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Preparation of Liposomes Modified with Lipopeptides Using a Supercritical Carbon Dioxide Reverse-phase Evaporation Method

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Abstract: Although liposomes are considered to be one of the most promising carriers for drug delivery systems (DDS), they have drawbacks such as insufficient drug-entrapment efficiency and long-term stability. The objectives of this study are to improve the trapping efficiency by addition of lipopeptides (LPs), and using a supercritical CO_2 reverse-phase evaporation (SCRPE) process, along with incorporation of PEG-modified phospholipids to improve long-term stability. In this study, bovine serum albumin (BSA) was used as a model drug substance for entrapment by liposomes.

Improvements in the entrapment efficiency and stability of liposomes were achieved by modification with LPs and use of a SCRPE preparation process. The BSA-entrapment efficiency of liposomes modified with cationic LPs with arginine residues, as a result of their ionic interactions, was six times that of liposomes prepared by the Bangham method. Use of a SCRPE method along with LP modification further enhanced entrapment and enabled spontaneous formation of unilamellar liposomes with long-term stability. Liposomes consisting of DPPC/Chol/C_{16}-Arg2/DSPE-PEG2000 (60/30/5/5), with up to 70% entrapment efficiency for BSA and a stability level of 90% for over 40 h, were obtained. DSC and SAXS analyses indicated that certain amounts of LP in the DPPC induced phase-transitional and structural changes in the lamellar membrane, and these changes improved the DDS carrier properties.

The SCRPE method provides organic-solvent-free liposomes, and the LPs for the liposome modification are derivatives of amino acids and fatty acids, which are sustainable and biocompatible materials. This study therefore suggests that there are opportunities for the development of novel DDS carriers with excellent performance and which address environmental concerns.

Key words: liposome, lipopeptide, entrapment efficiency, supercritical CO_2 reverse-phase evaporation (SCRPE), drug delivery system (DDS)

1 INTRODUCTION

Liposomes are considered to be one of the most promising carriers for drug delivery systems (DDS), as well as for cosmetics and in food applications[1-3]. However, liposomes, which consist primarily of phospholipids, are fragile during storage and after systemic or topical applications before reaching the target sight in the body. Another drawback of liposomes is insufficient drug entrapment. Many attempts have been made to overcome such problems by methods such as improvements to the processes used for liposome preparation and incorporation of other lipoids to improve the stability and entrapment efficiency.

Known methods for the preparation of liposomes are the Bangham method and reverse-phase evaporation methods; each has benefits and disadvantages[4-9]. One drawback of these methods is the need to use organic solvents in the preparation process to solubilize the lipids in dry films before hydration. Residual solvents, in particular chloroform, raise safety concerns because of their potential toxicity and the difficulty of removing them from liposome preparations. In our previous study, we succeeded in making liposomes free of organic solvents by using supercritical carbon dioxide ($scCO_2$). This method has been named the supercritical CO_2 reverse-phase evaporation (SCRPE)

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method[10, 11]. As the physicochemical properties of $scCO_2$ are similar to those of hexane, a lipid/water/$scCO_2$ complex forms an emulsion of "$scCO_2$ in water," and liposomes are obtained after depressurizing the CO_2 by spontaneous formation of a bilayer liposome structure. The glucose-entrapment efficiency in liposomes prepared by the SCRPE method was shown to be about five times that in liposomes prepared by the Bangham method[11]. Liposomes prepared using the SCRPE method can encapsulate water-soluble and oil-soluble materials synchronously in a short (ca. 40 min) preparation time. Large-scale preparation of liposomes by the SCRPE method is therefore relatively easy[10, 11] and is believed to be suitable for commercial production. Another advantage of the SCRPE method is a relatively low operational temperature (45℃); this preserves the bioactivities of the entrapped drug molecules. The SCRPE method is therefore a valuable method for preparing DDS carriers. However, there is still a need to improve the SCRPE method to increase the encapsulation efficiency of the liposomes. The objectives of this study are to improve encapsulation efficiency and increase liposome stability, using an SCRPE method, in order to develop advanced pharmaceutical applications.

It has been reported that liposomes modified with lipopeptides (LPs) enhance gene expression[12]. LPs consists of a lipid analog and a peptide, and their amino acid sequences can easily be tuned[12, 13]. LPs are therefore one of the most promising compounds for the preparation of functional liposomes. Taking the advantages of LPs into account, we have tried to prepare liposomes modified with LPs, using a SCRPE method, to increase the entrapment efficiency for bovine serum albumin (BSA) as a model drug substance. Investigation of the effects of LPs on the physicochemical membrane properties is another important objective of this study. Physiological phenomena occur not only by reactions between molecules, but also as a result of the influence of the biophysical environment.

In this study, we successfully made stable liposomes modified with LPs using the SCRPE method. We achieved a BSA-entrapment efficiency eight times that achieved with non-LP liposomes prepared by the traditional Bangham method. The stability was also significantly improved, especially as a result of polyethylene glycol (PEG) modification.

2 EXPERIMENTAL PROCEDURES

2.1 Materials

L-α-Dipalmitoylphosphatidylcholine (DPPC, 99.6% pure) and *N*-(carbonylmethoxypolyethylene glycol)-sn-glycero-3-phosphoethanolamine sodium salt (DSPE-PEG2000) were gifted by the NOF Co. (Tokyo, Japan). Ethanol (99.8% pure), used as the co-solvent, and cholesterol (Chol) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Bovine serum albumin (BSA, 98.0% pure, Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used to examine the trapping efficiency of the liposomes. Phosphate buffer saline (PBS, Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used as the solvent.

LPs with arginine (Arg) as a cationic binder for BSA were synthesized by Fmoc (9-fluorenylmethyloxycarbonyl group) solid-phase synthesis using an automatic peptide synthesizer (PSSM8, Shimadzu Co., Kyoto, Japan) (Fig. 1)[12]. C_{16}-Arg1 and C_{16}-Arg2 (conjugated C_{16} alkyl chains and one or two arginines) were synthesized by this process and used to modify DPPC liposomes and as a binder for capturing BSA. The LP synthesis was performed as follows: 68.0 mg of TGS-RAM (Shimadzu Co., Kyoto, Japan) resin were used; each reagent was added to the resin in turn[14, 15], and, finally, the LP was obtained in 60-70% yield.

2.2 Liposome preparation by the SCRPE method

Figure 2 shows a schematic diagram of the experimental apparatus for the SCRPE method, as reported previously[10]. DPPC, cholesterol, LP, and ethanol (7 wt% against CO_2) were sealed in the cell, and then CO_2 was introduced into the cell. The cell temperature was then raised to 45℃,

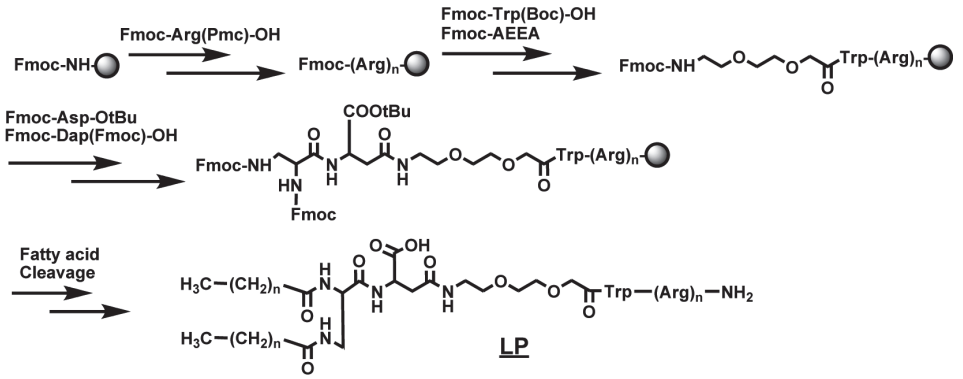

Fig. 1 Synthetic route of the LP using Fmoc-solid phase method.

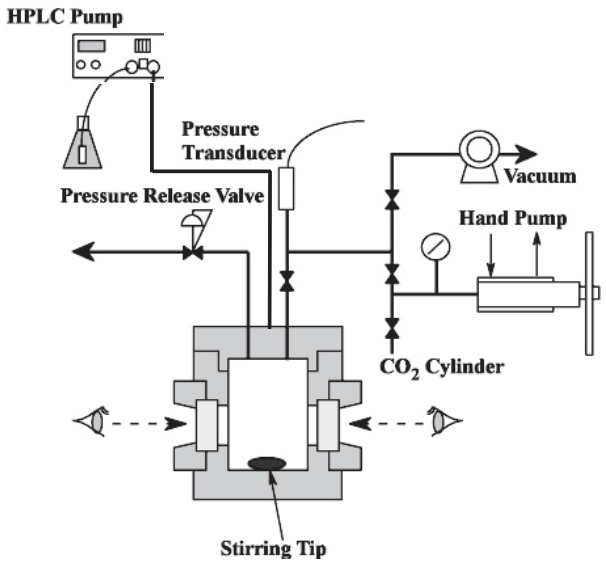

Fig. 2 Apparatus for preparation of liposome by SCRPE method

a temperature higher than the phase-transition temperature (41℃) of DPPC; the pressure was kept at 200 bar. After equilibrating for several tens of minutes, a BSA/PBS solution (3.0 mg/mL), used as the water-soluble model drug substance to be entrapped, was slowly (1.0 mL/min) introduced into the cell by an HPLC pump until the desired amount of solution was charged. The pressure was then reduced to release CO_2, thereby giving a homogeneous liposomal suspension. The interior of the pressure cell was stirred with a magnetic stirring tip during the experiments.

2.3 Liposome preparation by the Bangham method

The Bangham method was adopted as the conventional method used to compare the entrapping efficiency of liposomes prepared by the SCRPE method. DPPC, cholesterol, and LP were dissolved in a 1/1 chloroform/methanol solution in a test tube. The solvent was then removed by blowing nitrogen gas into the test tube, and the residual solvent was further dried overnight in vacuo at room temperature to give a thin lipid film on the wall of the test tube. BSA solution (3.0 mg/mL) was added to the lipid film, and the contents of the test tube were warmed to 40℃ for 10 min. The test tube was then shaken vigorously on a vortex mixer to yield multilamellar vesicles (MLV). The vesicles thus obtained were sonicated (CS-20, Shibata Scientific Technology Ltd., Saitama, Japan) for 5 min.

2.4 Incorporation of LP into liposomes

Liposomes (20 mM) modified with 5 mol% C_{16}-Arg1 were charged onto a gel-filtration column (Sepharose™ CL-4B, GL Science Inc., Tokyo, Japan). PBS was used as the mobile phase. Fractions of about 800 μL were collected and TritonX-100 was added to disintegrate the liposomes. The amounts of LP in the solutions were determined by the fluorescence of the tryptophan residue in the LP molecule by spectrofluorometry (RF-5300PC, Shimadzu Co., Kyoto, Japan).

2.5 Entrapping efficiency

The BSA-entrapping efficiency measurements were performed by placing the liposomal suspension on a gel-filtration column (mobile phase: PBS). Fractions of about 800 μL were collected and tested with a protein rapid assay kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan); the total volume of the fractions collected was 3 mL. The solutions were analyzed by the pyrogallol red method using a spectrophotometer (U-3310, Hitachi High-Technologies Co., Tokyo, Japan)

2.6 Measurement of retention efficiency

To determine the stability of the liposomes, the BSA-retention efficiency of the liposomes was measured. After preparation of the liposomes using the SCRPE method, a liposomal suspension was stored at 25℃ and then placed on a gel-filtration column in optional time. The liposomal fraction was added to a protein rapid assay kit and measured by a spectrophotometer (502 nm).

2.7 Transmission electron microscope observations of liposomes

A liposomal suspension was quickly frozen in liquid propane using a cryo-preparation apparatus (Leica EM CPC, Leica Co., Tokyo, Japan). The frozen sample was fractured in a freeze-replicating apparatus (FR-7000A, Hitachi High-Technologies Co., Tokyo, Japan) at −160℃. The fractured surface was replicated by evaporating platinum at an angle of 45°, followed by carbon evaporation at a normal incidence to strengthen the replica. The sample was then placed on a 400-mesh copper grid, after washing with acetone and water, and observed under a transmission electron microscope (TEM) (H-7650, Hitachi High-Technologies Co., Tokyo, Japan)

2.8 Measurement of particle size distribution of liposomes

The particle size distribution of the liposomes was measured by dynamic light scattering (DLS, Nicomp 380ZLS, Agilent Technologies, Tokyo, Japan) using an argon laser (532 nm).

2.9 Thermal analysis

Thermal analysis was conducted with a differential scanning calorimeter (DSC 8230, Rigaku Co., Tokyo, Japan). The scanning rate was 1℃/min, and pure water was used as a reference. A stainless steel pan was used as the sample vessel. The weights of the sample and reference were 10 mg.

2.10 Small angle X-ray scattering (SAXS) measurements

SAXS experiments were carried out using a SAXSess camera (Anton-Paar Co., Ltd., Graz, Austria) and a PW3830 X-ray generator (PANalytical Ltd., Almelo, Netherlands), operated at 40 kV and 50 mA. All samples were placed in a thin quartz capillary and set in a sample holder unit which controlled the temperature with an accuracy of 0.1℃ (TCS120, Anton-Paar Co., Ltd., Graz, Austria). An imaging plate was used to record the scattering data and the data were read by a cyclone storage phosphor system (Perkin-Elmer Co., Ltd., Waltham, MA, USA).

3 RESULTS AND DISCUSSION

3.1 Incorporation of LP into liposomes

Figure 3 shows the gel-filtration chromatogram of liposomes modified with LP (DPPC/C_{16}-Arg = 95/5 molar ratio), detected by the fluorescence chromophore of the tryptophan residue in the LP molecule; the liposomes were prepared by the SCRPE method. As shown in Fig. 3, most of the LP used for modification of the liposomes was incorporated into the liposomes; the incorporation ratio was ca. 90%. In a previous paper, another type of LP was incorporated in good yield (90%) into liposomes prepared by the Bangham method[12]. These results suggest that LPs are compatible with DPPC liposomes regardless of the preparation method.

3.2 Comparison of BSA-entrapping efficiencies

3.2.1 Modification of liposomes by cationic LPs

To enhance the BSA-entrapping efficiency, LPs with a strong cationic group on the Arg moiety were used as BSA binders. Table 1 shows the effects of incorporation of cationic LPs into liposomes prepared by the Bangham method. Liposomes modified with 5 mol% C_{16}-Arg1 significantly increased the entrapping efficiency, giving an efficiency six times that obtained with non-modified liposomes. Modification with 5 mol% C_{16}-Arg2 (peptide = Arg-Arg) showed a slight increase in BSA-entrapment efficiency because of the enhanced cationic properties. As the isoelectric point (pI) of BSA in water is 4.9, BSA has a negative charge under the entrapment conditions. BSA could therefore form a complex with the cationic LP, resulting in improved entrapment efficiency[16]. Incorporation of DSPE-PEG2000 into liposomes by the Bangham method might not show any incremental effect.

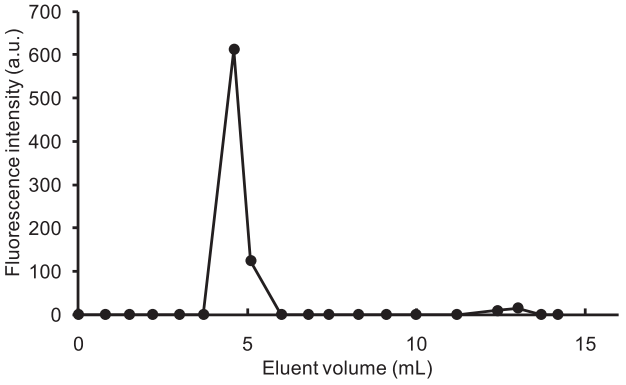

Fig. 3 Gel filtration chromatogram. (●) 20 mM liposome modified with 5 mol% C_{16}-Arg1 (DPPC/LP=95/5 molar ratio) prepared with SCRPE method, Tryptophan residue in LP was detected by fluorescence spectrometer (Ex:280 nm, Em:340 nm). Incorporation ratio was calculated to be weight conversion from standard curve of LP.

Table 1 Entrapping efficiency of BSA according to addition of LP and DSPE-PEG2000.

	Entrapment efficiency (%)	
	Bangham	SCRPE
Non-modified (DPPC/Chol=70/30)	5	20
C_{16}-Arg1 (DPPC/Chol/C_{16}-Arg1=65/30/5)	32	48
C_{16}-Arg2 (DPPC/Chol/C_{16}-Arg2=65/30/5)	37	68
C_{16}-Arg2/PEG (DPPC/Chol/C_{16}-Arg2/ DSPEPEG2000=60/30/5/5)	32	73

3.2.2 Effects of liposome preparation process

Incorporation of BSA into non-modified liposomes prepared by the SCRPE method was 20%, which was four times the incorporation achieved with the Bangham method (Table 1). Only slight increases in the entrapping efficiency were obtained by modification with cationic LPs of the SCRPE liposomes, although the entrapping efficiencies were much higher than those for liposomes prepared by the Bangham method for each type of LP incorporated. FF-TEM images of liposomes prepared by the SCRPE method show the formation of large unilamellar vesicles (LUV) (Fig. 4). In contrast, liposomes prepared by the Bangham method form multilamellar vesicles, as expected (no TEM photograph shown). This structural difference could be related to the higher entrapping efficiency of liposomes prepared by the SCRPE method[11]. The entrapping efficiency of liposomes modified with C_{16}-Arg1 was about 200% higher than that of non-modified liposomes prepared by the SCRPE method. Modification with C_{16}-Arg2 (peptide = Arg-Arg) showed a further increase in BSA-entrapment efficiency because of the enhanced cationic properties. Unlike the case for the Bangham method, addition of DSPE-PEG2000 to the liposomes slightly increased the entrapping efficien-

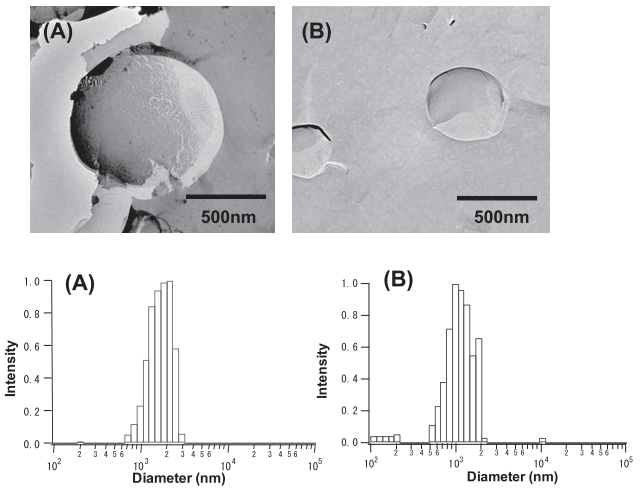

Fig.4 FF-TEM micrograph and size distributions by DLS of liposome.
(A); DPPC/Chol/C_{16}-Arg1=65/30/5 molar ratio (B); DPPC/Chol/C_{16}-Arg1/DSPE-PEG2000 =60/30/5/5 molar ratio Liposomes were prepared with SCRPE method.

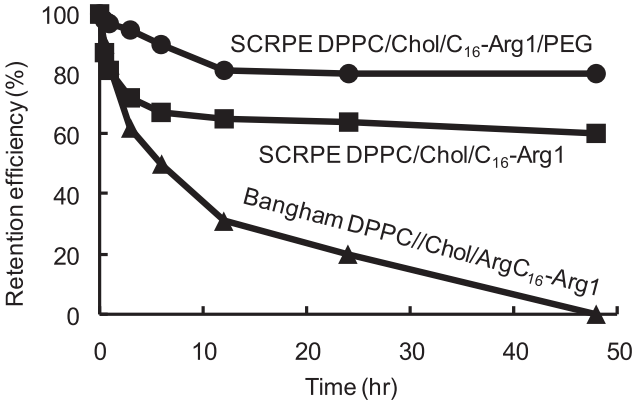

Fig. 5 Measurement of retention efficiency of BSA entrapped to the liposome at 25 °C.
(●) SCRPE, DPPC/Chol/C_{16}-Arg1/DSPE-PEG2000 =60/30/5/5 (in mol),
(■) SCRPE, DPPC/Chol/C_{16}-Arg1=65/30/5 (in mol), (▲) Bangham, DPPC/Chol/C_{16}-Arg1=65/30/5 (in mol). Lipid concentration of all liposomes is 5 mM.

cy, along with a reduction in particle size distribution, as shown in Fig. 4. The SCRPE method generates liposomes from a $scCO_2$/W emulsion[10, 11] by phase inversion under CO_2 purging. Thus the improvement in the entrapping efficiency and the reduction in particle size can be attributed to DSPE-PEG2000 behaving as a surfactant during the $scCO_2$/W emulsification[17, 18].

These results suggest that SCRPE is a useful method for liposome preparation because it produces organic-solvent-free liposomes in a single-step process. Furthermore, by using DSPE-PEG2000, the liposome particle size can be controlled.

3.3 Stabilization of LP liposomes

Liposome stability was evaluated by measuring the BSA-retention efficiency at 25℃. As shown in Fig. 5, liposomes prepared by the Bangham method and modified with C_{16}-Arg1 showed insufficient stability as complete release was found after 48 h. The SCRPE method improved the stability, and the entrapment efficiency remained constant at 60% up to 48 h. Incorporation of DSPE-PEG2000 dramatically improved the long-term stability, as shown in Fig. 5.

A combination of LP modification, the SCRPE process, and incorporation of a PEG-modified phospholipid with cholesterol brought us satisfactorily close to the goal of the study, with a BSA-entrapment efficiency of up to 70% and a release stability which was constant at 90% for over 40 h being achieved. These are promising indications that the approach used in this study could lead to the development of novel DDS for many applications. In order to achieve the final goal, we performed physicochemical analyses to determine the effects of LP modification on the structure and properties of the liposome membranes.

3.4 Physicochemical characterization of liposomes modified with LPs

The physicochemical membrane properties of liposomes modified with LPs were investigated by DSC and SAXS. Figure 6 shows the thermal analysis data for LP-modified liposomes prepared by the SCRPE method. In the case of DPPC-liposomes, there were hardly any differences be-

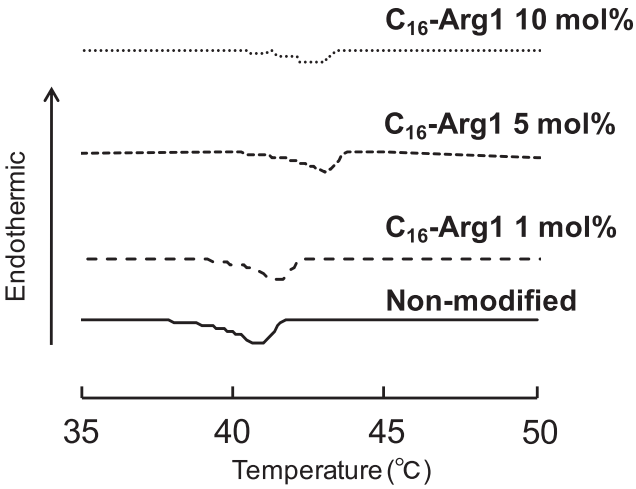

Fig. 6 Effect of modification ratio of LP into liposome in DSC heating curve; heating rate was 1°C/min and temperature range was from 25 to 60°C. Liposomes were prepared with SCRPE method.

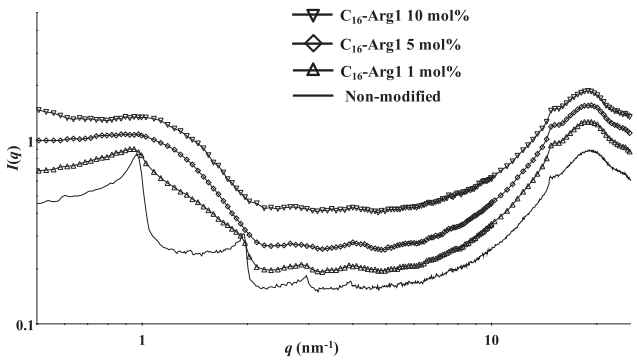

Fig. 7 Effect of the modification ratio of LP on nanostructure of liposome by SAXS. All data were measured at 25°C for 1h. Liposomes were prepared with Bangham method.

tween the DSC charts of those prepared by the SCRPE method and those prepared by the Bangham method[11]. In increasing the LP modification ratio, the peak indicating the phase-transition temperature shifted toward a higher temperature. The peak temperatures for liposomes modified with 5 mol% and 10 mol% C_{16}-Arg1 were about the same, but the latter showed some broadening. This result suggests that increasing the LP modification ratio perturbs the uniformity of the lamellar phase.

Figure 7 shows the SAXS measurement data for LP-modified liposomes prepared by the Bangham method. The absence of peaks at high q values indicates the molten state of the acyl chains[19]. At low q values, non-modified liposomes and those modified with 1 mol% C_{16}-Arg1 showed lamellar body patterns (multilamellar vesicles). As was the case for the DSC measurements, the scattering patterns for liposomes modified with 5 mol% and 10 mol% C_{16}-Arg1 were similar, with a broadening which indicated the formation of unilamellar vesicles. As liposomes prepared by the SCRPE method always forms unilamellar liposomes, as shown in Fig. 4, SAXS analysis of the structural changes were not conducted here. However, spontaneous formation of unilamellar vesicle structures under controlled conditions is a characteristic feature of the SCRPE method, and this could be the key to the development of efficient and stable LP-modified liposomes as desirable DDS carrier.

4 CONCLUSIONS

Improvements in the stability of liposomes and in their entrapment efficiency for a model drug molecule are achieved by modification with LPs and use of the SCRPE method as the preparation process. The ionic interactions of cationic LPs with arginine residues increased the BSA-entrapment efficiency to six times that of liposomes prepared by the Bangham method. Use of the SCRPE method, along with LP modification, further enhanced entrapment and enabled spontaneous formation of unilamellar liposomes with long-term stability. Liposomes consisting of DPPC/Chol/C_{16}-Arg2/DSPE-PEG2000 (60/30/5/5) showed a BSA-entrapment efficiency of up to 70% and stability at the 90% level for over 40 h. DSC and SAXS analyses indicated that certain amounts of LP in the DPPC induced phase-transitional and structural changes in the lamellar membranes; these changes improved the performance of the liposomes as DDS carriers.

The SCRPE method provides organic-solvent-free liposomes; the LPs for the modification of the liposome are derivatives of amino acids and fatty acids, which are sustainable and biocompatible materials. This study therefore suggests an opportunity for the development of novel DDS carriers with excellent performance and which address environmental concerns.

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