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Enhanced transdermal delivery of low molecular weight heparin by barrier perturbation

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ABSTRACT

The purpose of this work was to investigate the *in vitro* transdermal delivery of low molecular weight heparin (LMWH). Hairless rat skin was mounted on Franz diffusion cells and treated with various enhancement strategies. Passive flux was essentially zero and remained low even after iontophoresis ($0.065\,U\,cm^{-2}\,h^{-1}$) or application of ultrasound ($0.058\,U\,cm^{-2}\,h^{-1}$). A significant increase in flux across tape stripped skin ($4.0\,U\,cm^{-2}\,h^{-1}$) suggests the interaction of stratum corneum (SC) with LMWH which was confirmed using Differential Scanning Calorimetry and Fourier Transform-Infrared spectrophotometry. Maltose microneedles were then employed as a means to locally disrupt and bypass the SC. Transepidermal water loss (TEWL) and transcutaneous electrical resistance (TER) were measured to confirm the barrier disruption. Microneedles breached the SC resulting in increased TEWL, decreased TER and enhanced LMWH permeability ($0.175\,U\,cm^{-2}\,h^{-1}$). Microneedles when used in conjunction with iontophoresis had a synergistic effect on LMWH delivery resulting in enhancement of flux by 14.7-fold as compared to iontophoresis used alone. Confocal laser scanning microscopy substantiated the evidence about LMWH interaction with SC. In conclusion, LMWH was shown to interact with SC and therefore tape stripping or microneedles dramatically increased its delivery due to disruption of the SC skin barrier.

1. Introduction

Thromboembolic complications are the major reason for mortality in hip fracture patients (Salzman and Harris, 1976; Todd et al., 1995). Some studies reveal that one-third to one-half of all the patients are known to develop deep vein thrombosis (DVT) following hip fracture (Haake and Berkman, 1989; Sznitowska and Janicki, 2000). Most commonly the treatment begins with an anticoagulant like heparin or LMWH (Frydman, 1996). DVT therapy starts by hospitalizing patients for 5–7 days and administering heparin intravenously. After discharge from the hospital, oral warfarin or twice daily subcutaneous LMWH therapy is required for 3–6 months (Lensing et al., 1995; Charbonnier et al., 1998). A major disadvantage of outpatient warfarin therapy is the requirement for careful patient monitoring because of high protein binding and many unfavorable drug interactions (Hull et al., 1993).

Heparin does not cross the placental barrier to cause any side effects in the neonate, so it is a better alternative for a pregnant thrombotic patient. It is therapeutically effective at a concentration of 0.14 U/ml (Hirsh et al., 2001). However it binds to the endothelium and has a high affinity for plasma proteins resulting in a short half-life and unpredictable bioavailability (Hirsh and Levine, 1992). Hemorrhagic complications also are associated with heparin therapy (Schafer, 1996) and careful laboratory monitoring is required with multiple dose adjustments (Kroon et al., 1992). LMWHs are extensively used because of less adverse reactions and a greater antithrombotic activity than heparin (Zhu et al., 2003). They have proven their role as potent anticoagulants in the prevention and treatment of DVT (Harrison et al., 1998) and pulmonary embolism (Hyers et al., 1986). Two large trails (Koopman et al., 1996; Levine et al., 1996) have demonstrated the safety and efficacy of outpatient treatment with LMWHs.

The current use of anticoagulants is extensive and it was estimated that this multibillion dollar heparin market generated sales of US $ 3.7 billion in 2005. Unfortunately, the need for repetitive parenteral administration is still a major disadvantage.

Since LMWHs are produced by chemical or enzymatic degradation of heparin, they are termed as LMWH (Xiong, 1996). Both heparin and LWMH are high molecular weight hydrophilic polyanions (Ross and Toth, 2005) with poor oral bioavailability and are being administered parenterally. Poor oral absorption is a result of

Abbreviations: DSC, differential scanning calorimetry; DVT, deep vein thrombosis; FTIR, Fourier transform infrared spectroscopy; CLSM, confocal laser scanning microscopy; LMWH, low molecular weight heparin; TER, transcutaneous electrical resistance; PBS, phosphate buffer saline; SC, stratum corneum; TEWL, transepidermal water loss.

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ionic repulsion from negatively charged mucus and epithelial tissue (Dal Pozzo et al., 1989), destruction by gastrointestinal bacteria (Ahn et al., 1998) and, to a lesser extent, by the acidic conditions of the stomach (Jandik et al., 1996).

Transdermal drug delivery is one potential solution to this problem. Skin, the largest organ of the human body, offers a painless and compliant interface for systemic drug administration. It provides sustained and controlled delivery over long time periods with the feasibility of on-demand termination and an attractive alternative to injections (Bronaugh and Maibach, 1989). This route also offers an added advantage in the treatment of superficial venous thrombosis (Motlekar and Youan, 2006). Despite excellent therapeutic potential of LMWH and attractiveness of delivery via skin, only a few reports regarding heparin transdermal delivery have been published. It is evident that because of its relatively large molecular weight, negative charge and hydrophilic nature (Sznitowska and Janicki, 2000) passive transdermal delivery may not be feasible. Several physico-chemical driving forces therefore have been employed to deliver heparin or LMWH across the SC barrier. Ultrasound, electric fields like electroporation and iontophoresis, and combinations of ultrasound and iontophoresis have been used to increase the permeation of LMWH (Bos and Meinardi, 2000; Mitragotri and Kost, 2001; Pacini et al., 2006). Each of these procedures has limitations and these were discussed in more detail in the later part of this manuscript.

Another upcoming technology in transdermal research is the use of microneedles. Microneedles are tiny micron-sized structures that upon application can breach the SC and penetrate to the upper dermal layers. This delivery method is minimally invasive, pain-free and has lot of potential for drug delivery across skin. Recently, usage of soluble maltose microneedles which dissolve in the skin have been described (Kolli and Banga, 2008). In this present study soluble maltose microneedles were used to disrupt the SC barrier and the effect of barrier perturbation on the transdermal transport of LMWH was investigated.

2. Materials and methods

2.1. Materials

The LMWH with an average MW of 3000 Da was purchased from Sigma–Aldrich (St. Louis, MO, USA) and FITC-labeled heparin was procured from Polysciences, Inc. (Warrington, PA, USA). Microneedles were developed and supplied by Texmac, Inc. (Charlotte, NC, USA). The reagents used for the measurement of the anti-Xa activity (ACTICHROME®) were purchased from American Diagnostica, Inc., (Stamford, CT, USA). All other reagents were of analytical grade and used as supplied.

2.2. Animals

Male CD-hairless rats were obtained from Charles River (Wilmington, MA, USA) and were housed in the animal facility at Mercer University until used. The Institutional Animal Care and Use Committee of Mercer University approved the study.

2.3. Permeation studies

In vitro studies were performed with freshly excised hairless rat skin using vertical static Franz diffusion cells (PermeGear, Hellertown, PA, USA). The receiver chamber contained 5 ml of PBS (pH 7.4) and was constantly maintained at 37 °C temperature with a double water circulation jacket surrounding the lower part of the cell. The diffusion area of the skin was 0.64 cm^2. The donor compartment was loaded with 0.5 ml (1200 U/ml) of LMWH dissolved in PBS and was covered with parafilm to prevent evaporation. At regular intervals, 300 μl aliquots from receptor were collected and replenished with PBS. The samples were stored in a refrigerator until analyzed. The amount of LMWH permeated was corrected for the sample withdrawn at each time interval. All experiments were carried out in triplicates.

2.3.1. Iontophoresis

Iontophoresis protocols involved application of 0.5 mA/cm^2 dc current (Keithley Instruments, Inc., Cleveland, OH, USA) using Ag as an anode and Ag/AgCl as a cathode. All these experiments were conducted using static Franz diffusion cells as described in the previous section. Since LMWH is negatively charged at physiological pH, cathodal iontophoresis was carried out for 4 h. Also, iontophoresis was applied in conjunction with microneedles in a separate study.

2.3.2. Ultrasound

The Sonoprep® system (Sontra Medical Corporation, Franklin, MA, USA) was used to deliver low level of ultrasound energy (55 kHz) for short time periods (less than 60 s). The working procedure of the instrument was described earlier (Farinha et al., 2006). Briefly, a cylindrical ultrasonic horn with a replaceable housing is filled with PBS and 0.1% sodium dodecyl sulfate as a coupling buffer. Upon activation of Sonoprep®, the buffer is released and the ultrasonic horn vibrates to deliver ultrasound. The ultrasonic horn is positioned approximately 7.5 mm above the skin to deliver ultrasonic energy and occupies an area of 0.8 cm^2. During the application, electrical conductivity of the skin is measured, and an appropriate decrease in skin conductance leads to an auto-termination of the procedure. The ultrasound treated skin was then mounted on Franz cells for *in vitro* studies.

2.3.3. Stripping of the stratum corneum

Freshly excised hairless rat skin was placed on a soft surface and fixed with small pins. Transpore tape (3 M Transpore™ tape, St. Paul, MN, USA) with a surface area of approximately 4 cm^2 was applied on the SC surface of the skin. The tape was pressed gently and peeled immediately with a steady pull. Each skin section was stripped in alternating directions with 20 pieces of adhesive tape. To confirm the removal of SC, TEWL levels were measured before and after stripping. Stripped skin was used for permeation studies.

2.3.4. Microneedle application

Soluble maltose microneedles used for this study contained 28 microneedles per array (2 arrays were stacked together) each needle being 500 μm tall (Fig. 1). The design aspects of the maltose microneedles and their application for transdermal delivery have been described in detail (Kolli and Banga, 2008). Freshly excised skin from hairless rats was manually treated with maltose microneedles while applying a pressure of about 0.35–0.5 N. Once the needles were inserted into skin, the applicator was held in position for 1 min to let the microneedles dissolve in the skin and create microchannels. The treated skin was then mounted on the diffusion cells with SC side facing the donor.

2.4. Data analysis

All results are presented as means with respective standard deviation. Analysis of variance was used to assess the statistical significance of the observations. The cumulative amount of drug permeated through a unit area of skin was plotted as a function of time. The *in vitro* steady-state permeation flux was calculated from slope of linear portion of the plot.

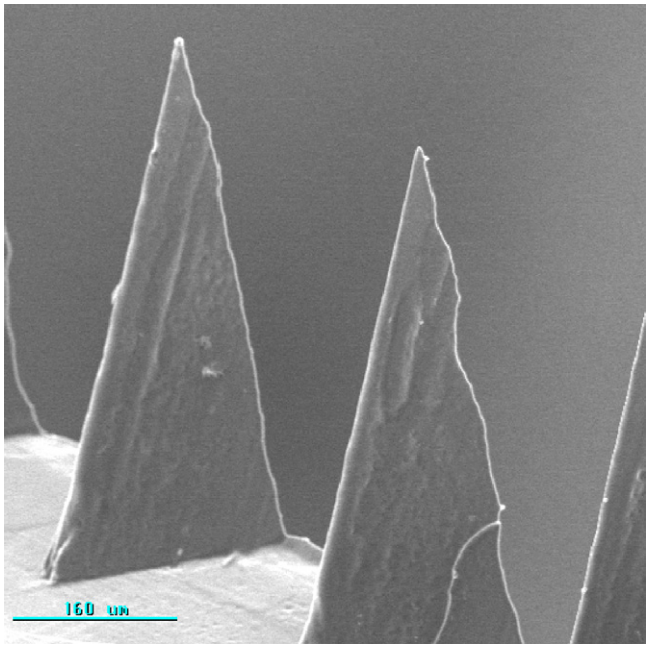

Fig. 1. Scanning electron micrograph image (X 150) of 500 μm long solid maltose microneedles in a single array.

2.5. Antifactor Xa activity measurements

The chromogenic assay (ACTICHROME®) kit was used for analyzing the LMWH levels (Jiao et al., 2002). In principle, LMWH catalyzes the reaction between factor Xa and antithrombin (AT-III). It binds with AT-III to form LMWH-AT-III complex and inhibits the clotting activity of factor Xa. The concentration of LMWH is directly proportional to factor Xa inhibition (Teien et al., 1976; Teien and Lie, 1977). Procedure involved addition of 200 μl of AT-III solution to 25 μl of sample and was incubated at 37 °C for 2 minutes. It was then added with 200 μl of factor Xa and incubated for 1 min. The mixture was further added with spectrozyme factor Xa and incubated for five more minutes. Finally the reaction was terminated by the addition of 200 μl of glacial acetic acid followed by 200 μl of filtered deionized water. The absorbance of the resulting solution was measured with a spectrophotometer (PerkinElmer, Uberlingen, Germany) at 405 nm. Student's *t*-test was performed for anti-Xa levels and the values were considered significantly different at $p < 0.05$.

2.6. Interaction studies

Differential scanning calorimetry (DSC) and Fourier transform infrared spectrophotometry (FTIR) studies were performed to investigate the possible interaction of LMWH with components of the stratum corneum.

2.6.1. Differential scanning calorimetry

DSC (DSC Q 100, TA instruments, New Castle, DE, USA) studies were performed in the temperature range from 0 to 300 °C with a heating rate of 2 °C/min where Indium was used as a reference material (Zidan et al., 2006). The procedure followed for the experiment was described earlier (Krishnaiah et al., 2002). In brief, SC was isolated from full thickness human skin (NDRI, USA) and incubated with LMWH solution (1200 U/ml) for 24 h. It was then removed and excess liquid was blotted; SC was desiccated for 24 h and DSC studies were carried out. DSC was performed for SC, LMWH and SC incubated with LMWH solution (5 mg) separately.

2.6.2. Fourier transform infrared spectroscopy

LMWH incubated SC samples prepared as described in the earlier section were investigated using a Nicolet 550 FTIR (Thermo Nicolet Corp., Madison, WI, USA). The dried SC sheets were mounted for spectral acquisition using a gripper that allows uniform contact of the sample with the crystal. The spectra were acquired from 100 scans with 4 cm^{-1} resolution. The scans were developed and corrected for distortion using Happ–Genzel apodization and baseline correction was performed for all the spectra.

2.7. Assessment of barrier integrity

Transepidermal water loss study was conducted under ambient conditions (room temperature of 25 °C and a relative humidity of 45%). The barrier function of SC was evaluated by measuring TEWL using a Cyberderm evaporimeter (Cortex Technology, Denmark). The probe was held on the skin until a stable reading was obtained (40 s) and measurements were recorded for the next 20 s. The measurements were performed in triplicates (Diez et al., 1991). Briefly, 10 minutes after pretreatment with microneedles, TEWL over the treated locations was measured and normal skin served as a control. The data was collected and processed using Desylab software (Cortex Technology, Denmark).

2.8. Transcutaneous electrical resistance measurements

The surface tension of the skin sample to be examined was reduced by alcohol swabbing and later the tissue was hydrated using PBS. It was then mounted over Franz diffusion cells with both the donor and receptor chambers containing PBS. The Ag/AgCl electrodes were placed on either side of the skin portion to measure the resistance in kΩ. The electrode above the skin surface was placed at a consistent length (2–3 mm) and submerged in the donor compartment during resistance measurement. The other electrode is positioned inside the sampling arm of receptor chamber.

2.9. Confocal laser scanning microscopy studies (CLSM)

A computerized Zeiss CLSM LSM510 (Goettinger, Germany) with X 10 objective was used to obtain the fluorescent images. Before examination, the hairless rat skin collected following *in vitro* experiments was blotted and SC surface was thoroughly wiped using saline and alcohol swabs. The skin samples were later placed on a microscope slide and then scanned using the CLSM. While obtaining fluorescent images, all samples were excited at 488 nm and X–Z sectioning has been used to determine the depth of permeation. Freshly excised skin was used as a negative control for the experiments.

3. Results

3.1. Permeation studies

The *in vitro* transdermal flux of LMWH following various enhancement strategies is shown in Fig. 2. The solid bar represents passive LMWH flux through hairless rat skin and it is apparent that there is only a low delivery even after 24 h. The open bar represents the flux of LMWH following cathodal iontophoresis. Though LMWH could be detected in the receptor compartment, the delivery was very low with a mean flux of 0.065 U cm^{-2} h^{-1}. Ultrasound, represented by the dotted bar in the graph, was used as another enhancement strategy. It demonstrated similar results with a low LMWH mean flux of 0.058 U cm^{-2} h^{-1} and it was lower compared to iontophoresis flux. The results of the permeation studies conducted across the tape stripped, microneedles pretreated skin and

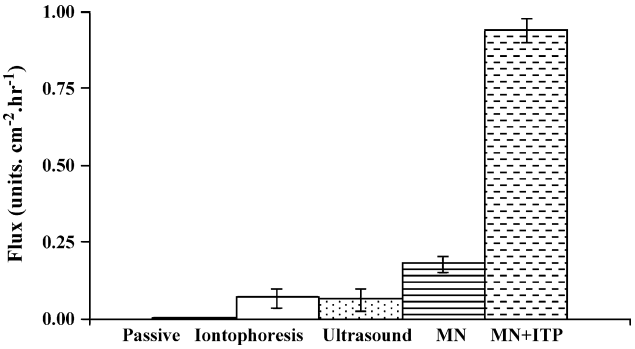

Fig. 2. Effect of various treatments on transdermal LMWH flux across hairless rat skin. Passive delivery is represented by solid bar, open bar represents the flux enabled by iontophoresis alone (0.5 mA/cm^2) and dotted bar shows the flux with ultrasound pretreated skin. Horizontal bar shows the flux following pretreatment with microneedles and dashed bar represents flux when microneedles were used in conjunction with iontophoresis.

microneedles used in conjunction with iontophoresis are discussed in Section 3.3.

3.2. Interaction studies

Though freshly excised hairless rat skin was used for the *in vitro* studies, it was difficult to isolate the SC exclusively from the hairless rat skin. Therefore the interaction studies were conducted using SC isolated from human skin.

3.2.1. Differential scanning calorimetry

Three endothermic transitions were observed before 130 °C for SC alone (Fig. 3). Since LWMH is a polysaccharide, an exothermic peak was detected at 225 °C. This transition indicates the transformation of LMWH from amorphous to a crystalline form. However, this transition vanished in the DSC thermogram of SC incubated with LMWH. The thermal profiles of SC pretreated with LMWH differed from SC alone. Two transitions before 100 °C vanished, a shift and lowering effect was observed for a transition at 130 °C.

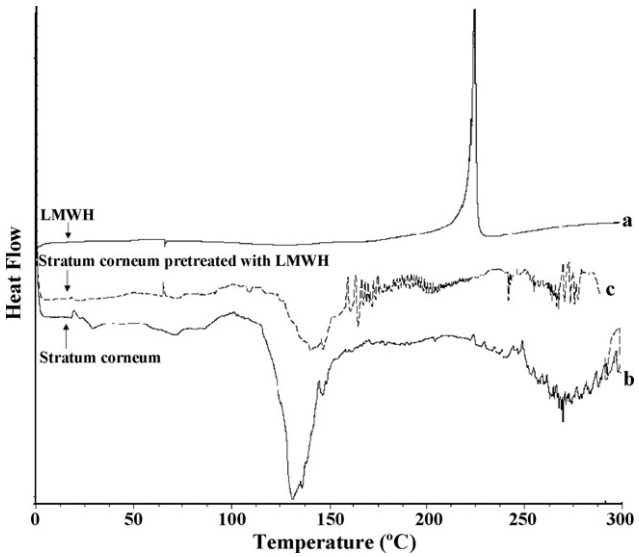

Fig. 3. DSC thermograms at a temperature range from 0 to 300 °C with a heating rate of 2 °C/min, (a) represents exothermic transition of LMWH alone, (b) illustrates the endothermic transitions of SC alone and (c) shows SC pretreated with LMWH transitions.

3.2.2. Fourier transform infrared spectroscopy studies

LMWH is a long polysaccharide with repeated disaccharide units of D-glucosamine linked by 1,4-interglycosidic bonds to uronic acid. The IR spectrum obtained for LMWH alone showed bands of main functional groups in the disaccharide units (COO^-, SO_3^-, OH, NH and C—O—C). These bands around 1600 and 1000 cm^{-1} correspond to stretching vibrations of the carboxylate and ether groups (Yang et al., 2006) (Fig. 4). The bands at 2850 and 2918 cm^{-1} correspond to symmetric and asymmetric C—H stretching vibrations of lipids in the SC (Krishnaiah et al., 2002). The peak at 3300 cm^{-1} is from the water molecule and the band at 1745 cm^{-1} represents C=O stretch (Brancaleon et al., 2001). The band at 1547 cm^{-1} characterizes the amide group and the band at 1380 cm^{-1} represents the CH_3 groups

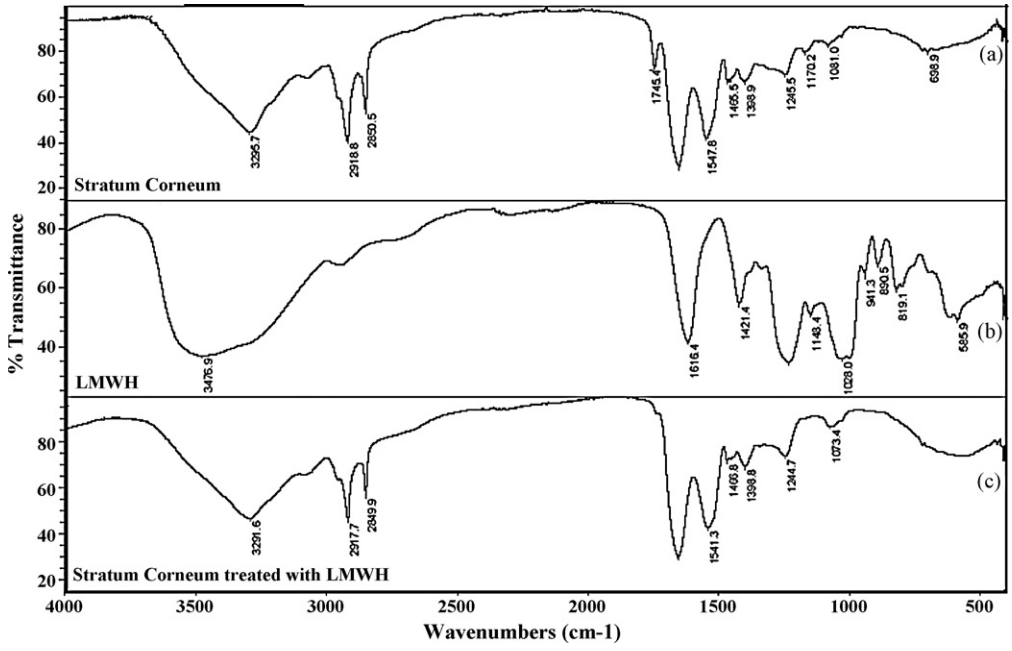

Fig. 4. FTIR spectra of (a) stratum corneum, (b) LMWH and (c) stratum corneum pretreated with LMWH at 4 cm^{-1} resolution with 256 scans.

(Brancaleon et al., 2001). The FTIR spectra for SC treated with LMWH band resulted in disappearance of peaks at 1745 and 1170 cm^{-1}.

3.3. In vitro permeation across tape stripped and microneedles pretreated skin

In vitro study with tape stripped skin resulted in increased transdermal transport of LMWH (mean flux of 4.069 U cm^{-2} h^{-1}). This was the highest flux among the techniques investigated. The skin permeability of LMWH was significantly increased after pretreatment with microneedles. A mean flux of 0.175 U cm^{-2} h^{-1} compared to undetectable permeation with untreated skin. The mean flux of LMWH was 14.7-fold higher than iontophoresis when the combined enhancement strategy of microneedles and iontophoresis was employed. The permeation was fivefold higher than microneedles alone and it was 16-fold higher than ultrasound alone. This combined strategy also reduced the lag time to 2 h from 8 h when microneedles were used alone. The results indicate a statistically significant increase ($P < 0.05$) in the permeation of LMWH through skin following treatment with microneedles and other treatments (iontophoresis and ultrasound) or passive delivery.

3.4. Assessment of barrier integrity

TEWL was measured before treatment and immediately following treatment with microneedles. TEWL values increased from

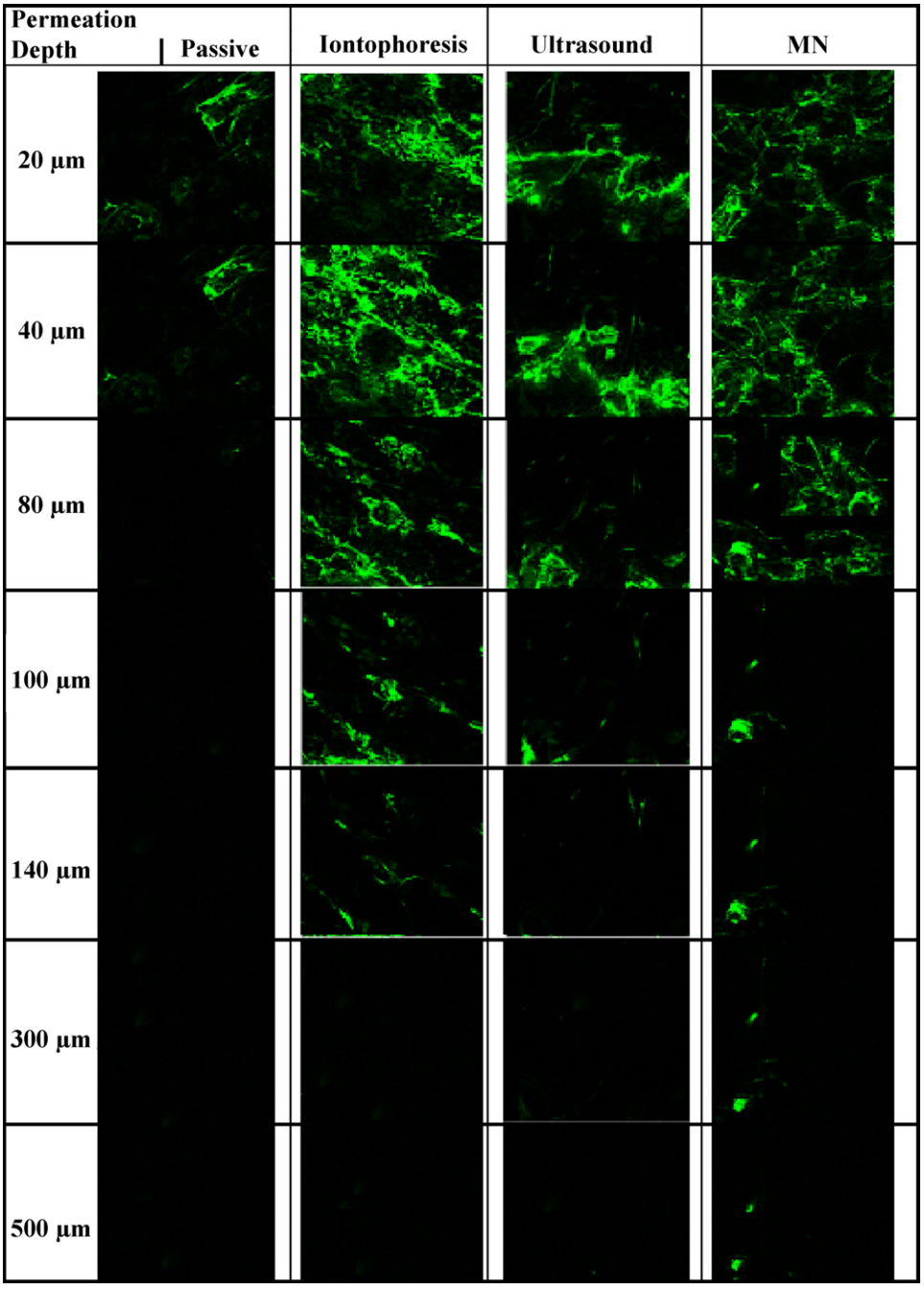

Fig. 5. Confocal microscopic images (X 10 objective) of the permeation of FITC-labeled heparin across hairless rat skin at various depths from the surface of the stratum corneum. Heparin transported across microchannel can be seen as a florescent green color up to a depth of 500 µm.

11.2 ± 1.4 to $19.7 \pm 3.4\,g\,m^{-2}\,h^{-1}$ immediately following pretreatment with microneedles as compared to TEWL values measured over intact skin. However, no significant difference ($P < 0.05$) between the control and TEWL measured at sites treated with the base of microneedle holder as a pressure control was observed. Also, TEWL increased by 13-fold (57.42 ± 4.42 $3.4\,g\,m^{-2}\,h^{-1}$) following tape stripping.

3.5. Transcutaneous electrical resistance measurements

TER of each skin preparation was taken following 15 min of equilibration with buffer. Reducing surface tension using alcohol swabs promotes more intimate contact of the skin surface being examined with PBS. All the experiments were performed on vertical static Franz diffusion cells with similar area of 0.64 cm^2. Untreated hairless rat skin was used as a control. It was observed that electrical resistance had dropped significantly following treatment with microneedles. The TER of hairless rat skin decreased from 21.7 ± 3.9 to 8.6 ± 1.1 kΩ following treatment with microneedles.

3.6. Confocal laser scanning microscopy studies

Following the *in vitro* study with FITC-labeled heparin using various enhancement protocols, the skin sample was imaged and recorded at increasing depths from the skin surface (Fig. 5). Freshly excised, untreated skin served as a negative control and no fluorescence was imaged either in SC or lower epidermal tissue. In contrast, in the region of skin that was subjected to *in vitro* studies, fluorescence was observed in the epidermal tissue. The passive skin samples displayed fluorescence intensity up to a depth of 40 μm. The specimens subjected to ultrasound treatment demonstrated fluorescence up to a depth of 100 μm. Iontophoresis resulted in increased permeation (depth 140 μm) of FITC-labeled heparin as compared to ultrasound. However, no fluorescence was seen beyond the epidermal tissue. These observations were similar to those seen with ultrasound treatment. In the case of pretreatment with microneedles where SC was breached and microchannels were created through the epidermis, fluorescence was observed beyond a depth of 500 μm. FITC-labeled heparin appeared to have migrated down through the SC, along the microchannels and into the lower epidermal tissue.

4. Discussion

DVT is the third most common cardiovascular disease after heart attack and stroke which can be prevented and treated by the use of LMWH. The safety and effectiveness of this agent makes it a potent anticoagulant (Hyers et al., 1986; Koopman et al., 1996; Levine et al., 1996; Zhu et al., 2003). Since LMWH can be administered subcutaneously once or twice daily without laboratory monitoring, many patients with DVT do not require hospital admission (Harrison et al., 1998). However, administration of this drug is a major distress for outpatients and could be improved by administering LMWH through an alternative route such as transdermal delivery.

Several attempts were made to deliver heparin across the skin but they could meet only with limited success. Even with the use of iontophoresis, the delivery was low (Prausnitz et al., 1995). Transdermal delivery, however, has been achieved with electroporation and ultrasound. Using electroporation, by applying short (1.9 ms), high voltage (150–350 V) pulses across skin (Prausnitz et al., 1995) a significant amount of heparin delivery was achieved. However, with an increase in current, the detrimental electrical irritation of skin also increases (Ledger, 1992). Also, the safety and drug stability issues associated with this method have not been addressed

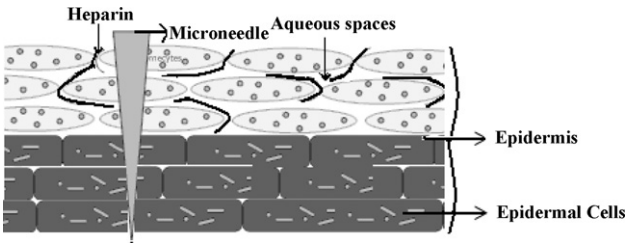

Fig. 6. Illustration of microchannel created following microneedles insertion and the possible entrapment of long polysaccharide LMWH across the corneocytes and epidermis.

(Prausnitz, 1997). LMWH was delivered across porcine skin using either ultrasound alone or the synergistic combination with iontophoresis (Le et al., 2000). Low frequency ultrasound was also used to deliver LMWH across porcine skin (Mitragotri and Kost, 2001).

Transdermal delivery of heparin was also demonstrated by using pulsed current iontophoresis (Pacini et al., 2006). However they used abrasion and shaving techniques in their procedure that resulted in a significant reduction in skin impedance and a decrease of SC thickness. While the method may not be practical to use, it provided evidence that heparin can be delivered through a disrupted or abraded SC.

Passive transdermal delivery of heparin was minimal and these observations were in accordance with earlier reports (Prausnitz et al., 1995). Iontophoresis was, therefore, employed to deliver LMWH. Iontophoresis is a non-invasive technique that uses electromotive force to propel charged drugs across the skin. LMWH is a candidate for iontophoresis since it is water-soluble and exists in a salt form with high charge density (Gangarosa et al., 1978; Lattin et al., 1991). Since it is an anionic drug, cathodal iontophoresis was carried out using Ag and Ag/AgCl electrodes. However, the delivery was not significant since low voltage iontophoresis cannot change the skin microstructure to generate any fresh and/or enlarged pathways for permeation across the skin and possible binding with SC (Prausnitz et al., 1995).

Ultrasound was also used as another enhancement strategy to deliver LMWH. It induces cavitation within the SC that leads to a disorder of the lipid bilayers and increases the permeability of the outermost skin layer thereby increasing drug transport (Le et al., 2000). Results in Fig. 2 reveal that LMWH delivery improved following ultrasound application as compared to passive.

Heparin was used in the earlier studies (Prausnitz et al., 1995) and is a class of molecules with a wide range of molecular fractions (MW range 5–30 kDa). As suggested earlier (Weaver et al., 1997) there is a possibility that during pulsing, some high molecular fractions can enter the aqueous spaces between two adjacent corneocytes, becoming trapped and resulting in negligible delivery (Fig. 6). Low molecular fractions, however, can permeate the skin more easily than larger ones (Prausnitz et al., 1995). LMWH was employed in the current study to avoid any possible hindrance due to entrapment. Few researchers have attempted to deliver LMWH across the skin using ultrasound (Le et al., 2000; Mitragotri and Kost, 2001) but the duration of ultrasound application is high. The current study employs the simple Sonoprep® system (Sontra Medical Corporation, Franklin, MA, USA), a portable and commercially available instrument. Also, the time of application is low and instrument terminates the ultrasound energy once the skin conductance reaches appropriate levels.

Some *in vitro* studies reveal that heparin interacts with the SC. This may be an additional reason for the minimal delivery across the intact skin irrespective of ultrasound or iontophoresis used as an enhancement strategy. An *in vitro* study conducted (Parisel et

al., 2003) on heparin interaction with skin cells revealed that heparin binds to cultured keratinocytes and melanocytes (epidermal cells) and fibroblasts (dermal cells). Moreover, they demonstrated the preferential interaction of heparin with keratinocytes which are abundant in SC. Heparin is a sulfated polysaccharide with GlcNAc6S moiety (disaccharide unit) and interaction with skin cells is likely because of the chemical structure (Hozumi et al., 2006).

Based on observations from passive, iontophoresis and ultrasound studies, an attempt was made to deliver LMWH across tape stripped skin. Delivery was enhanced significantly (flux of 4.07 U cm^{-2} h^{-1}) compared to passive, iontophoresis (63-fold higher) and ultrasound (70-fold higher). This increased flux was observed for tape stripped skin without the use of any other enhancement strategies in conjunction. This is most likely because SC is removed during tape stripping and the primary barrier for LMWH transport is bypassed but perhaps more importantly, the interaction of LMWH with the SC is also prevented. Increased TEWL levels following tape stripping indicated removal of SC. Tape stripping may provides an excellent means to enhance the transdermal delivery of LMWH but it cannot be a practical alternative. Microneedle technology provides an approach that can enhance delivery across skin by minimal disruption of SC and thereby creating microscopic channels for the drug transport. Therefore, the use of the microneedles could be a better alternative to deliver LMWH transdermally compared to other techniques.

Microneedle mediated transdermal delivery has received increasing attention in recent years. Detailed fabrication, manufacturing, design and characterization aspects of maltose microneedles used in this study were reported earlier (Miyano et al., 2005; Kolli and Banga, 2008). The technology avoids SC barrier resistance and possibly reduces LMWH interaction with superficial epidermis thereby allowing relatively free transport of LMWH. Maltose microneedles effectively breached skin barrier as measured by TEWL and TER studies. TER measurements were used earlier for assessment of skin integrity for *in vitro* dermal testing (Pinnagoda et al., 1990). TER measurements made across microneedles treated hairless rat skin samples reveal compromised barrier function. Decreased electrical resistance as compared to untreated skin indicates disruption of SC and thereby increased permeability. Microneedles used alone demonstrated significantly higher level than iontophoresis (2.7-fold higher) and ultrasound (3.0-fold higher). Microneedles pretreatment in conjunction with iontophoresis demonstrated a synergistic effect resulting in a mean LMWH flux of 0.942 U cm^{-2} h^{-1} and this was 5.4-fold higher than flux obtained with microneedles used alone. The enhanced delivery observed with the combined strategy may be attributed to creation of new large aqueous channels upon pretreatment with microneedles (Fig. 6) and the LMWH is forced through electrorepulsion under the influence of externally applied electric field. Such enhancement from a combination of microneedles and iontophoresis for antisense oligonucleotides (ISIS 2302) has been reported earlier (Wang et al., 2005).

In order to study the factors hindering LMWH transport across intact SC; DSC and FTIR studies were performed. DSC was used to measure temperature and heat flows associated with thermal transitions of biological macromolecules. In SC alone, the transitions before 100 °C represent melting of sebaceous lipids and rearrangement of intercellular lipid structure (Babita et al., 2006). Transition near 130 °C is a representation of proteins denaturation (Babita et al., 2006). The DSC profile of SC pretreated with LMWH showed loss of transitions before 100 °C and a lowering peak was observed at a transition of 130 °C. The disappearance and lowering of transitions indicate a possible interaction with SC components.

DSC results were supported by FTIR observations. FTIR was used earlier to determine the interactions of LMWH with positively charged compounds (Yang et al., 2006), for its quantification (Harada et al., 2005) and for dermal absorption studies (Merwe, 2005). FTIR profiles of SC pretreated with LMWH reveal that there is an interaction between SC components and LMWH derivatives (uronic and iduronic acid derivatives). Disappearance of peaks at 1745 and 1170 cm^{-1} might be a result of LMWH interaction with SC.

Confocal studies were performed to study the depth of permeation of LMWH following various enhancement strategies. CLSM offers controllable depth imaging and allows collecting serial optical sections from thick specimens. CLSM was used to visualize the penetration of model compound along the iontophoretic transport pathways (Turner and Guy, 1998). It was also investigated in the transport of fluorescent probes, on ultrasound pretreated skin (Alvarez-Roman et al., 2003). CLSM results in this study further substantiated the earlier observations from DSC and FTIR. These studies revealed that heparin remained localized in the SC or epidermal region of the skin irrespective of whether iontophoresis or ultrasound used as enhancement strategy. In contrast when skin was pretreated with microneedles fluorescence was observed beyond the epidermis and into deeper dermal layer. FITC-labeled heparin was observed to follow microchannels for transport across skin.

5. Conclusion

Transdermal delivery of LMWH would allow easier compliance as compared to parenteral administration. However, being a hydrophilic molecule, it does not cross the SC barrier and usage of enhancement strategies has limited value owing to its interaction with SC and epidermal components. This was demonstrated by DSC, FTIR and CLSM studies and further confirmed by tape stripping studies that resulted in a significantly increased delivery across the hairless rat skin. A more feasible and patient compliant approach (relative to skin stripping) would be usage of minimally invasive technique like use of microneedles to breach the SC barrier. Increased skin permeation of LMWH was observed following skin pretreatment with microneedles. Microneedles were shown to breach the SC basing on results from TEWL and TER studies. CLSM studies showed that skin treated with microneedles bypasses SC and hence reduces the interaction of LMWH, thereby improving its transdermal delivery. Additionally, when microneedles were used in conjunction with iontophoresis, permeability was increased further. These experiments have demonstrated that microneedles can be used to breach the SC to increase skin permeability of molecules like LMWH that interact with SC.

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