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Comparison of the Interfacial Properties of Span 80 Vesicle, W/O Emulsions and Liposomes

Keita HAYASHI,[1] Hiroshi UMAKOSHI[2] and Toshinori SHIMANOUCHI[3]*

[1] Department of Chemical Engineering, Nara National College of Technology,
22 Yata Yamatokoritama, Nara 639-1080, Japan

[2] Graduate School of Engineering Science, Osaka University,
1-3 Machikaneyama-cho, Toyonaka, Osaka 560-8531, Japan

[3] Graduate School of Environmental Life Science, Okayama University,
3-1-1 Tsushimanaka, Okayama, Okayama 700-8530, Japan

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Vesicles composed of sorbitan monooleate (Span 80) were prepared by a two-step emulsification method. The water-in-oil (W/O) emulsions generated in the first step of emulsification exhibited a quite dynamic and large hydrophobic difference between water and hexane. A subsequent emulsification yielded the vesicles with a fluid and flexible interface, as compared with that of liposomes. It was furthermore demonstrated that the two-step emulsification method has the advantage of the introduction of a PEG layer into the outer surface of the vesicles, while retaining the membrane properties of the vesicles.

1. Introduction

Phospholipids, detergents, and some polymers can form vesicles which are molecular self-assemblies with a lamellar structure, as reviewed previously in the literature [1]. Vesicles can encapsulate various materials such as fluorescence probes [2], drugs [3], genes [4], or peptides [5]. In the case of phospholipid vesicles (*liposomes*), many applications using vesicles have been investigated with respect to biosensors [6], drug delivery [7], gene expression [8] and (micro-) bioreactors [9]. Therefore, vesicle preparation is still an important issue for the design of functional systems using vesicles.

Vesicle preparation is fundamentally based on (i) the spontaneous swelling of lipids, (ii) solvent exchange, and (iii) a variation of lipid formulation, as previously reviewed [1]. In many cases, lipid formulation variation is associated with solvent exchange whose common feature is that the vesiculation is induced via a water-in-oil (W/O) or oil-in-water emulsion. Meanwhile, there is little known on the relationship between the variation of lipid formulation and its physicochemical properties, which are important to control such as encapsulation efficiency, adsorption of molecules and molecular function [10, 11]. Such an investigation would contribute to a better understanding of the preparation of vesicles with regard to their practical application.

In this study, sorbitan monooleate (Span 80) vesicles, prepared by a two-step emulsification method [12, 13] (Figure 1(a)-(c)) were employed as target systems because this process is likely to involve the above features. The interfacial properties of various systems such as vesicles and W/O emulsions were then investigated by using fluorescence probes. Next, the variation of the interfacial properties of Span 80

vesicles during the two-step emulsification process is discussed by contrasting them with a W/O emulsion and conventional liposomes. In addition, the two-step emulsification method allows modification of only the outer interface of the vesicles (Figure 1(d)), which would be advantageous in relation to the combination of the reaction system inside liposomes with the surface reaction on the vesicle membranes. Therefore, poly(ethylene glycol)(PEG) was used as the model ligand (e.g. peptide, lectin, antibody) as shown in Figure 1(d). The effects of pegylation on the vesicular surface were evaluated as compared with non-pegylated vesicle.

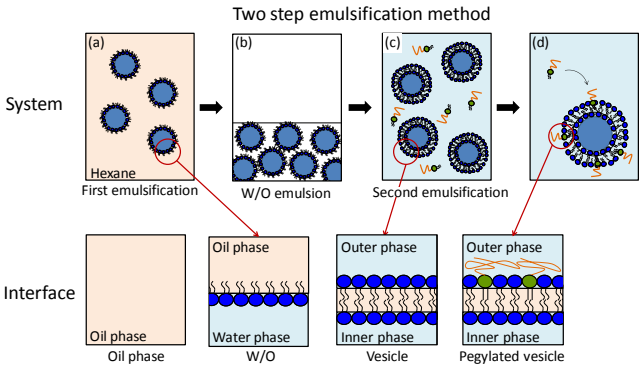

Figure 1. Schematic illustrations for the two step emulsification technique and the interface of various systems.

2. Experimental

2.1 Materials

1,2-Dimirystoyl-*sn*-glycero-3-phosphocholine (DMPC) and *N*-(carbonyl-methoxypolyethyleneglycol 2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (Figure 2) were purchased from NOF Corporation (Tokyo, Japan). Sorbitan monooleate (Span 80) was purchased from Wako Pure Chemical Ltd. (Osaka, Japan). Polyoxyethylene 23 lauryl ether (Brij 35) was purchased from Sigma-Aldrich (St. Louis, MO, USA). These lipids are shown in Figure 2. 8-Anilino-1-naphthalenesulfonic acid (ANS) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were obtained from Molecular Probes Inc. (Eugene, OR, USA). 6-Dodecanoyl-*N*,*N*-dimethyl-2-naphthylamine (Laurdan) and *N*,*N*,*N*-trimethyl-4-(6-phenyl-1,3,5-

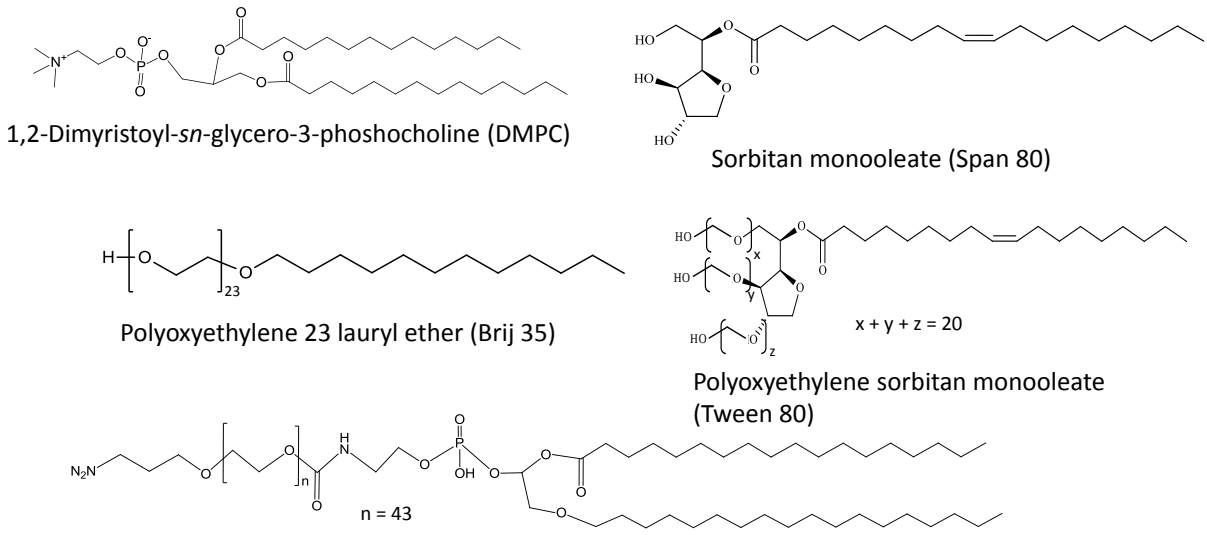

Figure 2. Chemical Structures of Lipids Used for Liposomes and Vesicles

hexatrien-1-yl) phenylammonium *p*-toluenesulfonate (TMA-DPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and AnaSpec Inc. (Fremont, CA, USA), respectively.

2.2 Pegylated liposome preparation

The DMPC liposome was prepared by the sonication method [14]. After hydration of a thin film of DMPC with a PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4; pH 7.3), the solution was ultrasonicated at 45 ºC. Brij 35 (10 wt%) was mixed in a DMPC suspension to prepare the hybrid liposome. The diameter was adjusted to 50 nm by using the extrusion method with a 50 nm polycarbonate filter.

2.3 Preparation of Pegylated Span 80 vesicles using two-step emulsification

Span 80 vesicles were prepared by the two-step emulsification method described in detail previously [13]. In short, 6 mL of n-hexane solution containing 300 mg Span 80 was added to 0.6 mL of inner-phase liquid (PBS), followed by the first emulsification for 6 min at 17,500 rpm using a micro-homogenizer NS-310E 2 (Microtec Co., Ltd., Japan). The solvent obtained from the water-in-oil emulsion was evaporated under reduced pressure, yielding a water-lipid emulsion to which 6 mL of PBS containing 96 mg of Tween 80 and 30 mg *N*-(carbonyl-methoxypolyethyleneglycol 2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine was added, followed by mixing with the homogenizer for 2 min at 3500 rpm to obtain the heterogeneous Span 80 vesicle suspension. The heterogeneous vesicle suspension was stirred for 3 h at room temperature, followed by storage overnight at 4 ^{o}C. The vesicles were then purified by ultracentrifugation (50,000 rpm at 4 ^{o}C for 120 min) in a Himac centrifuge CR15B (Hitachi Koki Co., Ltd., Japan). The purified Span 80 suspension was passed through a polycarbonate membrane (100 nm in pore size), and purified twice by ultracentrifugation.

2.4 Measurements of membrane properties

2.4.1 Polarization **of fluorescence probes**

The fluorescence polarization (*P*) of DPH and TMA-DPH was evaluated according to the previous reports [13, 15]. DPH or TMA-DPH dissolved in ethanol was added to a pre-formed vesicle suspension to give a lipid/probe molar ratio of 250 ($[(TMA\text{-})DPH]_{final}$ = 2 μM). Then, the mixture was incubated for at least 30min at room temperature under gentle stirring. The fluorescence polarization of the samples was measured with an FP 6500 spectrofluorometer (JASCO, Japan) equipped with a polarizer. The sample was excited with vertically polarized light (360 nm), and the emission intensity (430 nm) both parallel and perpendicular to the light was recorded. The temperature was kept at 37 ºC. Then, the fluorescence polarization (*P*) of DPH and TMA-DPH was calculated from the following equation:

$$P = \frac{I_{0°0°} - G \times I_{0°90°}}{I_{0°0°} + G \times I_{0°90°}}, \qquad G = \frac{I_{90°0°}}{I_{90°90°}}$$

where I_{xy} is the fluorescence intensity. The subscripts *x* and *y* for I_{xy} show the angle of the polarization plate of the exciting and emission sides, respectively.

2.4.2 Hydrophobicity of membrane surface

The measurement of the local hydrophobic environment of the lipid membrane has been reported in a previous paper [15]. The final concentration of lipid and ANS were 0.1 mM and 1.0 μM, respectively. The mixture was incubated at least for 30min at room temperature under gentle stirring. The fluorescence intensity of the samples was measured with an FP 6500 spectrofluorimeter (JASCO, Japan). The sample

was excited at 400 nm and the emission intensity was recorded at 470 nm. The temperature was kept at 37 ^{o}C.

2.4.3 Polarity of vesicle membranes

Laurdan fluorescence properties are extremely sensitive to the polarity around the molecule itself. The Laurdan emission spectrum has a red shift (Stokes shift) due to dielectric relaxation [16], giving the qualitative index for the hydrophobicity around Laurdan (inner membrane of vesicles as compared with ANS) [17]. When Laurdan was in a hydrophilic or a hydrophobic environment, maximum fluorescence intensity was observed at 440 and 490 nm, respectively [16]. The $GP_{(340)}$ value was suggested as a parameter in order to evaluate the hydrophilicity/hydrophobicity of the vesicular membrane [16]. The $GP_{(340)}$ value was calculated from the following equation:

$$GP_{(340)} = \frac{I_{440} - I_{490}}{I_{440} + I_{490}}$$

where I_{440} and I_{490} are the fluorescence intensities at 440 and 490 nm, respectively.

The emission spectra were measured by exciting wavelengths of 340 nm, according to the previous report [13]. The temperature was kept at 37 ^{o}C for the measurement of all samples.

2.4.4 Diameter distribution of vesicles

The size distribution of W/O emulsions, DMPC liposomes, and Span 80 vesicles were measured by using the dynamic light scattering mode of FPAR (Ohtsuka Electronics Co. Ltd., Japan).

3. Results and Discussion

3.1 Diameter distribution for each formulation

In the two-step emulsification method as shown in Figure 1, the water droplets in the emulsion are prepared in the first step. Without surfactants at the first emulsification step, a broad diameter distribution (the mean diameter: 510 nm) was observed as shown in Figure 3(a). The size of the coarse emulsion rapidly increased for several hours and underwent phase separation within a day (Figure 3(e)). Span 80 was then added to hexane / water to stabilize the coarse emulsion and the resulting size distribution is shown in Figure 3(b). The W/O emulsions stabilized by Span 80 are considered as the inverted micelle type (W/O emulsion/Span 80). The mean diameter was 431 nm. Flocculation or coalescence was not observed (Figure 3(e)).

Furthermore, the W/O emulsion/Span 80 was treated by a second emulsification step, followed by the extrusion method to adjust the size of particles to 100 nm in pore size. The size distribution curve was shifted to lower diameters (Figure 3(c)); the mean diameter: 123 nm). The Span 80 vesicles were stable for a long time (Figure 3(e)). This size distribution and stability was similar to DMPC liposomes (Figures 3(d) and (e)). It is noted that the size of DMPC (the mean diameter 102 nm) so prepared was generally larger than the membrane filter used (50 nm in pore size).

3.2 Dynamics of interfaces

The formation of Span 80 vesicles during the two-step emulsification process determined the interaction between the inverted micelles and between Span 80 vesicles. The interfacial structure of each system was investigated by using the rod-like fluorescence probes because their intramolecular rotational motion was sensitive to the ordered-structure of the interface. The intramolecular rotational motion of the

two fluorescence probes, DPH and TMA-DPH, was herein quantified by their fluorescence polarization (*P*) the reciprocal value of which represents the mobility of both [15, 18].

Figure 4 shows the (1/*P*) value of DPH and TMA-DPH in the variation of systems. Overall, the order of the (1/*P*) values of DPH and TMA-DPH were (1,2) > (3-6). Since Span 80 molecules can move not only at the interface between water and hexane but also in the bulk hexane phase, the intramolecular mobility of DPH and TMA-DPH is permitted, which is consistent with the large value of (1/*P*) in systems (1) and (2). In contrast, the supramolecular assemblies such as phospholipid vesicles (liposomes) and Span 80 vesicles indicated the low mobility of DPH and TMA-DPH. This is because the mobility of DPH and TMA-DPH orientated into the acyl chain region of the phospholipids or Span 80 was readily restricted. Furthermore, comparing DMPC liposomes (3,4) with Span 80 vesicles (5,6), the mobility of DPH was in the following order: (3, 4) < (5, 6). In contrast to DPH, the mobility of TMA-DPH was (3, 4) > (5, 6). The interface of Span 80 vesicles is composed of sorbitol as the headgroup. It was therefore considered that TMA-DPH orientated into the deeper region. In addition, no significant effect of the induction of the PEG layer to liposomes and Span 80 vesicles was observed. It is suggested that the mobility of the lipids was restricted in the formation of vesicles from the inverted micelles.

3.3 Hydrophobicity and polarity of interfaces

The intense mobility of lipids at the membrane interface resulted in the exposure of the hydrophobic environment of

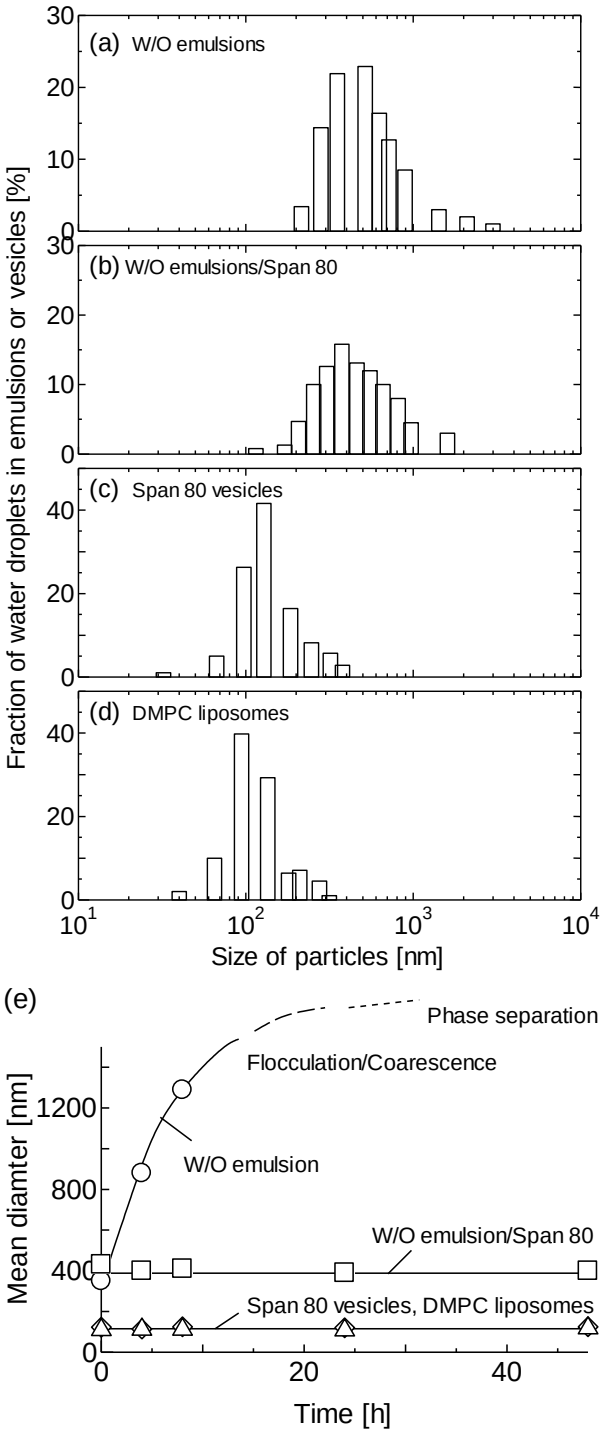

Figure 3. Size distribution of the coarse water droplet in the emulsion (a) without and (b) with Span 80. (c) Span 80 vesicles extruded, (d) DMPC liposomes extruded. (e) Time-course of mean diameter of each formulation prepared by Span 80 and DMPC. Key: (circle) W/O emulsion; (rectangle) W/O emulsion with Span 80; (triangle) Span 80 vesicle; (diamond) DMPC liposome.

membranes [16]. It has been reported that the hydrophobic fluorescence probe ANS can emit a large fluorescence intensity if ANS binds to the hydrophobic environment. The ANS fluorescence intensity for various systems was therefore measured as shown in Figure 5. The order of ANS intensity was (1, 2) > (3, 4) > (5, 6), indicating that Span 80 vesicles have a quite hydrophilic surface. Meanwhile, the pegylation of liposomes and vesicles indicated no remarkable change in hydrophobicity, implying no binding of ANS to the PEG layer or no difference in hydrophobicity between the PEG layer and the bulk aqueous phase under these experimental conditions.

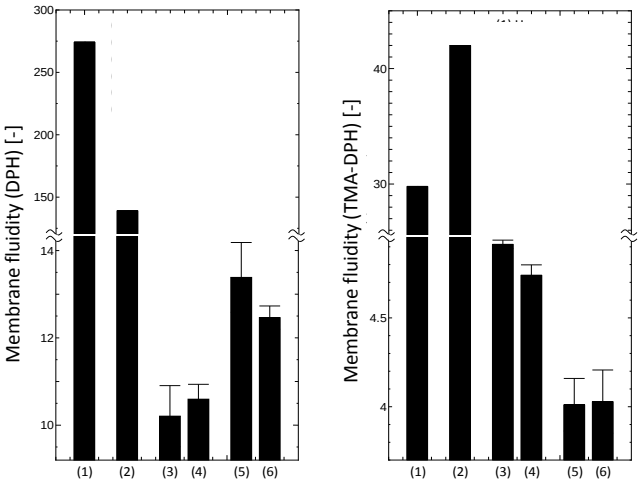

Figure 4. Mobility of (a) DPH and (b) TMA-DPH in various systems (37 ^{o}C). The values were evaluated by fluorescence anisotropy. (1) hexane; (2) W/O emulsion; (3) DMPC liposome; (4) Pegylated DMPC liposome; (5) Span 80 vesicle; (6) Pegylated Span 80 vesicle.

The polarity of each system was then investigated by using Laurdan. The spectrum of Laurdan for system (1) and (2) showed a peak at 435 nm (Figure 6(a)). In contrast, the peaks of the liposomes (3,4) and vesicles (5,6) were shifted to the longer wavelengths, suggesting that DMPC liposomes and Span 80 vesicles were more hydrophilic than hexane and the W/O emulsion. This result is compatible with that obtained in the ANS binding experiment (Figure 5). To further compare the polarity of the vesicle interfaces with liposomes, the generalized polarization (GP) values were then estimated. The order of the GP values (Figure 6(b)) was the same as that in the ANS binding experiment, indicating the large polarity (hydrophilicity) of the Span 80 vesicles. Meanwhile, the induction of the PEG layer resulted in no variation in the GP value for both the DMPC liposomes and Span 80 vesicles (Figure 6).

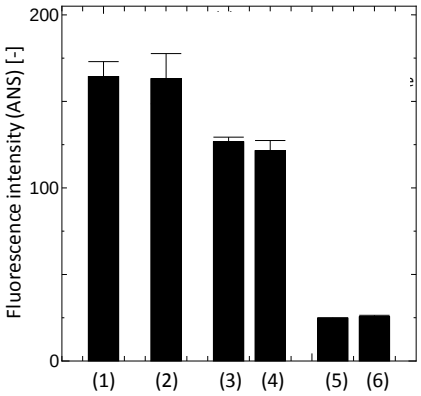

Figure 5. ANS fluorescence intensity for various systems. Measurements were performed at 37^{o}C. (1) hexane; (2) W/O emulsion; (3) DMPC liposome; (4) Pegylated DMPC liposome; (5) Span 80 vesicle; (6) Pegylated Span 80 vesicle.

3.4 Variation of interfacial property in a two-step emulsification

Based on the above results with respect to the interfacial properties of various systems, the influence of the two-step emulsification on the formulation of Span 80 is discussed by consulting Figure 1.

It has been reported that Span 80 is capable of forming emulsions of several hundreds of microns in diameter, in the water / *n*-tetradecane system of high Span 80 concentrations [19], suggesting the stable partitioning of Span 80 into the interface of the water / hexane system at the first emulsification step as shown in Figure 1(b). Actually, the hydrophobicity and other interfacial properties of the W/O emulsions in

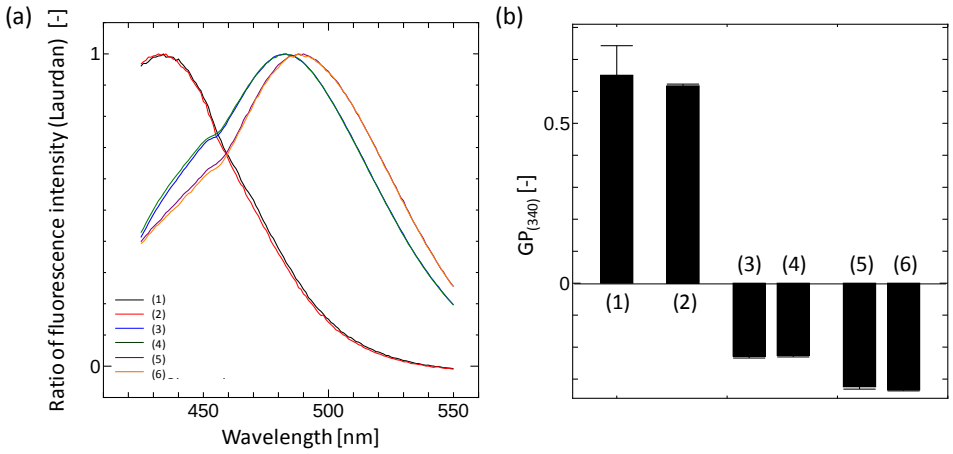

Figure 6. (a) Fluorescence spectra for laurdan in a variety of systems. (b) Generalized polarization value at 340 nm. Measurements were performed at 37 ^{o}C. (1) hexane; (2) W/O emulsion; (3) DMPC liposome; (4) Pegylated DMPC liposome; (5) Span 80 vesicle; (6) Pegylated Span 80 vesicle.

the absence of Span 80 (Figures 4-6) are advantageous for the induction of the flocculation or coalescence between W/O emulsions (Figure 3(e)). This is consistent with the conventional membrane fusion of liposomes [20] and the high interfacial excess energy of the O/W emulsions [21]. Addition of Span 80 in the first emulsification step reduces the interfacial excess energy of the W/O emulsions and prevents their flocculation/coalescence, although the inverted micelles showed high hydrophobicity and fluidity. This suggested stabilization of the O/W emulsions. The second emulsification process principally inherited this propensity, *i.e.* Span 80 vesicles obtained were so stable as to keep their size distribution. From the aspects of the interfacial properties, the variation of formulation for Span 80 along with the two-step emulsification process caused a reduction in hydrophobicity (Figure 5) and an increase in polarity (Figure 6), possibly resulting from the removal of hexane from the formulations of Span 80 at the second emulsification process. Taking into account the intense mobility of Span 80 headgroup (sorbitol) [13], water molecules were likely to be present in the deeper region of the vesicle membranes. This was probably because the sorbitol headgroup of Span 80 in the vesicle membranes could not prevent water from preventing into the deeper region of the membranes due to the weak dipole moment of sorbitol. As a consequence, the dynamics at the interface of Span 80 vesicles could be kept at a high level, as compared with that of the DMPC liposomes (Figure 4).

Another aspect of the two-step emulsification method is to be able to incorporate a model ligand such as PEG at the second emulsification step (Figure 1(d)), making it possible to modify the model ligand only at the outer surface of the vesicles in contrast to the conventional method [22]. As shown in Figures 4 to 6, the incorporation of the PEG layer into the membranes resulted in no large variation in surface properties for both liposomes and vesicles. Therefore, the two-step emulsification process succeeded in the modification of Span 80 vesicles at the outer surface, implying the effective utilization of the inner aqueous phase of the vesicles. In contrast, the PEG layer was incorporated into not only the outer but also the inner aqueous phase of DMPC liposomes although the induction of the stealth function can be achieved without variation of the membrane property [23].

4. Conclusion

The mobility of Span 80 at the interface is likely to be a key for the variation of its formulation and surface properties in the two-step emulsification process, as well as the incorporation of a PEG layer into the outer surface of Span 80 vesicles while maintaining the interfacial properties of the vesicles. Span 80 could stabilize the W/O emulsions obtained in the first emulsification step and prevented their flocculation / coalescence. This interfacial property of W/O emulsions was inherited in the second emulsification step. The present preparation method would afford the effective construction of a reaction system inside the vesicles combining with the reaction / transport system at the interface.

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