The Effect of Iontophoresis with and without Electroporation on the Penetration of High Molecular Compounds into the Stratum Corneum

Natsumi Hashimoto,¹ Shigeru Tatsuta,*,¹ Hisashi Kitamura,¹ Misa Katsuyama,² Kentaro Uchida,² Kenji Sugibayashi*,³,⁴

¹ Beauty and Personal Care Business Division, Living Appliances and Solutions Company, Panasonic Corporation, 2-3-1-2 Nojihigashi, Kusatsu, Shiga 525-8555, Japan: ² Product Analysis Center, Panasonic Corporation, 1048 Kadoma, Kadoma, Osaka 571-8686, Japan: ³ Faculty of Pharmaceutical Sciences, Josai International University, 1 Gumyo, Togane, Chiba 350-0295, Japan: ⁴ Faculty of Pharmacy and Pharmaceutical Sciences, Josai University, 1-1 Keyakidai, Sakado, Saitama 350-0295, Japan.

* To whom correspondence should be addressed.
Email: tatta.shigeru@jp.panasonic.com and sugib@josai.ac.jp
Summary

Both iontophoresis (IP) and electroporation (EP) can be utilized to increase the penetration of relatively high molecular pharmaceutical and/or cosmeceutical compounds into the stratum corneum (SC), the uppermost layer of the skin. However, few reports exist on which molecular weights are capable of penetrating the SC, although low molecular compounds of less than 500 Da have been found to readily permeate the skin barrier. In our investigation, we applied fluorescein amine-labeled sodium hyaluronate to porcine aural skin after treatment by IP alone or EP + IP. Each layer of the SC was then tape stripped several times. The stripped SC sheets were observed using a confocal laser scanning microscope to determine the relative amounts of sodium hyaluronate present. The results confirmed that the molecular weight of sodium hyaluronate that penetrated the SC was higher with EP + IP than with IP alone. A high correlation was also established between the quantity of sodium hyaluronate that penetrated and its molecular weight following combined EP + IP treatment.

**Key words**  electroporation; iontophoresis; sodium hyaluronate; stratum corneum penetration; molecular weight
INTRODUCTION

The stratum corneum (SC) of the epidermis is composed of corneocytes and intercellular lipids, with the intercellular lipids forming a lamellar structure consisting of water and lipids. This lamellar structure provides a highly effective barrier to different external stimuli and has the effect of preventing the penetration of various chemical substances into the skin. Therefore, even when a useful pharmaceutical or cosmeceutical ingredient is applied to the skin, it is prevented from penetrating the SC, and even if it is able to, the rate of penetration is extremely slow. It is also reported that the molecular weight and polarity of the chemicals applied to the skin affect the rate and depth of their penetration. Generally, the rate and depth of skin penetration decrease with increasing molecular weight. Chemical substances with a molecular weight of more than 500 Da therefore do not easily penetrate the skin. However, high-molecular-weight antibody drugs and medium-molecular-weight peptide drugs are an increasing focus of research attention, and a growing number of functional cosmetics containing polymer and high molecular components are being used in beauty and aesthetic medicine.

Non-invasive skin penetration-promoting technologies such as electroporation (EP) and microneedles have also attracted attention due to their ability to enhance the skin penetration of medium and high molecular weight molecules. EP can create micropores in the lamellar structure of SC intercellular lipids by generating a high electric field, and several drugs were successfully absorbed through the epidermis to system circulation. In addition, by using iontophoresis (IP) in combination with EP, the skin penetration of useful high molecular compounds can be further increased via the electroporated pores. EP significantly enhanced the in vitro skin permeation of
nalbuphine and its prodrugs, and the enhancement effect was more pronounced after IP application. The combined effect of EP and IP on the \textit{in vivo} percutaneous absorption was also reported using insulin. However, there are few reports on the relationship between the effects of EP and IP and the molecular weight of the penetrants. In addition, few reports were found on the SC penetration of high weight molecular compounds by the combined use of EP and IP. In the present study, using sodium hyaluronate of different molecular weights, we experimentally investigated the molecular weight range over which EP and IP can promote the SC penetration of typical medium to high molecular compounds.

\textbf{MATERIALS AND METHODS}

\textbf{Materials} Edible porcine aural skin (BSS005, LWD strain, National Federation of Agricultural Cooperative Associations) supplied by KAC (Kyoto, Japan) was used as the test model skin in conformity with the 3Rs for animal experiments. Fluorescein amine-labeled sodium hyaluronate (PG Research, Tokyo, Japan) was used as the test medium for high molecular weight compounds, prepared as 0.3 mg/mL aqueous solution. Seven kinds of fluorescein amine-labeled sodium hyaluronate with different molecular weights were used: 2,000 - 4,000 Da (average 3,000 Da), 5,000 - 10,000 Da (average 7,500 Da), 20,000 - 30,000 Da (average 25,000 Da), 40,000 - 80,000 Da (average 60,000 Da), 100,000 - 300,000 Da (average 200,000 Da), 600,000 - 1100,000 Da (average 850,000 Da), and 1200,000 - 1600,000 Da (average 1,400,000 Da).

\textbf{Iontophoresis and electroporation apparatuses} An IP device (made in-house at Panasonic Corporation) designed to output a square wave with a frequency of 3 kHz, a voltage of 10 V, and a duty of 50%, was used to ensure safety for human use. Figure
1a shows the monopolar IP device. It consists of a triangle electrode (base 35 mm × height 30 mm) having the treatment surface and a counter electrode (metal plate). The anode electrode was applied to the treated skin surface, whereas the electrical circuit ground was kept physically distant from the treated skin surface.

An EP equipment (made in-house at Panasonic Corporation) capable of outputting a square wave with a frequency of 1 kHz, a voltage of 37 V, and a duty of 50% was used to ensure safety for human use. Figure 1b shows the EP electrode configuration. Bipolar method was used. It consists of a comb-shaped electrode (1 mm width and 2 mm distance between electrodes), and copper rectangular electrodes (50 mm × 38 mm) were used for both the anode and the ground. An insulating layer made of polyimide or polyethylene terephthalate (PET) was provided on the surface of the electrode, enabling it to generate an electric field without permitting any current to flow on the treatment surface.
Fig. 1. Shapes of IP (a) and EP (b) devices and experimental setup (c) to determine skin penetration of sodium hyaluronate

(a) Shape of IP device: Monopolar method, consisting of a triangular electrode (base 35 mm × height 30 mm) having the treatment surface and a counter electrode (metal plate), (b) Shape of EP device: Bipolar method, consisting of a comb-shaped electrode (1 mm width and 2 mm distance between electrodes), and a copper rectangular electrode (50 mm × 38 mm) composed of an anode and a ground. An insulating layer made of polyimide/polyethylene terephthalate (PET) was provided on the surface of the electrode, and only an electric field was generated without current flowing on the treated surface, and (c) Experimental setup: (i) edible porcine aural skin, (ii) cotton cloth (7.5 mm × 10.0 mm, 0.4 mm thick) moistened with an aqueous solution (250 μL) of fluorescein amine-labeled sodium hyaluronate, and (iii) IP device or EP device.
**Determination of skin penetration-enhancing effects**  For the application of IP, a piece of cotton cloth (0.75 cm × 1.0 cm = 0.75 cm², 0.4 mm thick) moistened with an aqueous solution (250 μL) of fluorescein amine-labeled sodium hyaluronate of different molecular weights, was placed on the porcine aural skin which was layered on the metal plate, as shown in Fig. 1c. Thus, the dose became 0.10 mg/cm². The IP device was then applied to the top surface of the cotton cloth for six seconds. A voltage was applied between the IP electrode and the metal plate, and a current was passed from the surface to the bottom through the aural skin. Each molecular weight-sample of sodium hyaluronate was tested once.

For the combined EP + IP treatment, the aural skin was first treated with the EP device for three seconds, after which a piece of cotton cloth moistened with sodium hyaluronate solution (0.10 mg/cm²) was applied as shown in Fig. 1c. The IP device was applied for six seconds, in the same way as for the IP-alone treatment. The combined EP + IP treatment was also tested once for each molecular weight sample.

For both the IP-alone treatment and the combined EP + IP treatment, the aural skin surface was wiped three minutes after the procedure. The treated SC was then tape-stripped 10 times with pressure-sensitive adhesive (PSA) tape (D-100-D-Squame Standard Sampling Discs, Clinical & Derm, LLC, Dallas, TX, U.S.A.).

Bright- and dark-field observations were made using a confocal laser scanning microscope (FV10i, Olympus, Tokyo, Japan). The 7th section was used as the observation tape. Against the penetrated area (0.75 cm × 1.0 cm = 0.75 cm²) of fluorescein amine-labeled sodium hyaluronate, the ROI (region of interest) for the observation was set to 1,200 μm × 1,200 μm. The observed fluorescent area (%) as a proportion of the total area in the 7th stripped tape was defined as the penetrated
quantity of sodium hyaluronate.

RESULTS AND DISCUSSION

Figure 2 shows examples of confocal laser scanning microphotographs of the 7th section of stratum corneum, peeled off by tape stripping, containing the penetrated fluorescein amine-labeled sodium hyaluronate. The figure shows the data for the 7th stripped tape due to there being less variation in the 7th tape than in the 1st - 3rd tapes, and a higher value than seen in the 9th - 10th tapes. A similar comment on the variation in the 1st - 3rd tapes was found in the guideline for bioequivalence studies of generic products of topically applied formulations.8) The following data therefore comes only from the 7th tape.

When microscopically observed in a bright field, the stripped piece of SC was observed in white, whereas when observed in a dark field, the penetrated fluorescein amine-labeled sodium hyaluronate was observed in green (Fig. 2a and b, respectively, for both IP alone and EP + IP). The green color in Fig. 2b is hard to see because it is on a black background, so image-thresholding was carried out from green to red for Fig. 2a and b to Fig. 2c and d, respectively. Figure 2d (after image-thresholding) is thus much clearer than the corresponding Fig. 2b (before image-thresholding). Based on each binarized value, the amount of sodium hyaluronate penetrating the SC was determined from the fluorescent area, as fluorescein amine-labeled sodium hyaluronate per unit SC area.
**Fig. 2.** Microphotographs of the tape-stripped stratum corneum containing fluorescent sodium hyaluronate of molecular weight 2,000 - 4,000 Da (average molecular weight: 3,000 Da)

(a) Bright field, (b) Dark field, and (c) and (d) Post image-thresholding of (a) and (b), respectively.

Figure 3 shows the effect of the molecular weight of sodium hyaluronate on its penetration observed under each test condition. Semi-logarithmic plot and double-logarithmic plot were done in Fig. 3a and b, respectively. The Y-axis in Fig. 3a and b shows the fluorescent area (%) as a proportion of the total area in the 7th stripping tape as an index of quantity of penetrated sodium hyaluronate. Each data point
represents a single measurement. Highly scattered data were obtained in cases of high molecular sodium hyaluronate by only IP application (see Fig 3b), probably due to its low concentration in the SC sheet. The detection limit and quantification limit of the fluorescent area (%) were about 0.002 and 0.01%, respectively. Then, the penetration data having more than 0.01% show in blue diamond for EP +IP and red square for IP alone, and those less than 0.01% show in gray symbols in Fig. 3. It is clear, however, that the greater the molecular weight of sodium hyaluronate, the less hyaluronic acid penetrated the SC under both IP alone and EP + IP conditions. In addition, the penetrating quantity of sodium hyaluronate was generally greater with EP + IP than with IP alone. This result suggests that micropores were formed by the electric field generated by the insulated electrode only for 6-second application and that they increased the skin penetration of sodium hyaluronate. With EP + IP, the average molecular weight of sodium hyaluronate penetrating the SC increased about 5-fold for 3,000 - 7,500 Da and about 70-fold for 25,000 and 60,000 Da. Although penetration by IP alone markedly decreased when the average molecular weight of sodium hyaluronate exceeded 25,000 Da, with EP + IP, penetration into the SC was confirmed up to an average molecular weight of 200,000 Da. These results suggest that the larger the molecular weight, the greater the effect of EP on SC penetration. Since the quantity of sodium hyaluronate that penetrated markedly decreased at molecular weights exceeding 850,000 Da, the maximum permeable molecular weight was estimated to be about 200,000 Da. Only one measurement was done for each molecular weight of sodium hyaluronate. More experimental data must be added for detailed discussion.
**Fig. 3.** Effect of IP with and without EP on the penetration of sodium hyaluronate into the stratum corneum

(a) Semi logarithmic plot, (b) Double logarithmic plot. Symbols: blue diamond for EP + IP treatment and red square for IP alone. Gray symbols show data less than 0.01%.

Next, the amount of sodium hyaluronate penetrated for each molecular weight with the EP + IP treatment was subtracted from the quantity that penetrated with the use of IP alone to evaluate the effect of EP alone. The double logarithmic correlation was then determined between the subtracted value using the contribution by EP alone and the molecular weight of sodium hyaluronate. The results are shown in Fig. 4. Data for
fluorescein amine-labeled sodium hyaluronate with average molecular weights of 850,000 Da and 1,400,000 Da were omitted in the figure because of very low penetration %. A high correlation ($\gamma^2 = 0.97$) was confirmed between the two. Figure 4 was very closed to the data for EP + IP in Fig. 3b, which suggests much lower permeation by IP alone than the combined treatment of EP and IP.

**Fig. 4.** Effect of only EP treatment on the penetration of sodium hyaluronate ($\Delta$ penetration) into the stratum corneum

The amount of each molecular weight of sodium hyaluronate penetrated with EP alone ($\Delta$ penetration) was calculated by the difference between the penetration data by EP + IP and IP alone.
In this study, sodium hyaluronate was not directly measured, nor was the fluorescence spectrophotometer used to measure the fluorescent intensity of fluorescence-labeled sodium hyaluronate. We determined the fluorescence intensity area of fluorescence-labeled sodium hyaluronate in the stripped SC, as explained in Fig. 2, and used this area ratio (%) as an index of quantity of sodium hyaluronate present. The data in Figs. 3 and 4, although reliable and informative, therefore do not record the actual amount of sodium hyaluronate present. Our method, nonetheless, can be utilized to quantify compounds that are difficult to measure directly: further research is needed to determine the exact correlation between the direct measurement method and the present measurement method.

CONCLUSION

The short period of use of EP + IP increased the quantity of medium to high molecular weight chemicals that penetrated the SC and allowed molecular sizes of up to 200,000 Da to penetrate the skin. The EP + IP treatment far more effectively raised the SC penetration of medium to high molecular compounds than IP alone. Our results also showed a high correlation between the molecular weight of the penetrants and the quantity that penetrated the SC. In the near future, it will be necessary to consider more powerful application conditions of EP while continuing to prioritize safety.

Conflict of Interest The authors declare no conflict of interest.
REFERENCES

1) Potts R.O., Guy R.H., A predictive algorithm for skin permeability: the effects of molecular size and hydrogen bond activity, *Pharm. Res.*, 12, 1628-1633 (1995).

2) Bos J.D., Meinardi M.M., The 500 Dalton rule for the skin penetration of chemical compounds and drugs, *Exp. Dermatol.*, 9, 165-169 (2000).

3) Charoo N.A., Rahman Z., Repka M.A., Murthy S.N., Electroporation: an avenue for transdermal drug delivery, *Curr. Drug Deliv.*, 7, 125-136 (2010).

4) Shende P., Sardesai M., Gaud R.S., Micro to nanoneedles: a trend of modernized transepidermal drug delivery system, *Artif. Cells Nanomed. Biotechnol.*, 46, 19-25 (2018).

5) Fang J.Y., Sung K.C., Wang J.J., Chu C.C., Chen K.T., The effects of iontophoresis and electroporation on transdermal delivery of buprenorphine from solutions and hydrogels, *J. Pharm. Pharmacol.*, 54, 1329-37 (2002).

6) Tokumoto S, Higo N, Todo H, Sugibayashi K., Effect of electroporation and pH on the iontophoretic transdermal delivery of human insulin, *Int. J. Pharm.*, 326, 13-19 (2006).

7) Tokumoto S, Higo N, Todo H, Sugibayashi K., Effect of combination of low-frequency sonophoresis or electroporation with iontophoresis on the mannitol flux or electroosmosis through excised skin, *Biol. Pharm. Bull.*, 39, 1206-1210 (2016).

8) Sugibayashi K, Chemical disposition in skin, Chapter 3, in “Skin Permeation and Disposition of Therapeutics and Cosmeceutical Compounds”, ed, by Sugibayashi K., Springer Japan KK, 2017.