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Effects of alternating current frequency and permeation enhancers upon human epidermal membrane

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ABSTRACT

Previous studies have demonstrated the ability of AC iontophoresis to control skin resistance in different transdermal iontophoresis applications. The objectives of the present study were to (a) identify the alternating current (AC) frequency for the optimization of AC pore induction of human epidermal membrane (HEM) and (b) determine the effects of chemical permeation enhancers upon the extent of pore induction under AC conditions. Experiments with a synthetic membrane system were first conducted as the control. In these synthetic membrane experiments, the electrical resistance of the membrane remained essentially constant, suggesting constant electromobility of the background electrolyte ions under the AC conditions studied. In the HEM experiments, the electrical resistance data showed that higher applied voltages were required to induce the same extent of pore induction in HEM at AC frequency of 1 kHz compared with those at 30 Hz. Even higher voltages were needed at AC frequencies of 10 kHz and higher. AC frequency also influenced the recovery of HEM electrical resistance after AC iontophoresis application. An optimal AC frequency region for effective pore induction and least sensation was proposed. Permeation enhancers were shown to enhance pore induction in HEM during AC iontophoresis. The enhancers reversibly reduced the AC voltage required to sustain a constant state of pore induction in HEM during AC iontophoresis, consistent with the mechanism of lipid lamellae electroporation in the stratum corneum.

1. Introduction

Direct current (DC) of on/off pulses and alternating current (AC) have been previously investigated for transdermal iontophoresis applications (Bagniefski and Burnette, 1990; Miller et al., 1990; Pikal and Shah, 1991; Kumar et al., 1992; Thysman et al., 1992; Inada et al., 1994; Nakamura et al., 2001; Kinoshita et al., 2003; Haga et al., 2005). It has been suggested that pulsed DC and AC iontophoresis causes less skin irritation compared to traditional DC iontophoresis (Okabe et al., 1986; Li et al., 2003). AC at high frequency also has higher threshold of sensation and threshold of "let-go" than DC (Dalziel and Mansfield, 1950; Dalziel and Massoglia, 1956; Okabe et al., 1986) giving better safety profiles and allowing the use of higher electric current in transdermal iontophoretic transport. Also, DC iontophoresis can alter the electrochemical environment of the solution surrounding the electrodes (Li et al., 2004). Skin electrochemical burns may occur as a result of such solution changes during long iontophoresis application or when an inappropriate electrode design is used. Symmetric bipolar AC iontophoresis does not alter the solution surrounding the electrodes and thus can avoid this problem. It is therefore believed that a safe and high electropermeabilization state can be obtained with AC iontophoresis for transdermal drug delivery and analyte extraction.

Another advantage of AC iontophoresis is its ability to effectively reduce and maintain a relatively constant level of HEM electrical resistance (Song et al., 2002; Zhu et al., 2002, 2003). Flux variability has been observed during conventional constant DC iontophoresis *in vitro* (Green et al., 1991; Delgado-Charro and Guy, 1994; Singh et al., 1995; Zhu et al., 2002) and *in vivo* (Meyer et al., 1988; Li et al., 2003). This variability is related to the time dependent alterations of the pore pathways in skin that can occur during conventional constant current DC iontophoresis (Li et al., 2004). AC is more effective in sustaining a constant state of pore induction in HEM, and constant skin resistance AC iontophoresis has been shown to have less transdermal iontophoretic flux variability for neutral permeants than conventional DC (Zhu et al., 2002). The ability of AC to reduce and control HEM electrical resistance also makes this a useful method to improve ion-exchange membrane enhanced iontophoresis. Transdermal iontophoretic drug delivery typically has low transport efficiency. A previous study (Xu et al., 2009) has shown that the incorporation of an ion-exchange membrane having a charge opposite to that of the drug ion in an iontophoresis system can enhance transdermal iontophoretic transport (i.e., permeant transference number) by approximately two to four times when

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the electrical resistance of HEM was lowered to and maintained at 0.5 kΩ with AC. To optimize AC in this application, the interplay of AC frequency, AC voltage, and the extent of pore induction in HEM under AC should be understood. A method to achieve low HEM electrical resistance conditions with lower AC voltages such as by using chemical permeation enhancers is also desirable because this would reduce power consumption and might minimize adverse skin effects during iontophoresis.

Our laboratory has been trying to understand the major flux enhancing mechanisms of AC transdermal iontophoretic transport. These mechanisms include electrophoresis (direct electric field effect), electro-osmosis (convective solvent flow), and electric field-induced pore formation (electropermeabilization). In a previous study of the influence of AC conditions upon permeant transport across HEM, it was found that flux enhancement due to both electrophoresis and electro-osmosis increased when the AC frequency was decreased and, for AC iontophoresis at 2.5 V, the flux enhancement approached its maximum at 0.001 Hz (Yan et al., 2008). The effects of AC upon electropermeabilization in HEM were also investigated in this study but the electrical resistance results were inconclusive due to the limited scope of the study and the experimental design. Earlier studies on HEM electropermeabilization with AC were generally limited to low AC frequency such as 12.5 Hz (Li et al., 1999; Song et al., 2002). The effects of AC frequency and voltage upon HEM electropermeabilization have not been previously systematically studied.

Accordingly, the objectives of the present study were to (a) examine the effects of AC frequency and voltage upon the extent of pore induction in HEM during AC iontophoresis and (b) investigate the influence of skin permeation enhancers upon the AC voltage required to sustain a constant state of pore induction in HEM during AC iontophoresis. Unlike in the previous HEM studies that focused on electrophoresis and electro-osmosis under AC conditions, particularly flux enhancement at low AC frequencies, the present study was to explore the AC frequencies that would allow effective pore induction in the high AC frequency region and the synergistic effects of chemical permeation enhancers and AC electropermeabilization. In the present study, control experiments with synthetic membranes were first conducted to investigate a recently proposed AC ion flux enhancement mechanism (Shibaji et al., 2001); also, these control experiments were to serve as a baseline for the HEM studies. Then, the AC results with HEM were compared with the threshold currents of sensation and "let-go" reported in the literature (Dalziel and Mansfield, 1950; Dalziel and Massoglia, 1956). Lastly, the effectiveness of permeation enhancers in reducing the AC voltage required to sustain a constant state of pore induction in HEM was tested.

2. Materials and methods

2.1. Materials

Phosphate-buffered saline (PBS), pH 7.4 and 0.1 M ionic strength, was prepared from reagent grade chemicals and used in all experiments (0.077 M NaCl and 0.0074 M phosphate buffer). Sodium azide (0.02%) was added into PBS as a bacteriostatic agent. Millipore GVWP filters (0.22 μm pore diameter) were purchased from Millipore Corp. (Bedford, MA). Nuclepore membranes were polycarbonate membranes of approximately 7 μm in thickness and with a pore radius of 7.5 nm (Nuclepore Corp., Pleasanton, CA; Costar, Cambridge, MA; and Whatman Inc., Florham Park, NJ). 1-Hexyl-2-pyrrolidone (HP) was obtained from ISP Chemical (New Milford, CT). 1-Butyl-2-azacycloheptanone (BAZ) was synthesized at the Chemical Synthesis Facility at the University of Utah (Salt Lake City, UT). 1-Butyl-2-pyrrolidone (BP) and 1-octyl-2-pyrrolidone (OP) were purchased from Sigma Chemical Co. (St. Louis, MO). 1-Hexanol (HN) and 1-octanol (ON) were purchased from Fisher Scientific (Pittsburgh, PA). Split-thickness human cadaver skin (male and female, age between 21 and 74) was from the regions of the back and abdomen obtained from New York Firefighter Skin Bank.

2.2. Nuclepore control experiments

Baseline experiments were first carried out with synthetic membranes as the control. These control experiments were conducted with a composite membrane comprised of 50 Nuclepore membranes presoaked and sonicated in PBS before being mounted between two side-by-side diffusion half-cells (surface area around 0.7 cm^2). The junction between donor and receiver cells was clamped together and sealed with Parafilm. Experiments were conducted in the setup shown in Fig. 1. In the AC experiments, the membrane in PBS in a diffusion cell was electrically connected in series with a fixed resistor. Square-wave AC from 30 Hz to 100 kHz and 0.5–20 V in amplitude with a DC offset (0.15–0.5 V) was provided by a function generator (Model 4011, BK Precision, Yorba Linda, CA) and monitored with an oscilloscope. Ag/AgCl electrodes were the electrodes applying the AC electric field across the membrane. Two voltmeters were used to monitor the DC offset (the DC superimposed upon the AC to give a non-zero baseline in the voltage vs. time AC profile) across the fixed resistor and the membrane. The AC voltage across the system was continuously monitored by an oscilloscope during the experiment. The voltage drop across membrane was determined using the total voltage drops across the system and the fixed resistor. The DC offset current in the circuit (Fig. 1) was calculated from the voltage drop of the DC offset across the fixed resistor and the fixed resistor resistance value using Ohm's law. Membrane electrical resistance during AC was then calculated using the slope of the DC offset voltage drop across the membrane vs. the aforementioned DC offset current and Ohm's law. These electrical resistance values were then compared with those determined before and after iontophoresis. The electrical resistance before and after iontophoresis was determined by the application of 0.1 V and/or 0.2 V DC across the membrane and Ohm's law.

2.3. HEM preparation and setup

HEM was prepared by heat-separation using the split-thickness human skin (Peck et al., 1995). The membrane was mounted between two half-cells of a diffusion cell with a supporting Millipore filter (filter electrical resistance in PBS < 0.1 kΩ). A low electrical potential was applied across HEM with a potentiostat (JAS Instrument System, Inc., Salt Lake City, UT) or a waveform generator (Model 33220A, Agilent Technologies, Santa Clara, CA) to determine the initial electrical resistance of HEM using Ohm's law (Li et al.,

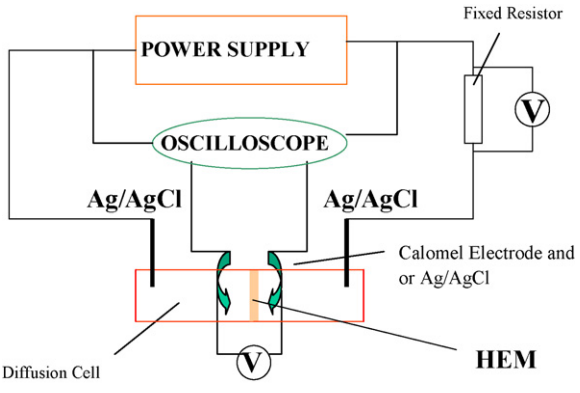

Fig. 1. Experimental setup and electric circuit.

1997). HEM with initial electrical resistance < 15 kΩ cm^2 was discarded and was not used in the present study. The initial resistance of HEM employed in the present study ranged from 16 kΩ cm^2 to 160 kΩ cm^2. Selected HEM was equilibrated in PBS at 37 °C for several hours to establish constant HEM electrical resistance before the start of an experiment. After equilibration, HEM was usually stable without any further significant changes in electrical resistance and permeability coefficients for polar permeants for up to 7 days (Peck et al., 1993).

2.4. AC experimental strategy

Table 1 lists the experiments performed in the present study. Briefly, the study of the effects of AC voltage and AC frequency upon HEM electropermeabilization was divided into two parts. The first part of the study was 10 s AC screening to identify the conditions that would be further investigated in the longer duration iontophoresis protocols and in the presence of chemical permeation enhancers. These short duration experiments allowed rapid and complete recovery of HEM after the application of the AC so that consecutive experiments with the same HEM sample having the same initial resistance could be carried out. This experimental design provided the advantage of minimizing the influence of HEM sample-to-sample variability (e.g., HEM thickness and HEM initial electrical resistance) by using data obtained from the same HEM sample as its own control. The effects of AC were then studied under the 3 h AC iontophoresis protocols of constant AC voltage or constant HEM electrical resistance, and the results were compared with the human data of sensation and "let-go" reported in the literature. These longer duration AC protocols examined the voltage and frequency effects under AC iontophoresis situations that are likely to be encountered in practice. AC experiments with chemical enhancers were then conducted after these HEM permeabilization results became available. HEM electrical resistance was used to evaluate the HEM barrier in all the experiments in the present study based on the inverse proportional relationship between HEM permeability and its electrical resistance shown previously (Inada et al., 1994; Peck et al., 1995; Li et al., 1999). The use of electrical resistance results conveniently allowed short duration iontophoresis experiments to provide the necessary information for designing the longer duration studies; it was considered impractical to conduct any meaningful transport experiments using a model permeant (e.g., due to transport lag time) in a multistage protocol for this purpose.

2.5. AC experiments of HEM

The same AC experimental setup described above (Fig. 1) and two iontophoresis protocols were used: constant voltage and constant HEM resistance iontophoresis. In the constant voltage experiments, square-wave AC of 9 V in amplitude and from 30 Hz to 10 kHz with a DC offset (0.15–0.5 V) was applied across the HEM system with a function generator (Model 4011, BK Precision, Yorba Linda, CA or Agilent Technologies Model 33220A, Santa Clara, CA) and monitored with an oscilloscope. Ag/AgCl was the current-driving electrodes. Two voltmeters were used to monitor the DC offset across the fixed resistor and HEM. The electrical resistance of the fixed resistor was about 10–30% of the resistance of HEM during AC application (the resistance of the fixed resistor ranged from 0.1 kΩ to 4.6 kΩ). The resistance of the fixed resistor was kept at minimum because it affected the AC waveform across HEM at high AC frequencies. For example, at AC frequencies of 10 kHz and 100 kHz, the resistance of the fixed resistor was ≤0.5 kΩ. In the experiments of AC frequency between 30 Hz and 1 kHz, calomel electrodes with Luggin capillaries (Li et al., 1997) were placed close to the HEM in the diffusion cells as the reference electrodes to determine the AC voltage and DC offset across the HEM (Fig. 1). In the experiments of high AC frequency (≥1 kHz), instead of the Luggin capillary calomel electrode system, Ag/AgCl electrodes were used as the reference electrodes. When the desired voltage was higher than 9 V, the output of the function generator was amplified by a custom-designed amplifier (University of Utah, Salt Lake City, UT). The electrical resistance of HEM during AC was determined by the slope of the DC offset voltage vs. DC offset current as described in Section 2.2. HEM electrical resistance before and after the experiment (the initial value and the recovery, respectively) was monitored by the four-electrode system. In the constant HEM resistance experiments, the experimental procedure was similar to that of the constant voltage AC except that the electrical resistance of HEM was maintained constant by varying the AC voltage as described previously (Zhu et al., 2003). The term AC voltage or AC electrical potential in the present paper represents the amplitude of AC (the maximum departure of the current from the mean).

2.6. AC experiments of HEM with enhancers

The experiments with enhancers were conducted in a diffusion cell setup as described in Fig. 1. The study was divided into two parts: (a) systematic study with six enhancers in a reproducible

Table 1
Summary of the experiments conducted and analyzed in the present study.

Experiment	Protocol	Purpose(s)
1. Nuclepore experiments	AC from 30 Hz to 100 kHz and amplitude of 0.5–20 V; 0.1 and 0.2 V DC	Examine the electromobility of ions and solution electrical resistance under AC
2. HEM experiments with short duration: constant voltage	AC from 30 Hz to 100 kHz and amplitude of 9 V for 10 s, multistage experiments using individual HEM samples as their own control	Screen and identify the AC frequencies to be tested in Experiment 3. Multistage protocol to reduce the effect of skin-to-skin variability.
3. HEM experiments with long duration: constant voltage	AC of 30 Hz, 1 kHz, 10 kHz, and 100 kHz and 9 V for 3 h	Examine the AC frequency and voltage effects on the extent of HEM permeabilization and recovery under long AC iontophoresis situations likely to be encountered in practice.
4. HEM experiments with long duration: constant HEM resistance	AC of 30 Hz, 1 kHz, 10 kHz, and 100 kHz and of variable amplitude to maintain HEM resistance at 1 kΩ for 3 h	Allow direct comparison with threshold AC data in the literature. Compare HEM recovery from the same starting electrical resistance of 1 kΩ. Identify the AC frequency and HEM resistance conditions to be studied in Experiment 6.
5. Enhancer AC experiments with short duration	AC of 1 kHz and variable amplitude to maintain HEM resistance at 3 kΩ for 40 min, multistage experiments using individual HEM samples as their own control, all enhancers were tested	Systematically examine the effects of the chemical enhancers under a controlled and reproducible condition. Examine enhancer reversibility. Identify the enhancer to be examined in Experiment 6. Multistage protocol to reduce the effect of skin-to-skin variability.
6. Enhancer AC experiments with long duration	AC of 10 kHz and variable amplitude to maintain HEM resistance at 1 kΩ for 3 h, the most effective enhancer from Experiment 5 was tested	Examine the effect of the enhancer on AC iontophoresis under the condition likely to be encountered in practice.

manner and (b) study with a selected enhancer under the condition of 1 kΩ AC for 3 h.

In the systematic study, a constant HEM resistance protocol of 3 kΩ and 1 kHz square-wave AC was selected. The rationale of selecting this AC iontophoresis condition will be discussed later in Section 3. HEM electrical resistance during AC was determined by the DC offset across HEM and that across the fixed resistance as described above. The electrical resistance of HEM before or after the AC application was also measured. The AC experiments carried out on each HEM sample were divided into five stages. In Stage I, AC was applied for 40 min to sustain the electrical resistance of HEM at 3 kΩ (±15%) in PBS. The AC voltage across HEM was monitored by the oscilloscope as described in the previous section. After the AC application in Stage I, HEM was allowed to recover overnight. In Stage II, before the application of AC, HEM was equilibrated in the enhancer solution (0.5% BAZ, 0.5% HP, 6% BP, 0.23% HN, 0.05% OP, or 0.022% ON in PBS) for 2 h. HEM equilibration was carried out by replacing the enhancer solution in both diffusion cell chambers with fresh enhancer solution 8 times 15 min each. These enhancer solutions were chosen according to the results of previous permeation enhancer studies showing that they provide a 10-fold passive flux enhancement across the lipoidal pathway of stratum corneum (Yoneto et al., 1995; He et al., 2003; Warner et al., 2003) in order to examine the effects of lipid fluidization on electropermeabilization. After equilibration, AC was applied to maintain HEM resistance at 3 kΩ as described in Stage I. After Stage II, the enhancer solution in the diffusion chambers was removed, the diffusion chambers were washed with PBS for 4 h (PBS in the diffusion chambers was replaced by fresh PBS solution every hour), and the membrane was allowed to recover overnight. Stage III was the repeat of Stage I. Stage IV was the repeat of Stage II, and Stage V was the second repeat of Stage I. The AC voltage required to maintain the electrical resistance of 3 kΩ was determined and compared. The electrical conductance of the enhancer solutions were also measured with a conductivity meter (Oakton Instruments, Vernon Hills, IL).

In the second part of the enhancer study, the effects of 0.5% HP upon HEM pore induction were determined under the AC condition of 10 kHz and HEM electrical resistance of 1 kΩ. Before the application of AC, HEM was equilibrated in 0.5% HP for 2 h as described above. AC was applied across HEM for 3 h to sustain the electropermeabilization state of 1 kΩ in PBS with HP, and the AC voltage needed to maintain 1 kΩ was recorded. The AC experiment in PBS without HP treatment was the control. The 10 kHz and 1 kΩ AC protocol and 0.5% HP were selected as the condition according to the results in the preceding AC and enhancer studies. The rationale will be discussed later in Section 3.

3. Results and discussion

3.1. Nuclepore membrane control experiments

A previous AC study (Shibaji et al., 2001) has shown flux enhancement of ion transport (up to twenty times) across a cellophane membrane under AC of high voltage and high frequency. This study has concluded that AC enhances membrane transport by increasing the electromobility of the ions as a result of the decrease in the hydrated ion radii under the AC electric fields. According to electrotransport theory (Erdey-Gruz, 1974), the effective electromobility of an ion would increase at the electric fields of 10^4 V/cm or greater when the ionic atmosphere around the ion cannot rearrange fast enough after the ion leaves its ionic cloud during iontophoresis (Wien effect). At higher electric fields such as greater than 10^9 V/cm, deviation from the Stokes' law is possible, leading to a further increase in ion mobility. Experiments to test the effects of AC upon ion electromobility are therefore important in order to properly interpret the electrical resistance data in the present study.

Table 2
Electrical resistance (kΩ) of Nuclepore membranes during AC iontophoresis at AC frequency of 100 Hz, 1 kHz, 10 kHz, and 100 kHz. Mean ± SD (n = 3).

Amplitude (V)	Frequency (Hz)			
	100	1k	10k	100k
0.5	0.73 ± 0.04	0.77 ± 0.03	0.73 ± 0.02	0.76 ± 0.02
5	0.76 ± 0.07	0.74 ± 0.04	0.74 ± 0.04	0.73 ± 0.04
15	0.77 ± 0.01	0.73 ± 0.06	0.73 ± 0.04	0.76 ± 0.03
20	0.71 ± 0.04	0.70 ± 0.04	0.73 ± 0.06	0.74 ± 0.05

In the present study, the effects of AC upon the electromobility of ions were examined with a synthetic membrane system—Nuclepore membranes. Table 2 presents the summary of the membrane electrical resistances at different AC frequencies during 0.5 V, 5 V, 15 V, and 20 V AC iontophoresis. The electrical resistance of the membrane system was found to be essentially constant during AC iontophoresis of 0.5–20 V at 100 Hz, 1 kHz, 10 kHz, and 100 kHz. These electrical resistance values were also essentially the same as those measured using DC before and after AC applications across the Nuclepore membranes. No significant alteration of the electromobility and of the effective Stokes–Einstein radii of the background electrolytes was observed under the AC conditions used in the present study. Hence, the results in these control experiments support the hypothesis that the decrease in HEM electrical resistance during AC iontophoresis is mainly a result of electric field-induced membrane alteration.

3.2. Effect of AC frequency on AC waveform across HEM

Fig. 2 shows the representative AC waveforms across HEM in the AC experiments from 1 kHz to 1 MHz and experimental setups of 0.46 kΩ or 4.6 kΩ fixed resistors (the resistance range of the resistors used in the present study) in series with HEM. The shapes of the AC waveforms in the figure resemble those of an electrical circuit of parallel resistor and capacitor and are consistent with the electrical behavior of HEM studied previously (Tregear, 1966; Yamamoto and Yamamoto, 1976; Kalia and Guy, 1995). At AC frequency up to approximately 1 kHz, the shapes of the AC waveforms across HEM and the output waveforms from the function generator were essentially the same in both the 0.46 kΩ and 4.6 kΩ resistor systems (data not shown). At higher AC frequencies (e.g., 10 kHz and 100 kHz), the shapes of the AC waveforms across HEM and those across the function generator began to differ. For example, at the AC frequency of 1 MHz, the AC profile across HEM was significantly altered compared to that across the function generator. This makes the direct comparison of the AC results at low frequency and frequency higher than 10 kHz difficult. Experiments were therefore not conducted at AC frequencies > 100 kHz and mechanistic analyses were not performed at AC frequencies > 10 kHz. The resistance of the fixed resistor in series with HEM was also observed to affect the AC waveforms across HEM, with the 4.6 kΩ resistor altering the AC profile across HEM more than that with 0.46 kΩ. Resistor of 0.46 kΩ was therefore selected and used in the AC experiments of 10 kHz and 100 kHz.

3.3. Short duration 10 s AC study

The effects of AC frequency on the extent of pore induction during 10 s AC were determined in the consecutive runs of different protocols with individual HEM samples. In these consecutive experiments, the membrane resistance was allowed to recover to a constant value after each AC application. When no further resistance recovery was observed within 2 h, AC of the next desired frequency was applied. By utilizing the same HEM sample in successive experiments, the influence of skin-to-skin variability was

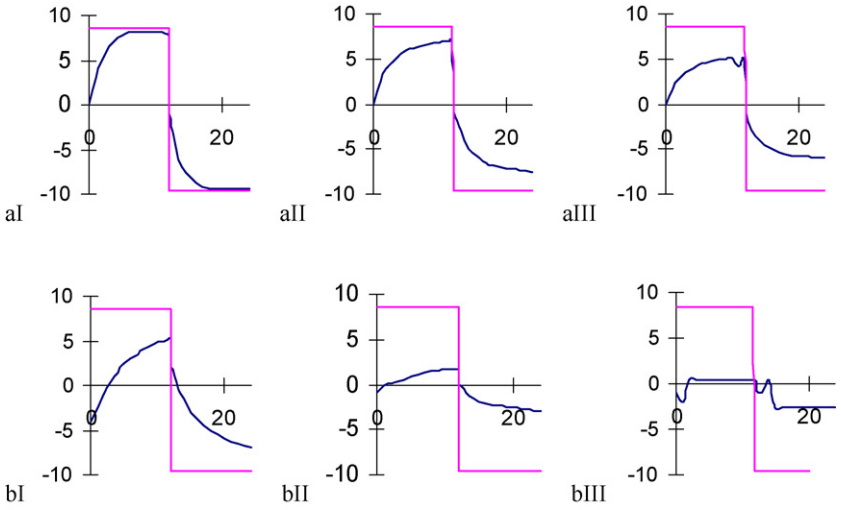

Fig. 2. AC waveforms across HEM (darker curves) at AC frequencies of (I) 10 kHz, (II) 100 kHz, and (III) 1 MHz when the resistances of the resistor in series of HEM were (a) 0.46 kΩ and (b) 4.6 kΩ. The lighter lines represent the square-wave outputs from the function generator.

reduced. When the results of the first and the last runs were the same, the effects of AC were deemed reversible and the same HEM was used as its own control. Otherwise, the data were discarded. Fig. 3 summarizes the 10 s 9 V AC iontophoresis data. The ratio of the extent of pore induction (E) of 30 Hz, 100 Hz, 300 Hz, and 10 kHz to that of 1 kHz is plotted against the applied AC frequency. The 1 kHz data were chosen as the baseline here because most previous AC transdermal transport studies in our laboratory were conducted at 1 kHz (Li et al., 2003; Yan et al., 2004, 2005). Ratios of the E values were used instead of the absolute value, so each HEM sample can serve as its own control. The number of HEM samples used to study the AC conditions is presented as n. The data in Fig. 3 show no significant differences between the E values at 100 Hz, 300 Hz, and 1 kHz; these E ratios are not significantly different from unity. Essentially, the same extent of pore induction was observed under AC of the same applied voltage at frequencies of 100 Hz, 300 Hz, and 1 kHz. At 30 Hz, the average E ratio is significantly greater than unity (90% confidence *t-test*). At 10 kHz, the average E ratio is less than unity (90% confidence *t-test*). These results suggest that the extent of pore induction at 30 Hz was greater than that at AC frequencies from 100 Hz to 1 kHz, which in turn was greater than that at 10 kHz. Increasing the AC frequency to 100 kHz further reduced the extent of pore induction, but because of the significantly differing AC waveform observed across HEM at this high frequency (see Section 3.2), a direct comparison between the 100 kHz data and those of the lower frequencies cannot be made.

3.4. Constant voltage AC 3 h study

Based on the results of the 10 s AC study, AC frequencies of 30 Hz, 1 kHz, and 10 kHz were selected to study the effects of AC frequency upon HEM pore induction in the 3 h AC experiments. AC of 100 kHz was also included for comparison. The changes of electrical resistance of HEM during AC iontophoresis of an applied voltage of 9 V at the four frequencies were determined. The recovery of HEM electrical resistance after each AC application was monitored for 1– 6 days until the HEM resistance recovered to a constant value with respect to time. Fig. 4 shows the representative electrical resistance profiles of HEM during 9 V AC iontophoresis. The data in the figure indicate that, in most cases, the drop of the electrical resistance followed a fast and then a slow phase: the electrical resistance of HEM dropped extensively in the first minute, changed slowly for a few minutes, and then became relatively constant over the remainder of the duration of the AC application. This supports the use of the short duration screening in Section 3.3. The steady-state HEM electrical resistance during 9 V 30 Hz AC was around 1.0–1.5 kΩ, cor-

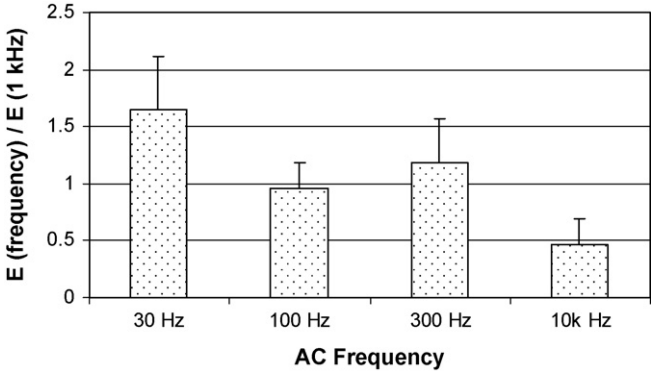

Fig. 3. Frequency effect on the extent of pore induction in HEM. E (frequency)/E (1 kHz) is the ratio of the extent of pore induction at the specific frequency to that at 1 kHz. Extent of electroporation (E) is the ratio of HEM electrical resistance before and at the end of the 10 s application of 9 V AC. Mean ± SD ($n \geq 4$).

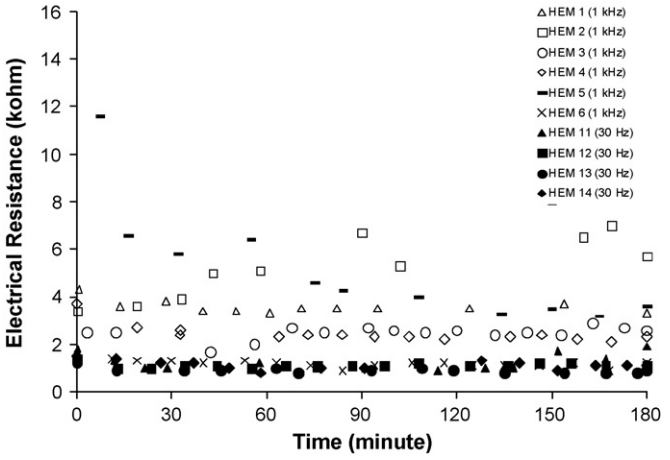

Fig. 4. Representative electrical resistance data of HEM during 9 V AC at 30 Hz (closed symbols) and 1 kHz (open symbols including dashes and crosses) for 3 h. Each symbol represents an individual HEM sample, and each data point represents the value at the measuring point.

Table 3
Summary of the 3 h AC studies [a] and the threshold AC currents.

Frequency	3 h AC of 9 V		3 h AC		Threshold AC current	
	Steady-state resistance (kΩ)	Fraction of resistance recovery	Voltage to sustain around 1 kΩ	Fraction of resistance recovery	"Let-go" (mA)[b]	Sensation (mA)[c]
30 Hz	1.1 ± 0.2	0.2 ± 0.2	9[d]	0.17 ± 0.16[d]	16	1.1
1 kHz	4 ± 2	0.6 ± 0.2	11 ± 1	0.25 ± 0.13	24	2.3
10 kHz	7 ± 5	0.9 ± 0.4	15 ± 3	0.46 ± 0.31	75	12
100 kHz	31 ± 25	0.9 ± 0.4	>40[e]	–[f]	–[g]	151

[a] Mean ± SD ($n \geq 4$). The diffusion cells had a diffusional area ≈ 0.7 cm^2 and 1 kΩ is equivalent to 0.7 kΩ cm^2.
[b] Obtained from reference (Dalziel and Massoglia, 1956).
[c] Obtained from reference (Dalziel and Mansfield, 1950).
[d] Same study as the 3 h AC of 9 V study.
[e] Cannot achieve 1 kΩ. The resistance values at 40 V (maximum AC output of the equipment) were 2–4 kΩ.
[f] Not applicable.
[g] Not available.

responding to up to around a 100-fold decrease in HEM electrical resistance with some HEM samples. These resistance values were close to those after the application of 100–1000 V short DC pulses on full thickness skin (around 0.5–1 kΩ) (Chizmadzhev et al., 1998) but significantly higher than those of stripped skin (around 20 Ω) (Pliquett et al., 1995). At 9 V and 1 kHz, the resistance values were around 1–8 kΩ. The recovery behavior of HEM electrical resistance was monitored after AC application. In most cases, longer recovery times and lower extent of electrical resistance recovery were observed after the application of the lower AC frequency. The first two columns of Table 3 summarize the results of the constant voltage AC experiments. It is of interest to point out that during the lowest frequency AC (9 V, 30 Hz AC), the resultant HEM electrical resistance values fell within a relatively narrow range (1.0–1.5 kΩ) and the scatter of the data was considerably less than that at the higher AC frequencies.

3.5. Constant resistance AC 3 h study

The objective of the constant resistance AC experiments was 2-fold. First, although poorer HEM resistance recovery was observed after the lower AC frequency applications than with the higher AC frequency, it was unclear if the poorer recovery was directly related to the lower resultant steady-state electrical resistance during AC or to some other concomitant aspect of the lower AC frequency. To gain further insight into this question, the effect of AC frequency upon the recovery behavior of HEM electrical resistance was examined by using a constant resistance AC approach. Secondly, these experiments were expected to provide the data for comparison between the results of the present study and threshold AC currents reported in the literature.

The experiments basically involved the following. AC was first applied to reduce and maintain the electrical resistance of HEM at around 1 kΩ (±20%)—the resistance observed in the 9 V constant voltage 30 Hz AC study which has relatively small variability compared to those under the other AC conditions—and the recovery of HEM electrical resistance after AC application was monitored. The resistance recovery data are summarized in column 5, Table 3. Although the data show a trend of improving HEM recovery with increasing AC frequency, large skin-to-skin variability was observed. To better understand the situation, Table 4 shows the individual HEM resistance recovery data. For low initial electrical resistance HEM, the fraction of HEM resistance recovery after the application of 9 V, 30 Hz AC was around 0.4. At 10 kHz, for the low initial HEM electrical resistance cases, the fraction of recovery after AC application was around 0.6–0.9. For HEM with high initial electrical resistance, poor recovery was observed after 10 kHz AC application. This is likely because of how the fraction of HEM resistance recovery has been defined—as the ratio of the resistance after AC application to the resistance before application (i.e., initial). Based on this definition, better recovery would be expected for HEM of low initial resistance than for HEM of high initial resistance. As an example, the formation of the same new pore in low initial resistance HEM would have less of an effect, percentage-wise, on the total HEM electrical resistance than in the case of HEM of high initial resistance. An analysis using resistance ratios therefore might become problematic here and could lead to a misleading assessment of the situation unless HEM samples of similar initial electrical resistances were used or the same HEM sample was used as its own control in the experiments. The data in Table 4 allow the comparison of HEM recovery behavior taking the initial electrical resistance into account. For the HEM samples having similar initial electrical resistances, there was generally better recovery when higher frequencies than when lower frequencies AC were used.

The fourth column in Table 3 presents the AC voltages used to induce and sustain HEM resistance of 1 kΩ at 30 Hz, 1 kHz, 10 kHz, and 100 kHz. When the AC frequency increases from 30 Hz to 10 kHz, the AC voltage required to induce and sustain the 1 kΩ condition increases. This trend is consistent with the resistance data of HEM in the 3 h 9 V AC study discussed above. At 100 kHz, the

Table 4
Initial electrical resistance of HEM before AC application and HEM resistance recovery after AC iontophoresis that maintained HEM resistance at 1 kΩ for 3 h.[a]

Protocol								
9 V, 30 Hz			9–13 V, 1 kHz			10–18 V, 10 kHz		
HEM sample	Initial resistance (kΩ)	Fraction of resistance recovery	HEM sample	Initial resistance (kΩ)	Fraction of resistance recovery	HEM sample	Initial resistance (kΩ)	Fraction of resistance recovery
11	53	0.17	30	33	0.36	18	25	0.50
12	28	0.42	31	40	0.33	19	23	0.84
13	83	0.14	32	83	0.15	20	23	0.68
14	100	0.03	33	83	0.12	21	71	0.13
17	111	0.04				22	91	0.16

[a] Same experiments as those presented in the 5th column of Table 3.

equipment used in the present study could not reduce the electrical resistance of HEM to 1 kΩ even at its maximum AC voltage output of 40 V (80 V peak-to-peak).

3.6. Comparisons of thresholds of sensation and "let-go" with the present AC results

The last two columns in Table 3 show the threshold AC currents of "let-go" (Dalziel and Massoglia, 1956) and the thresholds of sensation (Dalziel and Mansfield, 1950) at frequencies from 30 Hz to 100 kHz. The threshold currents of "let-go" are the 50 percentile sine-wave currents causing a person to "freeze," i.e., the maximum current under which a subject can control the muscle and be able to "let-go" his grasp of the electric source. The threshold currents of sensation are the 50 percentile sine-wave currents giving the perception of electricity when a subject was holding the current passing a copper wire. It should be noted that the threshold currents vary with the anatomic sites, electrical parameters, electrode and physiological conditions (Geddes et al., 1969; Katims et al., 1987). The types of the sensation were also different from low to high frequency AC. From 5 Hz to 2 kHz, sensations of "pricking or burning" to "vibration" and then "buzzing" were reported (Katims et al., 1987).

The data in Table 3 show a steep dependence between the threshold current of "let-go" and the AC frequency from 30 Hz to 10 kHz (5-fold; 16–75 mA). Note that AC of 1 V in the present 1 kΩ constant resistance iontophoresis experiments is equivalent to 1 mA. The AC currents for sustaining HEM at 1 kΩ were significantly lower than the threshold AC currents of "let-go" at all AC frequencies studied. For the threshold currents of sensation, the AC frequency region around 10 kHz is believed to be the optimal AC condition for effective electropermeabilization with the least sensation under the AC conditions tested in the present study; although the threshold current of sensation continues to increase with AC frequency above 10 kHz, the effect of HEM capacitance becomes significant (Fig. 2), leading to the inability of the AC to reduce HEM electrical resistance to 1 kΩ (e.g., 100 kHz data in Table 3).

3.7. Effects of enhancers upon AC iontophoresis

As described in Sections 1 and 2, the objectives of the present enhancer study differed from other iontophoresis studies (Rastogi and Singh, 2002; Smyth et al., 2002; Meidan et al., 2003; Wang et al., 2003) in two aspects. First, the objective was not to enhance the iontophoretic flux directly but to reduce the AC voltage required to sustain HEM electropermeabilization. Second, the enhancer studies can provide insight into the mechanism of HEM pore induction under AC by testing the hypothesis that AC electropermeabilization can be enhanced by chemical permeation enhancer induced lipid fluidization. In the first part of the AC enhancer study, AC frequency of 1 kHz was chosen because previous transdermal AC iontophoresis studies were mainly conducted at this frequency (Li et al., 2003; Yan et al., 2004, 2005). The HEM target resistance of 3 kΩ was used to ensure a high degree of reversibility of HEM from the effect of the AC electric field at this AC frequency and that the contribution of the pre-existing pores to the total resistance during AC was minimal.

Fig. 5 shows the representative data of the AC enhancer study with HEM. The AC voltages required to maintain the HEM electrical resistance of 3 kΩ during 1 kHz AC are plotted against time in Stages I, II, III, IV, and V of a single HEM sample. In all cases, (a) only minor AC voltage adjustments were required to maintain the HEM electrical resistance at the constant value after the initial period; (b) the enhancer effects were at least partially reversible; and (c) the HEM resistance value before each AC run was at least seven times higher than the target resistance of 3 kΩ. Table 5 summarizes the applied

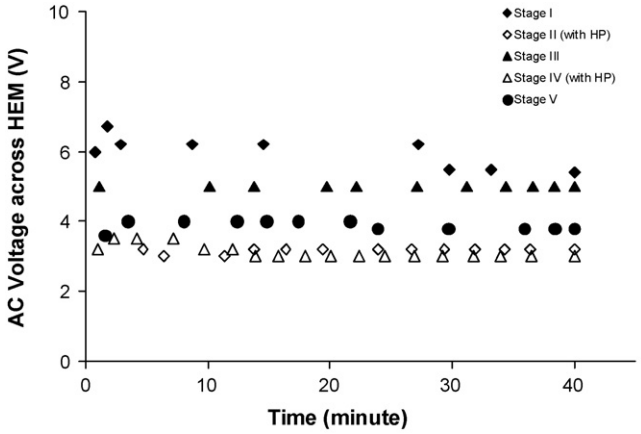

Fig. 5. Representative time profile of AC voltage across HEM to maintain HEM electrical resistance of 3 kΩ during 40 min 1 kHz AC in PBS with (open symbols) and without (closed symbols) the chemical enhancer 0.5% HP. Stages I, III, and V are the PBS controls. The initial electrical resistance of this HEM sample was 32 kΩ. Each data point represents the value at the measuring point.

AC voltage required to sustain a constant state of HEM resistance of 3 kΩ in PBS and in the enhancer solutions for the HEM samples studied. The mean of the results from different stages were first calculated for each HEM sample, and the mean and SD of all HEM results were presented. From the data in the table, lower AC voltages were required to induce and sustain a constant electrical resistance under the enhancer conditions. All the studied enhancers reduced the AC voltage required to provide the same HEM electropermeabilization state; i.e., the permeation enhancers increased the extent of HEM electropermeabilization under these conditions.

It should be noted that the enhancer concentrations used in the present study were relatively low: 0.23% HN, 0.022% ON, 0.5% BAZ, 0.5% HP, 0.05% OP, and 6% BP in PBS. Also, a cosolvent was not used. The conductivities of the enhancer solutions were not significantly different from those of PBS (data not shown). The enhancers under the conditions of the present study also did not significantly affect the initial electrical resistance of HEM. Changes in HEM electrical resistance were generally small before and after enhancer treatments (less than 10% in most cases; $n \geq 3$ donors for each enhancer). This was one of the major differences between the present and other studies (Rastogi and Singh, 2002; Smyth et al., 2002). Other differences include the previous DC vs. the present AC iontophoresis protocols. To our knowledge, the present study is the first to examine the effects of permeation enhancers at these concentrations in conjunction with constant resistance AC iontophoresis.

In the second part of the enhancer study, HP was selected as the enhancer according to the results in Table 5. The 10 kHz and

Table 5
AC voltage across HEM during constant resistance AC iontophoresis of 3 kΩ at 1 kHz and the ratio of the AC voltage with and without the enhancer.[a]

System	Average steady-state AC voltage	Voltage ratio (voltage PBS control/voltage enhancer)
PBS	5.9 ± 2.4	–
6% BP in PBS	2.3 ± 0.5	1.5 ± 0.2[b]
0.5% BAZ in PBS	4.5 ± 1.0	1.2 ± 0.1[b]
0.5% HP in PBS	2.8 ± 0.8	1.8 ± 0.3[c]
0.23% HN in PBS	4.7 ± 2.4	1.4 ± 0.1[c]
0.05% OP in PBS	4.1 ± 1.7	1.6 ± 0.7[c]
0.022% ON in PBS	5.3 ± 2.4	1.6 ± 0.2[c]

[a] Mean ± SD ($n \geq 4$ skin donors).
[b] Voltage of the control with PBS is statistically different from voltage with enhancer ($p < 0.01$).
[c] Voltage of the control with PBS is statistically different from voltage with enhancer ($p < 0.05$).

1 kΩ AC condition was chosen because this protocol was identified as effective in electropermeabilization with a relatively low degree of sensation (Table 3). The 3 h duration provided a situation representative of those usually encountered in transdermal iontophoresis in practice. Under this experimental condition, the AC voltages required to induce and sustain HEM electrical resistance of 1 kΩ at 10 kHz were 9 ± 3 V and 17 ± 6 V ($n = 3$ skin donors each condition) with and without 0.5% HP, respectively. The results of the PBS control experiments here (17 V) were consistent with those in the constant resistance AC 3 h study presented in Table 3 (15 V). HP of 0.5% assisted the AC electric field to maintain the HEM electroporation state and reduced the AC voltage by approximately a factor of two from that of the control; this is similar to the results of the corresponding 40 min 1 kHz 3 kΩ AC experiments in Table 5. As the electrical power is a product of the voltage and electric current and the electric current is proportional to voltage during constant resistance iontophoresis, the factor of two in voltage reduction would allow a 4-fold reduction in battery power consumption. This can also reduce the skin sensation related to electric current during AC.

It should be pointed out that a decrease in HEM electrical resistance may or may not affect the transference number in iontophoretic transport under constant current iontophoresis conditions. This is because the skin barrier properties such as pore size were not assessed in the present study. Consequently, increasing the extent of pore induction may not necessarily imply flux enhancement at constant current; however, the importance of increasing the battery life and possibly reducing the electric AC sensation should not be overlooked. The use of chemical enhancers in transdermal iontophoresis may help to reduce the energy required to perform constant skin resistance AC iontophoresis for controlling flux variability and enhancing delivery in ion-exchange membrane enhanced iontophoresis and to decrease the sensation related to the current passage during constant skin resistance AC for better safety and irritation profiles.

3.8. Mechanisms of pore induction with and without chemical enhancers under AC

The results in the 10 s and 3 h AC studies show that, similar to DC (Inada et al., 1994), the extent of pore induction during AC iontophoresis was strongly dependent on the applied AC voltage. This suggests that the mechanisms of pore induction in AC and DC iontophoresis are similar. AC frequency also affects the extent of pore induction: lower frequency AC provided a larger extent of pore induction than higher frequency AC. For HEM electrical resistance recovery after iontophoresis, increasing the voltage and duration of AC application and decreasing AC frequency decreases the extent of HEM resistance recovery. The effects of AC frequency upon pore induction and resistance recovery in HEM may be explained by the parallel resistor and capacitor model for HEM, in which HEM capacitive charging influences the exposure of the membrane to the electric field. In addition, depolarization/recovery of HEM during each AC square-wave pulse cycle occurs more often at high frequency than that at low frequency. The decrease in HEM electrical resistance during iontophoresis is believed to be due to the formation of pores or the changes of the existing pores in skin. Electroporation has been proposed to occur at the cells lining the appendageal ducts (Chizmadzhev et al., 1998). However, determining the location(s) of pore induction in skin is beyond the scope of the present study.

According to the electroporation theory proposed by Glaser et al. (1988) on lipid bilayers, the extent and rate of electroporation are related to the fluidity of the lipids. Previous findings in our laboratory on the influence of skin permeation enhancers are in accord with the view that these enhancers act by the mechanism of stratum corneum lipid fluidization to enhance transdermal permeation (Yoneto et al., 1995, 1996; He et al., 2003; Warner et al., 2003). Therefore, it is hypothesized that skin electroporation (i.e., skin electropermeabilization) can be reversibly enhanced by the lipid fluidization mechanism, and lower applied voltage would be required to induce and sustain a constant state of pore induction in HEM during AC iontophoresis in the presence of the permeation enhancers. Also, because these enhancers were expected to induce essentially the same fluidity increase in the transport rate-limiting domains of the stratum corneum intercellular lipid lamellae at their respective aqueous concentrations used in the present study (Yoneto et al., 1995, 1996; He et al., 2003; Warner et al., 2003), the similar effects observed under the enhancer conditions in the present study are consistent with the mechanism of lipid fluidization. The reversible nature of the enhancer effect further supports this hypothesis. Together, these findings suggest that pore induction during low to moderate voltage AC is related to electroporation of the lipid lamellae.

4. Conclusion

The effects of 9–40 V and 30 Hz–100 kHz AC upon HEM pore induction were studied. In the synthetic membrane control study, the electromobility of the background electrolyte ions under AC remained essentially constant at the voltage and frequency ranges investigated. In the HEM studies, the extent of pore induction during AC was dependent on the AC voltage and frequency. Higher AC voltage and lower frequency induced a larger HEM electrical resistance decrease. Longer recovery time and lesser extent of HEM resistance recovery were also observed after AC iontophoresis with higher voltage and/or lower frequency. Comparing the results in the present study with the threshold currents of sensation reported in the literature, the optimal AC frequency region for effective AC iontophoresis and the least sensation was determined to be around 10 kHz. Treatment of the HEM with the permeation enhancers under the present conditions did not affect the initial electrical resistance of HEM, but the permeation enhancers allowed the use of lower applied AC voltage for inducing and maintaining HEM electropermeabilization. The effects of the permeation enhancers were also shown to be reversible or at least partially reversible. This is consistent with the view that the chemical permeation enhancers are effective in reducing the energy required for electroporation of the stratum corneum lipids. In summary, the results of the present study on the effects of AC voltage and frequency should provide necessary information for the optimization of electropermeabilization utilizing AC. The results also demonstrate the effects of chemical permeation enhancers to enhance electropermeabilization. Such information is now available for controlling skin electrical resistance in the development of AC transdermal applications such as to help reduce flux variability during iontophoresis or to promote effective ion-exchange membrane enhanced iontophoresis *in vivo*.

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