Skin is the largest organ in our bodies, covering an area of approximately 2 m² and providing contact between our bodies and the external environment. The main function of skin is to act as a barrier to prevent the loss of tissue water and the external environment. The main function of skin is to act as a barrier to prevent the loss of tissue water and the external environment. The main function of skin is to act as a barrier to prevent the loss of tissue water and the external environment. The main function of skin is to act as a barrier to prevent the loss of tissue water and the external environment. The main function of skin is to act as a barrier to prevent the loss of tissue water and the external environment.

The average needle lengths range from 100 to 1500 μm. Although the implementation of these methods, including microneedles (MN), electroporation (EP), and sonophoresis (SN), are micron-sized needles that can breach the stratum corneum (SC), which is highly hydrophobic. Although SC is 10–20 μm thick, it only allows small, potent lipophilic drugs to permeate the skin. Most hydrophilic macromolecules, such as peptides and proteins, are unable to permeate passively. Therefore, enhancement techniques are required to assist in the transport of such macromolecules across the skin. 2,3) In this study, physical enhancement methods were intentionally highlighted, including microneedles (MN), electroporation (EP), and sonophoresis (SN). Although the implementation of these methods is different, they mutually seek to bypass or remove the SC. 5,6) MN are micron-sized needles that can breach the SC to create holes large enough for various molecules to pass through. The average needle lengths range from 100 to 1500 μm, which is long enough to pierce the SC and upper layers of viable epidermis. Nevertheless, the needles do not puncture the deeper dermis layer, where numerous nerve endings are located. In other words, the needles are minimally invasive and do not create a sense of pain. Interestingly, the needle holes are considerably larger than macromolecular sizes; therefore, the macromolecules can penetrate the skin effortlessly. Additionally, microneedle holes are thought to be aqueous, thereby facilitating hydrophilic macromolecule transport. 2,5) At present, MN technology may be effective in several possible clinical applications, including insulin delivery, transcutaneous immunizations, and cutaneous gene delivery. 6,7)

EP is the transitory structural perturbation of lipid bilayer membranes resulting from the application of high voltage pulses within a short time (μs-ms). Electric fields that are produced during current application induce structural rearrangements of the cell membrane. These rearrangements contribute to aqueous pore formation and provide a local driving force that facilitates the transport of hydrophilic molecules. Although EP is typically used on the unilamellar phospholipid bilayers of bacterial cell membranes to introduce a variety of molecules, such as DNA, EP also affects the SC, which is composed of multilamellar, intercellular lipid bilayer membranes. 7,8) To date, various molecules with a wide range of molecular weights and physicochemical properties have been successfully delivered using EP, including fentanyl, timolol, 8) salmon calcitonin, parathyroid hormone, 9,10) fluorescein isothiocyanate (FITC)-dextran (FD-4), 11,12) and DNA. 12)

SN, also known as phonophoresis, is the use of ultrasound to deliver therapeutic compounds through the skin. 13) This technique temporarily increases skin permeability; however, the basic mechanism is not still clearly understood. Ultrasonic energy can perturb mammalian tissue via several proposed mechanisms, i.e., thermal and cavitational effects. The former mechanism is attributed to the absorption of ultrasound energy, and the latter mechanism is caused by the collapse and oscillation of the cavitation bubble in the ultrasound field. 14) Acoustic cavitation is the proposed primary mechanism for SN. Given the distinct mechanisms, the SN frequencies are classified into 2 groups: (i) low-frequency SN (LFS; 20–100 kHz) and (ii) high-frequency SN (HFS; 0.7–16 MHz). Based on previous studies, LFS appears to more effectively increase skin permeability compared with HFS. A wider range of molecules, including small hydrophobic and large hydrophilic compounds, can be delivered by LFS compared with HFS. 15)

To date, the use of individual techniques alone and in...
combination have been widely investigated. MN pretreatment combined with iontophoresis (IP) has been studied by Wu et al. This method significantly enhances the flux of FD compared with MN pretreatment or IP alone. Later, Kumar, and Banga reported increased calcein and human growth hormone flux using the same combination. Furthermore, drug-loading dissolving MNs created from a methylvinylether and maleic anhydride (PMVE/MAH) copolymer demonstrated the potential for delivering bovine serum albumin (BSA) and insulin across neonatal porcine skin when used in conjunction with IP. However, this combination did not enhance the ability of small molecule-loaded MNs, e.g., theophylline, methylene blue, and fluorescein sodium, to permeate skin. Additionally, the combination of MN and EP, which is also referred to as in-skin EP, displayed a synergistic effect on FD-4 skin permeation compared with MN alone or conventional EP. In addition to the combined method described above, calcein and BSA skin permeation was enhanced using an array of hollow MNs in conjunction with LFS. The increased delivery rate and amount of drug delivered are attributed to SC fracturing using hollow MNs and increased flow rates of media containing solutions of each model drug.

Although each enhancement method and pair-wise combination promises improved skin permeation for a wide range of molecules, including macromolecules, the combination of these 3 methods has not been tested to the best of our knowledge. To increase the rate and amount of drug delivered, we combined the benefits of each method and carefully established the instrumentation; we named the combinatorial process MN+EP+SN. First, the MN array was used to pretreat the skin and break the SC barrier, resulting in holes large enough for molecules to pass through. An electric current was then applied to the MN array using an electrode to stimulate aqueous pore formation in the deeper skin layers. The ultrasonic energy created by the sonophoretic probe was provided to media containing the model drug solution, FD-4, to force the solution through the electroperforated holes. As a result, hydrophilic macromolecules were effectively transported through MN+EP-treated skin after SN application. The objective of this study was to investigate the in vitro skin permeation of the hydrophilic macromolecule FITC-FD (FD-4; molecular weight (MW) 4.4kDa) using 3 techniques: MN, EP, and SN. The effect of MN on skin permeation insertion force was investigated. Simultaneously, the effects of pulse voltage generated by EP and ultrasound intensity were determined. Thereafter, we evaluated the amount of FD-4 that permeated the skin using the following combinations of these 3 techniques under the appropriate conditions: MN+EP, MN+SN, EP+SN, and MN+EP+SN.

MATERIALS AND METHODS

Materials Fluorescein isothiocyanate (FITC)-dextran (FD-4; average molecular weight, 4.4kDa) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). All other chemicals were analytical grade and used without further purification.

Skin Preparation Porcine skin was used as an animal skin model in this study because it is a good model of human skin with regard to hair sparseness and physical properties. Porcine skin was obtained from a local slaughterhouse immediately after the animal was killed (Nakhon Pathom, Thailand). The excess subcutaneous fat was removed, and the skin was carefully trimmed to obtain a thickness of approximately 2.0–2.5 mm. The trimmed-skin thickness was measured using Vernier caliper.

MN Array Fabrication In this study, the MN arrays were classified into the following 2 categories depending on the enhancement method used: MN array and MN+EP array. The MN arrays used in the study were not associated with electric current, i.e., MN and MN+SN. In contrast, the MN+EP array was used in the following experimental groups: EP, MN+EP, and MN+EP+SN.

MN Array Preparation The MN arrays were prepared using 9 acupuncture needles (0.25×30 mm, DongBang Acupuncture, Inc., Boryeong, Korea). First, each MN was cut into 5 mm lengths and used to puncture a silicone sheet (15×15×2 mm), allowing 1000 µm of the needle tips to be exposed in the silicone sheet. The opposite end of the acupuncture needle was bent to obtain a perpendicular shape to anchor the needle. After piercing 9 needles, with 4-mm center-to-center spacing, the MN array was fixed on the silicone sheet using an adhesive tape to ensure that the needles remain stationary. A photograph of a needle tip in the silicone sheet and a schematic diagram of the MN array are shown in Figs. 1(a) and (b).

Preparation of the MN+EP Array To determine the combinatorial effect of MN and EP, the array was prepared as described above. However, before fixing the needles on the silicone sheet with an adhesive tape, copper wires, i.e., positive and negative line, were inserted under a perpendicular part of the needle so the MN+EP array could be used as an electrode in the EP experiments. To determine the effect of EP alone and eliminate the effect of MN, all of the needle tips were cut and filled until dull; these needles are referred to as blunted-MNs and were used to prepare the blunted-MN arrays following a method similar to that used to construct the MN+EP array. A schematic diagram of the MN+EP array is displayed in Fig. 1(c).

Characterization of the Microconduits Created by the MN Arrays The micropores created by the MN arrays were observed using a scanning electron microscopy (SEM, Cambridge, England). The MN-treated skin was dried and then covered with a thin layer of gold prior to observation at an accelerating voltage of 15 kV.

In Vitro Skin Permeation Studies In vitro permeation studies of FD-4 (2.5 mg/mL) were performed using a Franz diffusion cell apparatus. The diffusion cell has an average diffusion area of 2.022 cm², and the receptor compartment has an approximate volume of 6 mL. Before starting the experiments, the receptor compartment was filled with phosphate buffered saline (PBS, pH 7.4) and maintained at 32°C using a water bath. The solution in the receptor compartment was continuously stirred at 400 rpm using a magnetic stirrer. Each piece of treated porcine skin was placed on the receptor compartment by providing SC contact with the donor compartment. The donor and receptor compartments were then fixed using a clamp. The top of the donor compartment was filled with the model drug solution (1 mL). Moreover, the untreated porcine skin was used as a control. To investigate the cumulative permeation profiles, 500 µL of the model drug solution in the receptor compartment was sampled at 0.08, 0.25, 0.50, 1, 2,
After sampling, the solution was immediately replaced with the same volume of fresh PBS to maintain the sink condition. For better comparisons, the in vitro skin permeation studies were carefully performed in eight parallel sessions: (1) passive delivery, (2) MN alone, (3) EP alone, (4) SN alone, (5) MN+EP, (6) MN+SN, (7) EP+SN, and (8) MN+EP+SN.

**Skin Permeation Using MN** To determine the effect of the insertion force on model drug permeation, each skin was pierced using a homemade MN array with different forces (2.5, 5.0, 10, 20, and 30 N). Before starting experiments, a plastic container with smooth lid was filled with water to obtain the weight of 0.25, 0.5, 1, 2, and 3 kg. According to Newton's theory of gravitation (\( g = 9.8 \text{ m/s}^2 \)), these can be converted to 2.5, 5, 10, 20, and 30 N, respectively. Before the permeation studies, the plastic container with desired weight was carefully placed on a homemade MN patch and left it on the skin for 2 min. After that it was lifted from MN patch and MN patch was removed from the skin. Untreated skin was used as a control.

**Skin Permeation Using EP** EP was applied using a square wave pulse generator (ECM 830 Electro Cell Manipulator; BTX, San Diego, CA, U.S.A.). A homemade patch consisting of 9-blunted acupuncture needles connected with copper wire (blunted MN+EP array) was used as an electrode in order to eliminate the effect of needle. The skins were treated with pulse voltages of 50, 100, 200, and 300 V. The pulse duration and number of pulses were fixed at 1 ms and 99, respectively.

**Skin Permeation Using SN** A low ultrasonic frequency of 20 kHz was generated using an ultrasonic transducer (Vibra-cell™, VCX130 PB, Sonics and Materials, Inc., Newtown, CT, U.S.A.) and continuously applied. The radiating diameter of the transducer is 6 mm. Before starting the experiment, the donor compartment of the Franz diffusion cell was filled with the model drug solution (1 mL). The ultrasonic probe was positioned 3 mm above the skin inside the donor compartment during the ultrasound application. The intensities applied were 1.7, 3.9, and 6.1 W/cm².

**Skin Permeation Using the Combination of MN and EP** A homemade patch consisting of 9-acupuncture needles connected with copper wire (MN+EP array) was used as an electrode to evaluate the effect of pulse voltage on FD-4 permeation in MN+EP-treated skin. The skin was punctured using the MN+EP array with a force of 10 N for 2 min. Next, various voltages of electric current (50, 100, 200, and 300 V) were transferred to the skin using a pulse generator following conditions similar to those described above. An illustration of the in vitro skin permeation study using MN and EP is shown in Fig. 2(a).

**Skin Permeation Using the Combination of MN and SN** First, the MN array was used to create aqueous microconduits within the skin using 10 N for 2 min. The MN array was removed, and the skin was immediately placed on the Franz diffusion cell. Before starting the experiment, the diffusion cell was filled with the FD-4 solution (1 mL). The probe was placed 3 mm above the MN-treated skin, and ultrasonic energy was continuously applied to the skin using the following intensities: 1.7, 3.9, and 6.1 W/cm². An illustration of the in vitro skin permeation study using MN and SN is displayed in Fig. 2b.

**Skin Permeation Using the Combination of EP and SN** The blunted MN+EP array was used as an electrode in this experiment. EP was transferred to the skin using a 300 V pulse voltage. Pulse duration was fixed at 1 ms, and pulse number was fixed at 99. Next, the blunted-MN array was removed, and the skin was immediately placed on the Franz diffusion cell. The probe was placed 3 mm above the EP-treated skin. Ultrasonic energy was then applied through the FD-4 solution in the diffusion cell using 6.1 W/cm² for 2 min.
Skin Permeation Using the Combination of MN, EP, and SN

The MN+EP array was used as an electrode to evaluate the efficacy of this combination method. First, the skin was punctured with the MN+EP array using 10 N force. Next, electrical current was applied to the skin using a pulse generator. All electrical parameters, including pulse voltage, pulse time, and the number of pulses, were selected from the optimal protocol. The MN+EP-treated skin was mounted, and the FD-4 solution was instantly added to the diffusion cell. Ultrasonic energy was then applied using the optimized conditions. An illustration of this method is described in Fig. 2(a) (the first step) and Fig. 2(b) (the second step).

Quantitative Analysis

The FD-4 concentration in the receiver compartment was determined using a spectrofluorophotometer (RF-1501; Shimadzu, Kyoto, Japan). The fluorescent intensity was measured at the wavelength of 495 nm for emission and 515 nm for excitation. The concentrations were calculated from the fluorescent intensities using a calibration curve. All experiments were performed 4–6 times.

Confocal Laser Scanning Microscopy (CLSM) Studies

Before the observations, the treated skins were collected at the specified time following the permeation studies. The skins were washed with PBS (pH 7.4) and then wiped with tissue paper. The treated skin was placed on the microscopic slide, and the slide was scanned using an inverted Zeiss LSM 510 META microscope (Carl Zeiss, Jena, Germany).

Histological Examination of the Skin

Histological alterations in skins treated with various physical enhancement methods were evaluated. Each skin sample was fixed in a 10% formaldehyde solution for at least 3 d. The skin samples were dehydrated using ethanol and embedded in paraffin wax. The skins were vertically cut along the surface and stained with hematoxylin and eosin. The skin samples were then observed using light microscopy.

Mathematics of Skin Permeation

Fick’s first law of diffusion was employed to predict the rate at which substances penetrate the skin using different physical skin enhancement methods. The law assumes that the rate of transport of a drug passing through a unit area is proportional to the concentration gradient according to the following formula:

\[ J = -D \frac{\partial C}{\partial x} \]

where \( J \) is the rate of transfer per unit area, \( C \) is the concentration of the diffusing substance, \( x \) is the space coordinate, and \( D \) is the diffusion coefficient.\(^{22}\) Using the \textit{in vitro} permeation studies, the steady-state flux \( (J) \) of the drug can be calculated from the slope of the linear portion of the plot of the cumulative amount permeated per unit area against time.

Statistical Analysis

\textit{In vitro} drug permeation measurements were collected from 4 to 6 experiments. The values are expressed as the means±standard error (S.E.). The statistical significance of the differences in the amount of model drug permeated into the skin between the groups was examined using one-way ANOVA followed by Student’s \( t \)-test. The significance level is set at \( p<0.05 \).

RESULTS

Effect of MN Insertion Force on Skin Permeation

Insertion force is one of the factors that influences drug delivery and skin permeability.\(^{23}\) Before starting the \textit{in vitro} permeation studies using combination enhancement methods, the application forces were first optimized. The total cumulative amount of FD-4 that permeated MN-treated skin significantly increased as the insertion force increased from 2.5 to 10 N compared with the control group (Fig. 3). Although up to 30 N insertion force was applied, FD-4 permeation was not significantly enhanced with 10, 20, and 30 N insertion force (\( p<0.05 \)). It indicated that the 10 N insertion force was strong enough to overcome the resistance of skin, higher insertion forces would not affect the property of microneedle to puncture skin. The slope of the linear portion of the line plotted
from the steady-state values of the cumulative amount of FD-4 permeated and time (h) represents the permeation rate or the steady-state flux. Based on the data, an increase in the insertion force improves FD-4 flux (Table 1). As a result, a 10 N insertion force was therefore applied in further experiments.

**Evaluation of the MN Arrays Using SEM**
SEM imaging was used to visualize the surface of the MN-treated skin to ensure that the MN array can effectively breach the skin and create a hole. Figure 4(a) displays the micropores created by the MN array on the skin surface after piercing with 10 N force, and Fig. 4(b) shows an individual hole with a diameter of 100 µm created by MN.

**Effect of Pulse Voltage on Skin Permeation Using EP**
Pulse voltages and pulse times significantly influence the amount of drug delivered through EP-treated skins. To determine the effect of pulse voltage on FD-4 transdermal delivery, 99 pulses of 50, 100, 200, and 300 V were transferred using a blunted-MN array as an electrode and 100 ms pulse intervals. The results revealed that FD-4 skin permeation was undetectable at voltages below 200 V, which could serve as the voltage threshold of porcine skin under this protocol (data not shown). The FD-4 flux (µg/cm²h) in EP-treated skin after the application of 200 V and 300 V was 0.151 ± 0.047 (R²=0.975) and 0.216 ± 0.085 (R²=0.975), respectively. Furthermore, the effect of pulse voltages on MN-treated skin, i.e., the MN+EP method, was also investigated by maintaining a constant insertion force of 10N. FD-4 skin permeability significantly increased as pulse voltages increased from 100 V to 300 V, with the exception of 50 V and 100 V (p<0.05) (Fig. 5). The flux of FD-4 in MN+EP-treated skin was dramatically enhanced compared with MN alone (Table 2). Though the higher cumulative amount permeated attributed to an increase in voltage, the highest voltage that skin can be tolerate is 300 V. The skin was burned at 400 V. As a result, the combination of 10 N insertion force and 300 V was chosen for the next experiment.

**Effect of Ultrasound Intensity on Skin Permeation Using SN**
SN increases the skin permeability of several molecules by disturbing SC barrier function. The primary mechanism associated with this phenomenon is cavitation. Cavitation is dependent on various parameters, such as ultrasound frequency, intensity and application time. To evaluate the effect of ultrasound intensity (1.7, 3.9, and 6.1 W/cm²) on FD-4 skin permeation, LFS (20 kHz) was applied, and the application time was maintained at a constant of 2 min during the experiments. The results demonstrate no significant difference in the cumulative amount of FD-4 permeated after the application of all ultrasound intensities (p>0.05) (Fig. 6). The FD-4 flux (µg/cm²h) after the application of 1.7, 3.9, and 6.1 W/cm² was 0.335 ± 0.086 (R²=0.996), 0.178 ± 0.040 (R²=0.984), and 0.169 ± 0.040 (R²=0.967), respectively. The effect of MN pretreatment on FD-4 skin permeation following the application of various SN intensities is displayed in Fig. 7. The results show that the SN and MN combination dramatically enhances FD-4 skin

<table>
<thead>
<tr>
<th>Insertion force (N)</th>
<th>Flux (µg/cm²h⁻¹)</th>
<th>R²</th>
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<tr>
<td>2.5</td>
<td>0.48±0.14</td>
<td>0.996</td>
</tr>
<tr>
<td>5.0</td>
<td>1.37±0.36</td>
<td>0.997</td>
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<tr>
<td>10.0</td>
<td>2.31±0.55</td>
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<tr>
<td>20.0</td>
<td>2.44±0.86</td>
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<tr>
<td>30.0</td>
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<th>Pulse voltages (V)</th>
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<th>R²</th>
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<td>0a</td>
<td>2.358±0.551</td>
<td>0.995</td>
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<tr>
<td>50</td>
<td>5.832±0.454</td>
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<tr>
<td>100</td>
<td>5.588±0.737</td>
<td>0.996</td>
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<tr>
<td>200</td>
<td>6.190±1.493</td>
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<tr>
<td>300</td>
<td>7.582±2.226</td>
<td>0.996</td>
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*Fig. 4. Scanning Electron Microscope (SEM) Images of Pores Created by MN Array (a) and an Individual Hole Created in Porcine Skin by MN (b)*

*Fig. 5. In Vitro Permeation Profiles of FD-4 across Porcine Skin after Treatment with MN+EP Using Various Pulse Voltages: 0 V or MN Alone (■), 50 V (▲), 100 V (●), 200 V (▲), and 300 V (▲)*

Table 1. The Rate of FD-4 Permeation across Skins Treated with MN Using Various Insertion Forces

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<td>0.996</td>
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Table 2. The Rate of FD-4 Permeation across Skins Treated with MN Followed by EP Using Various Pulse Voltages

<table>
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*Fig. 5. In Vitro Permeation Profiles of FD-4 across Porcine Skin after Treatment with MN+EP Using Various Pulse Voltages: 0 V or MN Alone (■), 50 V (▲), 100 V (●), 200 V (▲), and 300 V (▲)*
permeability compared with MN alone; however, no statistically significant differences among all of the intensities used were observed ($p<0.05$). The FD-4 fluxes (µg/cm²/h) across MN-treated skin after treatment with 1.7, 3.9, and 6.1 W/cm² were $3.235\pm0.970 (R^2=0.995)$, $4.772\pm1.579 (R^2=0.995)$, and $3.554\pm1.211 (R^2=0.996)$, respectively. To compare skin permeation in the next experiments, an intensity of 6.1 W/cm² was used as the high ultrasound intensity.

**In Vitro Skin Permeation of FD-4 Using the Combined Strategies** In previous studies, the protocols used were carefully optimized and then selected to compare the FD-4 skin permeability. *In vitro* permeation profiles of FD-4 following treatment with several enhancement methods, i.e., MN, EP, SN, MN+EP, MN+SN, EP+SN, and MN+EP+SN, is presented in Fig. 8. These results demonstrated that FD-4 was not permeated by passive delivery (no treatment); however, the other techniques significantly enhanced FD-4 skin permeability ($p<0.05$). Although FD-4 could be detected in the receptor compartment following EP, SN, and EP+SN treatments, the cumulative amount was minimal. The total amount of FD-4 that permeated MN+EP-treated skin over 24 h was 20-fold higher compared with EP-treated skin and 3.6-fold higher compared with MN-treated skin. Similarly, the total amount of FD-4 that permeated MN+SN-treated skin was 6.3-fold and 2.1-fold higher compared with skin treated with SN or MN alone, respectively. The highest cumulative amount of permeated FD-4 was obtained when these 3 techniques were combined (MN+EP+SN). The total amount of FD-4 was 1.8-fold and 3.2-fold higher than MN+EP and MN+SN, respectively. The flux obtained from MN+EP was increased compared with MN and EP alone (Table 3). Likewise, MN+SN contributed to increased flux compared with MN and SN alone. The highest flux was observed with the combination of the 3 strategies, i.e., MN+EP+SN.

**CLSM Studies** CLSM was employed to visualize model compound penetration by providing controllable depth imaging and collecting sequential optical sections from the entire treated skin without sectioning. The FD-4 penetration through the skins following an hour of *in vitro* permeation studies was observed and recorded at increasing depths from the skin surface (Fig. 9). In the control group (untreated skin), minimal fluorescent signal was observed in the SC and lower epidermal tissue (data not shown). In contrast, the fluorescence intensity was localized around the microchannel where the SC was breached in MN-treated skin was observed. However, the fluorescence depth was not greater than 100 µm (Fig. 9(a)). Fluorescence was observed up to a depth of 220 µm in the skins treated with MN+EP, MN+SN, and MN+EP+SN. Furthermore, the hole size created by the combined techniques was greater than the hole size produced by MN alone (Figs. 9(b)–(c)).
Skin Histology  Figures 10(a)–(h) displays representative biological cross sections of porcine skin treated with the following enhancement methods: (a) untreated skin, (b) MN, (c) EP, (d) SN, (e) MN+EP, (f) MN+SN, (g) EP+SN, and (h) MN+EP+SN. These histological images indicate that no noticeable damage or changes in skin treated with all of the enhancement methods were observed. Both skin layers, i.e., epidermis and dermis, remained intact in all of the skin treatment groups (Figs. 10(b)–(h)). These results were similar to the normal appearance of untreated porcine skin (Fig. 10(a)).

DISCUSSION

The stratum corneum (SC), the skin’s outermost layer, is a barrier that prevents molecular transport across the skin.\(^{30}\) Therapeutic agents, such as peptides, proteins, and oligonucleotides, are difficult to deliver by conventional methods or topical delivery. However, advances in physical enhancement methods in the last decade has led to the development of powerful strategies to overcome the skin’s barrier function.\(^{31}\) MNs are micron-sized needles that can perforate the skin in a minimally invasive and painless manner, thereby creating aqueous transport pathways within the skin referred to as microchannels.\(^{23,32,33}\) Moreover, these microchannels have no limitation regarding the size of molecules that can pass. In terms of size, the microchannels are in the range of microns, while the macromolecules delivered are typically nanometers in size.\(^{17}\)

One of factors that challenge to development of MN-assisted delivery is skin elasticity.\(^{34,35}\) If the needle is not long enough to overcome the skin elasticity, it would provide an unsuccessful result. To date many research groups have reported the effect of MN length on skin permeability. The length has been varied between 25 and 2000\(\mu\)m.\(^{6}\) Their results demonstrated that the longer MN, the higher skin permeability would be achieved. Yan et al. evaluated the effect of MN length on the skin permeation of acyclovir. Their results showed that the permeation of acyclovir after pretreatment with 100–300\(\mu\)m MN was very low compared to control. In contrast, if the MN was lengthened up to 600\(\mu\)m, the permeation would be more effective. Furthermore, 1100\(\mu\)m-long MN showed the highest skin permeation.\(^{36}\) Teo et al. observed skin permeation of calcein after pretreatment with 130\(\mu\)m needle in vitro. However, this similar size of MN could not have an effectiveness to deliver insulin in vivo. They discussed that the length and tip sharpness have to be improved for systemic drug delivery.\(^{37}\) Martanto et al. have reported that only one third of the length of hollow MN can pierce the skin. MN was inserted to 1080\(\mu\)m from the skin surface, however it could penetrate the skin only 100–300\(\mu\)m.\(^{34}\) Though MN was relatively long, the tip of MN remained in viable epidermis. This could be implied that MN can breach SC without stimulating nerves.
locating in dermis layer. In clinical study, the relatively long MN, i.e., 620 µm, can successfully deliver naltrexone from its patch into systemic circulation. Furthermore, the only commercial product is Soluvia (Becton, Dickinson and Company). This product has 1.5 mm-long MN attached to 0.1 mL syringe that is prefilled with influenza vaccine. Above all, to assure that the length of MN will be long enough to penetrate SC and provide an efficiency to deliver macromolecules, we therefore fabricated MN patch with the length of 1000 µm.

In our experiments, a homemade MN array was successfully fabricated to evaluate the skin permeability of FD-4, a hydrophilic macromolecular compound. Additionally, the MN array was used in conjunction with the other enhancement strategies, i.e., EP and SN, to investigate the effects of the combined techniques.

Based on in vitro permeation studies, FD-4 can be successfully delivered using a homemade MN array. In contrast, FD-4 was not detected in the receptor compartment after passive delivery. As previously reported, a MN array with different dimensions overcome the SC barrier and improved FD-4 transport.16,19) The insertion force influences skin permeation. With increased insertion force, increased amounts of permeated FD-4 were obtained. This results could be attributed to the fact that different insertion forces create microconduits with different diameters and depths.36) SEM images also demonstrate that MNs successfully puncture and create obvious micro pores on the skin, indicative of SC barrier disruption.37,39–42) This finding could imply that these micro pores can serve as a significant pathway for the transportation of molecules across the skin. In addition, CLSM images also suggest that MN can effectively facilitate FD-4 transport into the skin as a result of SC opening.38)

EP (200–300 V) significantly enhanced the transdermal delivery of FD-4 compared with passive diffusion (p<0.05). This finding is possibly due to the fact that EP pretreatment promotes enhanced macromolecular transport through permeabilized skin by creating new aqueous pathways.27,28) The effect of pulse voltage was clearly demonstrated when EP was employed in conjunction with MN. The total cumulative amount of permeated FD-4 increased with increasing applied voltages via the MN+EP array.25,41–46) This results may be attributed to increased voltages that result in increased transport pathways.45)

SN is an enhancement technique that temporarily increases skin permeability, allowing several therapeutic agents to be delivered non-invasively. After 2 min of continuous SN application, the amount of FD-4 that permeated through the SN-treated skin was significantly increased compared with passive delivery (p<0.05). It should be noted that LFS, i.e., 20 kHz, can improve the transdermal delivery of hydrophilic macromolecules.37) Nevertheless, the range of intensities used in this study (1.7–6.1 W/cm²) did not affect FD-4 skin permeation through SN-treated skin.27) In addition, the total cumulative amount of FD-4 that permeated across the MN+SN-treated skin was similar among various applications of intensities. Based on previous studies, various SN mechanisms have been proposed, including thermal effect and cavitation. However, the latter is believed to be the predominant mechanism responsible for molecular transport.14) The collapse of cavitation disrupts the SC lipid bilayer membrane, resulting in enhanced FD-4 transport following SN application.15,41)

In the comparative studies, the amount of FD-4 that permeated the skin treated with either MN or EP alone was minimal. As expected, the MN+EP method displayed a synergistic effect on FD-4 skin permeation. These results are consistent with the studies of Yan et al., who report that the IN-SKIN EP technique increased FD-4 permeability compared with MN alone and conventional EP.39) Given that the SC displays increased electrical resistance compared with the underlying skin and deeper tissues, an electric current applied to the skin will be concentrated in the SC.7) To improve the electric field inside the skin, MN, which can perforate the skin and bypass the SC barrier, was employed in combination with EP to allow the electric current to reach the underlying skin. Our results obtained from in vitro permeation studies suggest that each MN can serve as an electrode for EP, thereby resulting in the formation of an electric field inside the skin. Interestingly, the microconduits created by the MN+EP array are larger and deeper compared with MN alone (see Fig. 9(b)). This finding might be attributed to the fact that decreased skin resistance with the MN+EP array could enhance the explosion of skin following EP application at the site of each needle tip.

The MN+SN strategy enhanced the transport of FD-4 compared with each technique alone. The synergistic outcome could be attributed to the creation of microconduits in the skin that could serve as a shunt to deliver macromolecules. Then, the collapse of acoustic cavitation generated by the sonophoretic probe could provide energy to the media containing FD-4 that promotes FD-4 diffusion into these channels.20) As previously reported, ultrasonic energy results in the creation of 1–2 µm diameter micropores on the skin. Using SEM, these pores were observed on the surface after LFS application.14) Based on these data, the combined method may serve as a useful technique for macromolecule delivery; the size of the microconduits created by MN appears to increase after 2 min of SN application (see Fig. 9(c)). When EP and SN were employed in combination, the total cumulative amount of permeated FD-4 remained low. It should be noted that MN significantly affects FD-4 skin permeation.

The best result was obtained using all of the enhancement methods in combination, i.e., MN+EP+SN. As discussed above, MN can effectively bypass the SC barrier. EP aids in the enlargement of the microconduits produced by MN+EP array. Finally, these microconduits are slightly further expanded following SN. For these reasons, the total amount of permeated FD-4 in MN+EP+SN-treated skin over 24 h was the highest compared with pairwise combinations or individual techniques. The application of all physical enhancement methods in our studies demonstrated safety under the specified conditions.47–50) However, the in vivo safety in clinical use has to be evaluated in further study.

CONCLUSION

To improve the rate and extent of drug delivery across the skin, physical enhancement methods, i.e., MN, EP, SN were used individually or in combination. The combined methods significantly enhanced FD-4 permeation in vitro porcine skin compared with individual methods. The combination of all 3 methods not only promotes FD-4 skin permeability. It could be concluded that MN+EP+SN could serve as an alternative strategy for transdermal drug delivery that does not
cause structural changes and skin damage.

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