Transdermal Delivery of Heparin by Skin Electroporation

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Therapeutic uses of compounds produced by biotechnology are presently limited by the lack of non-invasive methods for continuous administration of biologically-active macromolecules. Transdermal delivery would be an attractive solution, except macromolecules have not previously been delivered clinically across human skin at therapeutic rates. To increase transport of a highly-charged macromolecule (heparin), high-voltage pulses believed to cause electroporation were applied to skin. Using this approach, transdermal heparin transport across human skin *in vitro* occurred at therapeutic rates (100–500 μg/cm²h), reported to be sufficient for systemic anticoagulation. In contrast, fluxes caused by low-voltage iontophoresis having the same time-averaged current were an order of magnitude lower. Heparin transported across the skin was biologically active, but with only one eighth the anticoagulant activity of heparin in the donor compartment due to preferential transport of small (less active) heparin molecules. Flux, activity, and transport number data together suggest that high-voltage pulsing creates transient changes in skin microstructure which do not occur during iontophoresis. Safety issues are discussed.

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One of the greatest challenges in the use of biotechnology for human therapy is the continuous administration of biologically-active macromolecules of natural or biotechnological origin. Due to the short half-lives (minutes to hours) of many therapeutic compounds, conventional injections are often impractical. Oral delivery usually results in very low bioavailability, because macromolecules generally cannot cross the intestinal epithelium and are degraded in the liver and gastrointestinal tract. Encapsulation in implantable or injectable controlled release devices (e.g., microspheres) can protect macromolecules from degradation and elimination, but often renders them biologically inactive due to irreversible denaturation or aggregation[1]. Transdermal drug delivery potentially avoids some of these problems by continuously administering drugs across the skin and into the systemic circulation. However, transdermal transport of macromolecules is extremely difficult, due to the great barrier properties of human skin, due mainly to the multilamellar, intercellular lipid bilayers of stratum corneum[2–4]. Although a few drugs of low molecular mass (<400 Da) are delivered clinically, no macromolecule has previously been delivered clinically across human skin at therapeutic rates.

High-voltage electric field pulses have recently been shown[5,6] to increase transdermal transport of moderate-sized (<1,000 Da) compounds by up to at least four orders of magnitude. Experimental[7–15] and theoretical[16,17] evidence suggests these flux increases are caused by transient changes in skin microstructure by a mechanism involving electroporation of stratum corneum lipid bilayers. Electroporation involves the creation of transient aqueous pathways in lipid bilayers by the application of a short (μs to ms) electric field pulse[18–22]. Permeability and electrical conductance of bilayer membranes are rapidly increased by many orders of magnitude in both metabolically-inactive systems and living cells.

Heparin is a macromolecule (5,000–30,000 Da) widely used clinically for anticoagulation and prophylaxis of thromboembolism[23] and shows promise for other applications, such as control of unstable angina[24] and prevention of accelerated arteriopathies after angioplasty or by-pass surgery[25]. Because oral bioavailability is essentially zero, low-dose regimens are given by deep subcutaneous injection, while full-dose therapy is administered by continuous infusion, due to heparin's short half-life and the risk of bleeding complications[23]. A more convenient and patient-friendly method of continuous heparin administration would be desirable. We therefore investigated the possibility that skin electroporation might increase transdermal transport of macromolecules and thereby provide continuous delivery of heparin at therapeutic rates.

Results and Discussion

Increases in heparin transport caused by iontophoresis and high-voltage pulsing. We investigated the ability of electric fields to enhance transport of a highly-charged macromolecule (heparin) across human skin *in vitro* before, during, and after constant low-voltage and pulsed high-voltage electrical exposures. Under passive conditions (no electric fields) transdermal heparin flux was negligible. However, while applying short (τ = 1.9 ms), high voltage (150–350 V across skin) pulses to the skin at a rate of 12 pulses per minute, transdermal heparin flux was between 100–500 μg/cm²h (Fig. 1). This level of transport is therapeutically relevant (see below). After pulsing, the rate of heparin transport decreased, but remained elevated for at least 24 h (Fig. 1). Greater enhancement of transport was seen at larger voltages. Constant-current iontophoresis (0.1–1 mA/cm²), which moved the same total electric charge across the skin, also enhanced heparin transport, but to a much lesser extent (Fig. 2).

The high-voltage pulses used here were selected because they have been previously used with smaller molecules to provide dramatic enhancement of transdermal transport which was largely reversible[6]. The currents used during low-voltage iontophoresis were selected for two reasons. First, they bracket the maximum current density (~0.5 mA/cm²) which patients tolerate during clinical iontophoresis[26]. Second, the time-averaged current passed during high-voltage pulsing is in the range of 0.1–1 mA/cm². Therefore, both low- and high-voltage protocols have the same time-averaged current and therefore pass the same number of ionic charges across the skin, making comparisons of transport efficiency more direct (see below).

Elevated heparin flux after pulsing. Heparin flux remained partially elevated for several hours after high-voltage pulsing (Fig. 1). We considered two potential causes

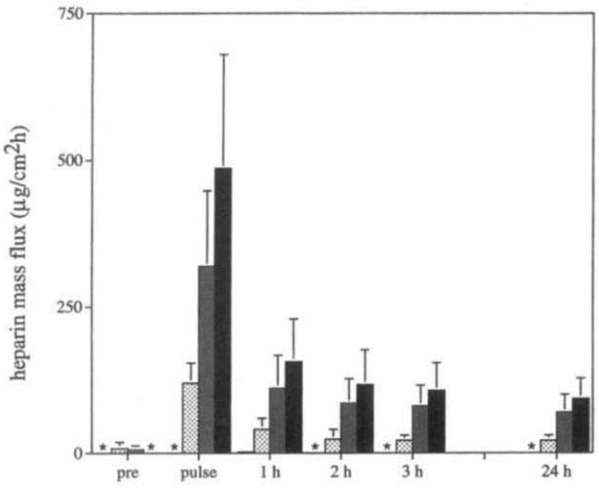

FIGURE 1. Transdermal heparin flux caused by high-voltage pulsing as a function of time and voltage. After 1 h of passive transport (pre), pulses were applied for 1 h at: (□) 0 V (control), (▨) 150 V, (▩) 250 V, and (■) 350 V across the skin. After pulsing, transdermal flux was measured for 24 h. High-voltage pulsing significantly increased transdermal heparin transport. Moreover, heparin flux decreased after pulsing, but did not return to pre-pulse values. Each point represents the average of 3–4 skin samples. Standard deviation bars are shown. Asterisk indicates a flux below the detection limit (of order 1 μg/cm²h).

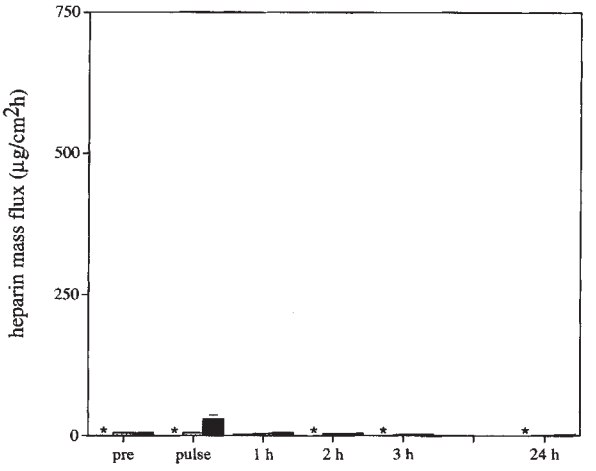

FIGURE 2. Transdermal heparin flux caused by low-voltage iontophoresis as a function of time and current density. Constant dc current was applied for 1 h at: (□) 0 mA/cm² (control), (▨) 0.1 mA/cm², and (■) 1 mA/cm². Low-voltage iontophoretic flux increases were approximately an order of magnitude lower than during high-voltage pulsing. Application of 1 mA/cm² is in excess of the maximum current density generally tolerated by patients clinically[26]. Each point represents the average of 3–4 skin samples. Standard deviation bars are shown. Asterisk indicates a flux below the detection limit (of order 1 μg/cm²h).

for this. First, an elevated post-pulse flux might come from heparin reservoirs created within the skin and filled during pulsing due to heparin binding or filling of aqueous spaces (such as stratum corneum keratinocyte interiors). This type of reservoir effect has been demonstrated for transdermal transport of other compounds[27]. Another potential cause is that pulsing altered skin structure, creating changes in skin permeability which persisted after pulsing.

Additional experiments were performed to better understand the cause of elevated heparin transport after pulsing (Fig. 3). To test the first hypothesis, heparin was placed in the donor compartment before and during pulsing. Immediately after pulsing, the donor compartment was emptied of heparin, rinsed well, and filled with saline. In this case, the only post-pulse source of heparin is reservoirs within the skin. As expected, during pulsing, heparin transport increased. Moreover, during the first hour after pulsing, a small flux was seen, possibly representing a small reservoir effect. However, at later times, no heparin was detected, suggesting that a reservoir effect cannot explain the observed post-pulse transport.

To test the second hypothesis, the skin was not exposed to heparin before or during pulsing. Immediately after pulsing, heparin was placed in the donor compartment. In this case, an elevated transdermal flux could not originate from skin reservoirs filled during pulsing, but would instead be caused by electrically-induced changes in skin permeability. In this experiment (Fig. 3), heparin flux was measured at all times after pulsing, which suggests that lasting changes in skin permeability occurred. However, these post-pulse fluxes were much less than those seen when heparin was present before, during, and after pulsing. This suggests that lasting changes in skin permeability cannot fully explain the observed elevation in post-pulse flux.

A possible explanation is that pulsing with and without heparin present may have different effects on skin permeability. In either case, electric field pulses can increase skin permeability by creating transport pathways across the skin. Previous experiments using smaller compounds have shown that these pathways disappear within hours after pulsing[5,6,10–12]. In contrast, when heparin is present during pulsing, it may become trapped in transport pathways and hinder their closing by an electrical repulsion effect[22]. This effect has previously been observed[28] during electroporation of Simian Cos-1 cells, where post-pulse membrane permeability was higher when DNA (another highly-charged macromolecule) was present during pulsing than when added immediately after pulsing.

Biological activity of heparin. Heparin transported across the skin was biologically active (Fig. 4). Moreover, heparin activity flux scaled directly with heparin mass flux. The specific activity of heparin transported during high-voltage pulsing (20±2 U/mg (mean ± standard deviation)) was higher than that transported during iontophoresis (9±4 U/mg). Both were significantly lower than the specific activity of heparin supplied in the donor compartment (165 U/mg).

Heparin removed from the donor compartment, after being exposed to electric fields but not transported across the skin, retained its full activity (data not shown). It is therefore unlikely that electric field-induced denaturation or other changes to heparin structure reduced the specific activity of heparin transported across skin. Instead, the possibility that skin acts as a molecular sieve could explain the observed result. This is plausible because heparin is a class of molecules generally ranging from 5,000 to 30,000 Da, where very small heparin molecules have weaker anticoagulation activity, and heparin fragments smaller than eight saccharides (~2500 Da) has no activity[23,29]. Given the skin's low permeability to macromolecules, small (less active) heparin molecules should cross skin more easily than large ones, which might explain the observed lower specific activity. Although the greater charge on large heparin molecules could increase their electrophoretic mobility, steric hindrance within skin is expected to outweigh this and make transport of small heparin molecules more favorable. This suggests that predominantly small heparin molecules were transported, but a portion of them were larger than 2500 Da.

A second possible explanation for low specific activity is that mass flux and activity flux measurements were made with

different populations of heparin. The bulk of heparin used, and that which determined biological activity, is from one source (Hepar Industries). Mass flux measurements were made using radiolabeled heparin from another source (New England Nuclear, NEN). Even small differences in the size distribution of heparin from these two sources could make a significant difference in their relative transport rates. Because all samples were lyophilized to remove tritium label which may have exchanged with water (see Experimental Protocol), radioactivity measurements should represent transported heparin and not represent dissociated label. A thorough analysis of the molecular weight distribution of Hepar and NEN heparin both before and after transport across skin could help explain these differences in heparin specific activity.

Differences between the activity of heparin transported by high-voltage pulsing and iontophoresis were also seen. Heparin with greater specific activity was transported by high-voltage pulsing (Fig. 4), which suggests that in this case larger heparin molecules were transported. However, the overall result is that therapeutically relevant amounts of active heparin were transported across human skin.

Heparin transport efficiency. The efficiency with which electric current transports a charged compound can be characterized by its transport number[15,30], which is the fraction of total current carried by that compound. In this study, current can be carried by heparin or small ions, such as sodium or chloride. 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) (Fig. 5). These values indicate that approximately 5% of the current was carried by heparin during pulsing, compared to only 0.7% during iontophoresis.

This significant difference in transport numbers implies that heparin transport was significantly less hindered during high-voltage pulsing than during iontophoresis. Our hypothesis is that high-voltage pulses cause transient changes in skin microstructure, 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[2-4] and therefore has less effect on $t_{heparin}$. Nevertheless, all measured transport numbers are significantly less than the maximum value ($t_{heparin}$ = 1, see Experimental Protocol), indicating that although heparin transport was less hindered during high-voltage pulsing, in all cases transport was significantly hindered. Unless the transport pathways are extremely large (i.e., much larger than a heparin molecule), this is expected.

Practical implications. The heparin fluxes measured here during high-voltage pulsing (100–500 μg/cm²h or 2–10 U/cm²h) are therapeutically relevant. For example, administration from a 100 cm² patch would result in a heparin delivery rate of 10–50 mg/h or 200–1000 U/h, based on mass flux and activity flux measurements, respectively. This is in the range sufficient for low-dose prophylaxis of thromboembolism (~500 U/h[23]) and full-dose anticoagulation therapy (700–2000 U/h[23]). Macromolecules have not been delivered clinically across human skin at therapeutic rates. Other than invasive methods (e.g., infusion pumps), there are no procedures for continuous delivery of macromolecules which have been approved by the U. S. Food and Drug Administration.

The elevated heparin fluxes observed after pulsing may not be desirable in some clinical situations. However, in others these persisting effects may be beneficial. For example, delivery of heparin at therapeutic rates may be possible with protocols which use fewer pulses or combine high-voltage pulsing with iontophoresis and/or other methods of enhancement. Moreover, if the hypothesis is correct that heparin and

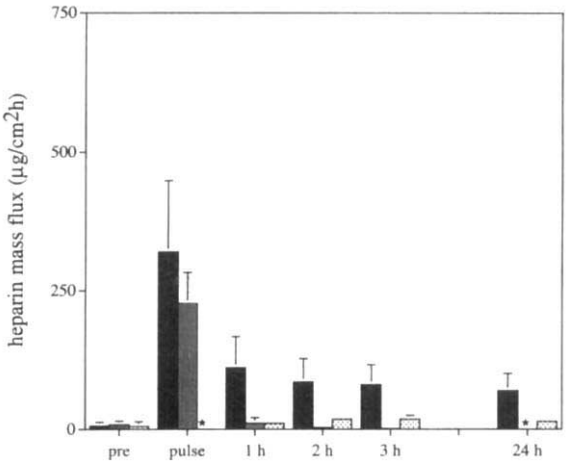

FIGURE 3. Transdermal heparin flux caused by high-voltage pulsing as a function of the time at which heparin was present. Pulses were applied at 250 V, where heparin was present in the donor compartment: (■) before, during, and after pulsing, (▩), only before and during pulsing, and (▨) only after pulsing. This figure suggests that elevated post-pulse fluxes seen when heparin is present before, during, and after pulsing (a) do not come from a reservoir in the skin filled during pulsing and (b) are not due to long-lived changes in skin structure caused solely by the electric field (see text for discussion). Each point represents the average of 4–5 skin samples. Standard deviation bars are shown. Asterisk indicates a flux below the detection limit (of order 1 μg/cm²h).

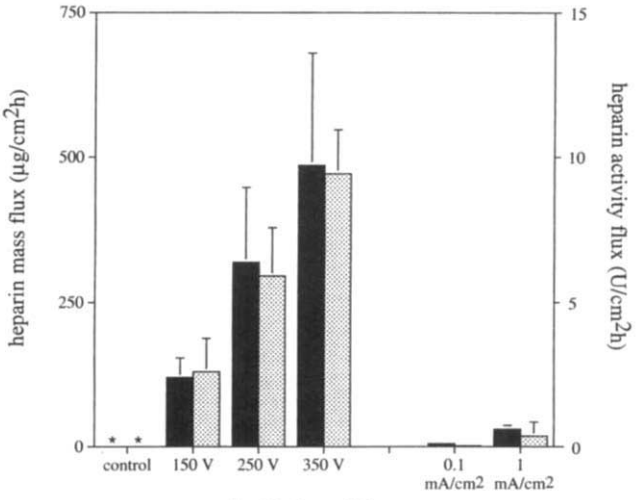

FIGURE 4. Heparin flux during electrical protocols determined by different assays. Heparin mass flux (■) was determined by radioactivity measurements, while biological activity flux (▨) was determined by the Whole Blood Recalcification Time assay. In all cases, active heparin was transported across the skin, albeit of lower activity than the heparin supplied in the donor compartment (165 U/mg, see text for discussion). The mean specific activity of heparin transported during high-voltage pulsing (20 U/mg) was higher than that transported during iontophoresis (9 U/mg). Each point represents the average of 3–4 skin samples. 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).

DNA are capable of keeping transport pathways open after pulsing, then this approach might be used for delivering other drugs through long-lived pathways kept open by highly-charged macromolecules. Although experiments were performed *in vitro*, these effects should also be seen *in vivo*, because they occur in the skin's stratum corneum, a tissue

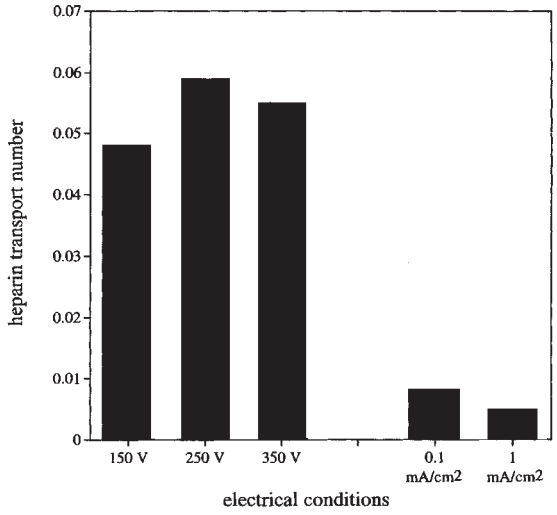

FIGURE 5. Heparin transport number during different electrical protocols. During high-voltage pulsing, transport numbers were approximately 0.05, while during low-voltage iontophoresis they were about an order of magnitude smaller. The transport number is a measure of the efficiency with which the electric field transports heparin. The larger transport numbers seen during high-voltage pulsing suggest the creation of larger aqueous pathways by the electric field.

whose properties change very little post mortem[2-4].

The activity data suggest that heparin with a smaller molecular mass is more easily transported across skin than larger heparin molecules. Therefore, low-molecular weight heparin formulations might be delivered transdermally at even greater rates. In fact, low-molecular weight heparins are preferred for some applications, since they have more predictable pharmacokinetics and dose-response relationships[29].

Electrical irritation of skin generally scales with current[26]. The transport number data show that for the same time-averaged current, high-voltage pulsing more efficiently transports heparin across the skin than iontophoresis. Consequently, delivery of a drug at a given rate using high-voltage pulsing should require a smaller time-averaged current than iontophoresis and therefore may be less irritating. However, pulse protocols and apparatus must be optimized and detailed *in vivo* studies need to be performed before issues of safety and irritation can be fully assessed.

Significantly, there is a strong clinical precedent for safely applying high-voltage pulses to skin. Common procedures which apply voltages up to hundreds of volts for durations up to milliseconds include transcutaneous electrical nerve stimulation, electromyography, and functional electrical stimulation[31]. However, long-term biochemical and pathological studies are needed to fully assess the safety and efficacy of protocols for skin electroporation.

Experimental Protocol

Transdermal flux measurements. The skin preparation methods and permeation apparatus have been described previously[6,15]. Briefly, heat-separated human epidermis was mounted in side-by-side permeation chambers containing well-stirred phosphate-buffered saline (PBS; pH 7.4, 150 mM total salts; Sigma, St. Louis) and maintained at 37°C. The use of cadaver skin is well established in studies of transdermal transport[32] and represents the most rigorous test, since animal skin is generally more permeable[2,3]. To assure initially intact skin barrier function, only skin samples were used which had at least 100 kΩ-cm² resistance and exhibited a passive heparin flux below our detection limit (of order 1 μg/cm²h). After allowing skin to hydrate in PBS for at least 1 h, a solution containing 200 mg/ml sodium heparin (165 U/mg; Hepar Industries, Franklin, OH) spiked with 1 μC/ml ^{3}H-heparin (New England Nuclear, Boston, MA) was placed in the donor compartment, facing the stratum corneum (0.7 cm² exposed skin), unless otherwise noted. Contents of the receptor compartment (facing the viable epidermis) were periodically removed, replaced with fresh PBS, and analyzed for both radioactivity and heparin biological activity. One milliliter of each receptor solution sample was lyophilized and resuspended in PBS, to remove ^{3}H label which may have exchanged with water. Analysis by calibrated liquid scintillation counting (model 2000CA, Packard, Downers Grove, IL) allowed measurement of heparin concentration in the receptor compartment and, thereby, calculation of transdermal fluxes. The remaining 2.5 ml of receptor solution was used for measurement of heparin activity.

Heparin activity measurement. Heparin anticoagulant activity was determined by measuring whole blood recalcification time (WBRT) using an automated coagulation timer (model 800, Hemochron, Edison, NJ)[33]. Freshly-drawn human blood was collected from an adult male volunteer in citrated tubes (buffered sodium citrate, Vacutaner, Becton Dickinson, Franklin Lake, NJ), maintained at 37°C, and used within 5 h. Human blood was obtained with informed consent and institutional approval. Blood (220 μl) and receptor solution (110 μl) were added to a warmed (37°C) round-bottom polystyrene tube (Falcon, Becton Dickenson, Lincoln Park, NJ) and allowed to co-incubate for >5 min at 37°C. Then, (a) 300 μl of the blood/receptor solution mixture was added to a warmed (37°C) Hemochron tube containing glass particle activators (P214; Hemochron), (b) 100 μl of warmed (37°C) 0.04 M $CaCl_2$ (Mallinckrodt, Paris, KY) was added to inactivate the citrate, and (c) the coagulation timer was started. The Hemochron tube was then mixed vigorously by hand and inserted into the well of the timer. The time at which a clot was detected was recorded as the WBRT. Heparin activity was then determined from WBRT using a standard curve generated from samples of known heparin activity. All samples were tested in duplicate.

Electrical protocols. Electrical protocols were applied for 1 h, using either exponential-decay electric field pulses (exponential decay time constant, τ = 1.9 ms; ECM 600, BTX, San Diego, CA) applied at a rate of 12 pulses per minute (ppm), or constant-current iontophoresis applied continuously using a constant-current power supply. Ag/AgCl electrodes[15] (In Vivo Metrics, Healdsburg CA) were used, each located approximately 3 cm from the skin. The negative electrode was in the donor compartment, while the positive electrode was in the receptor compartment. Reported voltages are average transdermal values determined during the first pulse of each hour-long sequence of pulses. Because significant voltage drops occurred within donor and receptor solutions and electrodes, the voltage applied across the electrodes was approximately three-fold higher than the transdermal voltage[6,11,34]. Moreover, because skin resistance decreased further as pulsing progressed, transdermal voltages during subsequent pulses were lower than the first-pulse values[11]. Over the range of voltages used for high-voltage pulsing (150–350 V), the range of transient, peak current densities was approximately 0.4–3.5 A/cm² (ref. 15).

Estimation of heparin electrophoretic mobility and transport number. Heparin electrophoretic mobility was estimated using a method described previously[15]. For this, heparin radius was estimated using an empirical relation[35] developed for dextran, another polysaccharide, $r = 0.488\ M_r^{0.437}$, where M_r is molecular mass and r is given in units of Ångstroms. Using estimates of average molecular mass ($M_{residue} = 317$ Da[36]) and charge ($z_{residue} = -2$ [ref. 36]) for each heparin glucose residue in the antithrombin-binding site, the valence of heparin was estimated using the relation, $z = z_{residue} \frac{M_r}{M_{residue}}$.

Using these values, heparin electrophoretic mobility for the range of molecular masses used (5,000–30,000 Da[23]), is estimated as $\mu_{heparin} \approx -1 \times 10^{-3}$ cm²/V s. This value should serve only as an order-of-magnitude estimate, because of the assumptions and simplifications that went into its determination. Transport numbers were calculated as described previously[15]. The maximum value of heparin transport number, $t_{heparin}$, occurs if heparin and small ion transport are both completely unhindered[15,30]. In this case, their transport numbers would scale with the product of electrophoretic mobility, concentration, and valence[30]. Because their mobilities are approximately equal (in water at 25°C: $\mu_{sodium} = 5.2 \times 10^{-4}$ cm²/V s[37], $\mu_{chloride} \approx -7.9 \times 10^{-4}$ cm²/V s[37], $\mu_{heparin} \approx -1 \times 10^{-3}$ cm²/V s (see above)), their relative concentrations were approximately 1:18 (17 mM heparin (for an average molecular mass of 12,000 [ref. 23]) and ~300 mM small ions), and their relative valences are 76:1 ($z_{heparin} = -76$ (see above, for $M_r = 12{,}000$) and generally $z_{ion} = -1$ or +1), then $t_{heparin,\ max}$ would approach unity if all transport were unhindered. Values of $t_{heparin,\ max}$ significantly less than unity indicate that transdermal heparin transport may be hindered.

Acknowledgments

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