Extension of the Scope of Anionic Phospholipid-Based Nanoformulation to Kaempferol and Indometacin

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Abstract
In this work, resveratrol was dispersed with anionic phospholipids of 1,2-dipalmitoyl-sn-glycero-3-phosphoryl glycerol (DPPG), 1, 2-dipalmitoyl-sn-glycero-3-phosphatic acid, and 1,2-distearoyl-sn-glycero-3-phosphoglycerol. Moreover, small-sized nanoparticles of kaempferol and indometacin were successfully prepared by using DPPG as a dispersion agent.

Keywords
phospholipid, nanoparticle, kaempferol, indometacin, resveratrol

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With the diversification of drug discovery in recent years, methods to disperse drug molecules into nanometer-sized particles plays a crucial role. For example, it is well known that properly size-controlled nanoparticles can target cancer tissue in blood administration by enhanced permeability and retention effect. Furthermore, it is known that small particles can be used as a drug delivery carrier showing high skin permeability in transdermal drug delivery. Recently, we have focused on a nanoformulation of drug molecules using naturally occurring anionic phospholipids. For example, we reported that small-sized resveratrol (Res) nanoparticles exhibiting high skin permeability could be prepared by dispersing Res with an anionic phospholipid of 1,2-dipalmitoyl-sn-glycero-3-phosphorylglycerol (DPPG). How applicable is this anionic phospholipid-based nanoformulation to other drug molecules? Obviously, this is the next fascinating challenge. In order to extend the applications of the anionic phospholipid-based nanoformulation, we herein confirmed that Res is dispersed not only with DPPG but also with anionic phospholipids of 1,2-dipalmitoyl-sn-glycero-3-phosphatidic acid (DPPA) and 1,2-distearoyl-sn-glycero-3-phosphoglycerol (DSPG). Furthermore, we successfully prepared small-sized kaempferol (Kae) and indometacin (Ind) nanoparticles (DPPG-Kae and DPPG-Ind, respectively) by dispersing them with DPPG.

Figure 1 shows phospholipids and drug molecules used in this study. Nanoparticles (Figure 2(A)) can be prepared by mixing a phospholipid and molecules to be encapsulated in water, raising the temperature higher than the phase transition temperature of the phospholipid, and then cooling to room temperature. As a typical method, DPPG powder (5.0 wt%) and Res powder (1 mM) were dispersed in water, heated at 60 °C, which is higher than the phase transition temperature of DPPG ($T_m = 41 ^\circ C$) for 15 minutes, and then at room temperature, and then ultrasonicated for 3 hours. As a result, Res powder, precipitated before the dispersion, was dispersed into a transparent aqueous solution after the mixing with DPPG (Figure 2(B)). In order to investigate systematically the effects of the anionic group of phospholipid, we utilized DSPG, which has the same hydrophilic part as DPPG, and a longer alkyl chain, DPPA, which has the same alkyl chain as DPPG and phosphoric acid as the hydrophilic part. We also checked the effects of zwitterionic 1,2-distearoyl-sn-glycero-3-phosphorylcholine (DPPC). As a result, a clear solution was observed when DSPG (Figure 2(C)) and DPPA (Figure 2(D)) were used, whereas a cloudy solution, due to large-sized particle of Res,
was observed when we used DPPC (Figure 2(E)), indicating that highly dispersible particles were produced when anionic phospholipids were used. In addition, among the anionic phospholipids, the most transparent solution was observed when DPPG was used.

Next, we tried to prepare nanoparticles of Kae and Ind using DPPG as well, as in the case of Res. Kae is one of the flavonols whose molecular structure is similar to that of Res. Kae is known to show antioxidant, anti-inflammatory, antimicrobial, anticancer, cardioprotective, neuroprotective, antidiabetic, antiosteoporosis, estrogen/antiestrogen effects, antianxiety, pain relief, and antiallergic activity. Indometacin is a nonsteroidal anti-inflammatory drug having anti-inflammatory and analgesic effects. As shown in Figure 3(A), clear dispersion was observed when Kae was dispersed with anionic DPPG, while a cloudy dispersion was observed in the case of zwitterionic DPPC (Figure 3). Consistent with the naked-eye observations, the sizes of DPPG-Kae and DPPC-Kae as analyzed with a laser diffraction particle size analyzer (SALD) were 144 nm and 9.4 µm, respectively (Figure 3(C)). Similar to the case of Kae, a clear solution was observed when Ind was dispersed with DPPG (Figure 3(D)), while a cloudy solution was observed when DPPC was used (Figure 3(E)). The particle sizes measured by SALD were 79 nm and 12.8 µm, respectively. The sizes of samples were measured once after the system became stable. We supposed that the DPPG-Kae and DPPG-Ind did not form well-organized aggregates because the DPPG nanoparticle containing piceid that we previously reported formed aggregates, not vesicles. We also confirmed that the DPPG-Kae and DPPG-Ind were stable, even in phosphate-buffered saline solution in which the sizes of the nanoparticles were 265 ± 8 nm and 98 ± 1 nm, respectively (evaluated by zeta average in DLS). In addition, we compared the size of DPPG-Kae and DPPG-Ind to those previously reported. As typical examples of Kae nanoparticles, chitosan, poly(amidoamine) PAMAM dendrimer, and polylactic acid-based Kae nanoparticles have been reported, and the size of the particles was 273, 250, and 210 nm, respectively. Therefore, the size of DPPG-Kae (144 nm) is smaller than these previous examples (Figure 4(A)). As typical examples of Ind nanoparticles, mesoporous silica, dextran, and hydrophobin protein-based Ind nanoparticles have been reported, and the sizes of the particles were 400, 292, and 250 nm, respectively. Also, in this case, the size of DPPG-Ind (79 nm) is the smallest among them (Figure 4(B)). That is to say, the DPPG-based nanoformulation can produce extremely small nanoparticles.

In this work, we successfully extend the scope of anionic phospholipid-based nanoformulation of hydrophobic molecules. First of all, we dispersed Res in aqueous solution not only by using DPPG but also by using DSPG and DPPA. Furthermore, we also succeeded in the preparation of small-sized DPPG-Kae and DPPG-Ind nanoparticles by dispersing

Figure 1. Molecular structures of phospholipids of 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG), 1,2-distearoyl-sn-glycero-3-phosphoglycerol (DSPG), 1,2-dipalmitoyl-sn-glycero-3-phosphatidic acid (DPPA), and 1,2-distearoyl-sn-glycero-3-phosphorylcholine (DPPC) and aromatic compounds of resveratrol (Res), kaempferol (Kae), and indometacin (Ind) used in this work.

Figure 2. (A) Synthetic illustrations for the preparation of Res nanoparticles. Photograph of the dispersions of DPPG-Res (B), DSPG-Res (C), DPPA-Res (D), and DPPG-Res (E). DPPA, 1,2-dipalmitoyl-sn-glycero-3-phosphatidic acid; DPPC, 1,2-distearoyl-sn-glycero-3-phosphorylcholine; DPPG, 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol; DSPG, 1,2-distearoyl-sn-glycero-3-phosphorylcholine; Res, resveratrol.
Kae and Ind in water with DPPG. In particular, Ind is supposed to inhibit cyclooxygenase 1 involved in Prostaglandin E2 production and suppress eosinophil proliferation and hyperactivity caused by Th2 cytokines, which is effective for skin diseases such as eosinophil pustular folliculitis. Therefore, small-sized DPPG-Ind nanoparticles in this study could be applicable as a transdermal DDS carrier by evaluating their skin permeability in the future.

**Experimental**

**General**

Ultrasonication was performed using a QSonica model ultrasonic homogenizer. Particle sizes were measured with a Horiba model LA-960 SALD. DPPG, DSPG, DPPA, and DPPC were purchased from Avanti Polar Lipids, and Res, Kae, and Ind from TCI.

**Preparation of Nanoparticles**

For the preparation of DPPG-Res, Res (1.0 mM) 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 when the solution turned clear. The resulting mixture was kept at room temperature for 1 hour and then ultrasonicated at 50 W for 3 hours keeping the temperature at 4 °C. DSPG-Res, DPPA-Res, and DPPC-Res were prepared in the same way, except for using DSPG powder (5.0 wt%), DPPA powder (5.0 wt%), and DPPC powder (5.0 wt%), respectively, instead of DPPG powder (5.0 wt%). DPPG-Kae and DPPG-Ind were prepared using the same method as DPPG-Res, except for using Kae (1.0 mM) and Ind (1.0 mM), respectively, instead of Res (1.0 mM).

**Declaration of Conflicting Interests**

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