Combined Effect of Low-Frequency Ultrasound and Iontophoresis: Applications for Transdermal Heparin Delivery

Long Le,[1] Joseph Kost,[2,3] and Samir Mitragotri[4,5]

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INTRODUCTION

Transdermal drug delivery offers several advantages over traditional drug delivery systems such as oral delivery and injection including elimination of first pass metabolism, minimization of pain, and possibility of sustained release of drugs (1). However, transdermal transport of molecules is slow due to the low permeability of stratum corneum, the uppermost layer of the skin. Therefore, it is difficult to deliver drugs across the skin at a therapeutically relevant rate. A possible solution to this problem is to increase the permeability of the skin using physico-chemical driving forces, referred to as penetration enhancers, for example, ultrasound (1–7), chemical enhancers (8,9) and electric fields (10,11). Although all these methods have been shown to enhance transdermal drug transport by themselves, some methods have been shown to work synergistically when applied simultaneously. For example, iontophoresis has been shown to operate in synergy with electroporation (12) or with chemical enhancers (13). In addition, ultrasound has also been shown to enhance transdermal drug transport synergistically with electroporation (14) or with chemical enhancers such as linoleic acid and Sodium Lauryl Sulfate (SLS) (8).

In this paper, we show that ultrasound under low-frequency conditions (~20kHz) enhances transdermal transport of heparin synergistically with iontophoresis. Heparin was chosen as a model molecule for this study since it has high therapeutic value. Specifically, it is one of the most commonly used antithrombotic agents. Furthermore, heparin is a negatively charged biopolymer and is an excellent candidate for testing the efficiency of ultrasound+iontophoresis for transdermal delivery of macromoleucles.

[1] University of Alabama, School of Medicine, Birmingham, Alabama, 35294.

[2] Department of Chemical Engineering, Ben Gurion University, Beer Sheeva, Israel 84105.

[3] Sontra Medical, Inc. Cambridge, Massachusetts 02139.

[4] Department of Chemical Engineering University of California, Santa Barbara, CA 93106.

[5] To whom correspondence should be addressed. (e-mail: samir@engineering.ucsb.edu)

MATERIALS AND METHODS

In Vitro Experiment

Pig skin was harvested from Yorkshire pigs immediately after sacrificing the animal according to methods described in Ref. (15). Sonophoresis experiments were carried out using a vertical Franz diffusion cell (skin area $=1.77$ cm^2, receiver volume $=12$ ml (3)). The receiver chamber was filled with PBS (Sigma Chemicals Co. (3)). Full thickness pig skin was mounted on the diffusion cell with the epidermis side facing up. The donor and the receiver compartments were clamped making sure there were no bubbles in the receiver chamber. Before applying ultrasound, the structural integrity of the skin was checked by measuring its conductivity as described in Ref. (3).

Ultrasound Application

Ultrasound was applied using a sonicator manufactured by Sonics and Materials, CT (model VCX 400). The ultrasound horn was positioned 1 cm above the skin sample. Surfactant solution containing 1% Sodium Lauryl Sulfate (negatively charged) or 1% Dodecyl Pyridinium Chloride (positively charged) in PBS (weight/volume %) was added to the donor chamber. Ultrasound was applied in a pulsed mode (5 seconds ON, 5 seconds OFF) at a frequency of 20 kHz and an I_{SATA} intensity of 7.4 W/cm^2 until the conductivity of the skin reached about 0.6 (kohm-cm^2)$^{-1}$. Ultrasound intensity and skin conductivity was measured separately using the procedure described in Ref. (15). The donor solution was changed every 2 minutes to minimize thermal effects on the skin. At the end of sonication, the donor chamber was rinsed thoroughly with PBS to remove residual surfactant. A solution of radiolabeled heparin (^{3}H labeled, obtained from New England Nuclear, average molecular weight of about 10,000, dissolved in PBS at a concentration of 10 μCi/mL) was then added to the donor compartment. Samples were taken from the receiver compartment at the end of 1 hour and 24 hours to measure the amount of heparin transported transdermally. These sampling times were chosen to measure the immediate and long-term effects of various treatments on transdermal transport. Although this protocol allows a basic determination of the effect of ultrasound and iontophoresis on transdermal transport, it does not allow a detailed evaluation of the effect of ultrasound on lag time and steady-state drug transport. Concentrations of heparin were measured using a scintillation counter (Packard Tricarb 2000 CA). Normalized flux of heparin was calculated based on the equation, $F_N = V\Delta C/\Delta t\,/(ACd)$, where V is the receiver volume, A is the skin area (1.77 cm2), $\Delta C/\Delta t$ is the measured increase in the heparin concentration in the receiver compartment over a period of time Δt, and Cd is the heparin concentration in the donor compartment at time zero.

Iontophoresis

In some experiments, iontophoresis was used to further enhance transdermal heparin transport. An Ag/AgCl disc electrode (1cm^2 area) was attached to a cap fitting directly into the opening of the donor compartment and was fully

immersed in the drug solution, 0.5 cm above the skin. It was made sure that no bubbles were trapped below the electrode to avoid disruption in the current flow. The electrodes in the donor and receiver chambers were connected to a constant current generator (Hewlett Packard) with the positive side in the receiver. Current density was adjusted to 0.45 mA/ cm^2. Iontophoresis was performed for 1 hour. When multiple cells were to be intophoresed, the cells were connected in series with the appropriate polarity orientation (positive electrode in the receiver compartment). Like passive transport experiments above, samples were taken and conductivity was measured at similar time intervals.

RESULTS AND DISCUSSION

Transport Enhancement Due to Ultrasound and Iontophoresis

Ultrasound was applied to the skin with two different surfactants (Sodium Lauryl Sulfate (SLS) and Dodecyl Pyridinium Chloride (DPC)) at a single ultrasound frequency (20 kHz) and intensity (7.4 W/cm^2). This set of ultrasound parameters was chosen since it corresponds to the most commonly used condition in our previous sonophoresis experiments (16). Two surfactants, SLS and DPC were chosen since they possess the same tail group but have oppositely charged head-groups, that is, SLS is negatively charged and DPC is positively charged. This allows us to investigate the effect of surfactant charge on transdermal heparin transport. We first describe the effect of ultrasound with SLS.

Application of ultrasound and SLS increased electrical conductivity of the skin. Skin conductivity before the application of ultrasound was about 0.01 (kohm-cm^2)$^{-1}$. Sonication was done until the skin conductivity reached a value of 0.6 (kohm-cm^2)$^{-1}$(an enhancement of about 60-fold). A typical application time required to achieve this resistance was 10 minutes. Ultrasound was then stopped, SLS was removed, and heparin was added to the donor compartment. Figure 1 shows the effect of various treatments on transdermal heparin flux. Case 1 shows flux for controls. The open bar shows normalized flux during the first hour of transport, and the hatched bar shows normalized flux over 24 hours. Case 2 shows transdermal heparin flux through ultrasound-pretreated skin. The data reveal that application of ultrasound enhanced long-term heparin flux by factor of 13 (compare hatched bars in case 1 and 2), although ultrasound had little effect on the transport during the first hour (compare open bars in case 1 and 2). Note that application of SLS alone for 10 minutes did not significantly increase skin permeability to heparin (data not shown). Thus, the enhancement of heparin transport was due to a combined application of ultrasound and SLS.

Case 3 in Figure 1 shows the effect of iontophoresis alone at a current density of 0.45 mA/cm^2 on transdermal heparin flux. Unlike ultrasound, application of electric current produced an immediate effect on transdermal heparin flux. Specifically, the normalized heparin flux during the first hour was about 15 times higher than controls (compare open bars in case 1 and 3). Note that iontophoresis was performed only for the first hour. However, the long-term transport after iontophoresis was about 10 times higher than in controls (compare hatched bars in case 1 and 3), suggesting that the enhancing effect of iontophoresis continued well beyond the time for which electric current was ON. The enhanced heparin transport during the initial 1-hour period when iontophoresis was ON is understandable due to the direct electrophoretic effect of the electric current on negatively charged heparin molecules. However, two possibilities could explain the continued drug transport following the termination of iontophoresis. First, the application of current—like sonication—induces structural changes in the skin, making it more permeable to the heparin molecules. Conductivity measurements support this idea by showing that the conductivity of the skin increased following one hour of iontophoresis and that the increase lasted over the next 24 hours (data not shown). The other possibility why significant heparin transport continued over 24 hours is that the one-hour application of current actively transported and built up a high concentration of heparin molecules in the skin. Once a high concentration of heparin in the skin was achieved, passive delivery could transport the drug more efficiently. It is not certain which of the two mechanisms are important. In this context, it is important to note that heparin itself interacts with the skin and results in increased transport in the presence of electric fields (17,18). Relevance of this property of heparin to data presented here should be further investigated.

Case 4 shows transdermal heparin flux during iontophoresis (0.45 mA/cm^2) across ultrasound-pretreated skin. Transdermal heparin flux during the first hour as well as over 24 hours is higher than that observed during ultrasound alone or iontophoresis alone. Specifically, long-term heparin flux during the combination of ultrasound and iontophoresis is about 2-fold higher compared to ultrasound alone or iontophoresis alone (compare hatched bars in case 4 with those in case 2 and

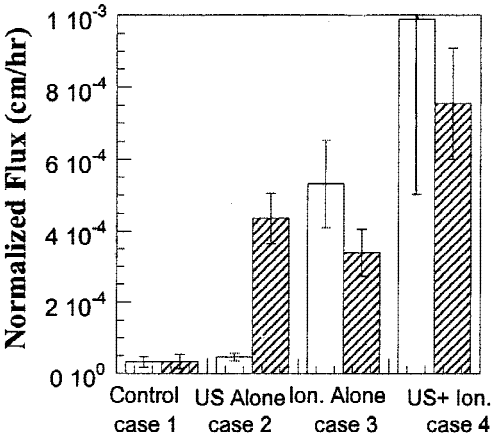

Fig. 1. The Figure shows the effect of various treatments on transdermal heparin flux. Case 1 shows transdermal flux for controls. Case 2 shows transdermal heparin flux after ultrasound pretreatment with SLS. Case 3 shows the effect of iontophoresis alone (0.45 mA/cm^2). Case 4 shows the effect of iontophoresis (0.45 mA/cm^2) across ultrasonically pretreated skin with SLS. The data in each case was collected over 5–6 experiments. Open bars show transdermal transport during the first hour. Hatched bars show heparin flux over 24 hours. The error bars are comparable to those observed in the case of passive transport.

3). Mechanistically, ultrasound pretreatment may disorder the lipid bilayers of the skin, thus opening new pathways for transport which may increase heparin transport. Further studies are required to support this hypothesis. Note that several studies have been reported in the literature that discuss the synergistic effect of surfactants and iontophoresis on transdermal drug transport. Specifically, cationic as well as anionic surfactants have been shown to enhance the effect of iontophoresis on transdermal transport. The study reported here differs from the literature data in that our experiments are focused on investigating the effect of ultrasound pretreatment (which was performed in combination with surfactants) on iontophoretic transport. Detailed investigation of the role of surfactants in this enhancement and its comparison with literature data is beyond the scope of this study.

Synergistic Effect of Ultrasound and Iontophoresis in the Presence of DPC

Although data in Figure 1 shows that application of ultrasound and SLS increased transdermal heparin transport, we thought that SLS and heparin molecules might compete for the same current during iontophoresis, affecting the maximal transdermal flux of heparin. The reason is that both SLS and heparin are negatively charged. On the other hand, if we use a positively charged surfactant, such as DPC, we may be able to further enhance transdermal transport of heparin since heparin has a negative charge. Hence, we performed additional experiments using DPC. The results of these experiments are shown in Figure 2. Case 1 shows transdermal heparin transport during controls (no treatment). Case 2 shows normalized heparin flux when ultrasound alone was applied with DPC. These results are comparable to those obtained for SLS (shown in case 2 of Figure 1). Specifically, application of ultrasound with DPC increased the steady state transport by about 10-fold, while the enhancement measured after 1 hour was about 3-fold. Case 3 shows the effect of iontophoresis (0.45 mA/cm^2) across ultrasound-pretreated skin (with DPC) on transdermal heparin flux. The enhancement of heparin flux (compared to passive transport in the absence of any treatment) was about 56-fold after 1 hour and 16-fold after 24 hours (note than iontophoresis was applied only for 1 hour). We also measured the effect of DPC alone (for 10 minutes) followed by iontophoresis (0.45 mA/cm^2) on transdermal heparin transport. No significant difference between this case and iontophoresis alone (0.45 mA/cm^2) was noted. That is, the enhancement after 1 hour (compared to passive transport in the absence of any treatment) was about 15-fold and that after 24 hours was about 10-fold (note that iontophoresis was applied for only 1 hour). No transport enhancement was obtained after a 10-minute skin contact with DPC alone (data not shown). For the sake of comparison, the corresponding data for SLS is also shown in Case 4 (reproduced from case 4 in Figure 1). The data shows that heparin transport during the first hour in the case of ultrasound pretreatment with DPC followed by iontophoresis is significantly higher than that in the case of SLS, thus supporting our hypothesis. Specifically, the enhancement after 1 hour in the case of ultrasound + iontophoresis (56-fold) is higher than the sum of those obtained during ultrasound alone (3-fold) and iontophoresis alone (15-fold). Thus, the effect of ultrasound and iontophoresis on transdermal heparin transport is truely synergistic. The enhancement measured after 24 hours was also higher in the case of ultrasound+iontophoresis (16-fold) compared to ultrasound or iontophoresis alone (10-fold each). However, the difference between various cases in smaller than that observed after 1 hour. This is understandable since iontophoresis was applied for only 1 hour.

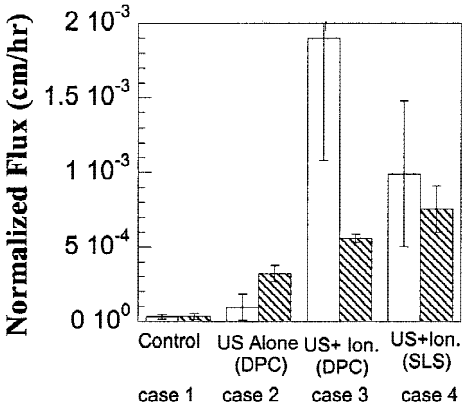

Fig. 2. The Figure shows the effect of various treatments on transdermal heparin flux. Case 1 shows transdermal flux for controls. Case 2 shows the effect of ultrasound alone (with DPC) on transdermal heparin flux. Case 3 shows the effect of iontophoresis (0.45 mA/cm^2) across ultrasonically pretreated skin with DPC. Case 4 shows the effect of iontophoresis (0.45 mA/cm^2) across ultrasonically pretreated skin with SLS (reproduced from Case 4 in Figure 1). The data in each case was collected over 5–6 experiments. Open bars show transdermal transport during the first hour. Hatched bars show heparin flux over 24 hours.

The data discussed so far shows that application of ultrasound with SLS and DPC enhances transdermal heparin transport. Heparin transport can be further enhanced by its combination with iontophoresis. We have previously shown that the enhancement of drug flux due to ultrasound depends on the choice of ultrasound conditions (15) as well as the choice and concentration of the surfactant (16). By optimizing these parameters, transdermal heparin flux can be further enhanced and should be investigated in future studies. We have also measured the activity of transdermally delivered heparin using Activated Clotting Time (ACT) tests and anti-Xa activity. Transdermally delivered heparin is biologically active as measured by both tests, although the activity of transdermally transported heparin accounts for only 25–40% of the total heparin (estimated based on radioactivity). The loss of activity was attributed to deactivation of heparin in the skin (Unpublished data). However, the remaining activity of heparin was still sufficient to induce significant biological effects based on in vivo experiments in rats.

Practical Implications of Combination of Ultrasound and Iontophoresis

Low-frequency ultrasound and iontophoresis have been individually shown to permeabilize skin (3,10). However, a combination of these two methods offers significant advantages over either of then alone. Specifically:

(1) **Enhancement of transdermal flux:** As shown in Figures 1 and 2, the combination of ultrasound and electric current offers a higher enhancement than that offered by each of them individually under the same conditions.
(2) **Reduction of the required voltage/current to achieve the desired flux:** Since ultrasonic pretreatment reduces skin resistivity, a lower voltage is required to deliver a given current during iontophoresis compared to that in controls.
(3) **Transdermal delivery of large molecules:** Since ultrasound pretreatment results in the formation of new transport pathways which allow transport of macromolecules, it should reduce the size-selectivity of skin permeability, thus allowing transdermal delivery of macromolecules.
(4) **Active control of transdermal drug transport:** The combination of ultrasound and iontophoresis may be used to develop a drug delivery method, where ultrasound pretreatment is used to permeabilize skin and electric current is used to control the flux. This method may be beneficial in achieving a rapid temporal control of transdermal flux.

At the same time, this method may require a relatively complex device for drug delivery. Future studies should focus on performing in vivo tests to assess the applicability of transdermal heparin delivery in vivo. Efforts should be focused on optimization of ultrasound, surfactant, and iontophoresis parameters to further enhance transdermal heparin transport. Mechanistic studies of the synergistic effect of ultrasound and iontophoresis should also be performed.

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