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# *"Novel Approach to Enhancing Skin Penetration of Active Ingredients by Cosmetic Film Formation on the Skin"*

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## 1. Introduction

Sensitive skin is a widely observed symptom. According to a recent comprehensive review report [1], more than 50% of the world's population complain of some sensitive skin symptoms. People having sensitive skin often suffer from itch, which is one of most distressing issues in daily life [2]. Itch is caused by activation of nerve fibers through physical or chemical stimulation of the skin, which subsequently induces scratching behavior. Scratching as a reaction to itch damages the skin and impairs the skin barrier function by disrupting the stratum corneum. Scratching also induces itching factors, such as histamine, substance P (SP), and a variety of inflammatory cytokines. Nerve endings in the skin have several types of itch-related receptors [3]. Thereby, scratching leads to a stronger itch. This vicious cycle is commonly referred to as the itch-scratch cycle, which imparts a severe and significant impact on patient's quality of life [4-6]. Recent results have shown that inhibiting interactions between SP and neurokinin 1 receptor (NK1R) is effective in alleviating chronic itch [7]. Recently, our research group has found that maltotetraose, hereafter abbreviated as MTO, suppresses itch through inactivation of NK1R [8,9]. However, MTO has inherently poor skin permeability because of its hydrophilic nature and large molecular weight. If MTO can be effectively delivered to the epidermis containing nerve fibers by overcoming these issues, mitigation of itching caused by sensitive skin symptom is expected.

For delivery of hydrophobic active ingredients in O/W emulsion formulations, several strategies have been explored to improve the skin penetration. One of the strategies is to reduce oil droplet particle sizes [10-12]. However, in the case of the delivery of hydrophilic active ingredients such as MTO, since the ingredients exist in the bulk (aqueous) phase, it is practically difficult to apply similar strategies. In addition, use of penetration enhancers potentially improves their penetration deep into the skin, but may simultaneously disrupt the skin barrier, which is considered unsuitable for people with sensitive skin. Therefore, to efficiently deliver the hydrophilic active ingredients such as MTO to the inner layers of the skin, we have focused on thin films formulations on the skin surface (referred to as cosmetic films). Many functionalities of cosmetics, such as permeability and texture, are closely linked with a physical or chemical state of the cosmetic film.

Recently, the surface morphology in the xy-plane on the skin has been studied to improve functionalities, such as texture and UV protection [13-15]. However, there have been very few studies that have focused on the permeability through the control of material distributions in the cosmetic film. In this study, we hypothesized that improving contact efficiency of the skin and an aqueous phase involved in the cosmetic film is crucial for the efficient delivery of the water-soluble active ingredients. Based on this hypothesis, we explored a new approach toward enhancing penetration by controlling the structural stability of the cosmetic film.

Here, we investigated the MTO skin penetration using mass microscopy. Mass spectrometry imaging (MSI) uses mass spectrometry to detect the components of interest without labeling [16]. MSI enables direct evaluation of the permeability of the targeted ingredients. In addition, a three-dimensional cultured human epidermis (CHE) contains tissues from the stratum corneum to the stratum basal [17]. CHE allows for an assessment of the MTO permeability into inner skin layers deeper than the stratum corneum. As a result, we find that optimization of the number of glycerol units in polyglycerol fatty acid ester (PGFE) surfactant, a component of the O/W emulsions, enhances the penetration of MTO. Furthermore, we have successfully analyzed the distribution of the aqueous phase in the cosmetic film, which greatly help elucidate the skin penetration enhancement mechanisms of MTO.

## 2. Materials and Methods

### 2.1 Skin permeability of MTO

#### 2.1.1 Permeability evaluation of MTO

We formulated the O/W emulsions with an average hydrophile-lipophile balance (HLB) of 6, 8, and 10 for the polyglycerol fatty acid ester (PGFE) surfactants at fixed mass concentration, while varying the mixing ratios of monoglycerin and decaglycerin as 1:0, 1:2, and 2:1. The skin permeability of each formulation containing 1.0% MTO was evaluated using the CHE (LabCyte EPI-MODEL 24; Japan Tissue Engineering, Japan). Phosphate-buffered saline (PBS) was used as a blank. The CHE were aseptically removed from a transport agarose medium, transferred into 24-well plates containing the assay medium, and pre-incubated overnight in a 5% $CO_2$ incubator at 37 °C. After pre-incubation, each sample was applied to the apical side of the CHE while the basolateral side was filled with PBS. After 6 hours in a 5% $CO_2$ incubator at 37 °C, the skin was rinsed with PBS and collected by cutting them out from the cup wells. The tissues were embedded in Super Cryoembedding Medium (Section-Lab, Japan), frozen on dry ice, and stored at -20°C.

#### 2.1.2 Sample Preparation for Matrix-assisted laser desorption/ionization (MALDI)-MSI Measurements

The frozen tissues were cut into 10 μm thick slices using a cryostat (CM1950, Leica, Germany) at -20 °C. The sections were gently mounted onto indium tin oxide (ITO)-coated glass slides (100Ω, Matsunami Glass, Japan). Before the matrix was applied to the samples, the slides were dried using a dry vacuum pump (DTC-21, ULVAC, Japan) for 15 minutes at room temperature. Subsequently, just before MALDI MSI (matrix assisted laser desorption/ionization mass spectrometry imaging) observation, the samples were coated with 2,5-dihydroxybenzoic acid solution (DHB, BRUKER, Germany) at 40 mg/mL dissolved in 50% methanol as a matrix, using an automatic sprayer (TM-Sprayer, HTX Technologies, USA). The spray parameters were set as follows: nozzle temperature, 80 °C; number of passes, 24; matrix solution flow rate, 0.05 mL/min; moving velocity, 1200 mm/min; track spacing, 3 mm; gas pressure, 10 psi; gas flow rate, 3 L/min; drying time, 10 s; and nozzle height, 40 mm.

### 2.1.3 Acquisition and data analysis of MALDI-MSI

The MALDI-MSI observations were conducted on a high-resolution microscopic imaging mass spectrometer (iMScope; Shimadzu, Japan), equipped with an atmospheric pressure-MALDI and a quadrupole ion trap Time-of-Flight analyzer. The mass spectra were acquired at m/z 400-700 in the positive ion mode with the scan pitch of 5 μm and a laser diameter of 5 μm, where m/z is the ratio of the mass of ion to its charge. The other setting parameters were set as follows: a repetition rate was 1000 Hz, the number of laser shots was 100, a detector voltage was 2.1 kV, the heat block temperature was 200 °C, and the curved desolvation line temperature was 200 °C. The MSI dates were analyzed by IMAGEREVEAL MS software (Shimadzu, Japan). Regions of interest (ROIs) were selected from the skin sections. An averaged signal intensity was obtained by averaging signal intensities inside a single ROI. All spectra were normalized to their respective total ion currents [18].

### 2.1.4 Hematoxylin and Eosin (H&E) Staining

The post-irradiated tissues were stained with Hematoxylin and Eosin (H&E) using a Hematoxylin and Eosin Stain Kit (Cat# HAE-1; ScyTek Laboratories, USA) to visualize structures of the epidermis. Before staining, the post-irradiated tissue sections were washed with acetone for a few seconds to get rid of the matrix. Stained tissues were photographed using a digital microscope (BZ-X810; Keyence, Japan).

### 2.2 Analysis of the cosmetic film by Confocal Raman microscopy

Confocal Raman microscopy (CRM; inVia Qontor, Renishaw, United Kingdom) was used to visualize temporal distributions of the aqueous and oil phases during a drying process in the cosmetic film on an artificial skin model. We utilized a synthetic skin (VITRO-SKIN, Florida Suncare Testing, USA) [19,20] as the skin model, which mimics the surface properties of human skin. The VITRO-SKIN has been claimed to have topography, pH, critical surface tension, chemical reactivity, and ionic strength properties close to those of human skin. To prepare the skin model for the imaging, the VITRO-SKIN membranes were incubated with a 15% glycerin aqueous solution in a closed and humidity-controlled chamber for 16 hours. The hydrated VITRO-SKIN membranes were removed from the hydration chamber and placed on slide glasses. Then, the cosmetic films were formed on the artificial skin models by applying the O/W emulsion formulations. Depth profiles were obtained at 0, 5, 10, and 15 minutes after the application of the emulsions. The CRM measurements were performed over an area of 20 μm × 15 μm, with a step size of 0.2 μm in a confocal mode using a 100× objective, with an excitation wavelength of 532 nm. The total time for each depth profile acquisition was approximately 300 seconds, with an exposure time of 0.03 s per spectrum. Analysis of the imaging data was conducted using WiRE software (Renishaw, UK) using principal component analysis (PCA), a multivariate analysis method for distinguishing spectral trends in the data.

## 3. Results

### 3.1 Permeability of MTO into CHE

The visualization of the MTO distributions in the skin tissues was conducted using the MALDI-MSI. First, ionization of MTO was confirmed. The MALDI-MSI measurements on the mixture of MTO solution and the matrix detected MTO at m/z 689.2 [M+Na]+ (Fig. 1). The mass spectrum did not overlap with the peaks of the matrix.

Next, the permeability of MTO into the skin tissues using the formulations containing the PGFE surfactants with different HLB values was visualized by attributing the peak of MTO. Fig.2(a) shows the H&E staining images of the skin sections and the visualization of the MTO distributions in the ROI area by MALDI-MSI. H&E staining enables observation of the skin layers deeper than the stratum corneum, which are considered to be the function areas of MTO. The layers deeper than the stratum corneum, identified by H&E staining, is enclosed with a dotted line in the ion image of MTO (Fig. 2(a)). The formulation containing PGFE with an HLB value of 10 (HLB10) showed markedly higher MTO permeability, reaching deeper layers than the stratum corneum. Furthermore, the comparison of MTO signal intensities in the mass spectra across the entire skin revealed that HLB10 exhibited the higher signal intensity than HLB6 and HLB8, suggesting enhanced penetration capabilities (Fig. 2(b)).

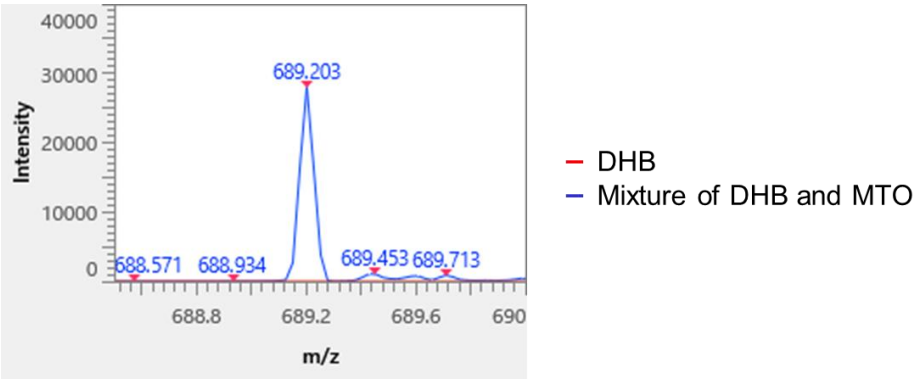


Figure 1. The evaluation of ionization ability of MTO. Average mass spectra obtained from the matrix (DHB) solutions with and without dissolved MTO.

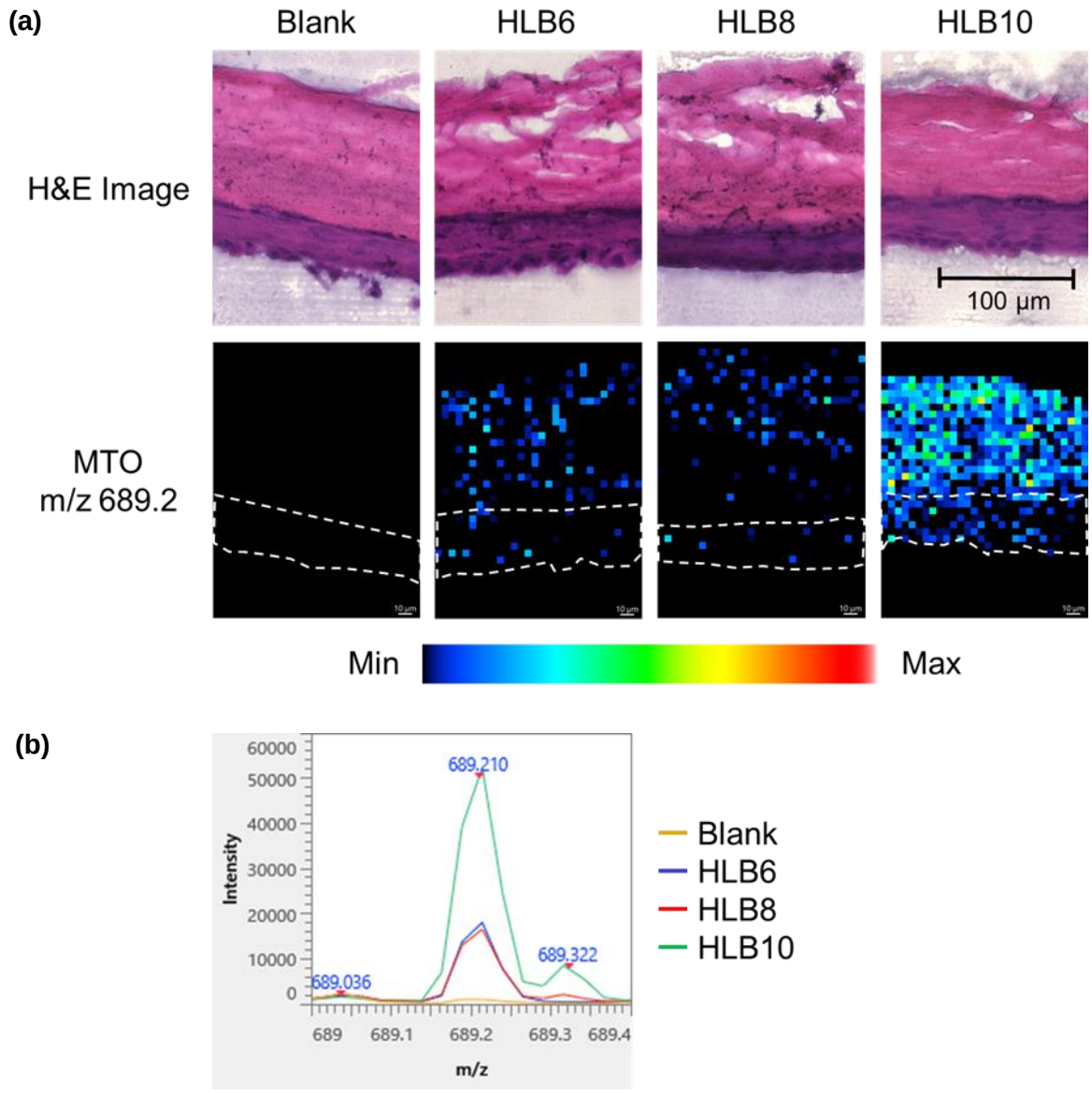


Figure 2. Visualization of the MTO permeability in the skin tissues using the O/W formulations containing PGFEs with different HLB values. (a) The H&E images and AP-MALDI-MSI ion images of MTO fragments at m/z 689.2 in the ROI area. (b) The mass spectra focusing on MTO fragments at m/z 689.2.

### 3.2 Temporal distributions of the aqueous and oil phases in the cosmetic film

We analyzed the structual changes in the cosmetic film by means of CRM during the drying process after the application of the O/W emulsions. We conducted time-lapse evaluations immediately after the application of the formulation for 15 minutes. In Fig. 3, the spatial distributions of the oil and aqueous phases in the cosmetic films are displayed, where white, red, and green areas represent the artificial skin, the oil phase, and the aqueous phase, respectively.

As shown in Fig. 3, the distributions of the oil and aqueous phases in the cosmetic film did not change dynamically in the formulations with HLB 6 and 8 of PGFEs. In contrast, as for the formulation with HLB 10, the aqueous phase exhibits a clear tendency to be localized at the

skin/cosmetic film interface with over time. This suggests that the localization of the aqueous phase should promote the penetration of the hydrophilic active ingredient to the skin when the aqueous phase preferentially adheres to the skin surface in an occluded environment.

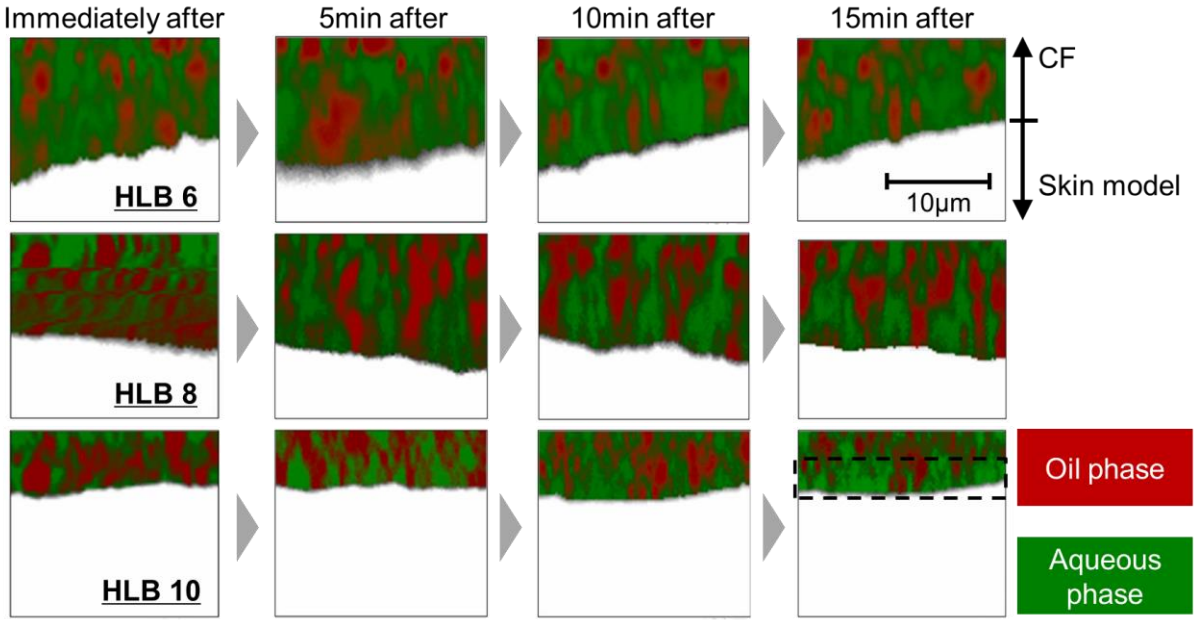


Figure 3. Effects of the HLB values of PGFE surfactants prescribed in the O/W emulsion formulations on the spatial distributions of the aqueous and oil phases in the occlusion layer of the cosmetic films. Time-lapse XZ cross-section images of the cosmetic film after applying the O/W formulations to the skin model are displayed for HLB 6, 8 and 10.

## 4. Discussion

Although MTO has been found to alleviate chronic itch by inhibiting the interaction between SP and NK1R [8,9], its large molecular weight and hydrophilicity pose challenges for skin permeability. In this study, to overcome these issues, we have developed a new technology for realizing an efficient delivery of MTO deeper into the skin. This goal has been accomplished by controlling the structural stability of the cosmetic film.

We found that the permeability of the MTO was improved in the O/W emulsion with HLB10, which demonstrated notable difference from those with HLB6 and HLB8. In addition, the MTO-permeability visualization showed that in the permeability-enhanced emulsion, MTO reached layers deeper than the stratum corneum, which are the functional expression layers of MTO. Furthermore, the CRM observation revealed a prominent tendency for the aqueous phase in the cosmetic film to be localized at the skin surface when the permeation-enhanced emulsions are applied. M. Nanjo et al. studied the stabilization of O/W emulsions using the membrane emulsification method [21]. They reported that when comparing the emulsification states using emulsifiers with the same HLB but different bulkiness of hydrophilic groups, the emulsifiers with bulkier hydrophilic groups resulted in lower monodispersity of the emulsified particles, leading to coalescence of oil droplets and phase separation. Therefore, we hypothesize that in the high-permeability emulsions, the stability is poor, and when the formulation is spread on the skin, the oil droplets coalesce and float within the film. Consequently, the aqueous phase is to be localized at the skin surface. This demonstrates that an increased contact frequency

of the water-soluble active ingredients on the skin promotes their penetration into the inner stratum corneum. In the near future, physicochemical evaluations of the emulsion structures should be conducted to obtain further insights into the coalescence process of the emulsions in the cosmetic films.

## 5. Conclusion

In this study, we potently focused on controlling the structural stability of the cosmetic film to efficiently deliver the hydrophilic active ingredients (MTO) to the skin inner layers. The permeability of MTO was markedly improved when the O/W emulsion formulated with PGFE having optimally large hydrophilic groups (HLB 10) was employed. The CRM imaging analysis on the spatial distributions of the oil and aqueous phases in the cosmetic film revealed a prominent tendency for the aqueous phase to be localized at the skin surface over time when the HLB values of the PGFE surfactants were optimally adjusted. This suggests that the increased contact frequency of MTO with the skin surface leads to the enhanced penetration of MTO into the inner stratum corneum. In fact, it has been clinically confirmed that the optimal formulation with HLB10 suppresses the itch. Our results unambiguously demonstrated the itch-relief efficacy of MTO [22]. This emerging technology that can avoid using any irritating permeation enhancers should be best suitable for personal care products targeting alleviation of sensitive skin problems.

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