Piceid Nanoparticles Stabilized by Anionic Phospholipids for Transdermal Delivery

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Abstract
Piceid, stilbenoid glucoside, is a representative resveratrol derivative. Because of a high tyrosinase inhibitory activity of piceid through resveratrol derivatives, transdermal delivery of piceid has been desired for taking advantage of the activity. Here we successfully prepared composite nanoparticles composed of anionic phospholipid of 1,2-dipalmitoyl-sn-glycero-3-phosphoryl glycerol (DPPG) and piceid by mixing them in water and a subsequent heating/cooling process. When small-sized fluorescently labeled DPPG-piceid (DPPG-FL-piceid) nanoparticles were added to rat skin tissue, FL-piceid molecules were localized in stratum corneum.

Keywords
piceid, DPPG, glycosylation, stratum corneum, nanoparticle

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Stilbene compounds as represented by resveratrol are one of the components of grapes and wine. They exhibit anticancer, anti-inflammatory, antiaging, and antiamyloid activities.¹⁻⁵ Piceid (Figure 1) is one of the resveratrol derivatives which can be isolated from Picea sitchensis or Polygonum cuspidatum. A preparation method using cultured plant cells has also been recently reported.⁶ One of the striking features of piceid is its very high tyrosinase inhibitory activity compared with other resveratrol derivatives.⁶ Tyrosinase is involved in the pigment adsorption associated with skin melanin formation and high activity which causes skin diseases such as melasma in the worst cases. Therefore, transdermal delivery of piceid would be a promising approach for curing the above-mentioned diseases. However, piceid tends to agglutinate in aqueous solutions due to its hydrophobic nature, preventing efficient penetration to skin tissue. Although resveratrol nanoparticles stabilized with surfactants or phospholipid for transdermal delivery have been reported,⁷,⁸ piceid nanoparticles for transdermal delivery have not been well studied. Anionic phospholipids are supposed to have high dispersibility because of strong electrostatic repulsion between nanoparticles, together with high biocompatibility.

In this study, we successfully created piceid nanoparticles stabilized with an anionic phospholipid of 1,2-dipalmitoyl-sn-glycero-3-phosphoryl glycerol (DPPG; Figure 1). Small-sized DPPG-piceid nanoparticles (~60 nm in diameter) could be prepared by ultrasonic treatment. Upon addition of the DPPG-piceid nanoparticles to rat skin tissue, piceid molecules localized in stratum corneum of the skin tissue were successfully observed. DPPG-piceid nanoparticles could be prepared by incubating water dispersed powder sample of DPPG and piceid above Tₘ of DPPG (Figure 2a). As a typical example, we dispersed DPPG powder (5.0 wt%) and piceid powder (0.04 wt%) and heated for 15 minutes at 60°C above the Tₘ of DPPG (41°C), and then cooled down to room temperature. Consequently, we observed that the piceid molecules, which formed precipitations without DPPG (Figure 2b), were well dispersed after the stabilization with DPPG to give a transparent solution (Figure 2c). We also confirmed the encapsulation of piceid into DPPG by absorption spectra, in which the absorption peak of piceid around 300 nm was clearly observed from the DPPG-piceid dispersion (Figure 2d, blue). This spectral profile is because of high dispersibility of DPPG-piceid nanoparticles because piceid powder hardly showed the peak due to the precipitation (Figure 2d, gray).

Tuning the particle size is important for designing drug delivery systems. Especially, small-sized nanoparticles are...
required for transdermal drug delivery because it needs to pass through narrow space between skin cells. For this purpose, we next tried to create small-sized nanoparticles. When we performed an ultrasonication treatment to the sample for 3 hours, the DPPG-piceid nanoparticles were fractionated to 60 nm-sized nanoparticles as confirmed by dynamic light scattering (DLS) analysis (Figure 2e). Transition electron microscopy (TEM) also confirmed nanoparticles with around 60 nm size (Figure 2f). Noteworthy, the size of DPPG-piceid nanoparticles in this work was remarkably smaller compared with previously reported resveratrol nanoparticles stabilized with neutral phospholipids (>100 nm).9,10 We consider that repulsive force between nanoparticles derived from anionic phospholipid of DPPG prevented fusion events between the nanoparticles.

For microscopic evaluation of skin permeability of DPPG-piceid nanoparticles, we next synthesized fluorescein isothiocyanate-labeled piceid (FL-piceid) (Figure 1). Figure 3 shows the synthetic scheme for FL-piceid. As a first step, bromoethylamine was reacted with piceid to introduce a versatile functional group of amine (piceid-amine). Then, FL-piceid was successfully synthesized by reacting the piceid-amine with FITC. The structure of the obtained FL-piceid was identified by nuclear magnetic resonance (NMR) and mass spectroscopy (MS).

Finally, we investigated the skin permeability of DPPG-FL-piceid nanoparticles (Figure 4a). We hybridized FL-piceid with DPPG (Figure 4b) in the same method as DPPG-piceid and incubated them with rat skin tissue placed on Franz diffusion cells. We prepared histological sections of the skin sample after 24 hours of incubation for fluorescent microscopic observation. Surprisingly, strong fluorescence due to localization of FL-piceid molecules was successfully observed (Figure 4d and f) as compared with the sample without DPPG-FL-piceid (Figure 4c and e). Although the molecular structure of FL-piceid is not exactly as same as that of piceid, we expected that DPPG-piceid nanoparticles would have rather high skin permeation capability because the molecular structure of piceid is much smaller than that of FL-piceid.

In this work, we have reported piceid nanoparticles stabilized by anionic phospholipids of DPPG. The DPPG-piceid

![Image](image_url)
nanoparticles could be easily prepared by heating followed by a cooling treatment to aqueous mixtures of DPPG and piceid. Upon addition of small-sized DPPG-piceid particles to rat skin tissue, the nanoparticles were localized in stratum corneum. Although piceid is an antioxidant agent that shows high tyrosinase inhibitory activity compared with other stilbenoids, transdermal delivery of piceid has been still challenging. The DPPG-piceid nanoparticles having the capability...
for localization in skin tissue demonstrated in this study would be a new candidate as skincare materials with antioxidant effects.

**Experimental**

**General**

$^1$H NMR spectra were recorded on a Bruker type Ascend-600 spectrometer. Electrospray ionization/MS (matrix-assisted laser desorption ionization-time-of-flight) was performed on Hitachi model Nanofrontier I.D. Ultrasonication was performed by using a QSonica model ultrasonic homogenizer. Particle sizes were measured by using a Malvern model Zetasizer Nano ZSP zeta potential analyzer (DLS). TEM was performed using a JEOL model JEM-1230 transmission electron microscope operating at an anode voltage of 80 kV, and the sample was stained with gadolinium acetate. DPPG was purchased from Avanti Polar Lipids.

**Synthesis**

To a solution of dimethylformamide (DMF, 2 mL) and acetonitrile (5 mL), a mixture of piccide (122 mg, 0.313 mmol), 2-bromoethylamine hydrobromide (192 mg, 0.938 mmol), and cesium carbonate (815 mg, 2.5 mmol) were added, and the mixture was stirred for 2 days at room temperature. The mixture was filtered to remove salts and rinsed with acetonitrile. The combined filtrate was removed under reduced pressure to obtain piccide-amine (76.0 mg, 0.175 mmol), which was used for the next reaction without further purification.

To a DMF solution (500 µL) of piccide-amine (60.7 mg, 0.140 mmol) and FITC-I (47.5 mg, 0.140 mmol) diisopropylamine (54.3 mg, 0.420 mmol) was added, and the reaction mixture was stirred for 17 hours at 25°C. After mixing with methanol (150 µL), the solvent was removed under reduced pressure to obtain the $^1$piccid (101 mg, 0.123 mmol). $^1$H NMR (600 MHz, dimethylsulfoxide [DMSO]-d$_6$): δ 3.12-3.80 (7H), 4.00-4.20 (4 H), 6.30-8.10 (18H). $^{13}$C NMR (600 MHz, DMSO-d$_6$): 40.3, 42,4, 54.4, 62.4, 63.5, 65.2, 65.9, 66.9, 102.2, 110.1, 110.3, 112.6, 117.4, 119.3, 124.6, 127.8, 129.0, 129.3, 130.8, 140.5, 141.1, 142.9, 148.0, 150.3, 152.9, 155.0, 159.2, 160.3, 161.7, 163.5, 167.7, 168.9, 169.7, 170.8, 171.1, 180.4, 189.6. ESI-MS: m/z 423.1 [M + H + Na]$^{2+}$.

**Preparation of DPPG-Piccid and DPPG-$^1$piccid Nanoparticles**

Piceid (0.04 wt%) was mixed with DPPG powder (5.0 wt%) in water and sonicated for 2 minutes to disperse homogeneously and then heated at 60°C for 15 minutes where the solution turned clear. The resulting mixture was kept standing at room temperature for 1 hour before use. DPPG-$^1$piccid was prepared in the same method except for using $^1$piccid (0.025 wt%) instead of piccide (0.04 wt%). To prepare small-sized DPPG-pced and DPPG-$^1$piccid

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**Figure 3.** Synthetic route of fluorescein isothiocyanate-labeled piccide ($^1$piccid).
nanoparticles, the samples were ultrasonicated at 50 W for 3 hours with keeping the temperature at 4°C.

**Transdermal Delivery**

In vitro skin permeation tests were performed using a vertical Franz diffusion cell with an effective diffusion area of 0.95 cm². Skin tissues were obtained from the abdominal hair of rats according to that reported previously. The subcutaneous fat and other extraneous tissues of rat skin were trimmed and removed. A piece of excised skin (area 3.14 cm², diameter 20 mm) was mounted on the Franz diffusion cell with the stratum corneum facing the donor compartment. One circular SS Nikasol or SS HGA patch (area 0.785 cm², diameter 10 mm) was applied to the stratum corneum side of the skin. The receptor compartment was filled with 3 mL of water and maintained at 32°C using a circulating water bath stirred with magnetic bars.

![Image of transdermal delivery](image)

**Figure 4.** (a) Synthetic illustrations for transdermal delivery of DPPG-FLpiceid nanoparticles. (b) Naked-eye observation of a dispersion of DPPG-FLpiceid nanoparticles. (c, d) Bright field and (c, f) fluorescence image of a sliced image of rat skin tissue 24 hours after incubation (c, e) without and (d, f) with DPPG-FLpiceid nanoparticles.
For microscopic observations, skin tissue was embedded into optimal cutting temperature compound, frozen, and cryosectioned.

Declaration of Conflicting Interests
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