

## Heparin Alters Transdermal Transport Associated with Electroporation

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**Short, high-voltage (HV;  $U_{\text{skin,max}} \approx 100\text{V}$ ) pulses have been shown to increase rates of transdermal transport by several orders of magnitude via a mechanism hypothesized to involve electroporation. We show that heparin, a linear, highly charged macromolecule, significantly alters the molecular transport capacity and lifetime of aqueous pathways across human stratum corneum (SC) created by such pulses. If co-transported during pulsing, heparin molecules can interact with the SC and other molecules, thereby altering ionic and molecular transport. We also observed an increase in post-pulse skin permeability and persistent lower skin resistance. Because most heparin molecules are long enough to span the five to six lipid bilayer membranes that separate corneocytes within the SC, these results can be explained by the hypothesis that heparin molecules were trapped within the skin, holding open pathway segments connecting adjacent corneocytes. These results support the skin electroporation hypothesis and provide the first demonstration of a chemical enhancer effect for transdermal transport by HV pulsing.**

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Many different methods are being investigated for transdermal drug delivery, including passive permeation with or without chemical enhancers<sup>1</sup>, ultrasound<sup>2</sup>, iontophoresis<sup>3</sup>, and electroporation.<sup>4–7</sup> In the latter case, short ( $\approx 1$  ms) pulses generating transdermal voltages of  $U_{\text{skin}} \approx 50$  to 100 V have been found to increase transdermal molecular transport by up to several orders of magnitude for a number of different compounds, including heparin. Our underlying hypothesis is that such pulses cause electroporation of the

multilamellar bilayer membranes of the skin's stratum corneum (SC). A specific hypothesis is that for transdermal voltages of about 50 to 100 V, essentially straight-through aqueous pathways are created within multilamellar bilayer membranes, such that adjacent corneocytes are connected, and large increases in molecular transport occur.<sup>4,8</sup> This hypothesis is an extension of individual cell membrane electroporation<sup>9</sup>: pores in the bilayer membranes between corneocytes connect to provide SC-spanning aqueous pathways (Fig. 1). Our results are consistent with this mechanism, and suggest that heparin may stabilize electroporation pathways.

### MATERIALS AND METHODS

*Skin preparation.* Side-by-side permeation chambers held heat stripped human epidermis, with an area  $A_{\text{skin}} = 0.7$  cm<sup>2</sup> exposed to solution.<sup>4,6,7</sup>

*Heparin and fluorescent tracer molecules.* The donor and receptor compartments are filled with phosphate-buffered saline (PBS; pH 7.4; 150 mM total salts, Sigma Chemical, St. Louis, MO). Sodium heparin ( $\geq 140$  U/mg; Sigma Chemical) was provided in the donor compartment of the chamber, at concentrations from 0 to 200 mg/ml. Heparin is a class of molecules, with a range of molecular weights (about 6,000 to 30,000 g/mol); the average MW of the heparin used in this study was about 20,000 g/mol.<sup>10</sup> The estimated range of molecular lengths is about 30 to 140 nm. Heparin is composed of repeating units of glucosamine and either glucuronic or iduronic acid residues, and because each of these disaccharide units has from one to three sulfates, the molecule has a large negative charge.<sup>11</sup> Two fluorescent water soluble molecules were also provided in the donor chamber, at a concentration of 1 mM: sulforhodamine (607 g/mol, charge =  $-1e$ ; red fluorescence) and calcein (623 g/mol; charge =  $-4e$ ; green fluorescence), so that the transport of molecules with nearly identical size but very different charge could be determined in a single skin preparation.

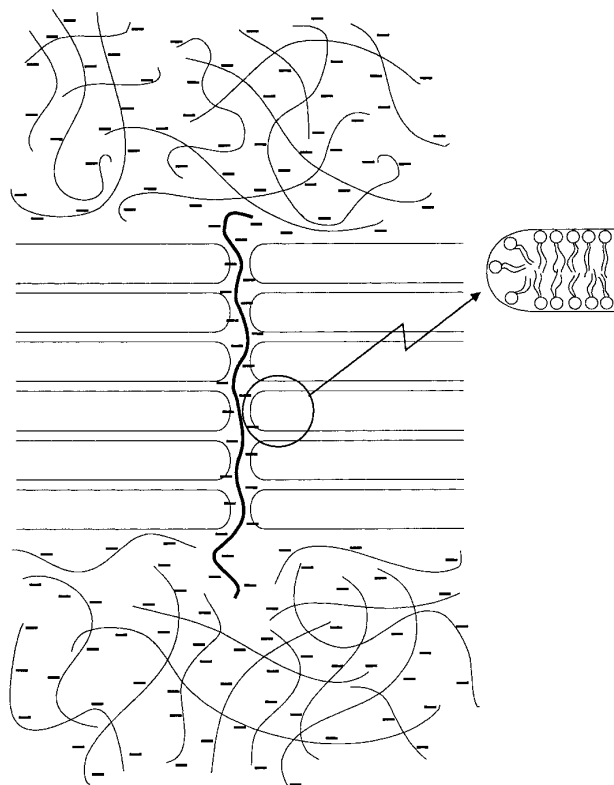
*HV pulse application (electroporation), iontophoresis, and skin resistance measurement.* High-voltage exponential pulses with peak amplitude of 1000 V and time constant  $\tau_p = 1$  or 2 ms were applied through stainless steel electrodes at 5 s intervals for one hour (720 pulses).<sup>4,6,7</sup> Iontophoresis conditions consisted of 1 mA/cm<sup>2</sup> for 1 hr. Skin electrical resistance,  $R_{\text{skin}}$ , was monitored as described previously.<sup>12</sup>

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**Transdermal molecular transport measurements.** Quantitative measures of both calcein and sulforhodamine fluxes for each skin preparation were determined using two color fluorescence measurements made in real time using a flow through system described previously.<sup>13</sup>

**Estimation of the number of aqueous pathways per participating corneocyte.** The "aqueous area",  $A_w$ , of a skin preparation is the effective area available for transport. Owing to Born energy and hindrance effects, this is not the total cross-sectional area of aqueous pathways, and is dependent on the molecule being transported. Similarly, the "fractional aqueous area" is  $F_w = A_w/A_{skin}$ . Previous experiments using similar pulsing conditions found  $F_w \approx 6 \times 10^{-5}$  for calcein and sulforhodamine.<sup>13</sup> If transport were uniform across the skin preparation, then  $F_w$  could be used to estimate the number of pathways per corneocyte. However, subsequent studies showed that molecular transport is concentrated within local transport regions (LTRs), and that all of the LTRs together occupy only a small fraction of  $A_{skin}$ ,  $f_{LTR} = A_{LTR}/A_{skin} \approx 0.1$  or less.<sup>6,14,15</sup> Therefore, the fractional aqueous area within LTRs is  $F_{w,LTR} = F_w/f_{LTR} \approx 6 \times 10^{-4}$ . Electrically created pathways are believed to have radii  $r_p \approx 1 \text{ nm}$ <sup>16</sup>, so the number of aqueous pathways per corneocyte is estimated to be  $N_p \approx F_{w,LTR}A_{cc}/(\pi r_p^2) \approx 10^5$ , where  $A_{cc} \approx 5 \times 10^{-9} \text{ m}^2$  is the area of a corneocyte.



**FIG. 1.** Illustration of the hypothesis that a linear macromolecule can enter an aqueous pathway that connects two adjacent corneocytes, and then become trapped, such that the pathway persists in some type of open state. The pathways, created by high-voltage pulsing, span the five to six bilayer membranes between the corneocytes, resulting in decreased resistance during pulsing. If some pathways trap a heparin molecule (thick, wavy line), altered post-pulse permeabilities and persistent, smaller  $R_{skin}$  are expected. The thinner, irregular lines within the corneocytes represent the negatively charged keratin matrix.

**TABLE 1**  
Maximum Transdermal Flux of Calcein and Sulforhodamine for Different Concentrations of Heparin and Different Time Constants of the Exponential Pulses

Protocol	Maximum calcein flux [ $\mu\text{g}/\text{cm}^2\text{h}$ ]	Maximum sulforhodamine flux [ $\mu\text{g}/\text{cm}^2\text{h}$ ]
$\tau_{\text{pulse}} = 2 \text{ ms}$ No heparin	17	16
$\tau_{\text{pulse}} = 2 \text{ ms}$ 200 mg/ml	5	26
$\tau_{\text{pulse}} = 1 \text{ ms}$ No heparin	12	10
$\tau_{\text{pulse}} = 1 \text{ ms}$ 0.1 mg/ml	12	11
$\tau_{\text{pulse}} = 1 \text{ ms}$ 1 mg/ml	8	9
$\tau_{\text{pulse}} = 1 \text{ ms}$ 10 mg/ml	3	6
$\tau_{\text{pulse}} = 1 \text{ ms}$ 200 mg/ml	3	17

*Note.* The peak voltage applied to the electrodes is approximately 1000 V, but due to a variable voltage divider effect a much smaller voltage ( $\approx 100 \text{ V}$ ) appears across the SC.<sup>12,13</sup>

## RESULTS AND DISCUSSION

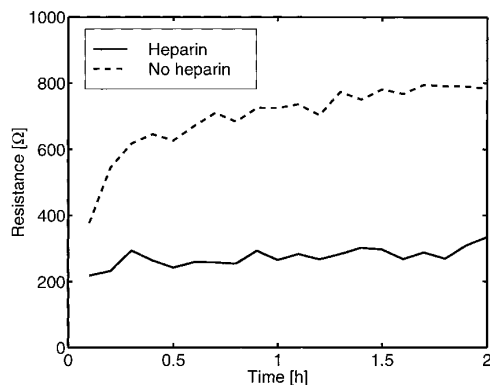
This study examined the effects of heparin on the transdermal transport of two charged fluorescent molecules during and after high-voltage pulsing, and on the transport of small ions after pulsing. The fluorescent molecules studied included a highly charged molecule (calcein,  $z = -4e$ ), and a similar size molecule with less charge (sulforhodamine;  $z = -1e$ ). As described below, transport of the small ions and singly charged sulforhodamine was increased by heparin, and transport of the highly charged calcein was decreased. For the largest heparin concentration (200 mg/ml), the maximum sulforhodamine transport increased by two-fold and the maximum calcein transport was decreased by three-fold, for both 1 ms and 2 ms pulses. Intermediate levels of heparin yielded smaller effects (Table 1).

The time course of skin resistance and sulforhodamine and calcein fluxes revealed significant dependence on the presence of heparin. Both with and without heparin, skin resistance dropped during pulsing and showed partial recovery after pulsing. Without heparin, post-pulse recovery was typically from  $R_{skin} = 400 \Omega$  to about  $800 \Omega$ . In contrast, with 200 mg/ml heparin  $R_{skin} \approx 300 \Omega$  initially and rose only slowly over time (Fig. 2). This persistent low resistance corresponds to a fractional aqueous area for small ions of order  $F_{w,ions} \approx 10^{-6}$ , far smaller than the peak value of order  $10^{-4}$  to  $10^{-3}$  during pulsing<sup>13</sup>. The observed lowering of the post-pulse transdermal electrical resistance is consis-

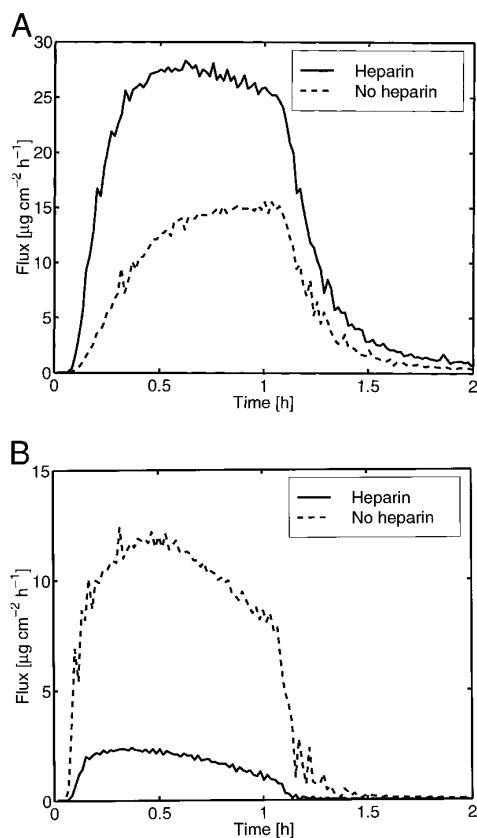
tent with the hypothesis that heparin is trapped in at least a fraction of the electrically created pathways. For one-hour exposure iontophoresis conditions, the post-treatment  $R_{\text{skin}}$  values were indistinguishable ( $\approx 10^4 \Omega$ ) with and without heparin.

Determined at the same time as the electrical measurements with the same skin preparations, the sulforhodamine flux was altered significantly (Fig. 3A). Both with or without heparin the flux rose rapidly after the onset of pulsing, but the maximum flux was achieved more rapidly with heparin ( $\approx 30$  min) than without ( $\approx 60$  min). Moreover, the maximum flux reached with heparin was higher than without. As in many previous experiments without heparin, when pulsing stopped (1 hr) the sulforhodamine flux decayed slowly, reaching a value approximately 2.5-fold larger with heparin than without. Again, these results are consistent with pathways that persist longer after pulsing. Subsequent experiments with other linear macromolecules have shown that transdermal transport of other small molecules can be similarly affected.<sup>17</sup>

The calcein flux was also altered significantly (Fig. 3B). Similarly to sulforhodamine, the calcein flux rose quickly with the onset of pulsing, both with and without heparin. Again, the maximum flux was reached more rapidly with heparin ( $\approx 8$  min) than without ( $\approx 30$  min). However, the maximum flux achieved with heparin was much lower than without. After pulsing stopped ( $t = 1$  hr), the calcein flux decreased slowly over about 30 min without heparin, but fell to below detection limits within 7 min with 200 mg/ml heparin. Unlike that of sulforhodamine, calcein flux was decreased by the presence of heparin. Both the diminished transport during pulsing and the rapid post-pulse cessation are consistent with electrostatic repulsion between the highly



**FIG. 2.** Effect of heparin on transdermal electrical resistance after pulsing. Immediately after pulsing without heparin (solid line; prepulse resistance  $8.2 \times 10^4 \Omega$ ), the resistance exhibited partial recovery, with  $R_{\text{skin}}$  rising from about 400  $\Omega$  to more than 600  $\Omega$  within 0.5 h, and reaching 800  $\Omega$  after 2 h. In contrast, with heparin at 200 mg/ml (dashed line; prepulse resistance  $1.3 \times 10^5 \Omega$ ) there was a small postpulse  $R_{\text{skin}}$ , and significantly less recovery, consistent with pathways held open by trapped heparin molecules.



**FIG. 3.** Transdermal flux of (A) sulforhodamine and (B) calcein with heparin (200 mg/ml) and without. Pulses were applied during the first hour (peak applied voltage 1000 V;  $\tau_{\text{pulse}} = 2$  ms). During the second hour, the flux decayed under passive conditions, and while measurable fluxes persisted for sulforhodamine, the calcein flux quickly reached values below the detection limit. The presence of heparin significantly altered the transport of both sulforhodamine and calcein, but in very different ways (see text).

negatively charged calcein and the negatively charged heparin trapped within aqueous pathways.

These experiments support the hypothesis that a linear macromolecule such as heparin can enter aqueous pathways created by HV pulses, and alter the transport of smaller, co-transported ions and molecules by (1) increasing the pathway lifetime, and (2) providing charge within the pathway which attracts or repels co-transported species. In single bilayer membranes, this "foot-in-the-door" hypothesis has previously been considered for cell membrane electroporation.<sup>18,19</sup> In skin, previous experiments that showed large increases in transdermal transport of heparin by HV pulsing provided hints that heparin may alter its own transport, but did not determine heparin's effect on the transport of other molecules.<sup>7</sup> A single macromolecule may hold open a pathway segment between two corneocytes if its length exceeds the thickness of five to six bilayer membranes ( $\approx 40$  nm). This condition is satisfied for most of the heparin molecules used here.

Electroporation of the multilamellar bilayer membranes between corneocytes is expected to involve essentially straight-through aqueous pathways ("pores") with radii of order  $r_{p,\min} \approx 1$  nm (Fig. 1), as this is much more energetically favorable than long, tortuous pathways between bilayers that go around the corneocytes.<sup>8</sup> Further, a linear, flexible molecule such as heparin should fit into such pathways.<sup>7</sup> To achieve lower post-pulse  $R_{\text{skin}}$  values by macromolecular trapping within straight-through aqueous pathways requires fifteen to sixteen corneocytes be connected. Each corneocyte originates and terminates many pathway segments,  $N_p \approx 10^5$  (see Methods). With such a large number of pathway segments entering and exiting the water-filled interior of each corneocyte, trapped heparin may be able to maintain persistent pathways spanning the entire SC, even if it can prolong the lifetime of only a small fraction of these segments. If heparin is trapped, and thereby keeps pathways open, it provides a possible basis for a persistently small  $R_{\text{skin}}$ . Due to its large negative charge, trapped heparin may also favor the entry of small, mobile cations (here  $\text{Na}^+$ ) into the pathways.

These experiments constitute the first test of the hypothesis that linear macromolecules can serve as chemical modifiers for transdermal ion and molecule transport associated with HV pulsing, and also provide support for the hypothesis that HV pulses cause electroporation within the multilamellar bilayer membranes of the SC, resulting in aqueous pathways connecting corneocyte interiors.

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