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ORIGINAL PAPER

Characterization and In-vitro Permeation Study of Stearic Acid Nanoparticles containing Hinokitiol

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Abstract Lipid nanoparticles containing hinokitiol (HKL) were prepared by a melt–emulsification method. Stearic acid was used as a lipid for the matrix material of the nanoparticles. According to results from transmission electron microscopy (TEM) and dynamic light scattering (DLS), most of the nanoparticles were less than 100 nm. When nanoparticles containing HKL were scanned on a differential scanning calorimeter, no endothermic peak of HKL was observed. This means that HKL in the lipid matrix of the nanoparticles is in a dissolved state. In an 18-h permeation study using hairless mouse skin mounted on a diffusion cell, the amount of HKL encapsulated in the nanoparticles transported to the receptor cell was five to ten times more than for HKL dissolved either ethanol or propylene glycol. Therefore, stearic acid nanoparticles strongly enhanced permeation of the skin by HKL.

Keywords Hinokitiol · Stearic acid · Nanoparticles · In-vitro permeation

Introduction

In order to promote the transdermal delivery of biologically active agents, extensive studies have been focused on delivery carriers such as lipid vesicles, micelles, nanoparticles. Among these, liposomes have been widely used to enhance the dermal delivery of hydrocortisone (an anti-inflammatory agent) [1], amphotericin B (an antifungal) [2], and retinoids [3]. Cationic vesicles were also found to be increase the anti-bacterial activity of triclosan [4]. In addition, they boosted the substantivity of minoxidil contained in a rinse-off type of hair-cleansing product, resulting in after-rinsing hair-growth promotion [5]. In a past decade, solid lipid nanoparticles (SLN) have attracted much interest of the scientist in the field of drug delivery. SLN has been reported to solubilize triptolide, an anti-inflammatory agent, and enhance the transdermal delivery and anti-inflammatory activity of the drug [6, 7]. Their use as a physical vector for gene delivery has also been proposed. According to the results, they enhanced in-vitro transfection activity by optimizing the cationic lipid and matrix lipid composition of SLN [8]. On the other hand, SLN significantly reduced the in-vitro toxicity of all-*trans* retinoic acid without loss of the antiproliferative effects against a wide range of cancer cell lines [9]. Furthermore, the chemotherapeutic potential of oral SLNs incorporating rifampicin, isoniazid, and pyrazinamide against experimental tuberculosis was evaluated. According to the results, in *M. tuberculosis* H37Rv-infected mice anti-cancer drugs incorporated in SLNs were much more effective than free drugs in terms of extermination of tubercle bacilli when administered orally [10].

On the other hand, hinokitiol (2-hydroxy-4-isopropyl-2,4,6-cycloheptа-2,4,6-triene-1-one, HKL), a natural product obtained from the essential oils of several kinds of trees, has been studied as an anti-microbial agents [11]. In addition to the anti-microbial activity, it inhibits the growth of tumor cells [12]. Furthermore, it is also reported to suppress ultraviolet B-induced apoptosis of mouse keratinocytes [13]. Owing to the anti-microbial activity and the inhibitory action on the apoptosis of keratinocytes, much attention is recently paid to the use of HKL as a hair-growth promotion agent [14, 15].

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In this study, stearic acid lipid nanoparticles containing HKL were prepared by a melt-emulsification method. The physical characteristics such as size distribution, apparent shape, microelectrophoretic behavior were investigated. In addition, the in-vitro skin permeation of HKL incorporated in the nanoparticles was investigated.

Experimental

Materials and Methods

Stearic acid was purchased from Aldrich. Hinokitiol was provided by LG Household and Healthcare. All other reagents were 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 Nanoparticles containing HKL

Stearic acid 4.0 g and hinokitiol 1.2 g were melted together at around 85 °C in a 20-mL beaker. In parallel, an aqueous solution of Tween 20, 80 mL (2.0%), was prepared in a 200-mL beaker and was then heated to the same temperature. While homogenizing the heated solution, the melt was slowly added. To obtain an oil-in-water emulsion, homogenization was continued for 20 min at around 85 °C. To solidify the oil droplets, the hot emulsion was carefully added to 267 mL of distilled ice water (0–5 °C).

Transmission Electron Microscopy

The nanoparticle suspensions were negatively stained using phosphotungstic acid solution (0.1%, pH 6.8) then transferred onto a formvar and carbon-coated copper grid. After drying the replica at room temperature, the image was observed on a transmission electron microscope.

Dynamic Light Scattering and Microelectrophoresis

The size distributions and the zeta potentials of nanoparticles were measured using ZetaPlus (Brookhaven Instrument). The content of nanoparticles in the suspension was adjusted to 0.03%. To avoid the effect of particle–particle interactions, the size distribution was measured immediately after shaking the suspension of lipid nanoparticles. When the effects of pH on the size distribution and the zeta potentials were investigated, the measurements were done 3 min after standing the suspension in a cuvette. pH was varied from 2.2 to 10.5 with 1 mmol L^{-1} NaOH and 1 mmol L^{-1} HCl solutions.

Differential Scanning Calorimetry

Stearic acid, HKL, and lipid nanoparticles with or without HKL were thermally scanned on a differential scanning calorimeter (TA instruments DSC 2010). The nanoparticles were scanned as suspensions in water and the other samples were in the dry state. Each sample of 1–5 mg weighed into aluminum DSC pans and was scanned from 20 to 85 °C at a heating rate of 2° min^{-1}.

In-vitro Permeation

Female hairless mice (type SKH) aged 6 weeks were sacrificed by cervical dislocation. The dorsal skin of each hairless mouse was excised and the adhering fat and other visceral tissue were removed. The skin was mounted onto Franz diffusion cells (0.636 cm^2 surface area) equipped with a 5 mL receptor compartment. Phosphate-buffered saline (PBS, pH 7.4) was used as the receptor content, thermostated at 37 °C, with stirring. Nanoparticle suspension or HKL solution (20 μL) was applied to the skin and then the receptor solutions, 20 μL, were assayed for HKL at predetermined time intervals using HPLC. The concentration of HKL in the donor cell was adjusted to 1%. The HKL assay was performed in a liquid chromatograph (Waters) equipped with a UV detector. A Microsorb-MV column was eluted with methanol at a flow rate of 1.0 μL min^{-1} and a sample of 15 μL was injected. The detection wavelength was 325 nm.

Results and Discussion

Transmission Electron Microscopy

Figure 1 shows the electron microphotograph of lipid nanoparticles containing HKL. The shape was almost spherical and the size was homogeneous and less than 100 nm. The nanoparticles were observed as white domains because the sample were pretreated by a negative staining technique. Phosphotungstic acid, a heavy metal for the staining, is water-soluble and it would stain the area

where nanoparticles do not occupy, because the stearic acid, comprising the matrix of the nanoparticle, is hydrophobic. Accordingly, electron beams go through the area where the nanoparticles are. However, the beams cannot pass through the areas where the heavy metals are. Therefore, the black and white contrasts were observed as shown in Fig. 1.

Dynamic Light Scattering

Figure 2 shows the size distribution of nanoparticles containing HKL. Most of the particles observed around 50 nm and a negligible amount occurred around 300 nm. Colloidal particles less than 100 nm do not follow Stoke's law. Instead, they are stably suspended and not influenced by gravitational force. In fact, the suspension of the nanoparticles was colloidally stable for at least 3 months. That is, no significant sedimentation or flotation was observed. Therefore, the nanoparticles could be applied to the water-based solution type of hair care products.

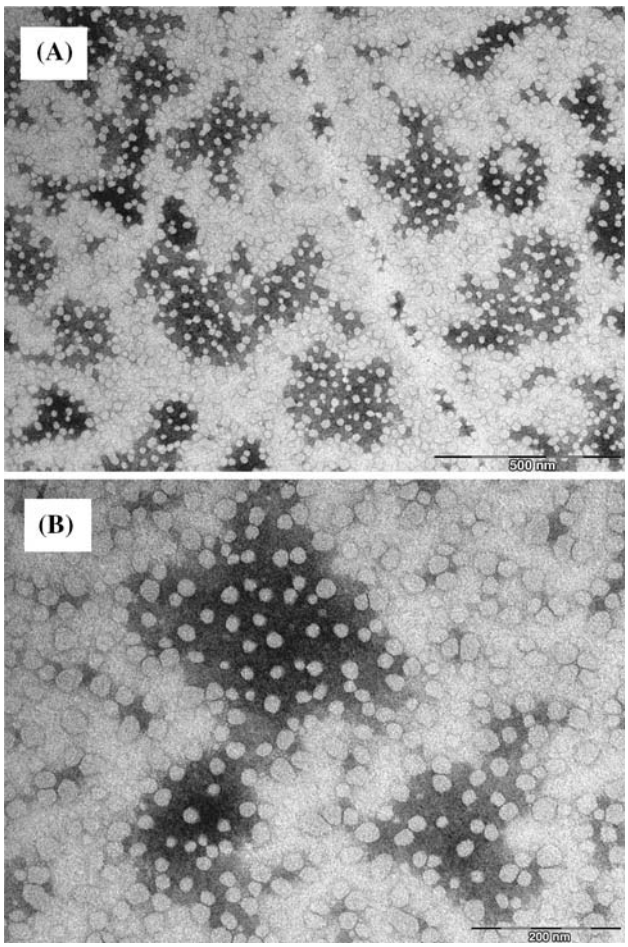

Fig. 1 Transmission electron micrographs of lipid nanoparticles containing HKL. The magnifications were ×50,000 (**a**) and ×100,000 (**b**)

Microelectrophoresis and Colloidal Stability

Figure 3 shows the zeta potentials of lipid nanoparticles with pH. The potential decreased with pH and the values are negative except at pH 2.0. The lipid nanoparticles are composed of stearic acid and HKL. Since HKL does not have an ionizable group, the zeta potentials of lipid nanoparticles depend mainly on stearic acid. Since the carboxyl groups of free fatty acids have a pK of approximately 5, fatty acids will be deprotonated at higher pH, such as 6, 8, and 10. COO^- of the fatty acids would account for the negative values of the zeta potentials.

Figure 4 shows the size variation of lipid nanoparticles in the suspension with pH. The mean size ranges from 200 to 500 nm. According to the electron micrographs, and results of dynamic light scattering, the size was less than 100 nm. In general, colloidal particles are likely to flocculate with time, resulting in larger aggregates, when the absolute values of the zeta potentials were less than 30 mV. In all the ranges of pH, the absolute values of the nanoparticles containing HKL prepared in this research were much lower 30 mV. It means that the repulsive forces between the nanoparticles were not high enough to prevent the aggregation of the particles. The size was observed to be less than 100 nm, when measured immediately after shaking the suspension. This means the particles exist as physical aggregates but not as fused lumps. In fact, the phase transition of the nanoparticles, 67 °C (see Fig. 5), is much higher than room temperature, where the size was measured. In this circumstance, the nanoparticles are in a solid state so that the fusion of the particles would hardly occur.

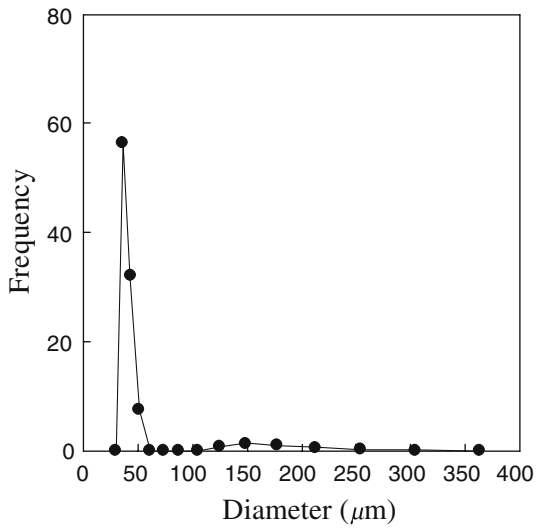

Fig. 2 Size distribution of lipid nanoparticles containing HKL, measured by the dynamic light scattering technique

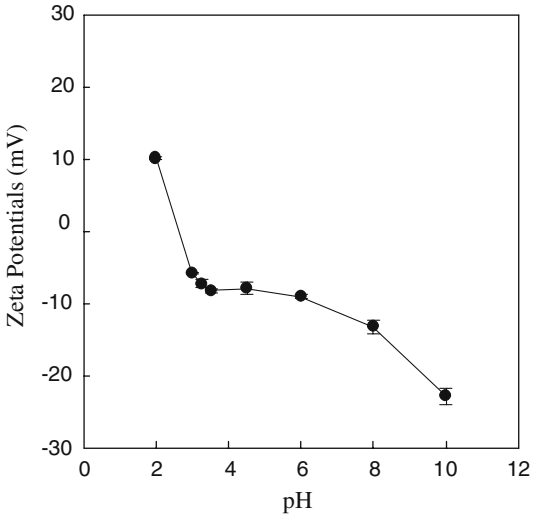

Fig. 3 Variation with pH in zeta potential of lipid nanoparticles containing HKL

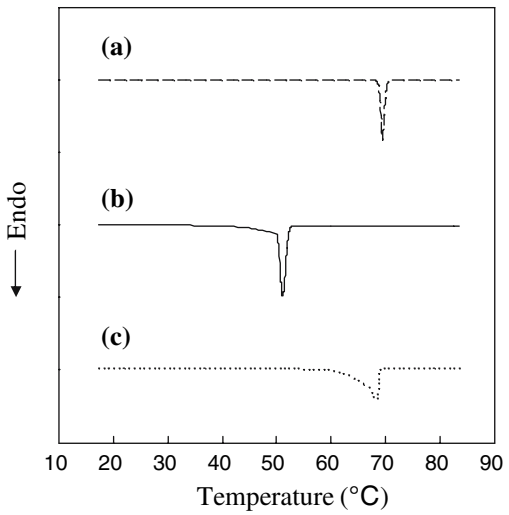

Fig. 5 Thermograms of stearic acid (**a**), HKL (**b**), and lipid nanocapsule containing HKL (**c**)

Differential Scanning Calorimetry

Figure 5 shows the thermograms of stearic acid, HKL, and lipid nanoparticles with or without HKL. Stearic acid exhibited an endothermic peak around 70 °C, because of melting of the solid crystal. Stearic acid nanoparticles in suspension also had an endothermic peak at almost the same temperature. This indicates that stearic acid nanoparticles are in the solid crystalline state. On the other hand, HKL had an endothermic peak around 50 °C and this is also due to melting of the solid crystals. In case of nanoparticle containing HKL, the peak of HKL disappeared. Since HKL is oil soluble, it should be solubilized in the oily matrix of the lipid nanoparticles. That is, the crystalline structure would be lost upon dissolution. This may explain why the peak of HKL is missing. The position of the peak is 2–3 °C lower than in case of nanoparticles containing HKL and the shape was somewhat broad. This is possibly because that HKL is dissolved in the stearic acid matrix so that it may disturb the close packing of the fatty acid.

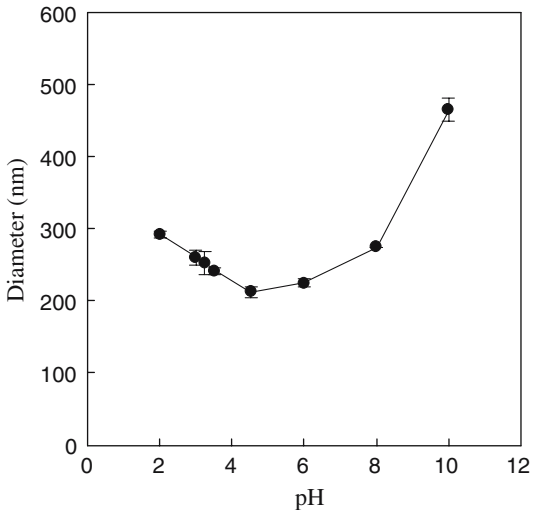

Fig. 4 Variation in size of lipid nanoparticles containing HKL with pH

In-vitro Permeation

Figure 6 shows the amount of HKL transported across hairless mouse skins at several time intervals. The flux of HKL increased with time. When HKL was dissolved in propylene glycol, the flux of HKL in 18 h was 3 mg cm^{-2} h^{-1}. When ethanol was used as a vehicle, it gave a higher flux than propylene glycol did. This is probably because ethanol disturbs the skin structure and plays a role as a permeation enhancer. Another possible reason is related to enhanced thermodynamic activity of HKL. Even though donor chamber is capped and sealed, ethanol evaporated and the concentration of the solution increased, resulting in an increased thermodynamic activity. On the other hand, when encapsulated in the lipid nanocapsules, the flux of HKL was much higher than for the solutions. In general, fatty acids are reported to enhance skin permeation of an active ingredient. They perturb the ordered structure of skin by hydrophobic interaction with skin lipids. In addition, nanoparticles less than 100 nm are known to readily penetrate the skin through the paracellular route (space between corneocytes) [16]. Because a significant proportion of the lipid nanoparticles prepared in this study is less than 100 nm, penetration should occur, although this has not yet been confirmed.

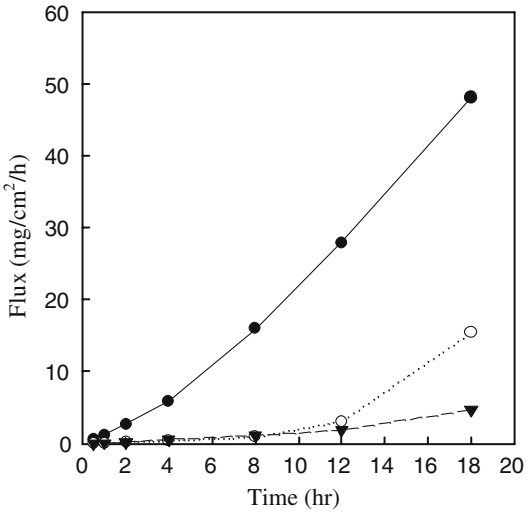

Fig. 6 Flux of HKL through hairless mouse skin at several time intervals. HKL encapsulated in lipid nanocapsules (*filled circles*), HKL dissolved in ethanol (*open circles*), and HKL dissolved in propylene glycol (*filled inverted triangles*)

Conclusion

HKL was incorporated in stearic acid nanoparticles by a melt–emulsification method. Most of the particles were less than 100 nm. The in-vitro permeation of HKL was markedly enhanced by the nanoparticles compared with HKL dissolved in either ethanol or propylene glycol.

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