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Molecular charge mediated transport of a 13 kD protein across microporated skin

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ABSTRACT

Transport of proteins across the skin is highly limited owing to their hydrophilic nature and large molecular size. This study was conducted to assess the skin transport abilities of a model protein across hairless rat skin during iontophoresis alone and in combination with microneedles as a function of molecular charge. The effect of microneedle pretreatment on electroosmotic flow was also investigated. Skin permeation experiments were carried out *in vitro* using daniplestim (DP) (MW, 12.76 kD; isoelectric point, 6.2) as a model protein molecule. The effect of molecular charge on protein transport was evaluated by performing studies in two different buffers – TRIS (pH 7.5) and acetate (pH 4.0). Iontophoretic transport mechanisms of DP varied with respect to molecular charge on the protein. The combination approach (iontophoresis and microneedles) gave much higher flux values compared to iontophoresis alone at both pH 4.0 and pH 7.5, however, the delivery in this case was also found to be charge dependent. The findings of this study indicate that electroosmosis persisted upon microporation, thus retaining skin's permselective properties. This enables us to explore the combination of microneedles and iontophoresis as a potential approach for delivery of proteins.

1. Introduction

Proteins and peptides are traditionally administered by parenteral route i.e. intravenously or subcutaneously because of poor bioavailability by most other routes (Shire et al., 2004). However, there are several alternative routes being explored with transdermal drug delivery being one of the attractive delivery methods. Passive diffusion of protein and peptide drugs across the skin is limited by their hydrophilic nature and high molecular weight (Schuetz et al., 2005b). Hence, enhancement methods like iontophoresis (Chaturvedula et al., 2005; Schuetz et al., 2005b), sonoporation (Weimann and Wu, 2002), electroporation (Tokumoto et al., 2006) and microneedles (Lin et al., 2001; Kolli and Banga, 2008), are being employed to increase the skin permeation of a wide variety of macromolecules.

Iontophoresis involves application of physiologically acceptable amounts of current to drive charged and neutral molecules across the skin. The two principle mechanisms governing iontophoresis are electromigration/electrorepulsion and electroosmosis. At physiological pH, skin is negatively charged and when a voltage difference is applied across the skin, there is a solvent flow seen from anode to cathode (Pikal, 2001). This bulk solvent flow is referred to as electroosmosis and is independent of the charge on the permeant. Thus, it can be used to transport charged as well as uncharged molecules. However, iontophoretic delivery is restricted to compounds of certain molecular weight (10–15 kD) (Turner et al., 1997; Banga, 2006; Cazares-Delgadillo et al., 2007).

The major rate-limiting barrier for transport of molecules across the skin is stratum corneum. As proteins are hydrophilic, their transport is highly hindered by the lipophilic stratum corneum. Efforts are on to overcome the barrier properties of skin by developing methods to breach stratum corneum in a transient and reversible manner. Microporation is one such approach being tried to effectively enhance the permeation of a wide range of molecules across the skin. Microneedles are minimally invasive, long enough to breach the stratum corneum but short enough not to reach the nerves in the deeper tissues, thus, not stimulating any pain receptors (Wang et al., 2005; Kolli and Banga, 2008). Combination of such methods where the barrier properties of the skin are compromised (microporation, electroporation) with iontophoresis might significantly improve skin permeation thereby making transdermal delivery feasible for a broad range of molecules. Combination strategies have been previously used by researchers to deliver insulin (Tokumoto et al., 2006), antisense oligonucleotides (Lin et al., 2001) and dextrans of various molecular weights (Wu et al., 2007).

The present study focuses on one such combination – microneedles and iontophoresis, to enhance the skin permeation of a model protein molecule. Skin transport studies were carried out using daniplestim as a model protein. It is an IL-3 receptor agonist consisting of 112 amino acid residues. It has a molecular weight of 12.76 kD and an isoelectric point of 6.2. This protein was chosen primarily for its physicochemical properties and not for its therapeutic potential.

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The purpose of the present study was (1) to assess the skin transport abilities of the protein during iontophoresis alone and in combination with microneedles as a function of molecular charge and (2) to study the effect of microneedle pretreatment on electroosmotic flow during iontophoresis.

2. Materials and methods

2.1. Materials

Sodium chloride (NaCl), sodium dihydrogen phosphate (NaH_2PO_4), di-sodium hydrogen phosphate (Na_2HPO_4), sodium acetate (CH_3COONa) and acetonitrile (HPLC grade) were obtained from Fisher Scientific (NJ, USA). Acetaminophen was purchased from Sigma–Aldrich (St. Louis, MO, USA). Silver wire (0.5 mm diameter) and silver chloride used in the making of electrodes were obtained from Sigma–Aldrich (St. Louis, MO, USA). All the solutions were prepared using de-ionized water.

Daniplestim (DP) was obtained as a gift from Pfizer, Inc. (St. Louis, MO, USA). It was provided as a lyophilized formulation in vials (1.0 mg/vial) consisting of TRIS/mannitol/sucrose (1.2/40/10), pH 7.5. The vials were reconstituted with de-ionized water prior to use to work at this pH.

2.2. Animals and skin preparation

Male hairless rats (8–10 weeks old) were used for all the experiments. They were obtained from Charles River Laboratories (Wilmington, MA, USA) and housed in the Mercer University animal facility until used. Rats were euthanized by CO_2 asphyxiation after which skin from the abdominal region was excised. The underlying subcutaneous fat was carefully removed and the cleaned skin was then used immediately to perform the *in vitro* studies. All the animal studies were reviewed and approved by the Mercer University Institutional Animal Care and Use Committee.

2.3. Iontophoretic permeation experiments

In vitro permeation experiments were carried out using vertical glass (static) Franz diffusion cells (PermeGear, Inc., Hellertown, PA, USA) having a diffusion area of 0.64 cm^2. The skin was clamped between the donor and the receptor compartments such that the epidermal surface always faced the donor chamber. The donor solution (300 μl) comprised of 0.08 mM (1 mg/ml) of DP and 15 mM of acetaminophen either in TRIS buffer (pH 7.5) or acetate buffer (pH 4.0). The receptor compartment (5 ml) consisted of phosphate buffer (10 mM, pH 7.4). A temperature of 37 °C was maintained throughout the duration of the experiment by means of a water circulation jacket surrounding the lower portion of the cell. Anodal iontophoresis experiments had 238 mM NaCl in the donor and cathodal iontophoresis studies had 150 mM NaCl in the receptor. A constant current (0.5 mA/cm^2) was applied for 6 h through Ag/AgCl electrodes connected to a power supply device (Keithley®, Keithley Instruments; Cleveland, OH, USA). About 500 μl of the sample was drawn at preset time points over a period of 24 h and replenished immediately with the same volume of receptor buffer. The samples were stored at 4 °C until analysis. All the experiments were performed in triplicate.

2.4. Effect of molecular charge

To evaluate the effect of molecular charge on the protein, iontophoresis was done in two different buffers (TRIS, pH 7.5 and acetate, pH 4.0) on either side of the isoelectric point (6.2). DP was originally obtained as a formulation in TRIS buffer, pH 7.5. The pH of the original formulation was modified by performing a buffer exchange into acetate buffer (pH 4.0). The buffer exchange procedure was carried out using dialysis cassettes (Pierce, Rockford, IL, USA) with a 2000 Da molecular weight cut off and a sample volume of 0.5–3 ml per cassette.

2.5. Protein stability

The effect of formulation pH on DP stability was evaluated by analyzing a 100 μg/ml solution of DP in TRIS (pH 7.5) and acetate (pH 4.0) buffer using reverse phase HPLC and size-exclusion chromatography. The stability of DP in the presence of skin and current application was also studied using HPLC. To mimic the *in vitro* permeation experiments, a 1 mg/ml DP solution was placed in the donor. Samples were collected at 0 h and at the conclusion of the study which was 24 h.

2.5.1. Size-exclusion chromatography

Alliance system (Waters Corp., MA, USA) equipped with a photodiode array detector (PDA) was used. The samples were eluted with a mobile phase consisting of 100% phosphate buffered saline (1×) on a Biosep-SEC-S 2000 column (Phenomenex; 30 cm × 4.6 mm). The flow rate was maintained at 0.5 ml/min and the detection wavelength was 214 nm. The injection volume was 50 μl.

2.5.2. Reverse phase HPLC

Alliance system (Waters Corp., MA, USA) equipped with a fluorescence detector was used. Gradient elution was performed with a mobile phase consisting of water, 1.5% trifluoroacetic acid and acetonitrile. DP was detected using an excitation wavelength of 276 nm and an emission wavelength of 340 nm. A C-4 column (Phenomenex; 15 cm × 4.6 mm; 5 μm) was used with a flow rate of 1.0 ml/min and an injection volume of 50 μl.

2.6. Skin microporation

The excised skin was porated manually with six layers of maltose microneedles stacked together as a single unit (27 needles/layer, Texmac Inc.) with an optimized pressure of approximately 0.625 kg/cm^2 for 60 s. Each individual needle was about 500 μm long with the length of the base and radius of curvature of the tip approximately around 200 and 3 μm respectively (Kolli and Banga, 2008).

2.7. Electroosmosis

Acetaminophen (15 mM) was used as a marker to study electroosmosis. It is a neutral hydrophilic molecule with negligible passive permeation; hence its transport through the skin upon iontophoresis is predominantly by electroosmosis (Schuetz et al., 2005a). The acetaminophen flux obtained was used as a measure of the degree of electroosmotic flow.

2.8. Tape stripping

This procedure was carried out to investigate the effect of stratum corneum removal on electroosmotic flow and compare it to microporation which involves removal of a small fraction of the stratum corneum layer. Stratum corneum was removed by using 3M transpore tape (about 30 strips) (St. Paul, MN, USA) (Surber et al., 2001). The removal of stratum corneum was ensured by transepidermal water loss (TEWL) measurements using a vapometer (Delfin Technologies, Inc., Stamford, CT, USA) (Grubauer et al., 1989). TEWL values increased relative to the control measurements

made prior to stripping and remained constant after about 20 strips.

2.9. Quantitative analysis

DP was quantified using a commercially available enzyme-linked immunosorbent assay (ELISA) human IL-3 kit (Duoset®) from R&D Systems (Minneapolis, MN, USA). The standard curve was in the range of 7.5–250 ng/ml. All the standards and samples were analyzed in duplicate.

Acetaminophen was quantified by high performance liquid chromatography (HPLC) using Alliance system (Waters Corp., MA, USA) equipped with a photodiode array detector (PDA) by a method which was previously reported (Schuetz et al., 2005a). The samples were eluted with a mobile phase consisting of 8% acetonitrile and 92% water adjusted to pH 3.5 with glacial acetic acid on a C-18 column (Varian; 25 cm × 4.6 mm; 5 μm). The flow rate was maintained at 1.2 ml/min and the detection wavelength was 243 nm. The injection volume was 50 μl.

2.10. Statistical analysis

Analysis of variance (ANOVA) was used. All the results are shown as a mean of the number of replicates used with respective standard deviation. The *in vitro* steady-state permeation flux was calculated from the linear portion of the cumulative amount *vs* time profile.

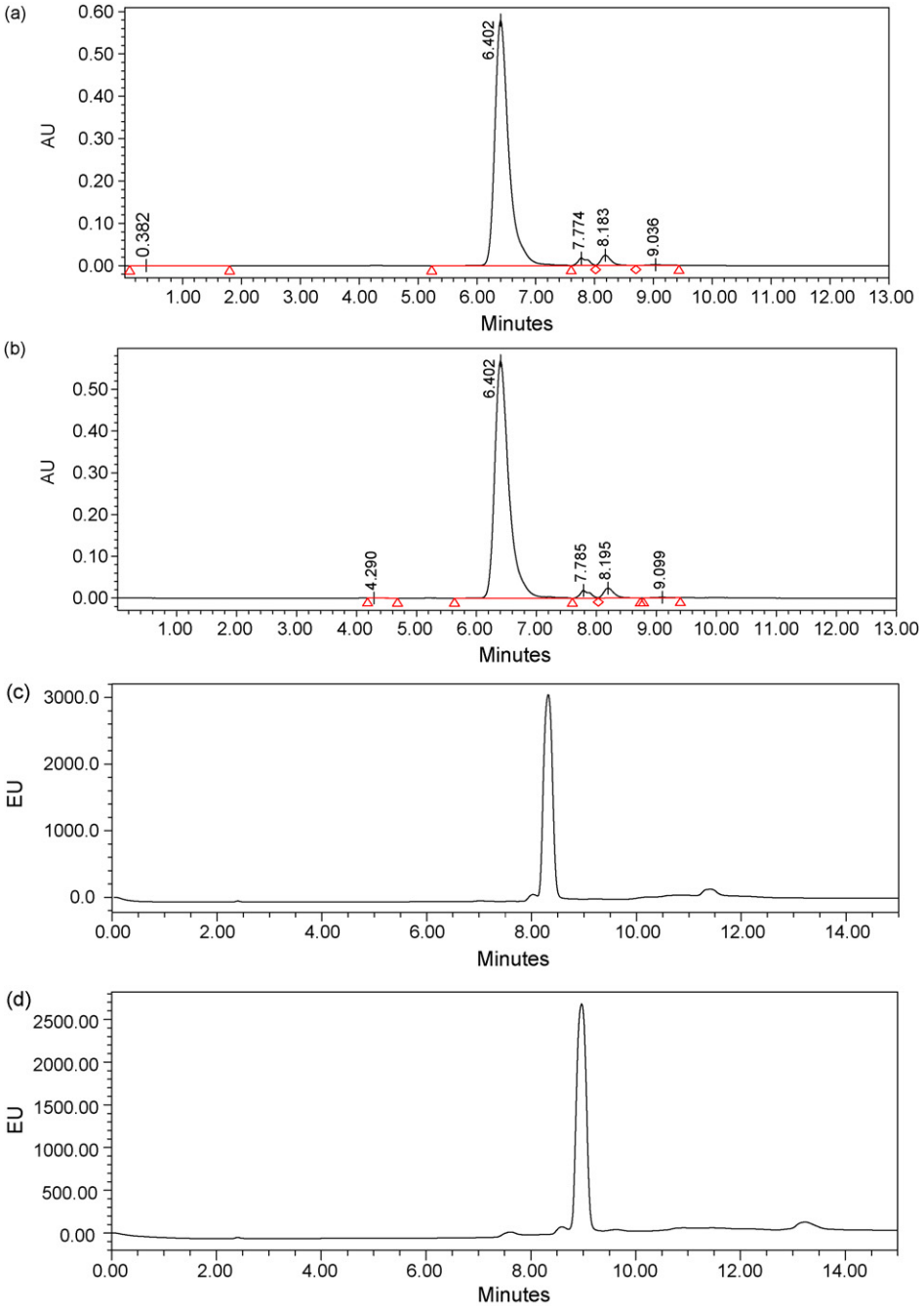

Fig. 1. Size-exclusion chromatograms of 100 μg/ml solution of DP in (a) TRIS buffer, pH 7.5 and (b) acetate buffer, pH 4.0. HPLC chromatograms of DP in (c) TRIS buffer, pH 7.5 and (d) acetate buffer, pH 4.0.

3. Results

3.1. Stability of daniplestim

SEC results (Fig. 1a and b) and HPLC analysis (Fig. 1c and d) confirmed the stability of daniplestim at both pH 7.5 and pH 4.0. The retention time and the peak areas were similar for both the formulations. Analysis of the donor solution at 0 and 24 h by HPLC further confirmed the stability of DP under experimental conditions (Fig. 2a–d). This suggests that daniplestim was stable during the permeation experiments.

3.2. Effect of molecular charge on mechanism of skin transport

The iontophoretic transport of DP was studied with two formulations having different pH values across intact and microporated skin. The permeation experiments were carried out with TRIS buffer formulation (pH 7.5, magnitude of charge on DP is approximately 3.1) and acetate buffer formulation (pH 4.0, magnitude of charge on DP is approximately 11.0). DP is negatively charged at pH 7.5, hence delivered under cathode (cathodal iontophoresis) and it is positively charged at pH 4.0, thus delivered under anode (anodal iontophoresis) (Fig. 3). The cumulative amount of DP delivered across the skin from pH 7.5 and pH 4.0 formulations by a combination of iontophoresis and microneedles and iontophoresis alone is shown in Fig. 4a and b respectively. There was no permeation seen at pH 7.5 with iontophoresis alone, however, at pH 4.0 about $555.3 \pm 9.9\,ng\,cm^{-2}$ was delivered. The cumulative amount permeated with iontophoresis across microporated skin at pH 7.5 and pH 4.0 was 717.8 ± 72.79 and $22{,}727.06 \pm 11{,}970.03\,ng\,cm^{-2}$ respectively (ANOVA, $p < 0.05$). There was approximately a 30-fold increase seen in the amount of DP that permeated from pH 4.0 formulation than pH 7.5 formulation with the combination strategy.

The steady-state flux of DP during 6 h of current application is shown in Fig. 5. At pH 7.5, the measured steady-state flux of DP for the combination of iontophoresis and microneedles was

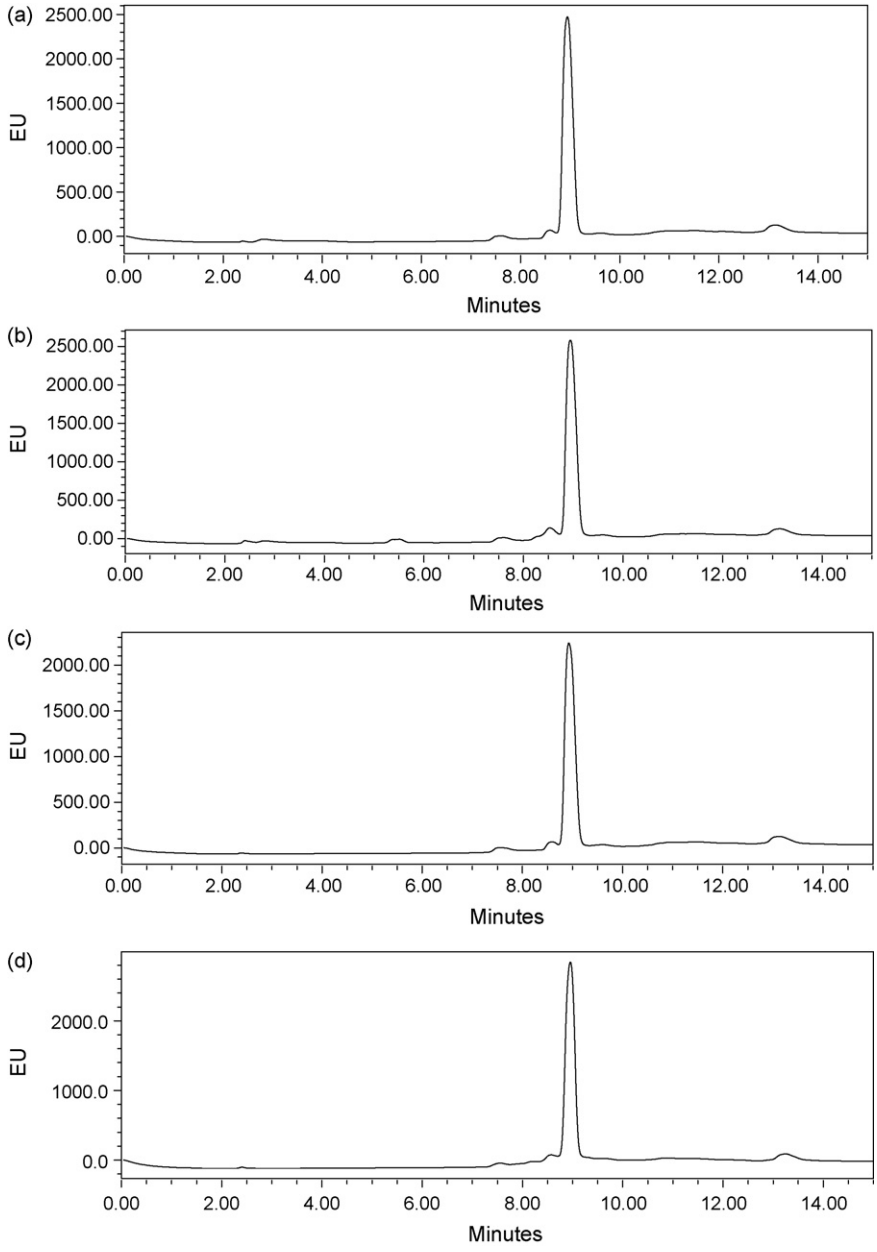

Fig. 2. HPLC chromatograms of DP at (a) 0 h and (b) 24 h in TRIS buffer formulation (pH 7.5) and (c) 0 h and (d) 24 h in acetate buffer formulation (pH 4.0).

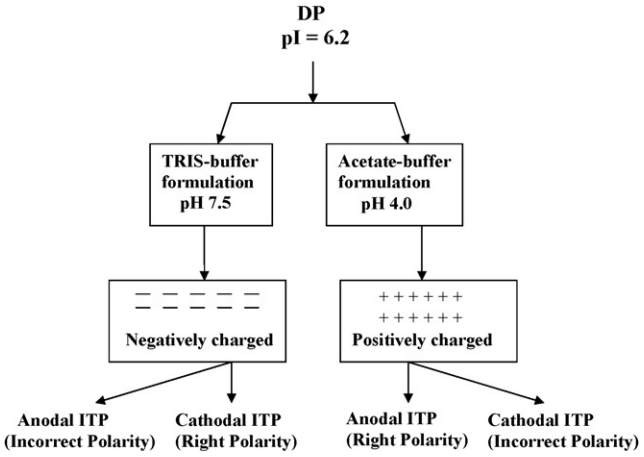

Fig. 3. Schematic showing the charge on DP in TRIS buffer and acetate buffer and the type of iontophoresis applicable.

$41.05 \pm 16.61\ ng\ cm^{-2}\ h^{-1}$. The steady-state fluxes of DP at pH 4.0 for iontophoresis alone and the combination strategy were 4.91 ± 2.27 and $781.49 \pm 625.94\ ng\ cm^{-2}\ h^{-1}$ respectively. Comparison of steady-state fluxes between the two formulations for iontophoresis across microporated skin shows that the flux was about 20 times higher at pH 4.0.

Acetaminophen (ACM) was added to the donor to measure the degree of electroosmotic flow (Schuetz et al., 2005a). Fig. 6a and b shows increase in the magnitude of electroosmotic flow as the skin pH is increased from 4.0 to 7.5. The steady-state ACM flux measured

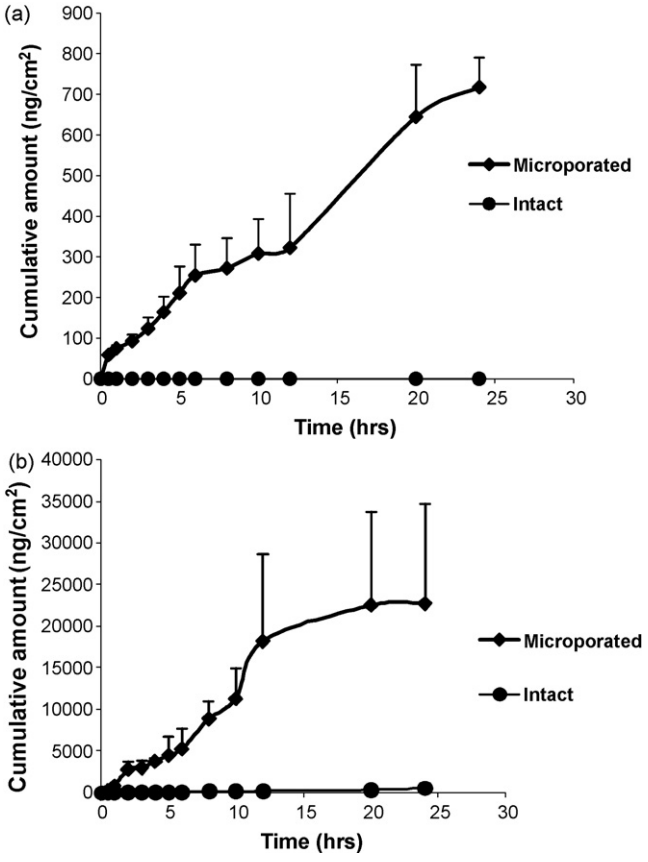

Fig. 4. Cumulative amount–time profile of DP over 24 h following 6 h of iontophoresis at 0.5 mA/cm^2 across intact and microporated skin: (a) TRIS buffer formulation (pH 7.5) (cathodal iontophoresis) and (b) acetate buffer formulation (pH 4.0) (anodal iontophoresis) (Mean ± SD) ($n = 3$).

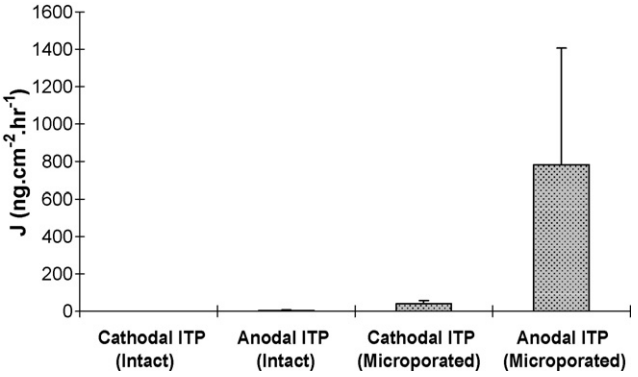

Fig. 5. Steady-state flux of DP during 6 h of iontophoresis at 0.5 mA/cm^2 across intact and microporated skin at pH 7.5 (cathodal) and pH 4.0 (anodal) (Mean ± SD) ($n = 3$).

under anode at pH 4.0 was $0.77 \pm 0.19\ \mu g\ cm^{-2}\ h^{-1}$ (Fig. 6a) and at pH 7.5 was $9.78 \pm 2.44\ \mu g\ cm^{-2}\ h^{-1}$ (ANOVA, $p < 0.05$) (Fig. 6b). To check for the reversal of direction of the electroosmotic flow at lower skin pH values, ACM flux was also measured under cathode. At pH 4.0, under cathode, the ACM flux was $2.1 \pm 0.12\ \mu g\ cm^{-2}\ h^{-1}$. The membrane might have reversed its charge at this pH, thereby causing reversal of electroosmosis. Also, there was an overall decrease in electroosmosis at pH 4.0 compared to pH 7.5 (ANOVA, $p < 0.05$).

3.3. Delivery under incorrect polarity of electrode

Daniplestim being negatively charged at pH 7.5 was delivered under cathode. However, to take advantage of electroosmosis and also to investigate if it was the dominant mechanism of skin transport, DP was delivered under anode (wrong or incorrect polarity) at pH 7.5 (Fig. 3). The cumulative amount–time profile of DP delivered under the incorrect polarity of electrode is shown in Fig. 7. The amount of DP permeated during iontophoresis

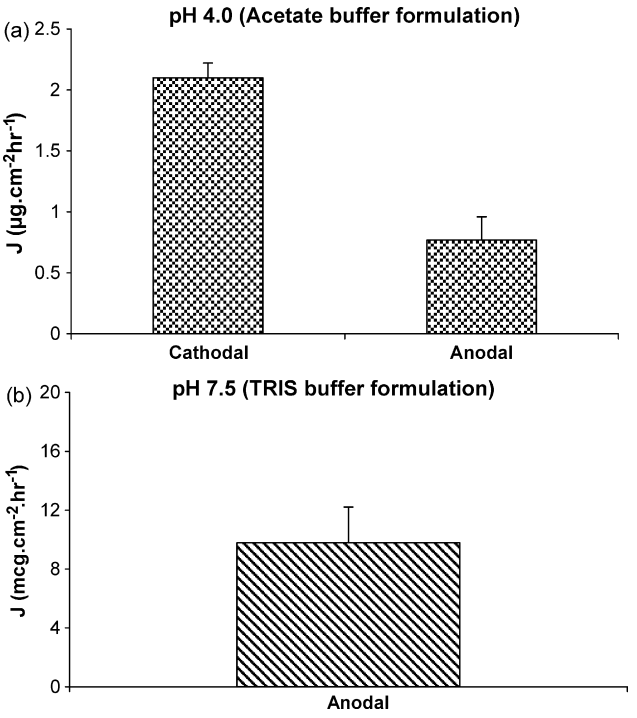

Fig. 6. Steady-state ACM flux during 6 h of iontophoresis at 0.5 mA/cm^2 across intact skin: (a) acetate buffer formulation (pH 4.0) under cathode and anode and (b) TRIS buffer formulation (pH 7.5) under anode (Mean ± SD) ($n = 3$).

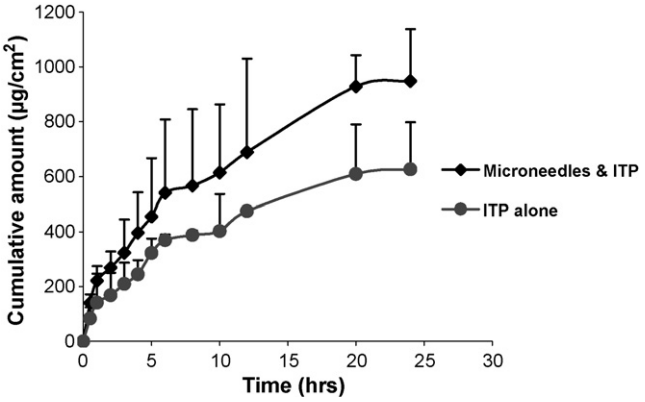

Fig. 7. Cumulative amount–time permeation profile of DP for 24 h following 6 h of iontophoresis at 0.5 mA/cm^2 across intact and microporated skin at pH 7.5 under anode (incorrect electrode) (Mean ± SD) (n = 3).

across intact and microporated skin was 626.29 ± 171.43 and 949.13 ± 189.49 ng cm^{-2} respectively (ANOVA, $p > 0.05$).

Comparison of steady-state fluxes of DP and ACM under cathode (right polarity with respect to the protein) and anode (incorrect polarity with respect to the protein) is shown in Fig. 8a and b. The steady-state flux of DP under anode across intact and microporated skin was 46.75 ± 16.99 and 63.55 ± 45.75 ng cm^{-2} h^{-1} respectively (Fig. 8a) (ANOVA, $p > 0.05$). The corresponding ACM fluxes were 9.78 ± 7.44 and 20.58 ± 5.16 μg cm^{-2} h^{-1} (Fig. 8b) (ANOVA, $p > 0.05$).

At physiological pH skin being negatively charged, electroosmotic flow occurs from anode to cathode. However, there was some ACM flux seen across microporated skin with cathodal iontophoresis at pH 7.5 (2.38 ± 0.335 μg cm^{-2} h^{-1}) (Fig. 8b). This is probably

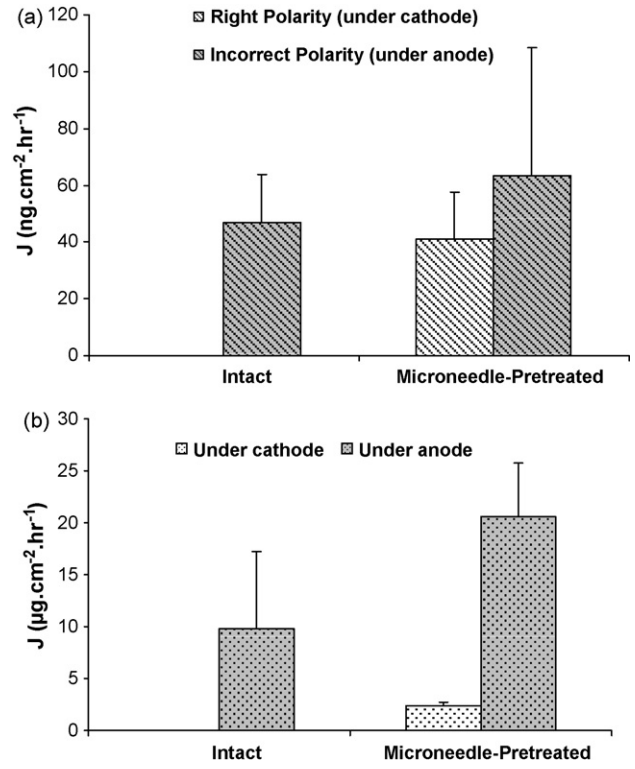

Fig. 8. Steady-state flux during 6 h of iontophoresis at 0.5 mA/cm^2 across intact and microneedle pretreated skin at pH 7.5 under cathode (right polarity with respect to DP) and anode (incorrect polarity with respect to DP): (a) DP flux and (b) ACM flux (Mean ± SD) (n = 3).

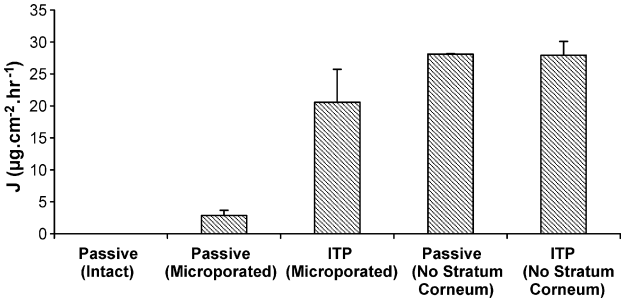

Fig. 9. Steady-state acetaminophen flux across (1) intact (passive), (2) microneedle pretreated (passive and 6 h of iontophoresis at 0.5 mA/cm^2) and (3) impaired skin (tape stripping passive and 6 h of iontophoresis at 0.5 mA/cm^2) (Mean ± SD) (n = 3).

due to the passive diffusion of ACM as a result of microporation and not due to electroosmotic flow.

3.4. Effect of pretreatment with microneedles on electroosmosis

Physical impairment of the skin barrier in combination with iontophoresis is one of the approaches being tried to enhance the delivery of high molecular weight drugs. The effect of pretreatment with microneedles and removal of stratum corneum on the transport of ACM flux is summarized in Fig. 9. Electroosmosis is a bulk fluid flow seen from anode to cathode at physiological pH, hence this study was carried out at pH 7.5 (Pikal, 2001). There was no passive permeation seen for ACM across intact skin. The passive permeation of ACM across tape stripped skin was significantly high (28.1 ± 0.075 μg cm^{-2} h^{-1}). However, application of current across the tape stripped skin did not further enhance the permeation of ACM (27.92 ± 2.16 μg cm^{-2} h^{-1}) (ANOVA, $p > 0.05$). This suggests that electroosmosis was completely abolished and tape stripping impaired the permselective properties of the skin. The measured steady-state flux of ACM across microporated skin (passive) was 2.86 ± 0.79 μg cm^{-2} h^{-1}. Iontophoresis across microporated skin further enhanced the flux of ACM by about seven times (20.58 ± 5.86 μg cm^{-2} h^{-1}) (ANOVA, $p < 0.05$), suggesting that electroosmosis persisted upon pretreatment with microneedles.

4. Discussion

Iontophoresis offers an attractive alternative delivery method for macromolecules over parenteral therapy; however, this method might be limited by the molecular size of the drug candidate (10–15 kD) (Turner et al., 1997; Banga, 2006; Cazares-Delgadillo et al., 2007). Use of micron size needles to create microchannels in the skin is one of the approaches being tested to increase the permeation of high molecular weight compounds (Prausnitz, 2004). The combination of microneedles and iontophoresis might further expand the horizon for therapeutic agents of any size to be delivered across the skin (Wu et al., 2007). The present study investigates into understanding the predominant transport mechanisms and their effect on the skin transport across microneedle pretreated skin.

Iontophoretic delivery depends on the properties of the permeant and also on the charge of the membrane (Merino et al., 1999). In the current study, the effect of formulation pH which in turn dictates the charge on the protein molecule was evaluated. At pH 7.5 there was no iontophoretic delivery of DP seen under cathode, however, there was some amount delivered under anode (incorrect polarity) at the same pH (Figs. 4a and 7). This suggests that electroosmosis is the predominant transport mechanism at this pH. However, this finding contradicts previous reports wherein the electrotransport of a similar sized protein (cytochrome c) was governed mainly by electromigration (Cazares-Delgadillo et al., 2007).

This might be because the transport mechanisms that operate depend on the physicochemical properties of the molecule and the skin model employed (Merino et al., 1999; Cazares-Delgadillo et al., 2007). Electroosmosis is believed to gain importance and the role of electrorepulsion is assumed to reduce with an increase in the molecular size (Guy et al., 2000). This probably holds true for DP as the delivery at pH 7.5 was seen only under anode. Also, the ACM flux measured further supports that electroosmosis was present and helped in the permeation of DP during iontophoresis at pH 7.5. Moreover, iontophoretic delivery of a large anion is thought to be more effective under anode than the cathode due to electroosmosis (Pikal, 2001).

DP was delivered *in vitro* at pH 4.0 by iontophoresis. However, the predominant mechanism of transport at this pH might be electrorepulsion and not electroosmosis. Reports from the literature suggest that at a given molecular weight, increase in molecular charge enhances electrorepulsion (Abla et al., 2005b). At pH 4.0, daniplestim has a higher molecular charge (~11.0) than pH 7.5 (~3.1), thus, the charge/molecular weight ratio is more at pH 4.0 (8.6×10^{-3}) than pH 7.5 (2.4×10^{-3}). Also, the steady-state ACM flux measured at pH 7.5 was approximately nine times higher compared to the flux at pH 4.0, thereby suggesting that there was a decrease in the magnitude of electroosmosis at pH 4.0. Highly charged species are believed to neutralize the skin's negative charge as they traverse through the iontophoretic transport pathways thus causing a decrease in electroosmosis (Guy et al., 2000). Electroosmosis is believed to decrease as the pH is lowered, especially with continued iontophoresis at high current intensities (Pikal, 2001). Electroosmosis might reverse its direction as the pH goes down to around 4.0 as the charge on the skin reverses from negative to positive (Pikal, 2001; Marro et al., 2001). However, the ACM flux measured in the reverse direction (under cathode) at pH 4.0 was still five times less compared to the flux at pH 7.5, suggesting a decrease in degree of electroosmosis. Also, there was no DP delivered at pH 4.0 under cathode (incorrect polarity of electrode) suggesting electrorepulsion is the major transport mechanism.

Acetaminophen was used as a marker to study electroosmosis. It is a hydrophilic neutral molecule with a pKa of 9.1. The high pKa of the molecule suggests that it is a very weak acid. Previous literature reports suggest that during the reverse iontophoresis of urea it behaved like a positively charged molecule due to the presence of some local positive charges (Degim et al., 2003; Sieg et al., 2004; Wascotte et al., 2007). From the chemical structure and pKa, ACM has the possibility of being a weak negative species at pH 7.5 and thus should have been delivered under cathode. However, this was not the case and ACM penetration was found under anode only. Similarly at pH 4.0 (acidic conditions), it would probably take up a proton and behave like a partially positive species. This should have shown delivery under anode but there was minimum permeation under anode and some permeation under cathode. Thus, partial charges might be effective in governing the transport for some molecules like urea (Degim et al., 2003). In contrast from the observations made in this work, ACM transport through skin was strictly governed by electroosmosis as a result of its neutrality. There is a definite possibility of ACM existing as a partial positive or negative species depending on the conditions used, but the charges might be too weak to have an effect on its skin transport.

Microneedle mediated transdermal delivery is expected to significantly enhance the permeation of macromolecules (Prausnitz, 2004). The microneedles used in this study are made up of maltose, a GRAS substance. Characterization of maltose microneedles with respect to their length, pore uniformity, length and diameter of the microchannel created, etc., has been done and reported by us previously. These needles are believed to go about 160 μm deep, effective enough to disrupt the stratum corneum and enter the epidermis without stimulating any nerve endings present in the dermis (Kolli and Banga, 2008). Transcutaneous electrical resistance measurements made by our lab previously have shown that the electrical resistance of hairless rat skin decreased about three times relative to normal skin following treatment with maltose microneedles (Lanke et al., 2009).

Several combination strategies to further improve the transport of molecules across the skin are currently being explored (Wang et al., 2005). Microneedles in conjunction with iontophoresis is one such promising combination with a potential to enhance the permeation of a wide variety of molecules (Lin et al., 2001; Wu et al., 2007). Iontophoretic transport mechanisms (electromigration and electroosmosis) are different across intact skin and impaired skin (Abla et al., 2005a). It was reported (Abla et al., 2005a) and also demonstrated in the current study that complete removal of stratum corneum by tape stripping abolishes electroosmosis, thus impairing the permselective properties of the skin. Microneedles, upon insertion into the skin, breach the stratum corneum to create microscopic channels in the skin so as to ease transit of molecules across the skin (Kolli and Banga, 2008). The effect of microporation on electroosmosis and thus, on skin permselectivity was evaluated in this study. The ACM flux across microneedle pretreated skin with iontophoresis was about seven times higher compared to the flux across microporated skin with no iontophoresis, thereby suggesting the presence of electroosmosis upon microporation. This finding is consistent with another report from the literature wherein the electroosmotic flow was found to exist upon microporation (Wu et al., 2007). It was also reported that localized barrier perturbation, where the treated area is very small relative to the total area of current application, might maintain the permselective properties of the skin (Abla et al., 2005a). Based on the diameter of the microchannels created (~55 μm), the microneedles used in this study would disrupt only about 0.6% of the total exposed area and hence, skin tends to retain its permselective properties (Kolli and Banga, 2008).

The combination of microneedles and iontophoresis always delivered higher amounts of DP compared to iontophoresis alone. Iontophoretic delivery is sensitive to the properties of the permeant and thus, the combination yielded different results with respect to different transport mechanisms dominating the two formulations. There was no significant difference between the amounts of DP delivered by the combination approach at pH 7.5 under cathode (right electrode) and anode (incorrect polarity of electrode). There was iontophoretic delivery seen for DP under anode at pH 7.5, however there was no great enhancement seen following microporation. On the contrary, at pH 4.0, there was almost 30-fold enhancement seen in the amounts of DP that permeated with iontophoresis and microneedle combination. Earlier in the discussion, it was concluded that electroosmosis is the predominant mechanism of transport at pH 7.5 and electromigration is the major mechanism at pH 4.0. This difference in iontophoretic transport mechanisms at pH 7.5 and pH 4.0 might explain the differences seen in the amounts of DP that permeated with the combination approach. Electroosmotic flow is seen as a bulk motion of the solvent that carries ions or neutral molecules with the solvent stream (Pikal, 2001). Electroosmotic flow is believed to occur through pores and the effective mean diameter of electrically conductive pathways within hairless rat skin was calculated to be approximately 36 Å (Ruddy and Hadzija, 1992; Pikal, 2001). It was shown in the current study that microporation does not impair electroosmosis and the presence of microchannels as a result of microporation might have further enhanced the electroosmotic flow passively. However, an increase in DP permeation was not seen probably because there was no driving force to push the molecule through these microchannels. Electroosmosis is a solvent flow seen from anode to cathode with no exertion of any kind of force on the molecule unlike electrorepulsion wherein the repulsive force drives the ions across the

skin. In the presence of microchannels, the effect of this additional force is magnified as a result of which the combination of iontophoresis and microneedles increased the permeation of DP by 30-fold at pH 4.0.

5. Conclusion

This work demonstrates the *in vitro* iontophoretic delivery of a 13 kD protein across hairless rat skin. The molecular charge present on the protein was shown to dictate the type of transport mechanism acting during iontophoresis. Pretreatment with microneedles did not impair electroosmosis and thus, the electrical properties of the skin were retained. Iontophoresis across microporated skin further enhanced the permeation of DP. The findings of this study suggest that combination of microneedles and iontophoresis saw maximum permeation of DP when electrorepulsion was the major transport mechanism. This finding might help in regulating the delivery of macromolecules across microneedle pretreated skin when combined with iontophoresis.

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