Two-step Emulsification Process for Water-in-Oil-in-Water Multiple Emulsions Stabilized by Lamellar Liquid Crystals

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Abstract: Multiple emulsions, also called complex emulsions or multiphase emulsions, include water-in-oil-in-water (W/O/W)-type and oil-in-water-in-oil (O/W/O)-type emulsions. W/O/W-type multiple emulsions, obtained by utilizing lamellar liquid crystal with a layer structure showing optical anisotropy at the periphery of emulsion droplets, are superior in stability to O/W/O-type emulsions. In this study, we investigated a two-step emulsification process for a W/O/W-type multiple emulsion utilizing liquid crystal emulsification. We found that a W/O/W-type multiple emulsion containing lamellar liquid crystal can be prepared by mixing a W/O-type emulsion (prepared by primary emulsification) with a lamellar liquid crystal obtained from poly(oxyethylene) stearyl ether, cetyl alcohol, and water, and by dispersing and emulsifying the mixture in an outer aqueous phase. When poly(oxyethylene) stearyl ether and cetyl alcohol are each used in a given amount and the amount of water added is varied from 0 to 15 g (total amount of emulsion, 100 g), a W/O/W-type multiple emulsion is efficiently prepared. When the W/O/W-type multiple emulsion was held in a thermostatic bath at 25°C, the droplet size distribution showed no change 0, 30, or 60 days after preparation. Moreover, the W/O/W-type multiple emulsion strongly encapsulated Uranine in the inner aqueous phase as compared with emulsions prepared by one-step emulsification.

Key words: water-in-oil-in-water multiple emulsion, two-step emulsification, poly(oxyethylene) stearyl ether, cetyl alcohol, liquid crystal emulsification

1 INTRODUCTION

Multiple emulsions, also called complex or multiphase emulsions, include water-in-oil-in-water (W/O/W)-type and oil-in-water-in-oil (O/W/O)-type emulsions. They have various industrial applications such as in pharmaceutical products, cosmetics, and food. Multiple emulsions are applied in drug delivery systems for sustained release of drug contained in the inner phase1-3, and in drug carriers for drug delivery4-7. In cosmetics, multiple emulsions are used to stabilize active ingredients8; they also improve the skin feel of cosmetic products and increase the permeability of active ingredients, owing to the presence of the innermost aqueous phase9-13). Functional foods utilizing multiple emulsions capable of sustained release of nutritional components in the innermost phase12-14 have been studied.

One-step emulsification15 and two-step emulsification are used to prepare multiple emulsions. The former generally uses phase inversion emulsification. Although used only occasionally, a method entailing the addition of an aqueous phase to a surfactant-rich oil phase forming a lamellar liquid crystal to give a multiple emulsion has been reported by Seiller and Martini16, who showed that thermodynamically stabilized multiple emulsions can be easily obtained by using almond oil, with purified soybean lecithin, poly(oxyethylene)sorbitan monostearate and poly(oxyethylene)sorbitan monooleate as emulsifiers, by adding water thereto to form a lamellar liquid crystal, and by dispersing it in the outer aqueous phase.

Although one-step emulsification is easy and convenient, it is not generally used because a) the compositions of the inner and outer phases inevitably become the same, b) the encapsulation rate of the innermost phase is not clear, and
c) formulations are limited. In contrast, two-step emulsification is widely used because d) a multiple emulsion is obtained in high yield, e) a multiple emulsion is prepared with good reproducibility, and f) the compositions of the inner and outer phases can be controlled comparatively freely.

On the other hand, an emulsion preparation method called liquid crystal emulsification has the benefit of using stable emulsions since emulsion droplets are covered by viscous lamellar liquid crystals. In this study, we propose a novel preparation method for multiple emulsions by combining liquid crystal emulsification with two-step multiple emulsification. By this method, we obtained stable W/O/W-type multiple emulsions.

2 EXPERIMENTAL
2.1 Materials
The following materials were used: poly(oxyethylene) stearyl ether having a poly(oxyethylene) chain with polymerization degree of 2 or 21; polyethylene glycol di(polyhydroxystearate) with a poly(fatty acid) chain number of 5-7; polyethylene glycol with a polymerization degree of 30; poly(oxypropylene) stearyl ether having a poly(oxypropylene) chain with a polymerization degree of 15. All the compounds were manufactured by Croda Japan K.K. We also used the following: a plant-derived squalane manufactured by Croda Japan K.K.; cetyl alcohol manufactured by Nikko Chemicals Co., Ltd.; stearic acid manufactured by NOF Corporation; 1,3-butyleneglycol manufactured by Kyowa Hakko Chemical Co., Ltd.; sodium chloride and Uranine manufactured by Wako Pure Chemical Industries, Ltd. and are both of special reagent grade; and water treated using a reverse osmosis membrane.

2.2 Methods
2.2.1 Preparation of W/O-type emulsions (primary emulsions)
Measured amounts of poly(oxyethylene) di(polyhydroxystearate), plant-derived squalane (about 1/5 of the total amount), and 1,3-butyleneglycol were placed in a beaker, and the mixture was dissolved by heating at 80 ℃. Similarly, 0.1% aqueous sodium chloride solution heated to 80 ℃ was added in small amounts with stirring in a homogenizer (POLY TRON PT 2100, Ishii Laboratory Works Co., Ltd.) to induce emulsification. After emulsification, the rest of the plant-derived squalane was slowly added, and the mixture was cooled to 40 ℃ by stirring in an ice-water bath to give the primary emulsions.

2.2.2 Preparation of W/O-W-type multiple emulsion
Using the method shown in Fig. 1, four types of emulsion (Emulsions A-D) were prepared. Emulsion A was prepared according to the scheme shown in Fig. 1(a). The amount of each component is shown in Table 2. Measured amounts of the oil solution and emulsifier, as shown in Table 2, were placed in a beaker, and the mixture was dissolved by heating in a hot-water bath at about 75 ℃ (Mixture 1). Then, measured amounts of 1,3-butyleneglycol, sodium chloride, and water were placed in a beaker, and the mixture was heated to 75 ℃ in a hot-water bath to give an aqueous phase.

The aqueous phase was added to Mixture 1 while stirring with a magnetic stirrer, and the mixture was emulsified in a homogenizer (10,000 rpm, 1 min). After emulsification, the mixture was cooled to 40 ℃ in cold water to give Emulsion A.

Emulsions B-D were prepared according to the scheme shown in Fig. 1(b). Measured amounts of the oil solution and emulsifier, as shown in Table 2, were placed in a beaker, and the mixture was dissolved by heating in a hot-water bath at about 75 ℃ (Mixture 2). Then, water was heated to 75 ℃ in a hot-water bath. The W/O-type emulsions were prepared by emulsifying the oil solution and emulsifier mixture (Mixture 2) in the aqueous phase (Mixture 3). After emulsification, the mixture was cooled to 40 ℃ in cold water to give Emulsion B. This method was repeated to prepare Emulsions C and D.

![Fig. 1 Schemes of preparation methods.](image-url)

<table>
<thead>
<tr>
<th>Table 1 Compositions of W/O-type emulsions.</th>
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</thead>
<tbody>
<tr>
<td>amount (wt %)</td>
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<tr>
<td>poly(oxyethylene) di(polyhydroxystearate)</td>
</tr>
<tr>
<td>plant-derived squalane</td>
</tr>
<tr>
<td>1,3-butyleneglycol</td>
</tr>
<tr>
<td>sodium chloride</td>
</tr>
<tr>
<td>water</td>
</tr>
</tbody>
</table>

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### Table 2: Compositions of Emulsion A-D.

<table>
<thead>
<tr>
<th>Emulsion A</th>
<th>wt %</th>
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</thead>
<tbody>
<tr>
<td>plant-derived squalane</td>
<td>5.0</td>
</tr>
<tr>
<td>poly(oxypropylene) stearyl ether</td>
<td>4.0</td>
</tr>
<tr>
<td>cetyl alcohol</td>
<td>1.0</td>
</tr>
<tr>
<td>stearic acid</td>
<td>1.0</td>
</tr>
<tr>
<td>poly(oxyethylene) di(polyhydroxystearate)</td>
<td>0.1</td>
</tr>
<tr>
<td>poly(oxyethylene) stearyl ether (polymerization degree of poly(oxyethylene) chain, 2)</td>
<td>3.0</td>
</tr>
<tr>
<td>poly(oxyethylene) stearyl ether (polymerization degree of poly(oxyethylene) chain, 21)</td>
<td>2.0</td>
</tr>
<tr>
<td>1,3-butylene glycol</td>
<td>0.2</td>
</tr>
<tr>
<td>sodium chloride</td>
<td>0.001</td>
</tr>
<tr>
<td>water</td>
<td>83.699</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Emulsions B-D</th>
<th>wt %</th>
</tr>
</thead>
<tbody>
<tr>
<td>poly(oxypropylene) stearyl ether</td>
<td>4.0</td>
</tr>
<tr>
<td>cetyl alcohol</td>
<td>1.0</td>
</tr>
<tr>
<td>stearic acid</td>
<td>1.0</td>
</tr>
<tr>
<td>poly(oxyethylene) stearyl ether (polymerization degree of poly(oxyethylene) chain, 2)</td>
<td>3.0</td>
</tr>
<tr>
<td>poly(oxyethylene) stearyl ether (polymerization degree of poly(oxyethylene) chain, 21)</td>
<td>2.0</td>
</tr>
<tr>
<td>W/O-type emulsion</td>
<td>6.3</td>
</tr>
<tr>
<td>water</td>
<td>82.7</td>
</tr>
</tbody>
</table>

sion, that is, the primary emulsion, was heated to 60°C in a hot-water bath. A small amount of water (x) was slowly added to Mixture 2 while stirring with a magnetic stirrer; the resulting mixture was continuously stirred (Mixture 3). The amounts of water (x) to be added when the total amount of emulsion was 100 g were as follows: x = 0 for Emulsion B, x = 4 g for Emulsion C, and x = 15 g for Emulsion D. The W/O-type emulsions were gradually added while stirring with a magnetic stirrer; the resulting mixture was then thoroughly mixed by continuous stirring (Mixture 4). Thereafter, stirring was terminated, and the remaining water was slowly added to the mixture. The mixture was emulsified in a homogenizer (10,000 rpm, 1 min). After emulsification, the mixture was cooled to 40°C in cold water to give Emulsions B-D. Additionally, W/O-type emulsions with Uranine (0.0001 g in 100 g of final emulsion) in the inner aqueous phase were prepared; the amount of water added to Mixture 2 was within the x range of 0-15.

2.2.3 Small-angle X-ray scattering (SAXS)

The structure of the lamellar liquid crystals was determined by small-angle X-ray scattering (SAXS). A SAXSsess camera (Anton Paar, PANalytical) used a PW3830 laboratory X-ray generator with a long fine focus sealed with a glass X-ray tube (K, wavelength of 0.1542 nm) (PANalytical). The SAXS apparatus was operated at 40 kV and 50 mA. A 1-mm-thick quartz capillary was filled with the samples for the measurements.

2.2.4 Microscopy and viscosity measurement of emulsions

The state of the emulsions was directly observed under an optical microscope. The liquid crystal structure was directly observed under a polarization microscope. The viscosity of the emulsions was measured using a TOKIMEC viscometer (rotor No. 4, rotation speed 12 rpm, measurement time 1 min).

2.2.5 Stability test of W/O/W-type multiple emulsions

The W/O/W-type multiple emulsions were held in a thermostatic bath at 25°C, and the stability 0, 30, and 60 days after preparation was evaluated by optical microscopy and the measurement of droplet size distribution using a laser scattering instrument (Shimadzu, SALD-7000) with volume reference.

2.2.6 Retention capacity in inner aqueous phase of W/O/W-type multiple emulsions

Each W/O/W-type multiple emulsion (0.5 g) prepared by two-step emulsification were placed in a beaker, and then phosphate buffer (pH 7.5, 10 mM, 20-30 mL) was added and dispersed in the solution such that the emulsion was not broken up. The amount of emulsion was adjusted to 50 mL using a measuring flask. Then, a portion of the emulsion dispersion was filtered (Whatman No. 1, particle retention capacity 11 μm), the obtained filtrate was passed through a syringe filter (Advantec, DISMIC-13, particle retention capacity 0.45 μm), and the W/O/W-type multiple emulsion was completely removed. The rest of the emulsion was treated by ultrasonication (about 15 min) to break it.

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Uranine in the filtrate and in the emulsion dispersion after ultrasonication was analyzed by high-performance liquid chromatography, and the encapsulation rate ($E$) of Uranine in the inner aqueous phase of the multiple emulsions was calculated by using

$$E = \left(1 - \frac{A_e}{A_f}\right) \times 100$$  \hspace{1cm} (1)

where $A_f$ and $A_e$ indicate the peak areas of Uranine in the filtrate and emulsion, respectively.

3 RESULTS AND DISCUSSION

3.1 Two-step emulsification of W/O/W-type multiple emulsions using lamellar liquid crystal

Micrographs of Emulsions A-D are shown in Fig. 2. Emulsions A-D were had from the same composition but were prepared by different methods. Emulsion A was obtained by one-step emulsification. Almost all the obtained emulsions were of the O/W type; only a few were of the W/O/W type. Emulsion B was obtained by adding W/O-type emulsions to an oil phase containing an emulsifier, and performing secondary emulsification. Although Emulsion B also formed W/O/W-type multiple emulsions in a few cases, most of these emulsions were of the O/W type. As reason for this is that the W/O-type emulsions added were broken up by the emulsifier in the oil phase, and thus did not form an inner aqueous phase. Unlike Emulsions A and B, Emulsion C was found to form W/O/W-type multiple emulsions, but also formed some O/W-type emulsions. The mixed W/O-type emulsions were separated in Emulsion D.

From the micrographs of Emulsions A-D, it is clear that the obtained emulsions are markedly different depending on the preparation method, even when the same components are used at the same amounts. In particular, although Emulsions B-D are different only in the amount of water ($x$) added to the oil phase (Mixture 2), the presence of conditions that enable an efficient preparation of W/O/W-type multiple emulsions was suggested as in the case of Emulsion C. A polarization micrograph of Mixture 4 ($x = 4$ g) is shown in Fig. 3.

Mixture 2 contains oil and emulsifiers. Mixture 4 ($x = 4$ g) changes into a white, highly viscous mixture that exhibits optical anisotropy. Such a phase change in a ternary system of higher alcohol, nonionic surfactant, and water, which are components of Mixture 4 ($x = 4$ g), has been reported by Fukushima et al.\(^{18-19}\), who note that the viscous phase with optical anisotropy is thought to be a lamellar liquid crystal. Therefore, Mixture 4 ($x = 4$ g) was subjected to small angle X-ray scattering (SAXS) (Fig. 4). As a result, $d$-spacing $d_1/d_2 = 1:0.5$, determined from the position of the observed peak, has confirmed that Mixture 4 ($x = 4$ g) is a lamellar liquid crystal.

We considered the production mechanism of W/O/W-type multiple emulsions using lamellar liquid crystals to be as follows. As shown in Fig. 5 (a), when $x = 0$, the addition of the W/O-type emulsion (primary emulsion) to a surfactant-containing oil phase to be used for the second emulsification broke the HLB balance of the W/O-type emulsions, and the emulsions also broke up. Sekine et al.\(^{20}\) developed a two-step emulsification process for O/W/O emulsions that utilizes a clay mineral, and efficiently prepared O/W/O-type multiple emulsions by increasing the molecular weight of the emulsifier for the second emulsification, thereby lowering the exchange rate of emulsifiers of O/W-type emulsions, which are primary emulsions; they also prepared the emulsifier for the second emulsification. When $x = 4$ g (Fig. 5 (b)), the emulsifier for the second emulsification formed a lamellar liquid crystal, which lowered the exchange rate of surfactants that form W/O-type emulsions. As a result, none of the W/O-type emulsions added were broken;
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instead, they dispersed in the lamellar liquid crystal as a medium. Thus, W/O/W-type multiple emulsions were efficiently prepared by dispersing and emulsifying the emulsion in the outer aqueous phase. Hence, this method can be considered a preparation method using liquid crystal emulsification. The emulsions prepared by liquid crystal emulsification show a lamellar liquid crystal structure at the periphery of emulsion droplets and characteristic optical property under polarization microscopy.

A polarization micrograph of W/O/W-type multiple emulsions for Emulsion C is shown in Fig. 6. Maltese crosses are confirmed in the polarization micrograph, and the periphery of the W/O/W-type multiple emulsions has been confirmed to be covered by a lamellar liquid crystal. As described above, Emulsions B and D were not multiple emulsions but O/W emulsions. To obtain multiple emulsions, W/O-type emulsions must be dispersed as fine droplets and, in the present study, those emulsion droplets must be covered by lamellar liquid crystals. As for Emulsion B, the W/O-type emulsions were added in an oil-emulsifier mixture so that the emulsifier affects the W/O-type emulsions and leads to destabilization. As for Emulsion D, lamellar liquid crystals were already diluted by excess water addition before addition of the W/O-type emulsions. Hence, no liquid crystals could cover the droplets of the W/O-type emulsions.

3.3 Effects of x on emulsion type and viscosity

Since the addition of W/O-type emulsions to a lamellar liquid crystal is one step in the two-step emulsification of W/O/W-type multiple emulsions using a lamellar liquid crystal, the amount of water to be added to the oil phase is important. Therefore, the amount of water to be added to the oil phase, x, was varied from 0 to 15 g, and the emulsions were prepared according to the scheme shown in Fig. 1 (b). The emulsions obtained were evaluated by microscopy and viscosity measurement (Fig. 7).

By one-step emulsification within the x range of 0-2, the viscosity of the emulsions showed no change. The emul-

![Fig. 4 SAXS spectrum of Mixture 4 (x = 4 g) (measurement temperature, 70°C).](image)

![Fig. 5 Emulsion preparation processes for (a) x = 0 and (b) x = 4 g.](image)

![Fig. 6 Polarization micrograph of W/O/W-type multiple emulsions (x = 4 g).](image)
sions prepared within this range were found by microscopic observation to have formed O/W emulsions. When $x = 2$ g, W/O/W-type multiple emulsions were confirmed to have been partially formed. Within the $x$ range of 3-4, a slight decrease in viscosity was confirmed. The microscopic observation confirmed the successful preparation of W/O/W-type multiple emulsions. For $x > 5$, a marked decrease in viscosity was confirmed. The microscopic observation of the emulsion confirmed the separation of the oil, and the marked decrease in viscosity was considered to be due to phase separation.

3.4 Stability of emulsions
The stability of the present W/O/W-type multiple emulsions was evaluated by measuring the droplet size distribution and microscopic observation at known time intervals.

The temporal changes in droplet size distribution for emulsions held at 25°C are shown in Fig. 8. The droplet size distributions 0, 30, and 60 days after the preparation were compared; results show no difference among the respective droplet size distributions.

An optical micrograph of the emulsion 60 days after the preparation is shown in Fig. 9. A multilayer structure was observed even at 60 days after the preparation and no change in the state of the W/O/W-type multiple emulsions was observed. These results reveal that the emulsions were of the W/O/W type and had relatively long-term stability.

Generally, the stability of emulsions becomes worse when the droplet size is larger and the size distribution is wider, owing to higher rates of creaming and Ostwald ripening. The average droplet size is about 10 μm as shown in Fig. 8, which is relatively large for stable emulsions. Also the droplet size distribution is apparently wide as shown in Fig. 9. However, through this novel preparation process, we could have reasonable stability as high as those for multiple emulsions prepared by membrane emulsifications, which allow stable multiple emulsions to be formed with relatively large emulsion droplets21, 22.

3.5 Uranine retention capacity in inner aqueous phase of W/O/W-type multiple emulsions
The retention capacity of the inner aqueous phase of W/O/W-type multiple emulsions was evaluated. Uranine was added as a probe to the inner aqueous phase of W/O-type emulsions to give W/O/W-type multiple emulsions. Uranine that diffused into the outer aqueous phase was measured by HPLC, and the encapsulation rate of Uranine in the inner aqueous phase was calculated by using eq. (1).

As a result, using one-step emulsification and $x = 0$, the encapsulation rate of Uranine in the inner aqueous phase did not exceed 10% and increased with increasing amount of water added. When $x = 4$ g, the encapsulation rate of Uranine was about 50% at maximum (Fig. 10). As demon-
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strated in Fig. 2. Emulsion B (x = 0) is of the O/W type and Emulsion C is of the W/O/W type. The results in Fig. 10 clearly reflect the difference in emulsion structure. Namely, water-soluble Uranine molecules can be encapsulated in the inner water droplets of W/O/W-type emulsions. The emulsions were held at 25°C, and the changes in the encapsulation rate of Uranine in the inner aqueous phase were confirmed. As a result, the encapsulation rate of Uranine in the inner aqueous phase showed no change even after 30 days (Fig. 11).

Encapsulation rate of Uranine in inner aqueous phase of W/O/W-type multiple emulsions. “1 step” on the horizontal axis is for the emulsions prepared by the method shown in Fig. 1(a). Numerical values indicate the x in Fig. 1(b).

<table>
<thead>
<tr>
<th>Urane encapsulation (%)</th>
<th>1 step</th>
<th>0</th>
<th>2</th>
<th>3</th>
<th>3.5</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 step</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>3.5</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>10</td>
<td>20</td>
<td>30</td>
<td>50</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>30 days</td>
<td>10</td>
<td>20</td>
<td>30</td>
<td>50</td>
<td>60</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 10 Encapsulation rate of Uranine in inner aqueous phase of W/O/W-type multiple emulsions (x = 4 g) immediately after preparation (0 days) and 30 days after.

Fig. 11 Encapsulation rate of Uranine in inner aqueous phase of W/O/W-type multiple emulsions (x = 4 g) immediately after preparation (0 days) and 30 days after.

4 CONCLUSION
A novel emulsification process for multiple emulsions combining liquid crystal emulsification with two-step emulsification was studied. W/O/W-type multiple emulsions were prepared by mixing W/O-type emulsions, which are primary emulsions, with a lamellar liquid crystal consisting of poly(oxyethylene) stearyl ether, cetyl alcohol, and water for secondary emulsification, and by dispersing and emulsifying the mixture in an outer aqueous phase. The amount of water to be added to the oil phase containing poly(oxyethylene) stearyl ether, and cetyl alcohol is an important parameter. A lamellar liquid crystal structure was present at the periphery of the W/O/W-type multiple emulsions prepared by this method. Moreover, by evaluating the stability of the multiple emulsions at 25°C, the droplet size distribution showed no change even 60 days after preparation, and the emulsion state showed no change as observed by microscopic observation. These findings indicate that the prepared W/O/W-type multiple emulsions are superior in temporal stability. Uranine was added to the inner aqueous phase of W/O-type emulsions, which are primary emulsions, to give W/O/W-type multiple emulsions. The maximum encapsulation rate of Uranine in the inner aqueous phase was about 50%, which is higher than that of such emulsions prepared by one-step emulsification.

References