The Influence of an Electric Field on Ion and Water Accessibility to Stratum Corneum Lipid Lamellae

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Purpose. To study ion transport through stratum corneum (SC) lipid lamellae under passive and iontophoretic conditions.

Methods. Iodide ion transport was measured by fluorescence quenching. Since the process involves diffusion of an iodide ion to the fluorophore located within the SC lamellae, the accessibility of iodide ions was measured. Moreover, the use of anthroyloxy fatty acid probes, provided information as a function of depth within the lamellae.

Results. Fluorescence quenching by iodide ions increased with iontophoretic current density, suggesting increased ion accessibility within the SC lamellae. In addition, at constant current, quenching decreased as the fluorophore was located deeper within the lamellae. This gradient in ion accessibility suggests that more iodide is found near the headgroup than near the core of the SC lipid lamellae. Results obtained in the absence of iodide also show increased water accessibility during iontophoresis.

Conclusions. These results show that in the presence of an applied electric field the SC lipid lamellae interior becomes more accessible to water and ions. These results imply that during iontophoresis, ion and water transport through human skin is associated, at least in part, with the SC lipid lamellae.

KEY WORDS: iontophoresis; passive diffusion; ion transport; drug delivery; fluorescence spectroscopy; stratum corneum lipid lamellae.

INTRODUCTION

The human skin consists of two distinct layers: the dermis and the epidermis. Spanning the epidermis and dermis are various skin appendages, such as sweat glands, sebaceous glands, and hair follicles (1). The outermost layer of the epidermis, the stratum corneum (SC), consists of keratinized cells (corneocytes), embedded in a continuous array of multiple lipid lamellae (2). Due to a high diffusive resistance, this extracellular SC lipid lamellar matrix forms the major barrier to the transport of water and ions through the human skin (3–5). Therefore, it is believed that under passive conditions (e.g. the application of a chemical gradient), the transport of ionized compounds predominantly involves aqueous shunt routes such as skin appendages (6,7). During iontophoresis, when an electrical potential gradient applied across the skin serves as the driving force for ion migration (8), transport through these shunt routes may play an even greater role (9–12). In addition, it has been proposed that ion transport may also occur through highly conductive pathways, not associated with the skin appendages (13). On the other hand, since appendages and other highly conductive pathways make up a very small percentage of the total skin surface (14), non-appendageal ion transport (involving the SC lipid lamellar matrix) may also contribute substantially to the net flux through skin under both passive and iontophoretic conditions. If the SC lipid route contributes to ion transport, then the accessibility of the lamellae interior to these charged species should increase. Consistent with this hypothesis, metal ions (15–17) have been localized within the extracellular spaces following passive diffusion and iontophoresis, however, due to the lack of spatial resolution associated with these techniques, the accessibility of ions to the interior of SC lipid lamellaes remained unresolved.

Fluorescence quenching is a useful technique to study the presence of ions in phospholipid membranes (18–21). With this technique, a quenching agent collides with a fluorophore resulting in depopulation of the excited state by non-radiative energy loss, which in turn causes a decrease of the fluorescence intensity and lifetime (22). Since quenching involves diffusion of the quencher to the fluorophore, this method reveals information on the accessibility of the fluorophore within the lamellae structure (18–21). The use of anthroyloxy fatty acids probes (n-AF), where the fluorophore, 9-anthroic acid, is esterfied to a known position (2, 6, 9, 12 or 16th) along a fatty acid acyl chain, has enabled us to evaluate the accessibility of the SC lipid lamellar structure as a function of depth (21). In the present study, the same technique is used to evaluate the accessibility of the SC lipid lamellae to iodide under passive and iontophoretic conditions.

MATERIALS AND METHODS

Chemicals

Hepes (4-(2-hydroxy ethyl)-1-piperazine ethane-sulfonic acid) and trypsin (type II) were purchased from Sigma Chemical Co. (St. Louis, MO), hexane, methanol, acetone and chloroform from Baxter Diagnostic, Inc. (McGaw Park, IL), sodium chloride (NaCl) from Aldrich Chemical Co. (Milwaukee, WI), potassium iodide (KI), potassium chloride (KCl), hydrochloric acid (HCl), and sodium thiosulfate ($Na_2S_2O_3$) from Fisher Chemical (Fair Lawn, NJ), and silver wire (Ag), 1 mm diameter, 99.9% w/w pure, from Johnson Matthey (Ward Hill, MA). All solvents were spectrophotometric grade. The fluorescence probes (Molecular Probes, Inc., Eugene, OR) were a series of 9-anthroyloxy derivatives (n-AF) where the fluorophore was esterfied on to the 2, 6, 9, or 12th methylene of stearic acid or on to the 16th position of palmitic acid.

Electrodes and Current Delivery Device

For all iodide iontophoresis experiments, a silver (Ag) anode and a silver/silver iodide (Ag/AgI) cathode were used. The cathode was prepared by sanding a Ag wire, washing it in acetone and cleaning it in 1 M HCl for 20 min. at 50°C. After rinsing with distilled water, the wire was anodically plated with AgI by immersion in a 0.5 M KI solution that contained 0.1% by mass of $Na_2S_2O_3$ to prevent formation of iodine (I_2), followed by application of a 0.3 mA current for 14 hours. For iontophoresis in the absence of iodide, a Ag anode and silver/silver chloride (Ag/AgCl) cathode were used. The cathode was

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electrolytically coated in 0.5 M KCl following the protocol mentioned above. The constant current for both electrode plating and iontophoresis was delivered by a Kepco APH 1000M power supply (Flushing, New York, NY), and was monitored using a 85 Multimeter (Fluke, Irvine, CA).

Instrumentation for Spectral Acquisition

All fluorescence measurements were performed at 20°C using a SLM-Aminco MHF 4850 spectrofluorometer (SLM Instruments, Inc., Urbana, IL) with a 360 nm wavelength long-pass filter (Oriel, Stratford, CT) on the emission side. Excitation was achieved with 350 nm wavelength light obtained from a Xenon arc lamp. Photo-bleaching of the fluorescence probe was prevented by using an excitation band pass of 1 nm and recording an emission spectrum within 30 seconds.

Skin Preparation

The individual AF probes were applied in a solution to the epidermal surface of split thickness human cadaver skin (approximately 2 mm thick), mounted in a side-by-side diffusion cell. The donor (epidermal) side of this cell contained Hepes buffer (20 mM Hepes, 50 mM NaCl, buffered at pH 7.4) with 2 μM AF probe, while the receiver side contained only Hepes. After a 4-hour incubation at room temperature, known to give a probe to SC lipid mole ratio of 1:300–400 (21), the skin was removed from this cell and thoroughly rinsed with Hepes buffer. For initial data acquisition, the skin was mounted in another custom-made, two compartment cell, which was placed in the sample compartment of the fluorescence spectrometer. This cell was made from non-conductive material and consisted of two triangular chambers, with matching 0.5 cm^2 holes in the inner walls (Fig. 1). The skin, with its epidermal side facing the donor compartment, was mounted between the two chambers, covering the hole. Both chambers could be filled from the top with 1.5 ml of fluid, depending on the experiment. The donor chamber had two UV transparent windows mounted at right angles. One allowed the beam of excitation light to enter the cell and illuminate the epidermal surface, the other allowed the emitted fluorescence light to leave the cell for detection.

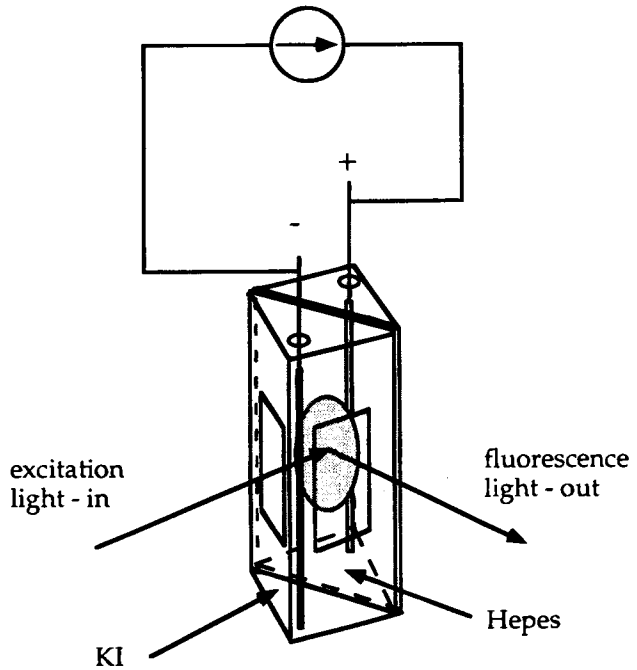

Fig. 1. Side-by-side diffusion cell used for on-line spectral acquisitions involving skin. In both compartments, containing Hepes or KI solution, electrodes could be inserted depending on the experiment. The skin is designated by the shaded area.

Quenching Under Passive Conditions

To record fluorescence spectra in the absence of a quencher, the skin was exposed to only Hepes buffer. The effect of quenching by passive transport was measured by refilling the donor chamber (in contact with the epidermal side of the skin) with KI solutions ranging from 0 to 6 M, all containing a small amount of $Na_2S_2O_3$. During the following 5 hours, an emission spectrum was recorded every 30 minutes.

Quenching Under Iontophoretic Conditions

To distinguish the effect of passive from iontophoretically enhanced ion accessibility, current was applied after the quenching under passive conditions (see above) had reached steady state. Iodide anions were introduced using a Ag/AgI cathode placed in the donor chamber, which contained 1.1 M KI, while a Ag anode was placed in the receiver chamber containing Hepes. Both electrodes were positioned at a distance of 8 mm from the skin. The effect of current density on iodide iontophoresis was evaluated at current densities of 100 and 300 μA/cm^2. During iontophoresis, spectra were recorded every 30 minutes until the fluorescence intensity reached a constant value and, hence, quenching had reached steady state.

The contribution of water (22) and Hepes buffer components to quenching was studied with 2-AF, following the same protocol as described above but using a Ag/AgCl cathode, Ag anode, Hepes buffer on the donor and receiver side, and a current density of 1000 μA/cm^2.

Spectral Analyses

Iodide acts as a collisional quencher for AF probes in phospholipid vesicles and SC (20,21) by a diffusive process, and therefore, fluorescence quenching is described by the Stern-Volmer relationship (22):

$$(Io/I) - 1 = Ksv[Q] \quad (1)$$

where Io is the fluorescence intensity of the unquenched fluorophore, and I is the intensity at quencher concentration [Q]. The slope of (Io/I)-1 versus [Q] is Ksv, the Stern-Volmer constant which reflects the quenching efficiency (22). The fluorescence intensities were determined from the peak height at the maximum of emission after spectra were corrected for light scattering by recording the baseline intensity around 600 nm.

Probe Migration During Iontophoresis

The probe, while highly lipophilic, has a carboxylic headgroup which is ionizable. Hence, we also investigated potential probe migration due to the applied electric field. In these experiments, 16-AF was applied to the epidermal surface of human cadaver skin using the protocol mentioned above. The skin

was then mounted in the two compartment cell (Fig 1.) which contained 1.1 M KI on the cathodal (epidermal) side and Hepes on the anodal side. A constant current of 1000 μA/cm^2 was applied across the skin for 16 hours using a Ag/AgI cathode and Ag anode. Afterwards, the skin was removed, thoroughly rinsed with Hepes buffer, and placed on a filter paper that was saturated with a 0.5% w/v trypsin solution. After overnight incubation at 20°C, the SC was then gently separated from the remaining skin. Both tissues were rinsed with distilled water, and stored under vacuum in a desiccator. The SC, remaining skin, both electrodes, and the solutions on the cathodal and anodal sides were separately extracted using a 2:1 chloroform-methanol solution. The samples were treated three consecutive times, each lasting six hours, with 1 ml of fresh extraction solution. The three extracts of each sample were then combined, dried with a nitrogen gas stream at 60°C, and resuspended in hexane. To quantify the concentration of the probe in each extract, the resultant fluorescence intensity at the emission maximum was determined for each sample and was compared with a calibration standard of 16-AF in hexane.

RESULTS

The data (Fig. 2) show that when the SC was exposed to the KI solution, the fluorescence intensity dropped, reaching a steady state value within 4 hours. These results further show that the magnitude of the intensity drop was proportional to the concentration of KI in the donor chamber as indicated by the linearity of the Stern-Volmer plots for all probes (Fig. 3). The slopes of these plots, which are the quenching efficiencies, showed a decreasing trend from 2- to 16-AF.

After iodide quenching under passive conditions had reached steady state, an electric current of 300 μA/cm^2 was applied across the skin. As a result of the current, the fluorescence intensity further decreased, reaching a minimal value after about 9 hours (Fig. 4). In the control experiment involving iontophoresis in Hepes buffer alone at 1000 μA/cm^2, the intensity also decreased (Fig. 4). However, this drop in intensity

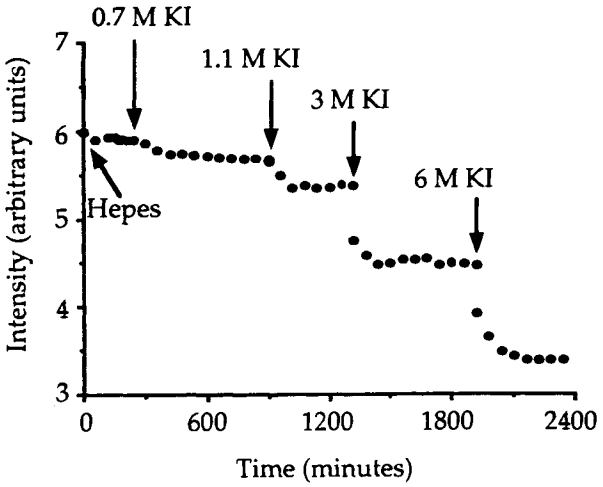

Fig. 2. The fluorescence intensity (arbitrary units) as a function of time (minutes) of 2-AF in SC during quenching under passive conditions. The effect of quenching was measured by subsequently exposing the SC to increasing concentrations of KI, starting with the absence of quencher (Hepes).

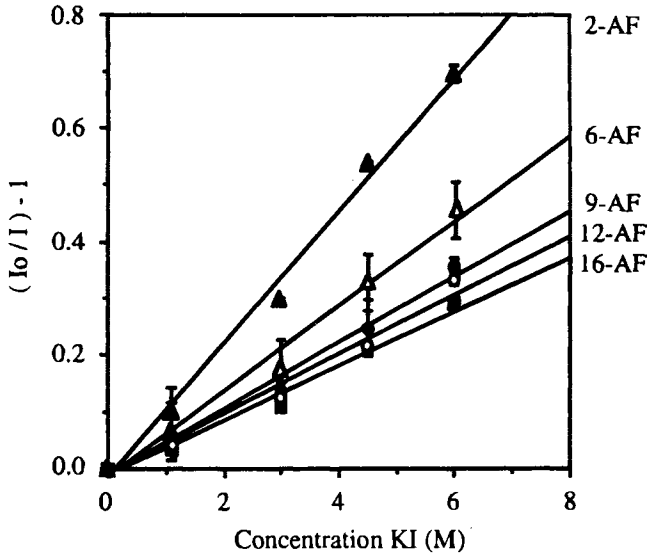

Fig. 3. Fluorescence intensity quenching ($(I_o/I)-1$) of AF probes in SC as a function of iodide (KI) concentration. Data represent the average ± S.D. and are obtained from three different pieces of skin. Symbols: closed triangles (2-AF); open triangles (6-AF); closed circles (9-AF); open circles (12-AF); closed squares (16-AF).

was about one-fourth that observed by iodide iontophoresis at a 30% lower current density. Fig. 5 shows the quenching results of 2, 9, 12-AF under passive conditions and iontophoresis at 100 and 300 μA/cm^2, together with the control. These results show that the magnitude of current-induced iodide quenching

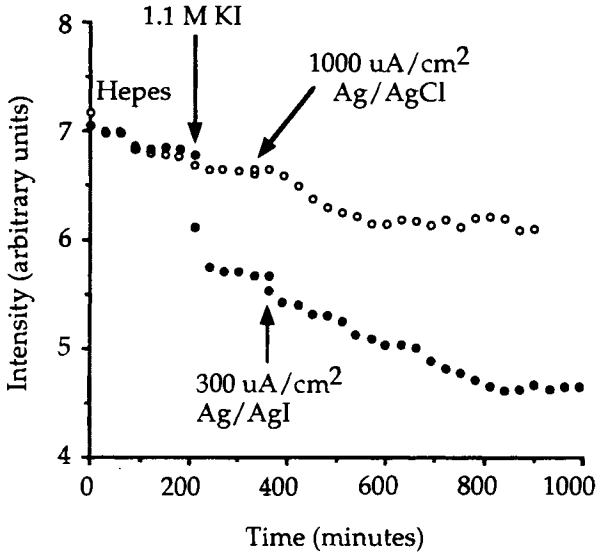

Fig. 4. The fluorescence intensity (arbitrary units) as a function of time (minutes) of 2-AF in SC during quenching under passive and iontophoretic conditions. Similar trends in data were also achieved with 9 and 16-AF. The closed circles (●) represent the experiment where quenching under passive conditions (1.1 M KI) was followed by iontophoresis (300 μA/cm^2 Ag/AgI). The open circles (○) represent the control experiment involving iontophoresis without iodide (1000 μA/cm^2 Ag/AgCl cathode). Both experiments started with exposing the SC to Hepes buffer.

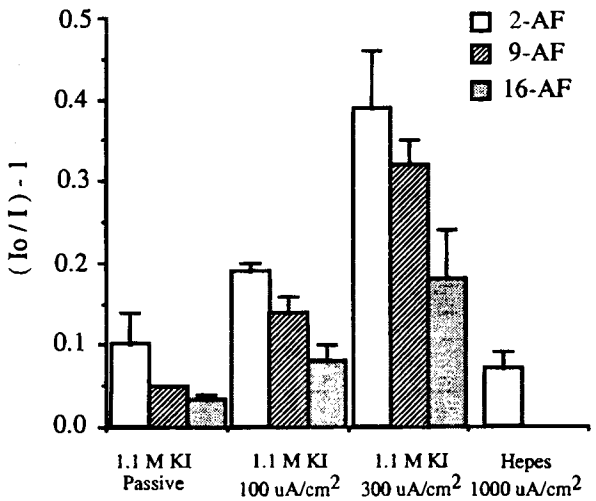

Fig. 5. Quenching results, (Io/I)-1, of 2, 9 and 16-AF using KI under passive conditions and iontophoresis at 100 and 300 μA/cm², together with the control involving iontophoresis in Hepes buffer alone at 1000 μA/cm².

decreased with increased depth of the fluorophore within the SC lamellae, and increased with current density.

The amount of probe within the skin, electrodes and donor and receiver solutions, was determined by extracting each with chloroform-methanol and measuring the resultant fluorescence intensity. These results show that following iontophoresis, the probe was only found in the SC, with a probe to lipid mole ratio of about 1:300–400, assuming an average molecular weight of 650 Da for extracellular SC lipids, and that lipids constitute 10% w/w of dry SC (23).

DISCUSSION

When applied to the epidermal surface of skin, AF probes, due to their lipophilic character, partition into the lipid lamellar matrix of the SC (24). Since extracellular lipids make up only about 10% w/w of dry SC (23), the probe could also partition into other parts of the epidermis. However, recent results (24) have shown that AF probes incorporated into skin exhibit a phase transition near 65°C, identical to that found in SC lipids and quite distinct from that seen in either keratin or epidermal phospholipids (18,24,25). In addition, the extraction of the probe from the SC using chloroform-methanol resulted in a complete loss in fluorescence (21). Since this solvent is known to remove extracellular SC lipids (23), these results strongly suggests that the probe was associated with SC lamellae. Finally, the fatty acid of the probe is identical to lipids found in the SC (23).

Quenching Under Passive Conditions

Iodide quenching of AF probes requires collision of the ion and the fluorophore during the lifetime of the excited state (22). Thus, the decrease in fluorescence intensity serves as an indicator of the close proximity of the ion to the fluorophore within the SC lipid lamellae. The results (Figs. 2 and 3) show that the decrease in intensity, and hence, increase in the magnitude of quenching, was proportional to the quencher concentration and depended on position of the fluorophore along the fatty acid acyl chain. The Stern-Volmer plot of the data (Fig. 3) shows a decrease in quenching efficiency from 2- to 16-AF. Since quenching efficiencies reflects ion accessibility, these results demonstrate that when the fluorophore was located further away from the fatty acid polar head group (i.e., deeper within the lamellae), it was less accessible to the quencher. This gradient in accessibility is consistent with results obtained with other lamellar lipid systems (18,20,21), and suggests that the AF probes were localized in a lipid lamellar structure where more iodide was present nearer the headgroup region than near the core. Most important, these results also show that iodide was found within the lamellae. Therefore, the combined results strongly suggest that under passive conditions the SC lipid lamellar matrix is accessible to iodide ions.

Quenching Under Iontophoretic Conditions

In iontophoresis, an electrical potential gradient applied across the skin serves as the driving force for ion migration through this tissue (8). Increasing the current at constant resistance will therefore increase ion migration accompanied by a flow of water. To ensure that any decrease in intensity during iontophoresis was only due to quenching and not to probe migrating out of the SC as a result of the applied electric field, the amount of probe in the SC was determined after iontophoresis at the highest current density studied. In these experiments, 16-AF was used since its high quantum yield enabled the detection of small amounts of probe. The results showed that the probe to lipid mole ratio in SC (1:300–400) was unchanged by iontophoresis, and that probe was not found in the remaining skin, electrodes or donor and receiver solutions, suggesting that AF probes did not migrate out of the SC due to an applied electric field. The retention of these probes within the SC even under an applied electric field is most likely due to their highly lipophilic character. Any decrease in fluorescence intensity during iontophoresis, therefore, must be due to quenching.

After quenching under passive conditions had reached a maximal value, current was applied to the skin so that any further changes in the fluorescence intensity could only be the result of the applied current (Fig. 4). The results showed that quenching increased in proportion to current density (Fig. 5), suggesting that as the current increased, more quenching agents entered the SC lipid lamellae. Iontophoretically enhanced quenching also decreased as a function of the position of the fluorophore along the fatty acid acyl chain (Fig. 5), qualitatively similar to results obtained under passive conditions (Fig. 3). Due to the combination of a Ag/AgI electrode with a KI solution on the cathodal side, iodide is the primary anionic current carrier through the skin. Hence, the additional quenching due to iontophoresis suggests that under an applied electric current SC lipid lamellae were more accessible to iodide.

Since water (22), and Hepes buffer components could also contribute to the observed quenching, iontophoresis in Hepes solution alone served as a control experiment. For these experiments, 2-AF was used since its fluorophore is the most accessible and, hence, most sensitive to quenching (18,20,21). As shown by the data (Fig. 4), Hepes iontophoresis using a current density of 1000 μA/cm² resulted in quenching, suggesting that SC lipid lamellae interior became more accessible to water and buffer components. However, since the magnitude of quenching was much smaller than that obtained with iodide iontophoresis at a 30% lower current density (Fig. 5), it can be concluded

that the contribution of water and buffer components to the quenching was small relative to iodide. The relative quenching efficiencies of water versus iodide are not known, however, preventing quantitative statements about the relative accessibility of water vs. iodide. Nevertheless, these results strongly suggest that during an applied electric field, the SC lipid lamellae interior becomes more accessible to water and ions. This would imply that during iontophoresis, ion and water transport through the human skin is associated, at least in part, with the SC lipid matrix.

The increased accessibility of the SC lamellae to water and ions is consistent with iontophoresis causing increased hydration of the SC (5,26). However, according to X-ray diffraction results (5), the increased hydration due to iontophoresis resulted in no significant swelling of the SC lamellae, suggesting that hydration did not alter the lipid lamellar structure. Similar results were obtained with infrared spectroscopy (IR) (26,27), whereby iontophoresis caused an increase in the SC hydration (as measured by oxygen-water absorbencies), yet no change in the carbon-hydrogen (C–H) stretching frequencies was observed. Since changes in C–H stretching frequencies correspond to altered SC lipid order (28), these results imply that iontophoresis does not affect the organization of SC lamellaes. Together with our results, these IR (26,27) and X-ray (5) data suggest that during iontophoresis, the SC lamellae interior becomes more accessible to water and ions, however, no significant structural alteration occurs. This is consistent with the relatively rigid structure of the SC lipid lamellae, even under fully hydrated conditions.

The lack of SC lipid lamellar alteration during iontophoresis is contrary to the conclusion drawn from published results involving skin impedance measurements (5). In these experiments, as current was applied across the skin, the impedance was measured as a function of temperature. Around 65°C, a major transition in the electrical resistance was observed, which corresponded to a solid-to-fluid phase change affecting the structure of SC lamellae, as observed using other techniques (24,28,29). As the current density was increased from 13 to 130 $\mu A/cm^2$, the impedance drop occurred at a lower temperature, suggesting a current-induced perturbation of the SC lamellae structure. However, the results from differential scanning calorimetry studies show that increased hydration shifts the SC lipid phase transition to lower temperatures due to water-dependent freezing-point depression (28). Therefore, an alternative explanation could be that the current-induced shift in SC lipid phase transition temperature results from increased hydration (3,26), and not from a perturbation of the SC lamellar structure, consistent with the results presented.

The results of the present study show that quenching of AF probes is a useful tool to study the presence of ions within SC lipid lamellae during iontophoresis. However, since quenching only indicates the presence of the ion in close proximity to the fluorophore, no detailed information is obtained on how the ion migrated into the SC lamellae. In addition, since the probes are located in SC lipid lamellae, there is also no information on how the ion migrated through other structures of the SC (e.g. paracellular, transcellular or appendageal), and to what extent ions in the SC lipids contribute to the total ion transport through the SC. Nevertheless, the results show that the interior of the SC lipid lamellae becomes more accessible to ions and water due to an applied electric field. This electrohydration may be essential to enhanced skin transport seen with both electroporation (30,31) and iontophoresis (9,30).

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