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ORIGINAL ARTICLE

In vitro skin permeation of hinokitiol loaded in vesicles composed of behenyltrimethylammonium chloride and stearic acid

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Abstract

Introduction: Behenyltrimethylammonium chloride (BTAC) and stearic acid (SA) could be associated with each other through salt bridges, and the associated BTAC/SA could build bilayer vesicles with the aid of hinokitiol (HKL). *Method*: The vesicles were prepared by a precipitation method and used to enhance the skin permeation of HKL. *Results*: In case the molar ratio of BTAC/SA/HKL was 1/1/0, no vesicle was observed on transmission electron microscope photos. When the molar ratio of BTAC/SA/HKL was 1/1/0.4, vesicle was observed together with some agglomerates. When the content of HKL increased to the ratios of 1/1/0.8 and 1/1/1.2, vesicles were exclusively observed. In vitro fluxes for 18 hours through hairless mouse skin of HKL dissolved in alcoholic solutions were less than 1 mg/cm^2/h. Whereas the fluxes of HKL encapsulated in the vesicles were about three times higher than that of HKL in the alcoholic solutions. *Conclusion*: The vesicles could be used for the hair growth promotion.

Key words: *Behenyltrimethylammonium chloride; hinokitiol; skin permeation; stearic acid; vesicles*

Introduction

The double-tailed amphiphiles can self-assemble to form bilayer vesicles when their packing parameter (*P*) is around 1[1,2]. Besides using two-tailed lipids (e.g., dipalmitoylphosphatidylcholine) for the preparation of vesicles[3], the bilayer vesicles can also be prepared by using two kinds of single-tailed molecules, which can be associated together and behave like two-tailed molecules. It is reported that *N*-[3-(dimethylamino)propyl]-octadecanamide (DMAPODA) and stearic acid (SA) can be associated with each other using a salt bridge between NH^+ of DMAPODA and COO^- of SA to form two-tailed molecule. The two-tailed molecule can form vesicles with bilayer structures more easily than the single-tailed molecule because of a higher packing parameter[4,5]. In addition, the vesicles with strongly positive charges could be classified into cationic vesicles, and they are reported to be more efficient than electrically neutral vehicles for the delivery of anionic compounds, such as DNA and drugs, in gene or drug delivery systems[6–8]. And they are also reported to show selectively target tumor endothelial cells[9]. Moreover, the vesicles that included ionizable groups in the bilayers are also pH-sensitive and widely used in drug delivery because of the simple preparation and active response to stimuli[10–12].

On the other hand, hinokitiol (HKL, 4-isopropyl tropolone, b-thujaplicin), which is isolated from the essential oils of trees, is known to have antifungal, antibacterial, and antiviral activities[13–15]. It is also reported to cure hair loss by suppressing factors that promote follicular apoptosis and stimulate new hair growth[16]. Thus, many in vivo experiments are reported to use it as a hair-growth promotion agent[17,18]. Furthermore, according to several reports, the skin-permeation property of HKL can be enhanced by using permeation enhancer, such as alcohol and lipid nanoparticles[19–21].

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In this study, behenyltrimethylammonium chloride (BTAC)/SA assemblies containing HKL were prepared by a precipitation method. The electronically associated molecules of BTAC/SA are believed to act as a two-tailed amphiphile. However, the addition of HKL was expected to help the BTAC/SA to form a two-tailed amphiphile with the rectangular shape for the assembling of the cationic vesicles. The effects of various concentration of HKL on the formation properties of assemblies were investigated. The physical characteristics such as apparent shape, particle size, and zeta potentials were measured. In addition, the in vitro skin permeation of HKL incorporated in the assemblies was also investigated.

Materials and methods

Materials

BTAC (M.W. 404.31) was gifted from SUNJIN Chemical Co. (Ansan, Korea). SA (M.W. 284) was purchased from Sigma (St. Louis, MO, USA). HKL (M.W. 164.2) was gifted from Osaka Chemical Co. (Osaka, Japan). Hydroxypropyl-β-cyclodextrin (HP-β-CD, M.W. 1500) and phosphotungstic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Water was distilled in a water purification system (Pure Power I$^+$; Human Corporation, Seoul, Korea) until the resistivity was 18 MΩ cm^{-1}. All other regents were in analytical grade.

Animals

Female hairless mice (type SKH) were obtained from Orient Bio (Seongnam, Korea). They were housed in suspended wire mesh cages in a room illuminated from 09:00 to 21:00 hours and kept at 20–25°C, with a rodent diet and water ad libitum.

Preparation of BTAC/SA/HKL assembly suspensions

The BTAC/SA/HKL assembly suspensions were prepared by precipitation method[22]. BTAC of 0.500 g (1.237 mmol), SA of 0.351 g (1.237 mmol), and varying amount of HKL (0.085–0.255 g) (0.518–1.553 mmol) were put together in a 50-mL round bottom flask so that the molar ratios of BTAC/SA/HKL were 1/1/0.4, 1/1/0.8, and 1/1/1.2. The mixture was dissolved into 3 mL of ethanol and then 5 mL of distilled water was added to the solution. Ethanol was evaporated from the solution in a rotary evaporator (Laborota 4000; Heidolph, Kelheim, Germany) thermostated at 45°C until all the alcohol was removed. Distilled water was added to the precipitates for the adjustment of the HKL concentration. The final pH was adjusted to 7.0 using 1 N HCl or 1 N NaOH.

Characterization of BTAC/SA/HKL assemblies

The shapes of BTAC/SA/HKL assemblies were observed by a transmission electron microscope (TEM, LEO-912AB OMEGA; LEO, Oberkochen, Germany) . When observed on the electron microscope, assemblies were negatively stained using phosphotungstic acid[23]. The size and the zeta potential of the BTAC/SA/HKL assemblies containing HKL were measured by varying pH on a particle size analyzer (Plus 90; Brookhaven, NY, USA). About 50 μL of an assembly suspension (10 mg/mL) was put into a cuvette and then it was filled up to 1.5 mL with distilled water, which was adjusted to pH 3.0–9.0 using 0.1 N HCl or 0.1 N NaOH. The suspension was equilibrated for 4 minutes at 25°C. The measurement was done with three runs where each run consists of 10 single gauging. Calorimetric property of the BTAC/SA/HKL assembly was investigated on a differential scanning calorimeter (DSC; DSC 2010, TA Instruments, New Castle, DE, USA). The assembly suspensions were centrifuged at 10080 *g* (HM-150IV; Hanil Science Industrial, Incheon, Korea) and the concentrated suspensions of 10–20 mg weighed into aluminum DSC pans, and they were scanned from 20°C to 110°C at a heating rate of 2°C/min. In parallel, pure BTAC, SA, and HKL were also investigated by DSC.

In vitro skin permeation

The dorsal skin of female hairless mice (type SKH) aged 6 weeks was mounted onto Franz diffusion cells (0.636 cm^2 surface area, mode name) equipped with 5 mL receptor compartment. HP-β-CD solution (0.2%) in phosphate-buffered saline (10 mM, pH 7.4) was used as the receptor content, thermostated to 37°C under stirring. HP-β-CD was included in the receptor as a solubilizer for HKL. About 200 μL of the BTAC/SA/HKL assembly suspensions was applied onto the skins and then the receptor solutions (300 μL) were assayed for HKL using HPLC at the predetermined time. HKL solutions in either ethanol or propylene glycol were used as controls for the permeation experiment. The HKL assay was performed in a liquid chromatograph (M600E, M7725i/Waters, 996PDA) equipped with a UV detector (0.05 AFUS, 325 nm)[19].

Results and discussion

Micrographs of vesicles

Figure 1 shows the TEM photos of BTAC/SA/HKL (1/1/0, 1/1/0.4, 1/1/0.8, 1/1/1.2) assemblies. When the ratio of BTAC/SA/HKL was 1/1/0, no evidence of vesicle was found. Particles like circles, vesicles, were found together with some agglomerates at the ratios of 1/1/0.4. As the contents of HKL increased so that the

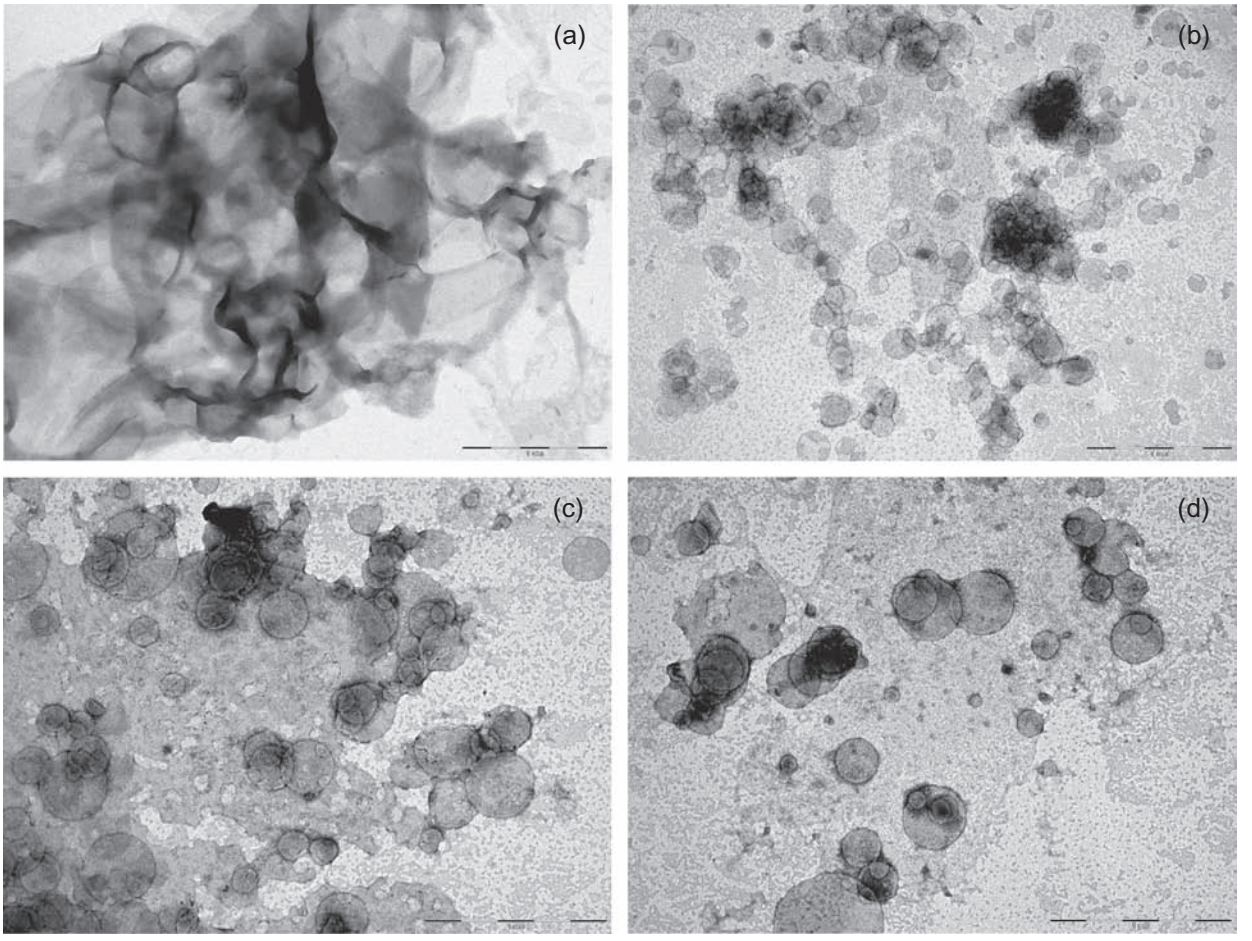

Figure 1. TEM photos of BTAC/SA/HKL [molar ratios, (a) 1/1/0, (b) 1/1/0.4, (c) 1/1/0.8, (d) 1/1/1.2] assemblies. Bars were 1 μm.

ratios of BTAC/SA/HKL are 1/1/0.8 and 1/1/1.2, only vesicles were observed. When amphiphiles are self-assembled in an aqueous phase, a critical factor to determine the type of the assemblies is packing parameter. Vesicles are known to be formed when the packing parameter is around 1. In terms of geometry, the longitudinal cross section of amphiphiles is required to be rectangle for the formation of bilayer vesicles[2].

In a previous work, DMAPODA, even though its packing parameter is much less than 1, could form vesicles with the aid of SA. The head of the fatty acid, carboxylate, was reported to attach to that of DMAPODA, amino group, by a salt bridge[4]. The associated molecules of DMAPODA/SA are believed to act as a two-tailed amphiphile. The association of SA will increase the volume of the hydrophobic tail of the amphiphile, so the associated molecules of DMAPODA/SA can form vesicles. The longitudinal cross section of the associated molecules will be close to a rectangle as the chain length of DMAPODA, C18, is the same as that of SA. Based on the same principle, SA could be associated with BTAC through salt bridging between the negatively charged carboxylate of SA and the positively charged quaternary amine of BTAC. However, the combination of BTAC/SA in equimolar ratio could not produce the vesicles (Figure 1a). The number of carbon in the hydrocarbon chain of BTAC is 22 and that of SA is 18. Therefore, the associated molecule will have two tails of which lengths are quite different. In this circumstance, the cross section of the associated molecules will be other geometry rather than a rectangle. This may account for why vesicles were hardly formed with BTAC and SA. On the other hand, when HKL was included in the preparation so that the ratios of BTAC/SA/HKL are 1/1/0.8 and 1/1/1.2, the vesicles were obtained. HKL is a lipophilic compound and it would readily be dissolved in the domains of the hydrocarbon chain. HKL could fill the spaces created by the difference in chain length between BTAC and SA, and it would help the BTAC/SA associate build bilayer vesicles.

Differential scanning calorimetry

Figure 2 shows the thermograms of HKL, SA, BTAC, and SA/BTAC/HKL (1/1/0.4, 1/1/0.8, 1/1/1.2) assembly suspensions. The melting points of HKL and SA were observed around 52°C and 72°C, and the softening temperature of BTAC was observed around 103°C. The suspension of BTAC/SA/HKL (1/1/0.4) assembly exhibited two peaks. The peak around 88°C would be ascribed to the phase transition temperature of bilayer membrane of vesicle. It was reported that the lipid bilayer membrane undergoes a phase transition from solid gel to liquid crystalline, and the phase transition temperature depends on the tail length of lipid[24]. As SA and HKL peaks disappeared in the thermogram of BTAC/SA/HKL (1/1/0.4) assembly, all of SA and HKL are thought to be employed in the formation of vesicles. And the peak around 103°C is due to BTAC, which did not participate in the formation of vesicles. Accordingly, it is believed that vesicles coexist with BTAC agglomerates at the BTAC/SA/HKL ratio of 1/1/0.4. In fact, not only vesicles but also agglomerates were observed on TEM photos (Figure 1b). On the other hand, only one peak around 78°C was observed with the suspension of BTAC/SA/HKL (1/1/0.8) assembly, and the peaks of SA and HKL were hardly observed. It means that all of SA and BTAC took a part in the formation of vesicles. The peak around 78°C is possibly because of the phase transition of the vesicle membranes. The phase transition temperature of vesicles having BTAC/SA/HKL (1/1/1.2) was observed around 65°C. The reason for decreased phase transition temperature with increasing the content of HKL might be that HKL is dissolved in the hydrocarbon domain of the bilayer membrane and it can fluidize the membrane. In a previous report, triclosan, a lipophilic compound, can fluidize the vesicle membrane of DMAPODA/SA, resulting in a decreased phase transition temperature[4].

Zeta potential and size

Figure 3 shows the change in the zeta potentials of BTAC/SA/HKL (1/1/0.4, 1/1/0.8, 1/1/1.2) assemblies with varying pH. When the ratio was 1/1/0.4, the zeta potential decreased from +57 to + 35 mV upon increasing pH from 3.0 to 9.0. The strong positive potentials are possibly because of the quaternary amines of BTACs. And the decrease in the potential with increasing pH is because the carboxylic group of SA will be deprotonated at higher pHs. When the contents of HKL increased so that the ratios were 1/1/0.8 and 1/1/1.2, the zeta potentials were markedly decreased at every pH tested. Following TEM photos (Figure 1) and thermograms (Figure 2), there were vesicles together with BTAC agglomerates at the ratio of 1/1/0.4. As BTAC has quaternary amine, the agglomerates might be responsible for higher zeta potentials at the ratio. On the other hand, all of BTAC turned out to participate in the formation of vesicles when the ratios were 1/1/0.8 and 1/1/1.2. In this circumstance, the positive charge of BTAC will be somewhat neutralized by the negative charge of SA. This may explain the reason why the zeta potentials were lower at the ratio of 1/1/0.8 and 1/1/1.2. Another reason would be that the surface charge density will decrease upon the dissolution of HKL into the hydrocarbon domain of BTAC/SA assemblies (HKL is electrically neutral).

Figure 4 shows the change in the size of BTAC/SA/HKL (1/1/0.4, 1/1/0.8, 1/1/1.2) assemblies with varying pH. When the ratio of BTAC/SA/HKL was 1/1/0.4,

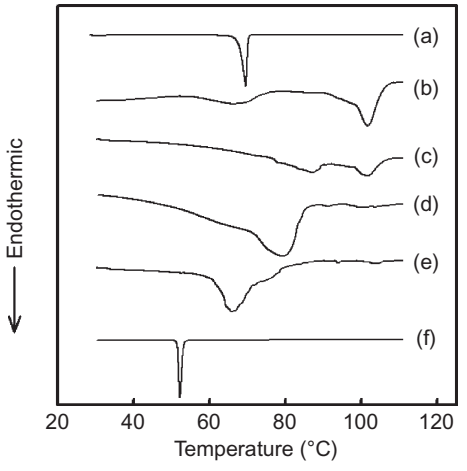

Figure 2. (a) Thermograms of SA, (b) BTAC, BTAC/SA/HKL [molar ratios, (c) 1/1/0.4, (d) 1/1/0.8, (e) 1/1/1.2] (f) assembly suspensions and HKL.

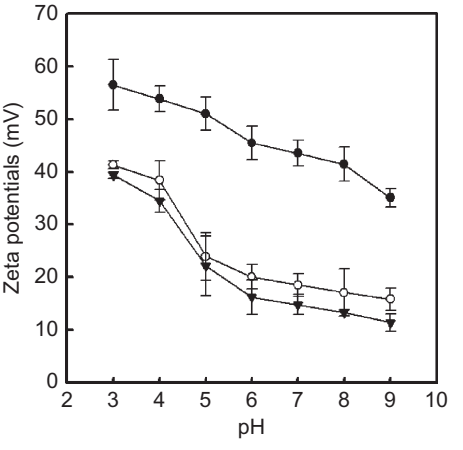

Figure 3. The change in the zeta potentials of BTAC/SA/HKL [molar ratios, (●) 1/1/0.4, (○) 1/1/0.8, (▲) 1/1/1.2] assemblies with varying pH.

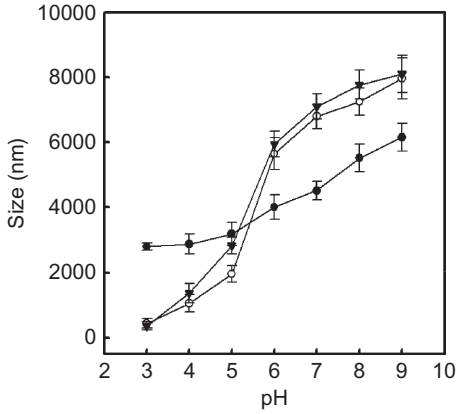

Figure 4. The change in the size of BTAC/SA/HKL [molar ratios, (●) 1/1/0.4, (○) 1/1/0.8, (▲) 1/1/1.2] assemblies with varying pH.

the size increased from about 2.7 to 6 μm upon increasing the pH from 3.0 to 9.0. When the contents of HKL increased so that the ratios of BTAC/SA/HKL are 1/1/0.8 and 1/1/1.2, the size increased from about 0.5 to 8 μm upon increasing pH from 3.0 to 9.0. The size of BTAC/SA/HKL (1/1/0.4) assemblies was bigger than that of the others (1/1/0.8 and 1/1/1.2) in acidic conditions. Following TEM photos (Figure 1) and thermograms (Figure 2), BTAC agglomerate was found together with vesicles at the ratio of 1/1/0.4 (Figures 1b and 2c). Thus, the agglomerates would be responsible for the bigger size. On the other hand, the size of BTAC/SA/HKL (1/1/0.4) assembly was smaller than those of the other assemblies (1/1/0.8 and 1/1/1.2) in neutral and alkali conditions. Referring to the zeta potentials of BTAC/SA/HKL (1/1/0.4) assembly in neutral and alkali conditions (Figure 3), the values were strong (around +40 mV) enough to prevent the aggregation of assembly particles. However, the zeta potentials of BTAC/SA/HKL (1/1/0.8, 1/1/1.2) assemblies (vesicles) in neutral and alkali conditions were weak (less than +20 mV) so that the vesicles may aggregate into lager clusters. It was reported that colloidal particles are stable against aggregation when the absolute value of the surface potential is greater than 25 mV[25].

In vitro skin permeation

Figure 5 shows the skin-permeation flux of HKL in BTAC/SA/HKL (1/1/0.4, 1/1/0.8, 1/1/1.2) assembly suspensions. The concentrations of BTAC/SA in the suspensions were kept constant (20 mg/mL), and accordingly HKL concentrations were 2.0, 4.0, and 6.0 mg/mL. The flux increased almost linearly with time regardless of the ratios of BTAC/SA/HKL. When BTAC/SA/HKL (1/1/0.4) assembly suspension was applied, the flux of HKL at 18 hours was only about

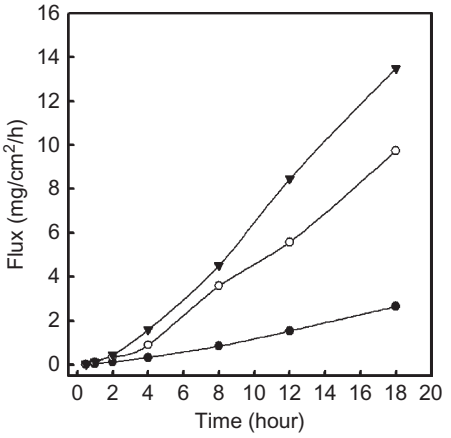

Figure 5. Skin-permeation flux of HKL in BTAC/SA/HKL [molar ratios, (●) 1/1/0.4, (○) 1/1/0.8, (▲) 1/1/1.2] assembly suspensions. The concentrations of BTAC/SA in the suspensions were kept constant (20 mg/mL).

2.5 mg/cm^2/h. When the contents of HKL increased so that the ratios were 1/1/0.8 and 1/1/1.2, the fluxes at 18 hours were about 9.7 and 13.5 mg/cm^2/h, respectively. The thermodynamic activity of HKL in the BTAC/SA/HKL (1/1/1.2) assembly suspension would be higher, because the contents of HKL in the assembly and the concentration in the suspension are higher than the others. Therefore, transfer from the assembly and from the suspension into skins would be more ready, resulting in a higher flux of HKL.

Figure 6 shows the flux of HKL in BTAC/SA/HKL (1/1/0.4, 1/1/0.8, 1/1/1.2) assembly suspensions, where the concentrations of HKL were kept constant (2 mg/mL) and accordingly BTAC/SA concentrations were 20, 10, and 6.67 mg/mL. As shown in Figure 6, HKL solutions in ethanol (2%, w/v) and in propylene glycol (2%, w/v) showed much lower flux than the suspensions.

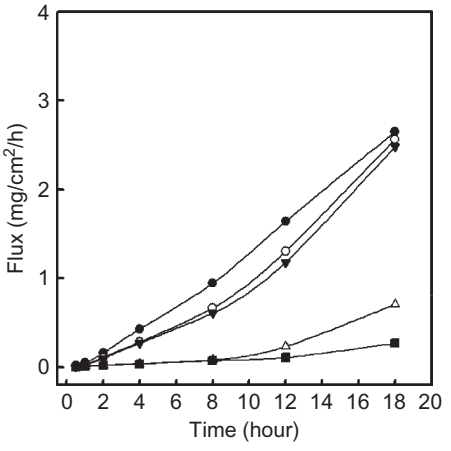

Figure 6. Skin-permeation flux of HKL in BTAC/SA/HKL [molar ratios, (●) 1/1/0.4, (○) 1/1/0.8, (▲) 1/1/1.2] assembly suspensions, (□) ethanol, and (■) propylene glycol solutions. The concentrations of HKL were kept constant, 2 mg/mL.

That is, BTAC/SA/HKL assemblies (which could be agglomerates or vesicle depending on the ratio of BTAC/SA/HKL) were much more effective in enhancing the skin permeation of HKL than the alcoholic vehicles. As the sizes of assemblies were much greater than the space between corneocytes, they would hardly penetrate into the skins. Furthermore, the phase transition temperatures of assembly (vesicle) membrane were higher than 37°C where the skin permeations were investigated, so the membranes were in solid gel states. Therefore, assemblies (vesicles) could hardly fit themselves into the space between corneocytes. How could the vesicles promote the permeation of HKL? Our speculation is that cationic BTAC will interact with anionic paracellular skin lipids to reduce the barrier function of the skin. But it is not clear yet. On the other hand, the fluxes were almost the same whatever the ratios of BTAC/SA/HKL were. As mentioned above, the promotion of HKL permeation may be due to the electrostatic interaction. According to Figure 3, there was no significant difference in the zeta potentials between the ratio of 1/1/0.8 and 1/1/1.2. This is why the skin-permeation properties of HKL were almost the same. In addition, the higher zeta potentials of the ratio of 1/1/0.4 resulted in a slightly higher flux in the early stage of skin permeation.

Conclusion

Vesicles composed of BTAC and SA in equimolar ratio were hardly obtained, but when HKL was included in the preparation so that the ratios of BTAC/SA/HKL are 1/1/0.8 and 1/1/1.2, vesicles were successfully formed. In vitro skin-permeation experiment was performed for 18 hours; the flux of HKL encapsulated in the vesicles was about three times higher than that of HKL in the alcoholic solutions. The BTAC/SA/HKL vesicles developed in this study could be used for the hair growth promotion and the treatment of bacteria-related skin disease.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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