (t_{total} / t_{int})$) was much shorter, less than 1 s. If only an electrical exposure in the presence of the keratolytic molecules was needed to create a microconduit, the present protocol could have been shortened to a very rapid (less than 1 s) procedure. However, the kinetics of keratolytic reactions within the corneocytes has not been studied. Electrochemical shortening our pulsing protocol to less than 3 min created no microconduits in the skin. We expect that by decreasing the time interval between pulses, the duration of microconduit creation procedure can be shortened.

Among the challenges of drug delivery [1], transdermal delivery of essentially any size molecule is a long-standing goal. This requires transport through the SC. Traditional, needle-based injection provides delivery of almost any water soluble compound, by creating a single large aqueous pathway in the form of the hollow core of a needle, through which drug is delivered by pressure-driven flow. The present study provides support for an alternate method of creating a low hindrance pathway through the SC for transdermal drug delivery and biochemical analyte extraction. If further study demonstrates that acceptable side effects are incurred during the creation and use of microconduits, then a basis for controlled, transdermal drug delivery of essentially any size water soluble molecule will have been established.

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