

In vitro evaluation of the effect of electrotreatment on skin permeability

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Summary

Background Stratum corneum composition and structure limit cutaneous bioavailability of pharmaceutical and cosmeceutical agents. Electrically assisted transport can increase the rate and extent of delivery; moreover, it also enables the administration of polar and charged molecules into the skin.

Aims The objective of this study was to investigate the effect of electrotreatment on skin permeability by measuring the cumulative delivery of caffeine and sodium ascorbyl phosphate. Furthermore, confocal microscopy was used to visualize the effect of electrotreatment on the penetration of calcein.

Methods Porcine ear skin was used for the *in vitro* permeation studies, which involved application of either the caffeine or sodium ascorbyl phosphate (NAP) containing gels using the roll-on supplied with the electrotreatment device.

Results Electrotreatment increased the amount of caffeine and NAP in the skin. Enhancement factors (EF) for NAP of 7.2 and 14.9 were observed following 20 min of electrotreatment and either immediate sampling or a further 60 min of passive diffusion compared with passive diffusion for either 20 or 80 min. The effect on caffeine permeation was less significant (EF = 2.1 for 20 min electrotreatment compared with passive diffusion for 20 min). The confocal microscopy images showed that electrotreatment significantly increased calcein permeation; fluorescence was observed deep into the viable epidermis – reaching depths of up to 60 to 80 microns.

Conclusions We have shown that electrotreatment increases skin permeability and the cumulative delivery of cosmeceuticals into the skin.

Keywords: caffeine, confocal microscopy, cutaneous bioavailability, electrotreatment, iontophoresis, sodium ascorbyl phosphate

Introduction

The excellent barrier function of the lipidic stratum corneum limits the entry of polar and charged molecules

into the skin.¹ In recent years, many different methods have been explored to reversibly impair skin barrier function including the application of electric fields. Examples of electrically assisted delivery (or electrotransport) techniques include electroporation and iontophoresis.^{2–5} The latter involves the application of a mild electric potential (typically ~10 V) to enable the controlled delivery of polar and charged molecules into and across the skin. There are two principal iontophoretic transport mechanisms: (i) electromigration, which is

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particularly important for the delivery of ions and depends on electric mobility^{5,6} and (ii) electroosmosis, an electrically induced solvent flow from the anode to the cathode (under physiological conditions) that can increase cation transport and is responsible for the passage of neutral molecules.⁷ The amount of drug (or cosmeceutical) delivered depends on the current intensity, the duration, and the area of current application. Of course, the physicochemical properties of the molecule also play a key role.^{6,8} Iontophoresis is particularly well suited to the treatment of dermatological conditions because it enables targeted delivery to the desired site and local action requires the administration of only small amounts of substance.

The electrotreatment device used in these studies (**tmt** systemTM, Mesoestetic Laboratories, Barcelona, Spain) combines the use of a low voltage to generate a constant direct *iontophoretic* current across the skin with the application of high-frequency *electroporating* voltage pulses. These are complementary techniques because iontophoresis acts on charged and neutral molecules, increasing their mobility by electromigration and electroosmosis, whereas electroporation creates microchannels or pores in the skin that enhance passive diffusion through the impaired barrier. These transient transport pathways can persist and facilitate passive permeation even after terminating the application of the electroporating voltage.

The general aim of this study was to show the increased permeability of skin towards topically applied formulations following electrotreatment. Caffeine and sodium ascorbyl phosphate (NAP), a phosphoryl ester precursor of ascorbic acid, were used as model compounds. Laser scanning confocal microscopy (LSCM) was used to visualize the effect of electrotreatment on the penetration of the anionic fluorophore calcein.

Materials and methods

Chemicals

Caffeine, NAP, ascorbic acid, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, disodium hydrogen phosphate, tetra-*n*-butylammonium sulfate, and perchloric acid were purchased from Fluka (Saint Quentin Fallavier, France). Sodium chloride, silver wire, and silver chloride, used for the fabrication of electrodes, DABCO (1,4-diazabicyclo-[2,2,2]-octane), glycerol, acetonitrile (Acetonitrile Chromasolv[®] for HPLC, and methanol were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). All solutions

were prepared using deionized water (resistivity > 18 M Ω cm).

Skin

Porcine ears were obtained from a local abattoir (Société d'Exploitation d'Abatage STAC, Chambéry, France) within a few hours of sacrifice. Skin samples were dermatomed (~750 μ m) the same day and stored at -20 °C for a maximum period of 2 months.

Experimental protocol

Permeation studies

In vitro permeation studies employed glass vertical diffusion cells (Disa, Milan, Italy). Samples of porcine ear skin were thawed at room temperature and then cut into discs of 2 cm diameter. The skin was mounted on the (lower) receptor chamber and kept in place using Parafilm (Fig. 1). Receptor chambers were filled with 4 mL of phosphate-buffered saline solution (pH 7.4), prepared with 16.8 mM Na₂HPO₄, 1.4 mM KH₂PO₄, and 136.9 mM NaCl.

100 μ L of each Mesogel were applied to the skin:

- Gel 1-CAF (Anti-celluliteTM program), containing caffeine
- Gel 2-NAP (Anti-agingTM program), containing NAP

The principal constituents of the Mesogel base are water, 70%; propylene glycol, 10%; hydroxypropylmethylcellulose (methocel, 4M), 2.4%; to this is added either Gel 1-CAF or Gel 2-NAP. Gel 1-CAF contains caffeine, 4%; cynara scholimus extract, 2%; phosphatidylcholine, 0.7%; sodium benzoate, 4%; and sodium desoxycholate, 4% as adjuvants to solubilize caffeine and phosphatidylcholine, respectively. Gel 2-NAP contains vegetal protein hydrolysate, 10%; argireline, 3%; NAP, 3%; and sodium hyaluronate, 0.2%.

The permeation experiments were performed using the steel roll-on electrode supplied with the device as the active electrode. The formulations are applied *in vivo* using gentle massage of the steel roll-on electrode, which serves as the applicator, releasing the formulation onto the skin *and* the source of the electric potential to enable electrotransport. The hemispherical geometry of the steel roll-on facilitates formulation application and enables it to be easily spread across the skin surface to be treated. However, it was not possible to replicate the circular massage motion *in vitro* because this can create small tears in the skin samples once they are placed in the diffusion cells.

The roll-on was connected to the electrotreatment control unit and was kept in contact with the Mesogel (100 μ L) containing the active ingredient on the skin surface for 20 min. A silver/silver chloride electrode was



Figure 1 Experimental set-up for *in vitro* permeation studies. Gel 1-CAF and Gel 2-NAP (100 μ L) were applied directly to the skin surface. Subsequently, the steel roll-on was placed in contact for the current application period with the device set at the appropriate polarity; the electrical circuit was completed by placing a return electrode in the sampling arm of the Franz cell, and in contact with the receiver phase.

inserted into the receptor chamber and used as a counter electrode to complete the electrical circuit, allowing the passage of electrical current through the formulation and the skin (Fig. 1). The current was monitored throughout the experiments by a digital multimeter connected in series. The applied gel was removed after the electrical treatment, or kept in contact with the skin for a further 60 min, allowing post-treatment passive diffusion of the permeant. Table 1 summarizes the treatment protocols employed.

The maximum electrical output of the electrotreatment device is 50 V, and this is regulated in terms of percentage power intensity; the predetermined programs function as a “black box”, in that the settings are fixed and cannot be changed by the operator. Caffeine was applied using the pre-programmed device setting suggested for use with Gel 1-CAF; the intensity setting for use with

this formulation is 12% (~ 6 V). In order to ensure that NAP (from Gel 1-NAP) was applied using a continuous negative voltage, we used the free program option on the device; this enables the user to select the duration and intensity rather than have it predetermined by the device. Visual inspection showed that the 30% intensity (~ 15 V) could be applied without risking any damage to the skin *in vitro*. The duration and frequency of the pulses for caffeine were 160 microseconds and 1724 Hz, respectively, and for NAP, the corresponding values were 320 microseconds and 1200 Hz, respectively. Passive diffusion experiments using the same experimental setup, but in the absence of an electric potential, were performed as controls.

A sample of the receiver solution was withdrawn at the beginning and at the end of the experiments in order to evaluate the total amount of caffeine or NAP permeated in the receptor chamber. At the end of the experiment, the gel was removed, the cell was dismantled, and the skin was carefully washed, with an 80 : 20 mixture of distilled water and ethanol, to remove any residual formulation and then thoroughly dried with paper. Skin discs corresponding to the area of formulation application (0.6 cm^2) were cut from each sample and placed in pre-weighed plastic tubes, which were then weighed again to determine the amount of tissue. Then, they were extracted with the appropriate validated method to quantify the amount of active agent retained within the membrane.

Experiments were carried out with $4 \leq n \leq 8$ replicates.

LSCM experiments

The effect of electrotreatment on skin permeability was visualized using LSCM and calcein as a hydrophilic fluorescent probe. A 10 mM calcein solution was diluted 1 : 10 in the Mesogel (Anti-flaccidity™ program). The resulting fluorescent gel with a final calcein concentration of 1 mM (100 μ L) was applied on the epidermal surface of skin samples mounted in Franz diffusion cells as previously described. The steel roll-on active electrode was kept in contact with the gel, and a return silver electrode was inserted in the receptor chamber. As for the NAP experiments, the free program (level 5) and 30% maximum power were used to apply a negative potential for 20 min. A negative polarity was used because calcein is a polyanionic molecule (-4). Passive diffusion experiments, lasting 20 min, were performed as controls.

At the end of the experiments, the cell was dismantled, and the skin was carefully washed with a 20% ethanol solution and gently dried with paper. The skin was placed on a glass slide, covered by a mixture of 2.4 mg/mL DABCO in phosphate-buffered saline (pH 7.5) and

Table 1 Experimental conditions adopted for the electrotreatment experiments

Formulation	Program*	Polarity	Power†	Treatment duration (min)	Post-treatment passive permeation (min)	Control passive permeation (min)
Caffeine (Gel 1-CAF)	"Anti-cellulite"	+	12%‡	20	0	20
NAP (Gel 2-NAP)	"Free"	-	30%§	20	60	80
				20	0	20
				20	60	80

*Program and †Power refer to the device settings – the former selects the type of treatment and the latter refers to the intensity of the voltage that is applied; this is expressed as a percentage of the maximum power intensity.

‡Caffeine was applied using Gel 1-CAF; the device has a pre-programmed intensity setting for use with this formulation (12%).

§In order to ensure that the NAP (from Gel 1-NAP) was applied using a continuous negative voltage, we used the free program option on the device; this enables the user to select the duration and intensity rather than have it predetermined by the device. Visual inspection showed that the 30% intensity could be applied without risking any damage to the skin.

glycerol 50 : 50. A cover slide was applied. The permeation of the fluorescent probe as a function of depth within the skin was visualized using a Laser Scan Microscope 510 Meta (Carl Zeiss, Jena, Germany). The system was equipped with an argon lamp laser (with excitation lines at 488, 568, and 647 nm). Calcein excitation and emission wavelengths were 488 and 543 nm, respectively. The confocal images were obtained with an Achroplan 40×0.8 dry objective and analyzed using Zeiss LSM Image Browser software. The images represent the average of eight repeated scans of a small portion of skin optically sectioned in an XZ-plane.

Analytical methods

Skin extraction protocol – validation

The extraction methods for caffeine and NAP were validated in blank experiments and by spiking the skin samples with a known amount of drug. The HPLC system consisted of a 600E Controller Pump, a 717-plus Autosampler Injector and an In-line Degasser, a UV 2487 dual λ Detector (Waters, Saint-Quentin Yvelines, France).

Caffeine

Extraction of caffeine contained in the porcine ear skin discs was performed using 1 mL of a mixture comprising 85% perchloric acid 1 N solution and 15% acetonitrile for 1 h at a temperature of 50 °C. A Supelcosil™ LC-18 HPLC column (5 μ m particle size; 300×4.0 mm; Supelco, Saint Quentin Fallavier, France) was used for caffeine separation and an 85 : 15 mixture of water/acetonitrile as mobile phase; the flow rate was 1 mL/min. The UV-Detector was employed with a detection wavelength of 276 nm (loop 100 μ L). During control experiments, the extraction conditions adopted for caffeine gave a satisfactory recovery from the skin of 105.1% \pm 8.2%.

The limit of detection and limit of quantification were 0.09 and 0.27 μ g/mL, respectively.

NAP

NAP extraction from the treated skin was carried out using 1 mL of a 94% distilled water and 6% methanol mixture, extracting at room temperature, to avoid NAP hydrolysis, for 90 min. The Supelcosil™ LC-18 HPLC column (5 μ m particle size; 300×4.0 mm; Supelco) was employed for NAP analysis. The mobile phase was a 95 : 5 mixture of a buffer, consisting of 2.72 g/L KH_2PO_4 , 3.48 g/L K_2HPO_4 , 1.70 g/L tetra-*n*-butylammonium hydrogen sulfate (pH 7) and acetonitrile at a flow rate of 1 mL/min. The detection was performed at a wavelength of 245 nm (loop 100 μ L). In control experiments, the extraction conditions adopted for NAP gave a satisfactory recovery from the skin of 93.7% \pm 6.5%. The limit of detection and limit of quantification were 0.12 and 0.36 μ g/mL, respectively.

Statistical methods

Data were expressed as mean \pm standard deviation. Outliers, determined using the Grubbs test, were discarded. The results were evaluated statistically using analysis of variance (ANOVA); Student's *t*-test was used to compare two data sets. The level of significance was fixed at $P < 0.05$.

Results and discussion

Permeation studies

Caffeine

Caffeine is a low molecular weight hydrophilic neutral molecule (MW 194.14; log *P* – 0.07). Its small size

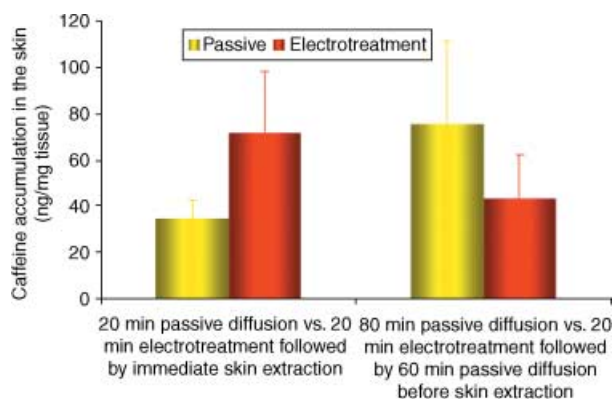


Figure 2 Skin accumulation of caffeine following 20 min of either passive diffusion or electrotreatment (conditions: Gel 1-CAF; 12% intensity). A statistically significant difference (denoted by $**P < 0.05$) was observed between the two treatments for the 20 min application time (EF = 2.1). However, there was no difference when 80 min passive permeation was compared with 20 min electrotreatment followed by 60 min passive permeation (mean \pm standard deviation).

facilitates its passive diffusion in the membrane. Because it is uncharged, any enhancement of its transport due to electrotreatment can be attributed to electroosmosis and increased passive diffusion through micropores created by electroporation.

We quantified caffeine accumulation in the skin after using two experimental conditions: (i) passive and electrically assisted delivery for 20 min and (ii) 80 min of passive diffusion was compared to 20 min electrotransport followed by 60 min passive diffusion (Fig. 2). Under these conditions, there was a statistically significant difference (2.1-fold increase; $P < 0.05$) in caffeine accumulation after 20 min electrotreatment (12% intensity) compared with 20 min passive diffusion (71.56 ± 26.69 and 34.67 ± 7.94 ng/mg tissue, respectively). Because the mean sample application area (0.6 cm^2) and thickness ($750 \mu\text{m}$) of the skin samples were known and assuming a density of 1 g/cm^3 , then it is possible to estimate the total mass of the skin tissue ($\sim 45 \text{ mg}$)⁹; thus, the total amount of caffeine present in the skin following 20 min electrotreatment (12% intensity) was $\sim 3.22 \mu\text{g}$ (corresponding to a cumulative delivery per unit area of $5.37 \mu\text{g/cm}^2$). Considering that $100 \mu\text{L}$ of the Mesogel (containing 4% caffeine) was applied to the skin, and assuming that the density of the gel was 1 g/cm^3 , this implies that $\sim 0.08\%$ of the applied dose permeated into the skin. There was no statistical difference observed between 80 min passive diffusion and 20 min electrotreatment followed by 60 min passive diffusion. The longer duration of the passive diffusion means that

any initial differences due to improved kinetics with electrotreatment are masked and together with the intrinsic variability of skin permeation experiments make it impossible to demonstrate any advantage of electrotreatment.

The data presented above refer to the amounts determined within the skin samples where it was certain that the skin was undamaged – in these cases, no caffeine was detected in the receiver compartment, a reasonable assumption given the small amount of formulation that was applied ($\sim 100 \mu\text{L}$) and the relatively short permeation times used in these experiments.

Previous investigations into caffeine permeation and penetration *in vitro* and *in vivo* have found lower transport rates than the present study^{10–12}: (i) Feldmann and Maibach found a cumulative caffeine permeation *in vivo* of $\sim 3.5 \mu\text{g}$ after 12 h following application of $4 \mu\text{g/cm}^2$ of ^{14}C -labeled caffeine over 13 cm^2 (after 120 h, $47.56\% \pm 20.99\%$ of the applied sample was detected in the urine)¹⁰; (ii) Zesch *et al.* studied cutaneous bioavailability of ^{14}C -labeled caffeine from compositionally different ointment formulations *in vitro* and *in vivo* – their *in vitro* studies showed that 30 min after applying 30 mg ointment containing 0.1% caffeine over a 7 cm^2 surface area, cumulative permeation in the skin ranged from 0.89 to $2.24 \mu\text{g/cm}^2$; *in vivo*, upon application of 100 mg of 0.1% ointment over 28 cm^2 , cumulative permeation of caffeine after 30 min was $0.67 \mu\text{g/cm}^2$,¹¹ (iii) Chambin-Remoussenard *et al.* used HPLC to quantify caffeine in the stratum corneum after application of acetone and emulsion-based caffeine formulations (2.22 and $2.37 \mu\text{g/cm}^2$, respectively) – cumulative amounts of caffeine recovered from the horny layer following a 30 min application over a 5 cm^2 area were 0.037 and $0.212 \mu\text{g/cm}^2$ for the acetone and emulsion formulations, respectively.¹²

NAP

The effect of electrotreatment on NAP accumulation was investigated in a second series of experiments (as described above). NAP is a low molecular weight anion and is primarily transported by electromigration from the cathode with a smaller contribution due to enhanced passive permeation through the microchannels created by electroporation.¹³

Figure 3 shows the results from *in vitro* studies that compared the amount of NAP recovered from the skin following electrotreatment using Gel 2-NAP with that found after simple, passive treatment. The formulation was applied using two different conditions: (i) passive and electrically assisted delivery for 20 min and (ii) 80 min of passive diffusion was compared with 20 min electrotransport followed by 60 min passive diffusion. Electrically assisted delivery is clearly superior as evidenced by the

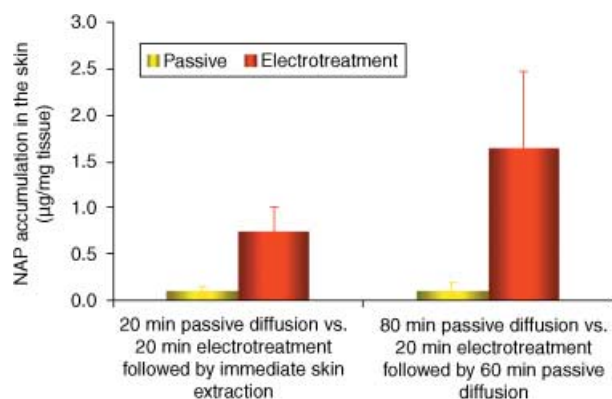


Figure 3 Electrotreatment (conditions: free program; 30% intensity) significantly increased the cumulative delivery of NAP (a precursor of ascorbic acid) in the skin compared with passive diffusion. Data shown from two experimental conditions: (i) 20 min electrotreatment vs. 20 min passive diffusion and (ii) 20 min electrotreatment followed by 60 min passive permeation vs. 80 min passive permeation (mean \pm standard deviation).

7.2-fold (0.10 ± 0.04 and 0.74 ± 0.27 $\mu\text{g}/\text{mg}$ tissue, respectively) and 14.9-fold (0.11 ± 0.08 and 1.64 ± 0.82 $\mu\text{g}/\text{mg}$ tissue, respectively) increases in the amount of NAP recovered from the skin after the respective treatments. Using the same approach as described in the preceding section for caffeine, it is possible to estimate the total amount of NAP present in the skin sample and hence the fraction of the applied dose that was delivered. The analysis shows that after 20 min electrotreatment (30% intensity), the total amount of NAP in the skin and the fraction of the dose delivered were 33.3 μg and 1.1%, respectively. After 20 min electrotransport followed by

60 min passive diffusion, the corresponding values were 73.8 μg and 2.46%, respectively. Given the application area of 0.6 cm^2 , these values correspond to cumulative delivery per unit area of 55.5 and 123 $\mu\text{g}/\text{cm}^2$ for the two electrotreatment protocols.

Although the assay method was capable of distinguishing between NAP and ascorbic acid, only the former was detected in the skin samples; this may be due to the latter's instability.

Comparison of the caffeine and NAP data shows that transport of the latter was an order of magnitude greater. This was due to the greater efficiency of electromigration as a transport mechanism and the use of the higher driving potential.

LSCM experiments

Confocal microscopy is an extremely powerful method to visualize the permeation of fluorescent molecules through the skin. Because this technique captures light from different horizontal (or "XY") focal planes, it can be used to monitor the fluorescence of a suitable probe molecule from different layers within the skin – ranging from the uneven skin surface and the upper layers of the stratum corneum deep into the viable epidermis. Using the appropriate software it is then possible to treat the images "digitally" to extract information from each "horizontal" plane to build a vertical "XZ" cross-section, a so-called "optical slice."

Due to its physicochemical properties, calcein, an anionic fluorophore, is unable to diffuse passively across the stratum corneum. Thus, in the control experiment, calcein was localized at the skin surface, unable to penetrate into the lipophilic stratum corneum (Fig. 4). In contrast,

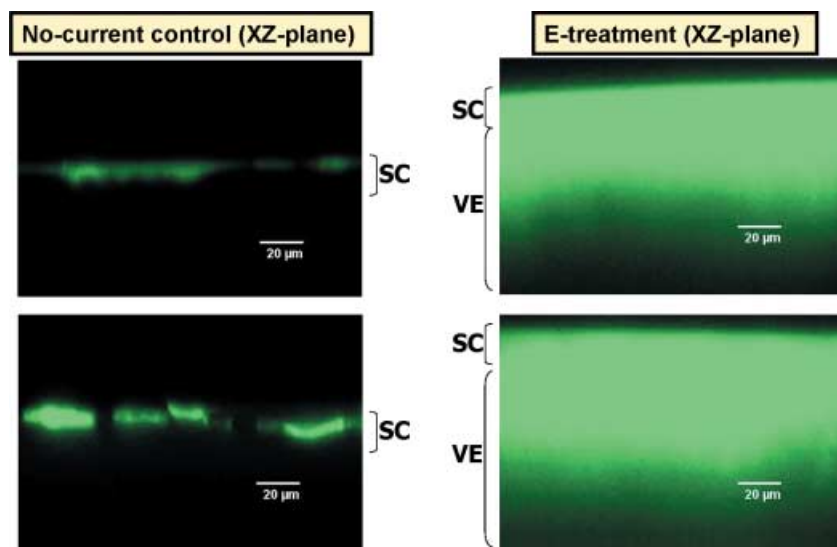


Figure 4 Representative confocal microscopy images showing the effect of 20 min current application on the permeation of calcein into the skin. Electrotreatment (E-Treatment) clearly enabled the anionic fluorescent marker to reach much deeper into the skin (Series II), penetrating into the lower epidermis. In the control samples, calcein fluorescence is localized at the skin surface. The "XZ-plane" signifies that the image represents a vertical cross-section through the skin sample.

electrotreatment increased skin permeability and enabled calcein to penetrate much further, reaching deep into the viable epidermis – as evidenced by the uniform degree of fluorescence observed reaching depths of up to 60 to 80 microns within the membrane. Permeabilization of the stratum corneum by electrotreatment might also facilitate the entry of hydrophilic substances by increasing the membrane's water content helping to solubilize polar and charged permeants.

Conclusions

The results have shown that electrotreatment is an effective means to increase the delivery of both neutral (caffeine) and charged (NAP) cosmeceuticals into the skin. However, it is more efficient for the transport of charged molecules, such as NAP, because electromigration is a more effective driving force than either electroosmosis or enhanced passive diffusion through micropores.

The LSCM images clearly show the permeabilizing effect of electrotreatment on barrier function – as evidenced by the dramatic increase in calcein fluorescence, which can be detected at much deeper levels within the viable epidermis. As is the case with NAP, calcein is an anion under physiological conditions and benefits from electromigration to drive it across the skin.

Acknowledgments

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References

- 1 Scheuplein RJ, Blank IH. Permeability of the skin. *Physiol Rev* 1971; **51**: 702–47.
- 2 Smith EW, Maibach HI, eds. *Percutaneous Penetration Enhancers*, 2nd edn. Boca Raton, FL: CRC Press; 2006.
- 3 Naik A, Kalia YN, Guy RH. Transdermal drug delivery: overcoming the skin's barrier function. *Pharm Sci Technol Today* 2000; **3**: 318–26.
- 4 Prausnitz MR, Mitragotri S, Langer R. Current status and future potential for transdermal drug delivery. *Nat Rev Drug Discov* 2004; **3**: 115–24.
- 5 Kalia YN, Naik A, Garrison J, Guy RH. Iontophoretic drug delivery. *Adv Drug Deliv Rev* 2004; **56**: 619–58.
- 6 Abia N, Geiser L, Mirgaldi M, et al. Capillary zone electrophoresis for the estimation of transdermal iontophoretic mobility. *J Pharm Sci* 2005; **94**: 2667–75.
- 7 Pikal MJ. The role of electroosmotic flow in transdermal iontophoresis. *Adv Drug Deliv Rev* 1992; **9**: 201–37.
- 8 Abia N, Naik A, Guy RH, Kalia YN. Effect of charge and molecular weight on transdermal peptide delivery by iontophoresis. *Pharm Res* 2005; **22**: 2069–78.
- 9 Anderson RL, Cassidy JM. Variations in physical dimensions and chemical composition of human stratum corneum. *J Invest Dermatol* 1973; **61**: 30–2.
- 10 Feldmann RJ, Maibach HI. Absorption of some organic compounds through the skin in man. *J Invest Dermatol* 1970; **54**: 399–404.
- 11 Zesch A, Schaefer H, Stüttgen G. The quantitative distribution of percutaneously applied caffeine in the human skin. *Arch Dermatol Res* 1979; **266**: 277–83.
- 12 Chambin-Remoussenard O, Treffel P, Bechtel Y, Agache P. Surface recovery and stripping methods to quantify percutaneous absorption of caffeine in humans. *J Pharm Sci* 1993; **82**: 1099–101.
- 13 Huh CH, Seo KI, Park JY, Lim JG, Eun HC, Park KC. A randomized, double-blind, placebo-controlled trial of vitamin C iontophoresis in melasma. *Dermatology* 2003; **206**: 316–20.