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Modulated iontophoretic delivery of small and large molecules through microchannels

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ABSTRACT

The objective of this work was to modulate transdermal drug delivery by iontophoresis though skin microchannels created by microneedles. Calcein and human growth hormone were used as a model small and large molecule, respectively. In vitro permeation studies were performed on porcine ear skin under three different settings: (a) modulated iontophoresis alone, (b) pretreatment with microneedles and (c) combination of microneedles pretreatment and modulated iontophoresis. For modulated iontophoresis, 0.5 mA/cm^2 current was applied for 1 h each at 2nd and 6th hour of the study. Methylene blue staining, calcein imaging and pore permeability index suggested maltose microneedles created uniform microchannels in skin. Application of iontophoresis provided two peaks in flux of 1.04 μg/(cm^2 h) at 4th hour and 2.09 μg/(cm^2 h) at 8th hour of study for calcein. These peaks in flux were significant higher when skin was pretreated with microneedles ($p < 0.05$). Similarly, for human growth hormone, modulation in transdermal flux was achieved with combination of microneedles and iontophoresis. This combination also provided significant increase in cumulative amount of calcein and human growth hormone delivered as compared to microneedles or iontophoresis alone ($p < 0.05$). Therefore, iontophoresis can be used to modulate drug delivery across skin microchannels created by microneedles.

1. Introduction

The skin is largest and one of the easily accessible organ of human body. It provides a potential site for topical or transdermal administration of wide range of drugs. However, passive permeation of drugs across the skin is limited to small and moderately lipophilic molecules. For large and hydrophilic drug molecules, various passive and active enhancement strategies such as chemical enhancers, iontophoresis, ultrasound, electroporation, microneedles and microdermabrasion are being studied to enhance their transdermal permeation (Banga, 2011). Microneedles and iontophoresis are two such active enhancement techniques that have been widely used to deliver number of drug molecules transdermally (Badkar et al., 2007; Guy, 2010; Katikaneni et al., 2011; Prausnitz and Langer, 2008; Vemulapalli et al., 2011).

Iontophoresis involves the use of small amount of physiological current to deliver charged and neutral drug molecules into and across the skin. The mechanism of iontophoresis is based on the "like repels like" principle where a positively charged drug molecule is repelled into the skin by placing an anode on the skin while a cathode is used to deliver negatively charged drug molecule across the skin. Iontophoresis can provide rapid delivery of drug molecules both locally and systemically. This technique has been proven to be effective in delivering ionized and hydrophilic drug molecules through the skin, which could not be delivered passively earlier. This technique has been widely studied both by pharmaceutical scientists and physical therapy professionals to deliver a wide range of drug molecules via skin or nails (Chaturvedula et al., 2005; Costello and Jeske, 1995; Dubey and Kalia, 2011; Dutet and Delgado-Charro, 2010; Kigasawa et al., 2011; Takasuga et al., 2011; Vemulapalli et al., 2011). Iontophoresis has been used extensively for broad range of applications such as treatment of hyperhidrosis, topical anesthesia, diabetes diagnosis, etc. (Annaswamy and Morchower, 2011; Chia et al., 2011; Dixit et al., 2007; Ozaki et al., 2011; Sieg et al., 2004).

As the amount of drug delivered through the skin using iontophoresis is proportional to the current strength and duration of current application, this approach can provide programmable and controlled drug delivery to obtain therapeutic levels. By controlling the current parameters a drug delivery profile can be modulated for an indication as well as dose can be titrated for a patient. Some of the marketed iontophoresis devices include: Dupel® (Empi, Inc., St Paul, MN, USA), Phoresor® II (Iomed, Salt Lake City, UT, USA), Iontophor-PM/DX (Life Tech, Inc., Houston, TX, USA) and Drionic® (General Medical Co, Los Angles, CA, USA). One of the key advantages of an iontophoresis device is the ability to program a dose and an "on-demand" button allowing the patient to administer the dose when needed. The Iontophor-PM/DX is a

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fully programmable device that allows users to enter and store personalized protocols. Iontophoresis has been largely successful in delivery of small and hydrophilic drug molecules but its usage has been limited to deliver macromolecules such as proteins of size larger than about 13 kDa (Banga, 2011). It has also been suggested that smaller and more hydrophilic ions travel faster than larger ions. This limitation has led to investigation of this technique in combination with other enhancement methods such as microporation/electroporation, ultrasound and use of chemical enhancers (Boinpally et al., 2004; Katikaneni et al., 2010; Pillai and Panchagnula, 2003; Rastogi et al., 2010; Sugibayashi et al., 2000).

Microneedles are micron-sized needles, which upon insertion in the skin form microchannels that serve as aqueous pathways for delivery of wide range of therapeutic molecules including proteins. Microneedles, being micron-sized, do not reach the nerve endings in the dermis, and are thus painless and minimally invasive (Bal et al., 2008; Birchall et al., 2011; Gill et al., 2008; Gupta et al., 2011; Mikolajewska et al., 2010). There is no size limit for delivery of macromolecules through these microchannels, as the size of these microchannels is in microns and maximum dimension of a typical macromolecules administered into body are in nanometers (Banga, 2011). There are minimal chances of irritation and infection caused by these needles (Donnelly et al., 2009) and they offer a patient friendly route of drug administration compared to hypodermic needles. A broad range of therapeutic molecules including macromolecules such as proteins (Donnelly et al., 2010; Kumar et al., 2011; Song et al., 2010) have been delivered using this technology and it is being widely studied by both academic institutions and industry (Burton et al., 2011; Harvey et al., 2011; Pettis et al., 2011; Roxhed et al., 2008). Recently Intanza® (Sanofi Pasteur), a microneedle based influenza vaccine has been approved by FDA; it delivers the vaccine to dermis which is one of the highly immunogenic site, thereby eliciting a strong immune response.

To the best of our knowledge, this is the first reported study on combination of "modulated" iontophoresis with microneedles for transdermal drug delivery. The main aim of this work was to modulate the delivery of small and large model molecules by combination of iontophoresis and microneedles. Iontophoresis can drive molecules across skin microchannels which cannot otherwise be delivered by iontophoresis alone (typically, those larger than about 13 kDa). The effect of current density and duration of application were also investigated. In this study, calcein was used as a model of hydrophilic and small molecular weight drug. Calcein is widely used in literature as model for transdermal transport studies because of its fluorescence, size (623 Da) and electrical charge ($z = -4$) (Bahia et al., 2010; Park et al., 2005; Pliquett et al., 1996; Ueda et al., 2009; Xie et al., 2005). Human growth hormone (hGH) was used as a model protein. It has molecular weight of around 22 kDa and an isoelectric point of 4.9 (Li, 1982). This protein was chosen primarily for its physiochemical properties, as passive transdermal permeation of such macromolecule is not possible. There are many studies reported to enhance transdermal delivery of hGH using physical means such as utilizing microneedles, thermal ablation, etc. (Ito et al., 2008; Lee et al., 2011; Levin et al., 2005). In this study, hGH was delivered across the skin microchannels created by microneedles and iontophoresis was used in combination to modulate this delivery.

2. Materials and methods

2.1. Materials

Maltose microneedles were developed and supplied by Elegaphy, Inc. (Otsu, Japan). Calcein (Fluoresoft-0.35%) for imaging studies was obtained from Holles Laboratories, Inc. (Cohasset, MA, USA). Methylene blue dye used in micropore characterization was obtained from Eastman Kodak Co. (Rochester, NY, USA). Human growth hormone was received as a gift from Pfizer, Inc. (Chesterfield, MO, USA). Silver wire (0.5 mm diameter), silver chloride used for the preparation of electrodes and calcein for permeation studies were purchased from Sigma–Aldrich (St. Louis, MO, USA), Iontophoresis power supply unit (Model 2400 SourceMeter) was purchased from Keithley Instruments (Cleveland, OH, USA). Full thickness skin was isolated from pig ears supplied from Pelfreeze Biologicals (Rogers, AR, USA), human growth hormone ELISA kits were purchased from DRG International, Inc. (Mountainside, NJ, USA).

2.2. Methods

2.2.1. Characterization of maltose microneedles

The maltose microneedles used in this study have been previously imaged using a scanning electron microscope by our group (Kolli and Banga, 2008). To characterize the pores created by these microneedles methylene blue staining and calcein imaging were performed. Full thickness pig ear skin was treated with maltose microneedles and stained with 1% (w/v) methylene blue dye solution for visualization of the microchannels. Excess dye was removed with alcohol swabs and images were taken using a stereomicroscope (Hi-Scope KH2200, Hirox Co., Japan). These pores were further characterized for pores uniformity by determining the relative skin permeability of calcein through these micropores. The procedure employed for calcein imaging has been reported elsewhere (Kolli and Banga, 2008). Briefly, calcein solution was applied to full thickness pig ear skin treated with maltose microneedles for one min. The excess dye was wiped off with alcohol swabs. A fluorescent image was taken using a digital camera (Canon, Taiwan) attached with a macro lens and a fluorescence filter. These images were analyzed using Fluoropore software, which gave a value termed the pore permeability index (PPI), a number representative of the calcein flux into each pore. The pores created by these microneedles were also observed by Hitachi S-3700N variable pressure scanning electron microscope (VP-SEM) at accelerating voltage of 15 kV and low vacuum of 50 Pa.

2.2.2. Iontophoresis protocol

For all iontophoresis experiments, anode and cathode were prepared in house. A planar coil of silver wire was prepared manually and used as anode. The cathode was custom made by coating a melt of silver chloride on a fine silver wire. The coating procedure was continued until a uniform and sufficient coat of silver chloride was obtained. Current was supplied using Keithley 2400 source meter. The cathode was placed in the donor chamber and the anode was inserted into the receptor compartment through the sampling arm to perform cathodal iontophoresis. A constant direct current (DC) of 0.5 mA/cm^2 was applied for 1 h. This iontophoresis protocol was used for calcein and hGH permeation studies.

2.2.3. Histology of skin

The purpose of this study was to verify if iontophoresis caused any structural changes in the skin. Full thickness porcine ear skin was mounted on vertical static Franz diffusion cells. The receptor compartment was filled with phosphate buffer (pH 7.0) and donor chamber was filled with 500 μL phosphate buffer solution. Cathodal iontophoresis was performed on the mounted skin as per the iontophoresis protocol mentioned earlier. Following iontophoresis, skin was embedded in OCT medium and stored overnight at −80 °C. The cryo-sectioning of frozen skin was done using a cryomicrotome to attain skin section of about 10-μm thicknesses. These sections

were then stained with hematoxylin and eosin (H&E) and images were observed using a microscope (Leica DM750).

2.2.4. Calcein permeation study

In vitro permeation studies were performed on full thickness pig ear skin using vertical Static Franz type diffusion cells (Permegear, Inc., Hellertown, PA, USA). The temperature of the receptor was maintained at 37 °C throughout the experiment. The study included three sets of experiment groups ($n \geq 3$ each): (a) only microneedle pretreated skin, (b) modulated iontophoresis alone and (c) combination of microneedle pretreatment and modulated iontophoresis. For microneedle treated skin, maltose microneedles were inserted in the skin for a minute and the treated skin was mounted on the receptor compartments (effective area of diffusion was 0.64 cm^2) with the epidermal surface facing the donor chamber. For iontophoresis only, intact full thickness skin was mounted on the receptor compartments. The donor chambers were then placed on the mounted skin and filled with 500 μL of 1 mM calcein solution. To maintain sink condition receptor compartment was filled with phosphate buffer pH 7.0. For iontophoresis only and combination treatment sets, cathodal iontophoresis was performed (calcein has charge of −4 at physiological pH). Direct current (DC) of 0.5 mA/cm^2 was applied at 2nd and 6th hour of the study for 1 h each (iontophoresis at 2nd–3rd and 6–7th hour). The current density of 0.5 mA/cm^2 was selected as it has been reported to be well tolerated in humans (Burnette and Ongpipattanakul, 1988). The duration of current application was 1 h as our group has previously studied that an hour of iontophoresis was sufficient to significantly increase drug delivery through the skin (Banga, 2011; Sachdeva et al., 2010). We have also reported that the flux recovers back to normal level in 2–3 h after iontophoresis (Conjeevaram et al., 2002). Thus we kept interim period of 3 h between two iontophoresis application. To study permeation of calcein, samples (0.3 mL) were withdrawn from the receptor compartment at predetermined time points and replenished with the same volume of fresh receptor buffer. Samples obtained were protected from light and were analyzed using Synergy HT microplate reader (BioTek, Winooski, VT, USA) at 480 nm excitation and 520 nm emission wavelength.

2.2.5. Human growth hormone permeation study

In vitro permeation study design was similar to calcein permeation study. However, hGH being a macromolecule cannot be delivered by iontophoresis alone, and thus this permeation study included only two treatment sets ($n \geq 3$ each), microneedle pretreated skin and combination of microneedle and modulated iontophoresis. Full thickness pig ear skin was treated with maltose microneedles and mounted on the receptor compartment as described earlier. The donor compartment was placed and filled with 500 μL of 10 mg/mL hGH formulation. As hGH has an isoelectric point of 4.9, it is negatively charged at physiological pH. Therefore, cathodal iontophoresis was performed in combination treatment group. A constant current power supply of 0.5 mA/cm^2 was applied at 2nd and 6th hour of the study for 1 h each. Samples (0.3 mL) were withdrawn from the receptor compartment at predetermined time points and replenished with the same volume of fresh receptor buffer. Samples obtained were analyzed using enzyme linked sorbent assay (ELISA) quantitation kit. Briefly, the kit contained microtiter wells coated with immobilized sheep anti-hGH antibody. The wells were incubated with 50 μL of test samples and 100 μL of enzyme conjugate reagent solution (mouse monoclonal anti-hGH antibody in horseradish peroxidase) for 45 min at room temperature. This allowed the test samples to react with antibodies resulting in hGH molecules being sandwiched between the solid phase and enzyme-linked antibodies. After incubation, the wells were washed with distilled water to remove unbound antibodies and incubated with 100 μL of TMB reagent solution in dark for 20 min, resulting in development of blue color. The color development was stopped with addition of 100 μL of stop solution and optical density was measured at 450 nm using microtiter plate reader.

2.2.6. Variation of current strength and duration

The objective of this experiment was to assess the calcein flux across full thickness skin by utilizing modulated iontophoresis of varied current density and time in combination with microneedles. The experimental set up consisted of two sets of experiments, modulated iontophoresis alone and combination of modulated iontophoresis with microneedles. For modulated iontophoresis, a current density of 0.8 mA/cm^2 was applied for 15 min at 1st hour followed by current of 0.4 mA/cm^2 at 5th hour of study for 2 h (5–7th hour). The rest of experimental set up was similar to previous study. Samples were withdrawn at predetermined time intervals and amount of calcein permeated into receptor compartment was measured.

2.3. Statistical analysis

Student's *t*-test and analysis of variance (ANOVA) was used to determine the statistical significance of data obtained from experiments. Mean of replicate measurements ($n = 4$) with corresponding standard error (SE) was used to plot each graph.

3. Results and discussion

Iontophoresis and microneedles are two enhancement methods for transdermal delivery of hydrophilic molecules and macromolecules. Iontophoresis has been utilized to increase the permeation of several ionized drug molecules with poor absorption/permeation through skin. It also allows opportunity for controlled and programmed drug delivery, by altering the electrical parameters such as current and time. However, actual range of drug molecules this technique can deliver is limited. Therefore, a combined use of iontophoresis with other physical or chemical enhancement methods has been studied. In this study, we have investigated the possibility of combined use of iontophoresis with microneedles for modulated delivery of model molecules calcein and human growth hormone through porcine ear skin. We have used full thickness porcine ear skin for our experiments as it has been widely used as an in vitro model for human skin (Herkenne et al., 2006; Jacobi et al., 2007; Kong and Bhargava, 2011; Luzardo-Alvarez et al., 1998; Nicoli et al., 2010; Sekkat et al., 2002). Also, it has been reported in literature that some properties of pig ear skin such as, epidermal thickness, composition, lipid content and general morphology, are comparable to human skin (Sekkat et al., 2002; Simon and Maibach, 2000).

3.1. Characterization of maltose microneedles

The microneedles used in this study were made up of maltose, a carbohydrate and a GRAS substance. Maltose microneedles, upon insertion into skin, dissolved creating micron sized pores. The length of the microneedles used in this study was 500 μm and each array consisted of three stacked layers of microneedles; each layer has 27 microneedles. Microchannels created by these microneedles were visualized by methylene blue staining as the area of the skin breached by microneedles took up the methylene blue dye whereas the rest of the skin remained impermeable. Fig. 1(A) is a picture of full thickness pig ear skin stained with methylene blue following treatment with three-layered microneedles. Almost all of the microneedles successfully created microchannels and uniformity of these microchannels was characterized by calcein imaging studies. Fig. 1(B) shows the full thickness skin treated with

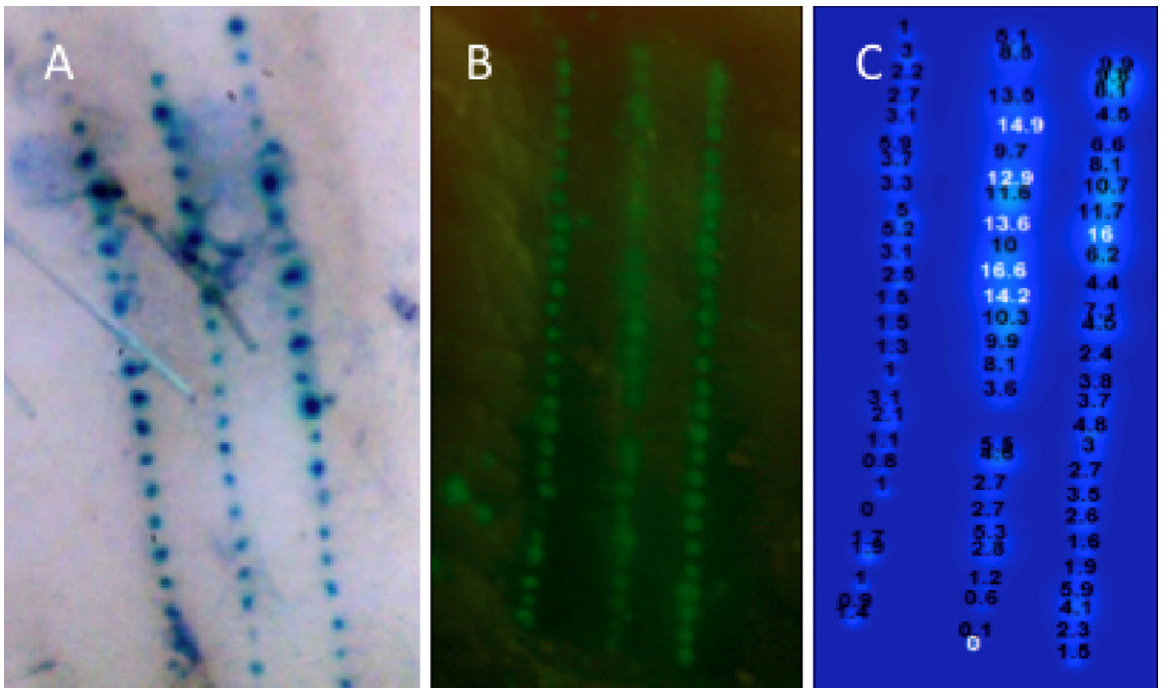

Fig. 1. Visualization of microchannels created by maltose microneedles. (A) Stereoimicroscope image showing three rows of microchannesl stained by methylene blue. (B) Fluorescent image of microchannesl after exposure to calcein. (C) These pores were assigned a value called the pore permeability index (PPI) representing the amount of calcein diffused through each pore.

maltose microneedles followed by exposure to calcein. This image was further processed by Fluoropore software, which assigned the PPI value [Fig. 1(C)] for each microchannel, representing the relative amount of calcein that diffused through each microchannel. Fig. 2 shows the histogram of PPI distribution with a mean value of 5.1 and standard deviation of 4.22 for all microchannels. This histogram indicated PPI distribution was narrow and the microchannels created were uniform. These results are supported by scanning electron microscope image of microporated skin. The surface area of a microchannel created by microneedles was about 2000 μm^2 (Fig. 3). Therefore one array of microneedles would disrupt approximately an area of 0.162 mm^2, which would be approximately 0.25% of total exposed area of skin. We have extensively characterized these maltose microneedles in past and assessed their ability to enhance drug transport across rat skin. We have studied the dimensions and geometry of these microneedles by scanning electron microscopy. The radius of tip was calculated to be 3 μm, indicating microneedles were sharp enough to penetrate the stratum corneum (Kolli and Banga, 2008). Another important issue to consider while working with microneedles is closure of the microchannels following microporation. If the microchannels close during the drug application, outcome may be sub-therapeutic levels in body or if pores remain open for long, it may cause complications such as infection or contamination. We have reported

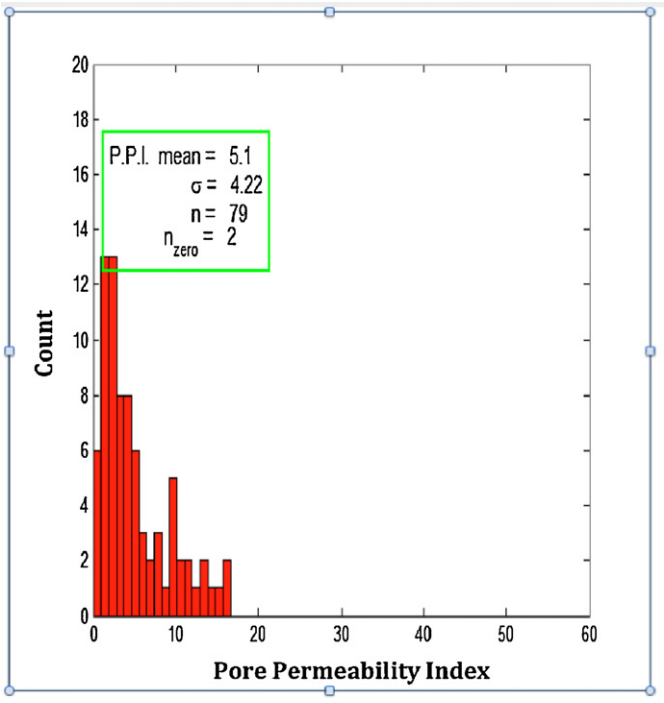

Fig. 2. Histogram showing the distribution of the pore permeability index (PPI) with respect to number of microchannels; the mean PPI was found to be 5.1 which indicate the average calcein flux through the microchannels.

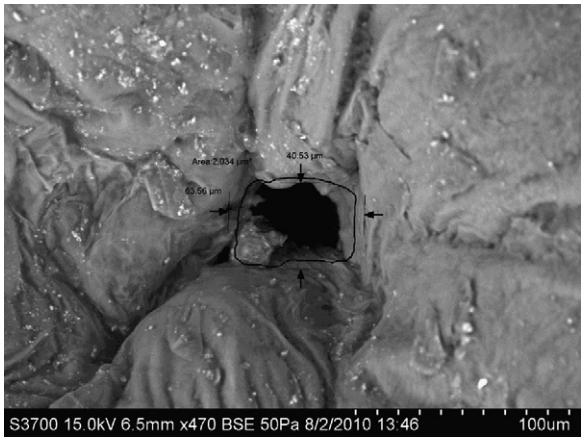

Fig. 3. Scanning electron microscope (SEM) image of a pore created by maltose microneedles on porcine ear skin.

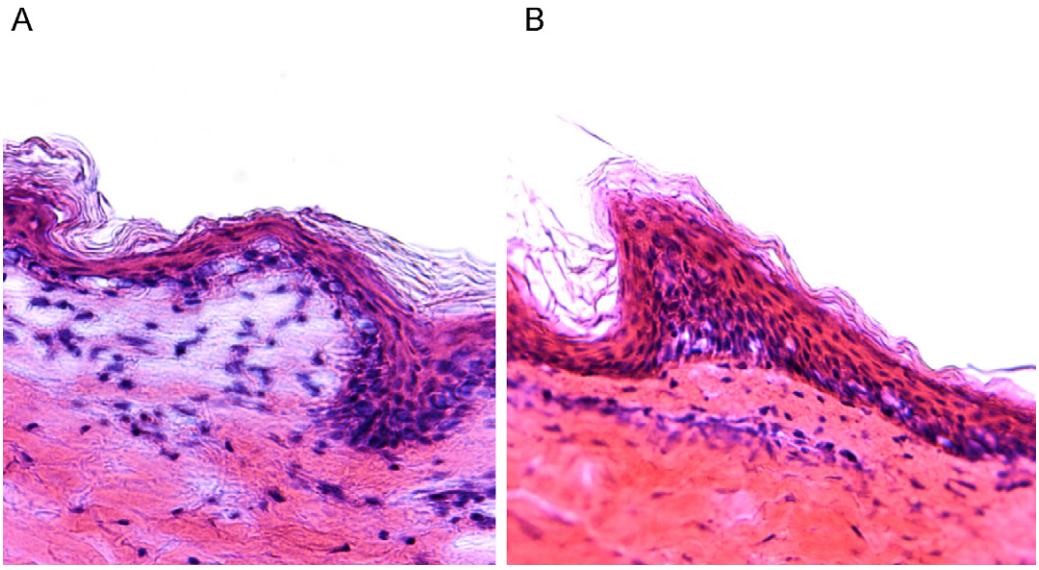

Fig. 4. Histology of porcine ear skin after H&E staining. (A) Control: untreated skin sample. (B) Skin sample post-iontophoresis (current density of 0.5 mA/cm^2 for 1 h). Epidermis had uniform thickness without any visible disruption. No morphological difference was observed between control and iontophoresis treated skin.

using in vivo studies that microchannels created by these maltose microneedles close within 15 h, if open to environment. However, if pores were occluded with a solution such as PBS buffer, the microchannels remain open for up to 72 h (Kalluri and Banga, 2011). These findings suggest that the microchannels allow drug delivery while drug solution is applied and pores start to close once solution is removed. We have also demonstrated earlier that these maltose microneedles are able to deliver large model molecules such as IgG (MW = 19.27 kDa) and monoclonal antibodies (MW = 150 kDa).

3.2. Skin histology

Iontophoresis is a non-invasive active transdermal drug delivery technology used to increase delivery of drug across the skin. The microscopic examination of skin sections after hematoxylin and eosin staining indicated no significant difference between iontophoresis treated and controlled skin. Fig. 4(A) shows control skin (no iontophoresis treatment) and Fig. 4(B) refers to iontophoresis treated skin section. In both sections intact stratum corneum (SC) can be seen along with other layers of epidermis. The partial detachments of stratum corneum at some places can be observed in iontophoresis treated as well as in control and is a common histology artifact. No visible difference in dermis was observed in control and iontophoresis treated skin. Overall, both layers (epidermis and dermis) of skin and appendages (hair follicles and sebaceous glands) did not show any alteration in shape or morphological damage by iontophoresis. Pacini et al. (2006) have reported similar results on living rat skin following iontophoresis. Lee et al. (1998) observed some separation of lipid bilayers in stratum corneum after iontophoresis but they attribute these change in SC intercellular structure to hydration and occlusion during experiment. Thus histological examination of iontophoresis treated skin did not reveal any sign of tissue or cell damage.

3.3. Calcein permeation study

We studied calcein transport across microporated full thickness skin, intact skin with iontophoresis and microporated skin in combination with iontophoresis (Figs. 5 and 6). Calcein is a small molecular weight (622.5 Da) compound and negatively charged at

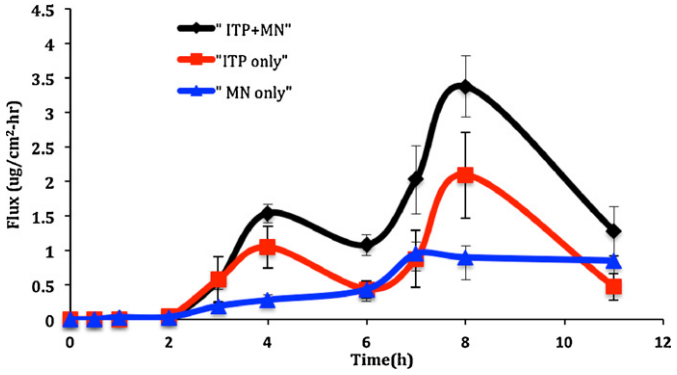

Fig. 5. Flux of calcein across full thickness pig ear skin with modulated iontophoresis alone, microneedle only or both in combination, iontophoresis current density of 0.5 mA/cm^2 was applied for 1 h at time 2 h and 6 h (mean ± SE, n = 4).

physiological pH. Because of the stratum corneum's hydrophobic character, transdermal delivery of hydrophilic drug is challenging. Therefore calcein a hydrophilic and highly charged molecule was selected as a model small drug in this study. Fig. 5 shows

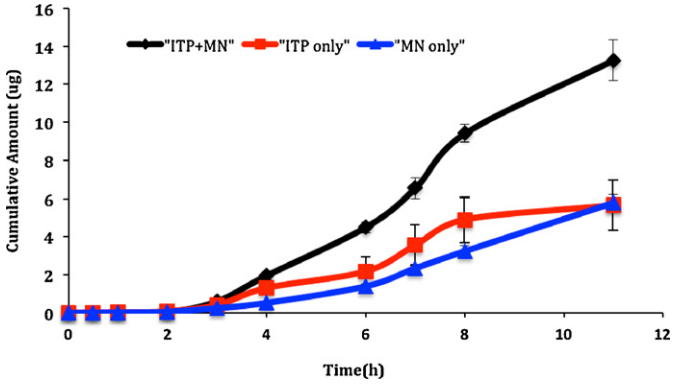

Fig. 6. Cumulative amount of calcein delivered through full thickness pig ear skin with modulated iontophoresis alone, microneedle only or both in combination, iontophoresis current density of 0.5 mA/cm^2 was applied for 1 h at time 2 h and 6 h (mean ± SE, n = 4).

flux of calcein across full thickness porcine skin for all the conditions tested. There was no measurable permeation of calcein in initial 2 h under all three conditions. In microporated skin, the flux was found to increase slowly from 2nd hour till 8th hour. The initial lag period of 2 h may be the time taken by calcein to permeate through the microchannels into the skin and further in the receptor chamber. In case of iontophoresis alone, calcein being a hydrophilic compound was unable to permeate through intact skin till the current was applied (at 2nd hour of the study). Similar observation has been reported by Turner and Guy (1997) who investigated the impact of physicochemical properties of molecule on efficiency of iontophoretic transport across the skin using calcein as a model molecule. They also observed negligible passive delivery of calcein into the skin and enhanced delivery under cathodal iontophoresis. At pH 7.4, calcein has molecular charge (−4.0) and we observed enhanced calcein permeation under cathode (negative electrode). This suggests electorepulsion is the major transport mechanism responsible for calcein transport under iontophoresis. As seen in Fig. 5, there was a significant flux when iontophoresis was applied followed by gradual decrease in flux once the current was turned off. However, it should be noted that flux does not recover completely once iontophoresis was terminated, this support earlier observation by Turner et al. that pretreatment of skin with current cause subsequent increase in passive permeation of calcein. Thus, it is believed that the permeability barrier of the skin is compromised for a significant period following iontophoresis. The modulated iontophoresis protocol resulted in two peaks in flux of 1.29 μg/(cm^2 h) at 4th hour and 2.09 μg/(cm^2 h) at 8th hour of study. This shows that delivery can be planned and controlled by repeating the iontophoresis protocol at different time intervals. Therefore, this approach can be utilized to achieve the delivery profile of a drug that needs variation in therapeutic concentrations at different time period. Further, same iontophoresis protocol was applied on skin pretreated with microneedles. A significant higher flux, higher than that achieved by iontophoresis alone, is attained when current was applied and declined after termination of iontophoresis. However, the flux value after termination of iontophoresis was still significantly higher than that obtained by iontophoresis alone or microneedle treatment alone ($p < 0.05$). The peak value of flux obtained at 4th hour and 8th hour using the combination of modulated iontophoresis and microporation was significantly higher than that achieved by modulated iontophoresis alone ($p < 0.05$). This combination provided highest flux that was almost two times higher than that achieved by iontophoresis alone and four times higher than steady flux achieved by microneedles alone.

Fig. 6 shows the comparison of corresponding cumulative amount of calcein delivered by three experimental conditions. The cumulative amount of calcein delivered by microneedle treatment in combination with modulated iontophoresis was higher than that delivered by either of the enhancement technique alone. This difference remains to be statistically significant after 4th hour of study ($p < 0.05$). As seen from Fig. 6, the cumulative amount of calcein delivered by combination of microneedle and iontophoresis in 11 h was more than two times higher than delivered by iontophoresis alone. Finding from Wu et al. supports these results, where they have demonstrated that microneedle pretreatment significantly increased iontophoretic transport of Fluorescein isothiocyanate dextrans. It was reported that post iontophoresis passive permeability was also enhanced with microneedles pretreatment (Wu et al., 2007). Microneedles, upon insertion into the skin create microchannels and the effect of electrorepulsive force is increased in presence of these microchannels. Thus, modulated iontophoresis in combination with microneedles allow for enhanced delivery of calcein and opportunity of delivery modulation by varying current parameters.

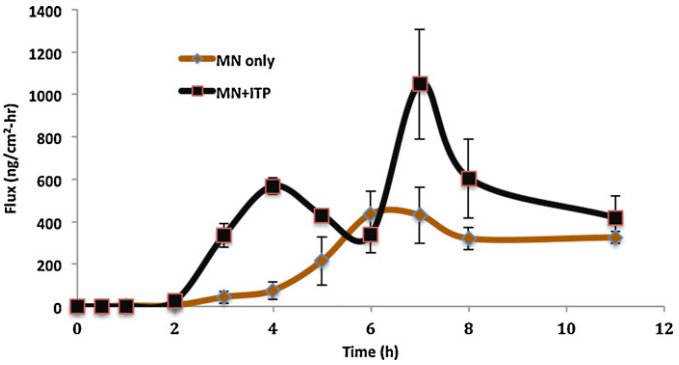

Fig. 7. Flux of hGH across full thickness pig ear skin with microneedle only or in combination with modulated iontophoresis, 0.5 mA/cm^2 current was applied for 1 h at time 2 h and 6 h (mean ± SE, $n = 4$).

3.4. Human growth hormone permeation study

Human growth hormone (hGH, somatotropin) is a single-chain polypeptide comprising 191 amino acids. It is synthesized, stored, and secreted by the anterior pituitary. hGH level vary throughout the day and night as pulsatile release of hGH occurs in 4–8 discrete bursts throughout day and night. The secretion profile is modulated through a complex neuroendocrine control system along with other physiological stimulators such as sleep, hypoglycemia and exercise. Usually, with the onset of deep sleep or after 15–20 min of vigorous exercise, growth hormone levels rise. Pharmaceutically, hGH is used to treat growth disorders and hormone deficiencies in both children and adults. Growth hormone is generally administered by intramuscular (IM) or subcutaneous (SC) injection. The ideal dosing schedule remains open to question; some authors propose single SC injection at night than morning injection, in an attempt to mimic the physiological diurnal rhythm (Toogood et al., 1997). However, this is far from physiological release profile and results in non-pulsatile GH profile. Therefore, modulated transdermal delivery of hGH may allow programmable release profile to mimic the circadian rhythm of the body leading to improved efficacy of externally supplied hGH. In this study, strategy of combined use of modulated iontophoresis and microneedles for programmable delivery of hGH was explored.

The hGH permeation studies were performed using microneedle alone and microneedles in combination with iontophoresis. In the past we have demonstrated that only iontophoresis cannot deliver interferon alpha 2b (MW = 19.271 kDa) through intact skin (Badkar et al., 2007). It is also well established that the molecular size limit for iontophoretic transport of molecules through intact skin is around 13 kDa. So in this study we have not studied permeation of hGH through iontophoresis alone. As seen in Fig. 7, there was a lag period of 2 h before any measurable amount of hGH could be detected in the receptor compartment. Delivery through microporated skin resulted in steady state flux of about 400 ng/(cm^2 h) after 7 h. Lee et al. have studied hGH delivery using a dissolvable microneedle patch in hairless rats and this treatment was well tolerated by rat skin (Lee et al., 2011). This supports the feasibility of microneedle technology for delivery of hGH. The combination of microneedle and modulated iontophoresis provided similar drug delivery profile as observed with calcein delivery. The hGH flux increased significantly when current was applied followed by steady decrease in flux once the current was stopped. The modulated iontophoresis protocol resulted in two peaks in hGH flux at 4th and 7th hours of study. Fig. 7 also shows that the flux recovered within 2 h and increased to slightly higher level when consecutive current was applied. Fig. 8 shows the cumulative amount of hGH delivered by combination of enhancement techniques was

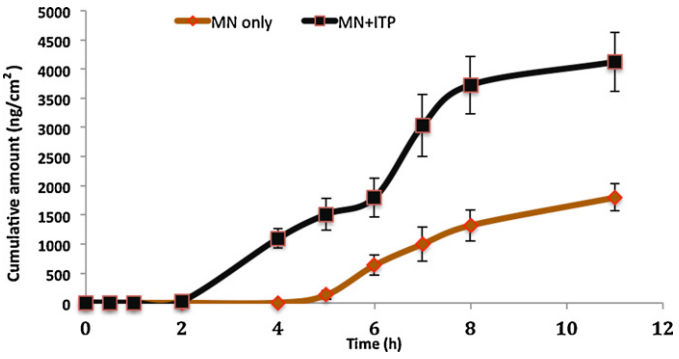

Fig. 8. Cumulative amount of hGH delivered through full thickness pig ear skin with microneedle only or in combination with modulated iontophoresis, 0.5 mA/cm^2 current was applied at 2nd and 6th hour for 1 h (mean ± SE, $n = 4$).

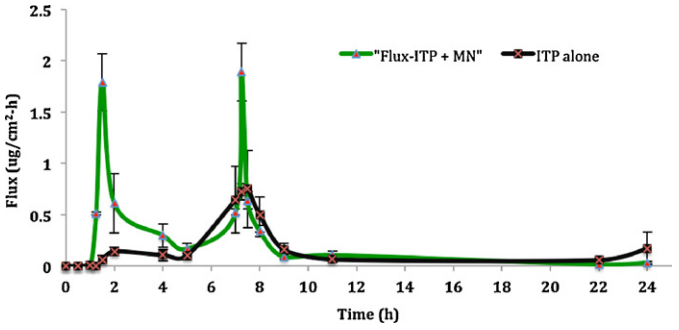

Fig. 9. Flux of calcein across full thickness pig ear skin with modulated iontophoresis only or in combination with microneedles, iontophoresis of 0.8 mA/cm^2 was applied at 1st hour for 15 min and 0.4 mA/cm^2 current was applied at 5th hour for 2 h (mean ± SE, $n = 4$).

significantly higher than delivered by microneedle alone ($p < 0.05$). Thus, the results indicate that delivery of hGH can be enhanced, controlled and programmed by adjusting iontophoresis protocol.

3.5. Variation of current strength and duration of current

This study was performed to study the effect of current density and the duration of current application. Fig. 9 shows the flux of calcein for both of the experimental sets. For modulated iontophoresis alone, there was a slight increase in flux of calcein when current of 0.8 mA/cm^2 was applied for 15 min and flux recovered partly following termination of current. However, a significant increase in flux was observed when 0.4 mA/cm^2 current was applied for 2 h and gradually recovered once current was terminated. While in case of microneedle pretreated skin, a sharp rise in flux was observed after application of 0.8 mA/cm^2 current for 15 min, the flux recovered and increased to a similar peak when current of 0.4 mA^2/h current was applied for 2 h. The peaks obtained using iontophoresis in combination with microneedle were statistically different than that achieved by iontophoresis alone ($p < 0.05$). The transdermal flux of calcein at different current densities is given in Table 1. It should be

Table 1
The in vitro transdermal delivery flux of calcein across pig ear skin.

Iontophoresis protocol	Maximum flux (μg/(cm^2 h))
Cathodal iontophoresis across intact skin	
0.8 mA/cm^2 for 15 min	0.143 ± 0.04
0.4 mA/cm^2 for 120 min	0.75 ± 0.36
Cathodal iontophoresis across microporated skin	
0.8 mA/cm^2 for 15 min	1.79 ± 0.27
0.4 mA/cm^2 for 120 min	1.89 ± 0.28

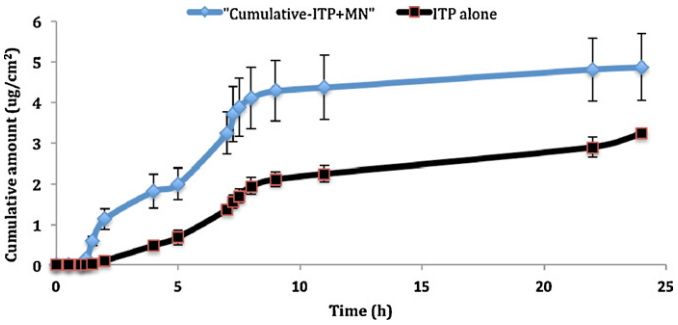

Fig. 10. Cumulative amount of calcein delivered through full thickness pig ear skin with modulated iontophoresis only or in combination with microneedles, iontophoresis of 0.8 mA/cm^2 was applied at 1st hour for 15 min and 0.4 mA/cm^2 current was applied at 5th hour for 2 h (mean ± SE, $n = 4$).

noted that high current density of iontophoresis for short duration failed to provide a sharp peak in flux on intact skin while same current protocol provided a sharp peak in flux with microporated skin, perhaps because small driving force was enough for large amount of calcein to diffuse through aqueous microchannels. For intact skin, once the duration of current was extended to 2 h, low current density was sufficient enough to transport calcein through preexisting aqueous pathways such as sweat ducts and hair follicles. Another reason can be non-linear transport of charged molecule across skin because of diverse nature of skin as reported by Pliquett et al. They propose that aqueous pathways created by application of higher current, have very non-linear transport behavior depending on their size. Several of the newly created pathways are smaller than preexisting ones (hair follicle and sweat ducts) and transport of calcein is more obstructed and transport rate per pathways is smaller than preexisting pathways (Pliquett et al., 2000). However, in this case no concluding remarks can be made regarding linearity or non-linearity relation between flux and applied current density, as time of application was not equal. Interestingly, for combination of iontophoresis and microneedle, the peak in flux obtained at high current density-short duration was not statistically different than that achieved at low current-longer duration. This effect can be attributed to the fact that microchannels provided major pathway for calcein transport and amount of calcein permeated was not merely influenced by electric driving force. The cumulative amount of calcein delivered by iontophoresis alone and iontophoresis in combination with microneedles is shown in Fig. 10. The difference is statistically significant ($p < 0.05$) at all time points. However, for microporated skin, the amount of calcein delivered in initial 5 h was about the same as amount delivered in next 5 h (5–10th hour). This suggests that small duration of high current density iontophoresis delivered same amount of drug across microporated skin as longer duration of low current density iontophoresis. Therefore, either high current density or longer duration current can be used to achieve the same dose.

4. Conclusions

In this study, maltose microneedles were characterized and used to microporate full thickness pig ear skin to evaluate drug delivery of a model small (calcein) and large molecule (human growth hormone). It was found that modulated transdermal delivery of small as well as large molecule is possible upon microporation of the skin in combination with iontophoresis. The modulated iontophoresis protocol resulted in peaks in flux with application of current and gradual decrease with termination of current. The flux obtained using same iontophoresis protocol on microneedle-pretreated skin was significantly higher than on intact skin. The iontophoresis protocol also delivered higher cumulative amount

of calcein across microporated skin than intact skin or only microporated skin. Therefore, transdermal delivery of small as well as macromolecules can be enhanced, controlled and modulated by using iontophoresis in combination with microneedle technology. This approach can be utilized to tailor the delivery profile of a drug that needs variation in therapeutic concentrations with time, and possibility to provide on demand personalized dosing for individual patients. We also observed that small duration of high current density or longer duration of small current density iontophoresis can be used to achieve the same dose. Thus, current density and time can be used appropriately to program a desired drug delivery profile.

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