Preparation and Properties of Nonionic Vesicles Prepared with Polyglycerol Fatty Acid Esters Using the Supercritical Carbon Dioxide Reverse Phase Evaporation Method

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Abstract: Previously, we reported a new preparation method for liposomes and niosomes (nonionic vesicles) using supercritical carbon dioxide (scCO$_2$) as the solvent (scRPE method). In this study, niosomes were prepared from polyglycerol fatty acid ester (PG ester)-type nonionic surfactants. These surfactants are made from naturally derived materials and are neither harmful to the human body nor to the environment. Niosomes were prepared using the scRPE method with ethanol as the co-solvent. Through this method, decaglycerol distearate (DG2S) and decaglycerol diisostearate (DG2IS) formed niosomes. On the other hand, decaglycerol monostearate (DG1S), which has a high hydrophilic-lipophilic balance (HLB) value, yielded a solution of spherical micelles, and decaglycerol tristearate (DG3S), which has a low HLB value, yielded a gel-like solution. Niosomes of DG2IS had higher trapping efficiencies and dispersion stabilities than those of DG2S because the membrane fluidity of the DG2IS niosomes was greater than that of the DG2S niosomes. The niosomes obtained in the present study are candidates for cosmetic and pharmaceutical applications because they are formed from nonionic surfactants derived from natural sources, and prepared using the scRPE method, which avoids the use of harmful organic solvents.

Key words: nonionic surfactant, polyglycerol fatty acid ester, supercritical carbon dioxide reverse phase evaporation method, niosome, liposome

1 Introduction

Liposomes have phospholipid bilayers with an aqueous phase at their core$^1$. They have been used in drug delivery systems, cosmetics, as biomembrane models, and as microcapsules for chemical reactions$^{2-8}$. One of the most interesting properties of liposomes is that they retain water-soluble substances in the inner aqueous phase and oil-soluble substances in the surrounding bilayer$^9$. Although liposomes are expected to have a wide range of uses in industry, they have several problems concerning their physical and chemical stabilities. One useful approach to reduce these problems is to use nonionic surfactants, instead of phospholipids, to form vesicles. Compared to liposomes, vesicles made of nonionic surfactants are more suitable for industrial applications because of their high physicochemical stabilities and low cost. Furthermore, there are a large number of nonionic surfactants available for the design of nonionic vesicles, known as niosomes, because many combinations of hydrophilic and lipophilic groups can be made. For example, niosomes prepared using polyoxyethylene alkyl ethers, sucrose fatty acid esters, polyglycerol fatty acid esters, sorbitan fatty acid esters, and polyoxyethylene sorbitan fatty acid esters have been reported$^{11,12}$. However, the preparation of liposomes or niosomes requires the use of harmful organic solvents such as chloroform and acetone. These organic solvents are necessary to dissolve the phospholipids or nonionic surfactants$^{13-15}$. When vesicles are used as drug carriers...
and in cosmetics, the use of organic solvents must be minimized. Recently, we reported a new, environmentally friendly method, the supercritical carbon dioxide reverse phase evaporation method (scRPE method), for the preparation of liposomes or niosomes in a single step using supercritical CO$_2$ (scCO$_2$) \cite{16,17}. However, niosome formation by the scRPE method has been demonstrated only for nonionic surfactants with polyoxyethylene as the hydrophilic group \cite{18,19}. In pharmaceuticals, cosmetics, and food products, consumers prefer to use products made from safer materials, especially plant-derived materials. Thus, petroleum-derived materials such as polyoxyethylene-type nonionic surfactants should be avoided. However, there are few reports of niosomes prepared with non-polyoxyethylene-type nonionic surfactants.

In this study, the scRPE method was used to prepare niosomes with polyglycerol fatty acid ester (PG ester)-type nonionic surfactants. PG ester-type nonionic surfactants are widely used as food additives because they are safe to be used for such purposes. In this paper, we report the effects of the molecular structures of nonionic surfactants on niosome formation. In addition, we investigated their physicochemical properties, including trapping efficiency, by cryogenic transmission electron microscopy (cryo-TEM), fluorescence anisotropy (FA), differential scanning calorimeter (DSC), and dynamic light scattering (DLS).

## 2 Experimental

### 2.1 Materials

Decaglycerol monostearate (DG1S, NIKKOL Decaglynn 1-SV), decaglycerol distearate (DG2S, NIKKOL Decaglynn 2-SV), decaglycerol diisostearate (DG2IS, NIKKOL Decaglynn 2-ISV), and decaglycerol tristearate (DG3S, NIKKOL Decaglynn 3-SV), listed in Table 1, were provided by Nikko Chemicals (Tokyo, Japan) and were used as PG ester-type nonionic surfactants. These materials are derived from plant, in which a primary source is palm. They were used as purchased without further purification.

### 2.2 Methods

#### 2.2.1 scRPE Method

Figure 1 shows a schematic illustration of the experimental apparatus for the scRPE method. The desired amounts of nonionic surfactant and ethanol were sealed in the pressure cell, and CO$_2$ was introduced. The cell temperature was then raised to 60°C and maintained at the desired CO$_2$ pressure. After allowing a few minutes for equilibration, an aqueous solution of 0.2 M glucose, which was used as a model of a water-soluble drug, was slowly (0.1 mL/min) introduced using a HPLC pump until a total volume of 5 mL, and the surfactant concentration reached 10 mM. The pressure was then reduced by releasing CO$_2$, yielding a suspension. The interior of the cell was stirred with a magnetic stirring tip during the preparation process.

### Table 1 Polyglycerol fatty acid ester-type nonionic surfactants used in this study.

| Chemical structure of nonionic surfactants | HLB* |
|-------------------------------------------|------|
| Polyglycerol fatty acid ester             |      |
| $\text{H}_2\text{C} = \text{CHCH}_2\text{O} \bigg| \text{CHCH}_2\text{C} = \text{O} \bigg| \text{CH}_2\text{CHCH}_2 \bigg| \text{OR} \bigg| \text{OR} \bigg| \text{OR}$ |
| $n = \text{ca.} 10$ \hspace{0.5cm} $R = \text{H or stearyl, isostearyl (esterification degree = 1 to 3)$ |
| Decaglycerol monostearate (DG1S)          | 12.0 |
| Decaglycerol distearate (DG2S)            | 9.5  |
| Decaglycerol tristearate (DG3S)           | 7.5  |
| Decaglycerol diisostearate (DG2IS)        | 10.0 |

* HLB values were given by suppliers.
2.2.2 Evaluation of niosome suspensions
To determine the trapping efficiency, glucose was used as a model for a water-soluble drug and entrapped in the niosomes. The glucose-loaded niosome suspension was dialyzed against water using a cellophane tube (Viscase Scales Co.) to remove unentrapped glucose. Then the niosomes inside the tube were destroyed by adding ethanol. The amount of glucose in the solution was determined by the mutarotase-GOD method\(^{20,21}\) using a spectrophotometer (UV-260, Shimadzu Co.).

The hydrodynamic diameter of the niosomes was estimated using a NICOMP 380ZLS particle size analyzer equipped with a 5-mW He-Ne laser at a constant detector angle of 90°. The obtained scattering data were fitted using a number-weighted NICOMP-mode analysis to estimate the diffusion coefficient of the surfactant assemblies in suspension. The hydrodynamic diameter was obtained from the diffusion coefficient using the Stokes-Einstein equation.

The formation of niosomes was confirmed by cryogenic transmission electron microscopy (cryo-TEM). A small amount (3–5 \(\mu\)L) of sample suspension was placed on the surface of a copper TEM grid, covered by a holey carbon film, that was held by a pair of self-locking tweezers mounted on a spring-loaded shaft with a cryo-preparation system (LEICA EM CPA2, LEICA Microsystems). The sample drop was blotted with a filter paper to form a thin liquid film on the grid (<300 nm) and immediately plunged into liquid ethane cooled by liquid nitrogen (<−170°C). The grid was transferred onto the tip of a cryospecimen holder (CT-3500, Oxford Instruments) cooled by liquid nitrogen. Specimens were kept below −170°C and imaged in a transmission electron microscope (H-7650, Hitachi Science Systems, Ltd.) at an accelerating voltage of 120 kV with a low electron dose.

DSC measurements were performed with a differential scanning calorimeter (DSC-60, Shimadzu Co.) at a heating rate of 5 K/min. A stainless steel pan was used as the sample vessel. The reference sample was a quantity of distilled water with the same weight as that of the sample.

The microviscosity of the bilayers was investigated through fluorescence polarization measurements using 1,6-diphenyl-1,3,5-hexatriene (DPH) as the fluorescence probe. DPH was dissolved in tetrahydrofuran, and the resultant solution was added to each of the niosome suspensions to give a molar ratio of DPH to surfactant of 1:100. The DPH-containing suspensions were then incubated at 37°C for 2 h to solubilize the probe, and the fluorescence intensity was measured with a fluorescence spectrophotometer (RF-5000, Shimadzu Co.). The excitation and emission wavelengths were 350 and 450 nm, respectively. Fluorescence polarization \(P\) was calculated using the following equation.

\[
P = (I_p - I_v) / (I_p + I_v)
\]

\(I_p\) and \(I_v\) are the fluorescence intensities of the emitted light polarized parallel and vertical to the polarization of the source light, respectively.

3 Results and discussion
3.1 Solubility of nonionic surfactants in sc\(\text{CO}_2\) and dependence on ethanol addition
In our previous study\(^{19}\), we found that, to form niosomes using the scRPE method, the nonionic surfactants must be completely dissolved in sc\(\text{CO}_2\), and the hydrophilic-lipophilic balance (HLB) of the surfactants must be in a range of 9.5 to 9.9. All nonionic surfactants used in the present study were poorly soluble in sc\(\text{CO}_2\) at 60°C without the use of co-solvents. Furthermore, the trapping efficiency of the suspensions obtained with the scRPE method without co-solvent was almost 0%. Thus, the addition of ethanol as co-solvent\(^{17}\) was required for the formation of niosomes using these nonionic surfactants. In pharmaceuticals and cosmetics, ethanol addition is permitted within legal limits. Figure 2 shows a phase diagram of DG2S, DG2IS and DG3S in sc\(\text{CO}_2\) as a function of the amount of ethanol at 60°C. In this figure, the presence of one phase indicates complete dissolution of the surfactants in sc\(\text{CO}_2\); in contrast, two phases indicate that a portion of the surfactant added cannot dissolve in sc\(\text{CO}_2\). DG1S did not completely dissolve in sc\(\text{CO}_2\) below 200 bar (the upper \(\text{CO}_2\) pressure limit of the present equipment), even if ethanol was added in the surfactant suspension. On the other hand, DG2S was soluble in sc\(\text{CO}_2\) at pressures greater than 130 bar when the concentration of ethanol was greater than 7.5 wt.%. In the case of DG2IS, complete dissolution occurred at ethanol concentrations greater than 2.5 wt.% and at \(\text{CO}_2\) pressures

![Fig. 2](http://example.com/figure2.png)

**Fig. 2** Phase diagram of polyglycerol fatty acid ester-type nonionic surfactants (DG2S, DG2IS and DG3S) in sc\(\text{CO}_2\) as a function of the amount of ethanol at 60°C. DG1S was insoluble in sc\(\text{CO}_2\) at every pressure of \(\text{CO}_2\) and ethanol concentration investigated in this study.
greater than 120 bar. For the DG3S system, dissolution occurred at ethanol concentrations greater than 5.0 wt.% and at CO₂ pressures greater than 105 bar. These results indicate that the solubility of the surfactants in scCO₂ increases with increasing esterification of the PG ester-type surfactants. It is known that solvent strength of scCO₂ is close to that of hexane and the solubility of surfactants in hexane is increased with their hydrophobicity. On the basis of the above results, in the present study, niosomes were prepared at 60°C with a 7.5 wt.% ethanol concentration and a CO₂ pressure of 130 bar, allowing complete solubilization of DG3S, DG2S, and DG2IS in scCO₂.

3.2 Preparation of niosomes using PG ester-type non-ionic surfactants

Preparation of niosomes using PG ester-type nonionic surfactants was carried out using the scRPE method under the conditions described in Section 3.1. Table 2 shows the results of visual observations, average particle sizes, and trapping efficiencies for the PG ester-type nonionic surfactant suspensions made just after their preparation. Using DG2S and DG2IS, turbid suspensions were obtained, suggesting the formation of niosomes. In contrast, DG1S and DG3S yielded translucent and gel-like solutions, respectively. All samples passed through a filter with a pore size of 5 μm before DLS measurements in order to remove dust in the samples. The DG1S sample turned transparent after filtration probably because large DG1S crystals with the size above 5 μm were also removed. The average particle size for the resultant sample obtained by DLS was about 9 nm, which suggested the formation of spherical micelles. In addition, the trapping efficiency for the translucent DG1S sample without filtration was almost 0%, which suggested that niosomes were not formed in the DG1S sample. The DG2S and DG2IS aggregates had particle sizes of 340 and 220 nm, respectively. In addition, the trapping efficiency values for DG2S and DG2IS were 5 and 20%, respectively. Figure 3 shows cryo-TEM micrographs for molecular assemblies formed by DG2S and DG2IS. Hollow structures observed in the cryo-TEM micrographs strongly suggest the formation of niosomes in the DG2S and DG2IS systems. On the other hand, although DG3S dissolved in scCO₂ with ethanol co-solvent, the formation of niosomes could not be confirmed, and a gel-like solution was obtained. We previously reported²⁹ that the optimum HLB values for niosome formation with nonionic surfactants range from 9.5 to 9.9. For DG2S and DG2IS, whose HLB values are in or close to this range, niosomes were formed using the scRPE method. Because the HLB value (7.5) of DG3S is outside of the optimum HLB range, lamellar structures were not stable and did not form. In the case of DG1S, the HLB value (12.0) is greater than the upper limit of the optimum HLB values for niosome preparation. These findings indicate that two factors, i.e., high solubility of the PG ester-type nonionic surfactants in scCO₂ on addition of ethanol and choice of nonionic surfactants with an HLB value within the optimal HLB range, are required for the formation of niosomes by the scRPE method. These requirements for PG ester-type nonionic surfactants are similar to those for ethylene oxide-type nonionic surfactants, as reported in our previous study²⁸.

Table 2 Appearance, average particle sizes, and trapping efficiency for PG ester-type nonionic surfactant suspensions using the scRPE method.

| Visual observation | DG1S | DG2S | DG3S | DG2IS |
|--------------------|------|------|------|-------|
| Transparency       | Translucent liquid | Turbid liquid | Gel | Turbid liquid |
| Particle size (nm) | 9 | 340 | N.A. | 220 |
| Trapping efficiency (%) | 0 | 5 | N.A. | 20 |

Fig. 3 Cryo-TEM micrographs for (a) DG2S and (b) DG2IS niosome suspensions (10mM).
3.3 Comparison between DG2S and DG2IS niosomes

Figure 4 shows the average particle sizes of DG2S and DG2IS niosomes as a function of time after the sample preparation. The particle sizes of the DG2IS niosomes remained almost constant for at least one week. In contrast, those of the DG2S niosomes grew gradually from 350 to 1500 nm, suggesting that the dispersion stability of the DG2IS niosome suspension was greater than that of the DG2S niosomes. Figure 5 shows DSC curves for the DG2S and DG2IS niosome suspensions. An endothermic peak was observed at 42°C for the DG2S niosome suspension, indicating a phase transition from gel state (Lγ) to liquid-crystalline state (Lα). On the other hand, there was no endothermic peak for the DG2IS niosome suspension in the temperature range of 25 to 70°C. Figure 6 shows the fluorescence anisotropy of DPH incorporated into DG2S and DG2IS niosomes as a function of increasing temperature. The fluorescence anisotropy in DG2S niosomes gradually decreased from 0.14 to 0.12 until the temperature reached 40°C; then, it rapidly decreased, reaching 0.08 above 45°C. This suggested that the membrane fluidity was rapidly changed from low to high at a temperature between 40°C and 45°C. In the case of the DG2IS niosome suspension, the fluorescence anisotropy was almost constant, at 0.07, which is almost equal to that of the Lα state of DG2S, from 5 to 50°C. Thus, the DG2IS niosome was an Lα state in this temperature range. In general, branch-type alkyl chains have a low melting point below 0°C. Thus, the Lα-Lβ phase transition of DG2IS niosome probably occurs below 0°C. These results, as well as those from DSC measurements, indicate that the membrane fluidity of DG2IS niosomes was greater than that of DG2S niosomes at 25°C. The cryo-TEM image of the DG2S niosomes (Fig. 3a) showed that these particles had a non-spherical shape, which was likely to be flat lamellar plates. In general, rigid bilayers form flat lamellar structures, not vesicles (niosomes), because of their high bending energy. In contrast, niosomes formed of DG2IS, which has greater membrane fluidity, were almost spherical (cryo-TEM image in Fig. 3b). The high membrane fluidity of the DG2IS niosomes results in a stable dispersion with high trapping efficiency.

4 Conclusions

In this study, niosomes using PG ester-type nonionic surfactants were prepared by the scRPE method. The addition of ethanol as a co-solvent was necessary for complete dissolution of the PG ester-type surfactants in scCO₂. For DG2S and DG2IS, niosomes were successfully formed by the scRPE method in the presence of 7.5% ethanol. These nonionic surfactants have HLB values in the optimal range, allowing formation of stable lamellar structures in aqueous media, and this is an important factor to obtain niosomes using the scRPE method. Niosomes prepared with DG2IS have both higher trapping efficiencies and dispersion sta-
bilities than DG2S niosomes because of the higher membrane fluidity of DG2IS niosomes. This membrane fluidity arises due to the branched hydrophobic moieties in the molecule. The findings obtained in this study give useful information on the preparation of niosomes using nonionic surfactants derived from natural sources using an environmentally friendly method.

References
1) Bangham, A. D.; Standish, M. M.; Watkins, J. C. Diffusion of univalent ions across the lamellae of swollen phospholipids. J. Mol. Biol. 13, 238-252 (1965).
2) Balon, K.; Riebesehl, B. U.; Mueller, B. W. Determination of lipid partitioning of ionizable drugs by titration. J. Pharm. Sci. 88, 802-806 (1999).
3) Colic, M.; Morse, D. The elusive mechanism of the magnetic ‘memory’ of water. Colloids Surf. A 154, 167-174 (1999).
4) Montero, M. T.; Hernandez-Borrell, J.; Keough, K. M. W. Fluororquinoxolone-biomembrane interactions: monolayer and calorimetric studies. Langmuir 14, 2451-2454 (1998).
5) Komatsu, H.; Okada, S. Increased permeability of phase-separated liposomal membranes with mixtures of ethanol-induced interdigitated and non-interdigitated structures. Biochim. Biophys. Acta 1237, 165-175 (1995).
6) Imura, T.; Yanagishita, H.; Ohira, J.; Sakai, H.; Abe, M.; Kitamoto, D. Thermodynamically stable vesicle formation from glycolipid biosurfactant sponge phase. Colloids Surf. B: Biointerfaces 43, 115-121 (2005).
7) Yokouchi, Y.; Tsunoda, Y.; Imura, T.; Yamauchi, H.; Yokoyama, S.; Sakai, H.; Abe, M. Effect of adsorption of bovine serum albumin on liposomal membrane characteristics. Colloids Surf. B: Biointerfaces 20, 95-103 (2001).
8) Lian, T.; Ho, R. J. Y. Trends and developments in liposome drug delivery systems. J. Pharm. Sci. 90, 667-680 (2001).
9) Kaneda, Y. Virosomes: evolution of the liposome as a targeted drug delivery system. Adv. Drug Delivery Rev. 43, 197-205 (2000).
10) Marjan, J.; Xie, Z.; Devine, D. V. Liposome-induced activation of the classical complement pathway does not require immunoglobulin. Biochim. Biophys. Acta 1192, 35-44 (1994).
11) Manosroi, A.; Wongtrakul, P.; Manosroi, J.; Sakai, H.; Sugawara, F.; Yuasa, M.; Abe, M. Characterization of vesicles prepared with various non-ionic surfactants mixed with cholesterol. Colloids Surf. B: Biointerfaces 30, 129-138 (2003).
12) Nasseri, B. Effect of cholesterol and temperature on the elastic properties of niosomal membranes. Inter. J. Pharm. 300, 95-101 (2005).
13) Uchegbu, I. F.; Vyas, S. P. Non-ionic surfactant based vesicles (niosomes) in drug delivery. Inter. J. Pharm. 172, 33-70 (1998).
14) Batsru, S.; Korn, E. D. Single bilayer liposomes prepared without sonication. Biochim. Biophys. Acta 298, 1015-1019 (1973).
15) Szoka, F. Jr.; Papahadjopoulos, D. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. Proc. Natl. Acad. Sci. U.S.A. 75, 4194-4189 (1978).
16) Otake, K.; Imura, T.; Sakai, H.; Abe, M. Development of a New Preparation Method of Liposomes Using Supercritical Carbon Dioxide. Langmuir 17, 3898-3901 (2001).
17) Otake, K.; Shimomura, T.; Goto, T.; Imura, T.; Furuya, T.; Yoda, S.; Takebayashi, Y.; Sakai, H.; Abe, M. Preparation of Liposomes Using an Improved Supercritical Reverse Phase Evaporation Method. Langmuir 22, 2543-2550 (2006).
18) Ri, K.; Yamaguchi, S.; Wongtrakul, P.; Hashimoto, S.; Otake, K.; Okkubo, T.; Sakai, H.; Abe, M. Preparation and Characterization of Nonionic Surfactant Vesicles Using Supercritical Carbon Dioxide. Mater. Technol. 23, 340-347 (2005).
19) Yamaguchi, S.; Tsuchiya, K.; Sakai, K.; Abe, M.; Sakai, H. Preparation of Nonionic Vesicles Using the Supercritical Carbon Dioxide Reverse Phase Evaporation Method and Analysis of Their Solution Properties. J. Oleo Sci. 65, 21-26 (2016).
20) Miwa, I.; Okuda, J.; Meada, K.; Okuda, G. Mutarotase effect on colorimetric determination of blood glucose with -D-glucose oxidase. Clin. Chim. Acta 37, 538-540 (1972).
21) Okuda, J.; Miwa, I. Enzymatic micro-determination of D-glucose and its anomer. Protein, Nucleic Acid, Enzyme 17, 216-224 (1972).