Supporting Information for:

Nanomorphology Tuning in Doxorubicin-Loaded Liposomes *via* Cooling-Induced Doxorubicin-Crystallization and Membrane Phase Transition

Koki Nishimura[†,1], *Taiki Fujimoto*[†], *Taisei Tokumoto*[†,1], *Kenjirou Higashi*[1,†,*], *Ryo Hidaka*[†], *Keisuke Ueda*[†], *Takeshi Morita*[§], *and Kunikazu Moribe* [†]

[†]Graduate School of Pharmaceutical Sciences, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8675, Japan

[§]Graduate School of Science, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan

[1] These authors contributed equally to this manuscript.

[*] Corresponding author. Tel.: +81-43-226-2866; Fax: +81-43-226-2867.

E-mail: ken-h@faculty.chiba-u.jp (K. Higashi).

Contents

Materials

Hydrogenated soybean phosphatidylcholine (HSPC), mainly composed of distearoylphosphatidylcholine (DSPC), was purchased from NOF Corporation (Tokyo, Japan). Cholesterol and doxorubicin hydrochloride (DOX-HCl) were obtained from Sigma-Aldrich Corporation (St. Louis, MO). The chemical structures of HSPC, cholesterol, and DOX-HCl are shown in Figure S1. Sepharose® 4 fast flow and Wako phospholipid C kit were purchased from GE Healthcare (Buckinghamshire, UK) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively. All other materials were of reagent grade.

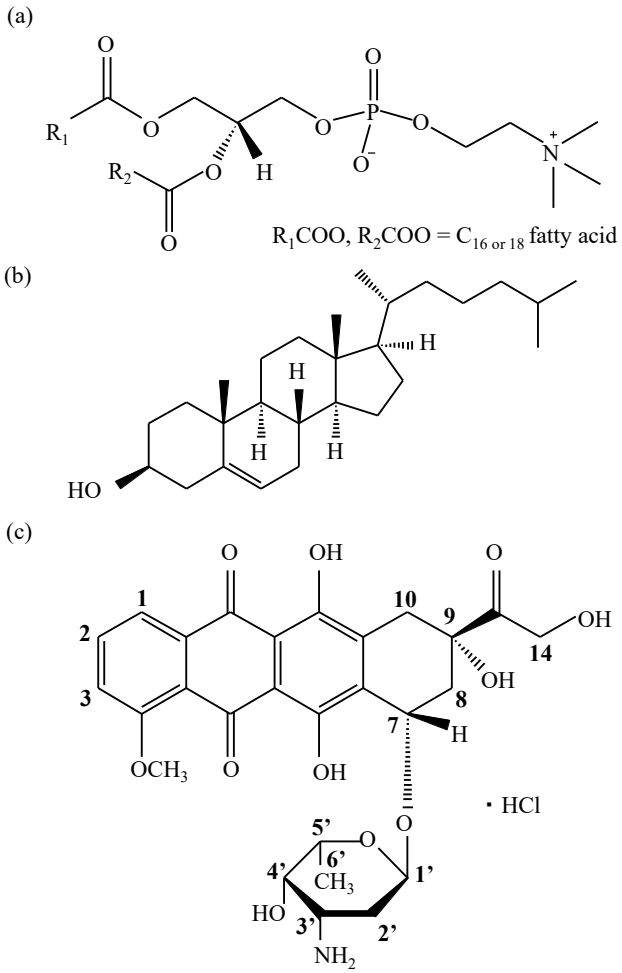
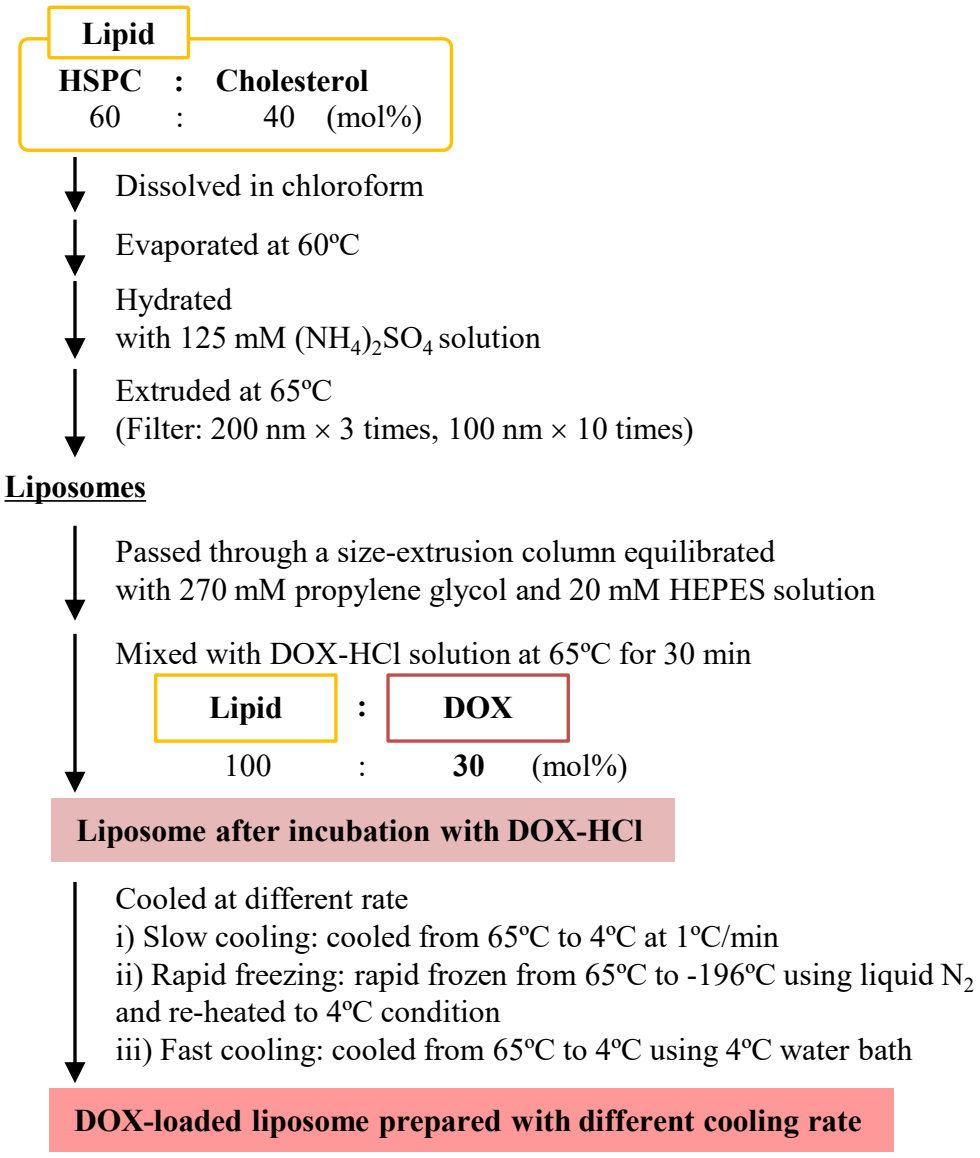


Figure S1. Chemical structures of (a) hydrogenated soybean phosphatidylcholine (HSPC), (b) cholesterol, and (c) doxorubicin hydrochloride (DOX-HCl). The number in (c) was used for the peak assignment in ^{1}H NMR.

Preparation of *approx.* 100 nm DOX-loaded liposomes by different cooling rates

Liposomes were prepared using the hydration and extrusion method. DOX was loaded into the inner aqueous phase by applying ammonium sulfate gradients across the lipid bilayer, known as the active-loading method (Scheme S1)[13]. HSPC and cholesterol were dissolved in chloroform at a molar ratio of 60:40. Chloroform was completely removed using a rotary evaporator and vacuum-dried to prepare a lipid-thin film. The lipid thin film was hydrated with 125 mM ammonium sulfate solution at 65°C. It was extruded through a 200 and 100 nm polycarbonate filter 3 and 10 times, respectively, at 65°C using an extruder (Northern Lipids Inc., Burnaby, Canada) to obtain a liposome suspension with a particle size of about 100 nm.

A size extrusion column (Sepharose® 4 fast flow) was used to replace the external medium of liposomes with isotonic 270 mM propylene glycol/20 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) solution (pH 6.8) and to establish the ammonium sulfate gradient. Total lipid concentration was quantified using a Wako phospholipid C kit. A certain amount of DOX-HCl was dissolved separately in 270 mM propylene glycol/20 mM HEPES (pH 6.8) to prepare a DOX-HCl solution. Then, the DOX-HCl solution was mixed with liposome suspension at a ratio of 30 mol% against the total lipid concentration for 30 min at 65°C to load DOX (as DOX-sulfate) in the inner aqueous phase. The liposome suspension after incubation was cooled to 4°C to prepare the DOX-loaded liposome. Three different cooling methods were used as follows: (i) slow cooling, cooling from 65°C to 4°C at 1°C/min using a water bath; (ii) fast cooling, cooling from 65°C to 4°C using 4°C water bath, (iii) rapid freezing: freezing from 65°C to -196°C using liquid N_2 and subsequent heating to 4°C.



Scheme S1. Preparation method of DOX-loaded liposomes prepared with different cooling rates.

The particle size distribution for DOX-loaded liposomes prepared with different cooling rates was evaluated by dynamic light scattering (DLS). Figure S2 shows the mean volume diameter (MV), polydispersity index (PDI), and particle size distribution. All the liposomes had an MV of approximately 100 nm with unimodal distribution with a PDI less than 0.15, regardless of the cooling method.

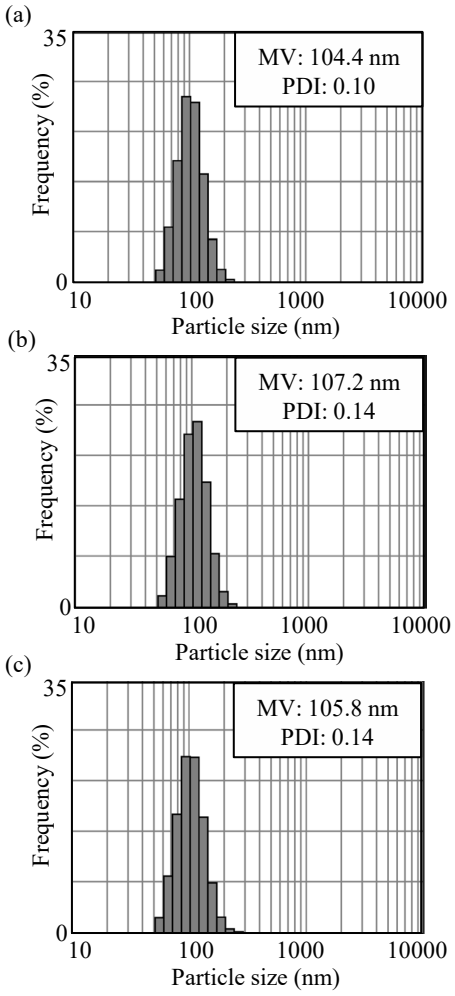


Figure S2. Particle size distributions of DOX-loaded liposome prepared with different cooling rates: (a) slow cooling, (b) rapid freezing, and (c) fast cooling. The figure shows the mean volume diameter (MV) and polydispersity index (PDI).

The encapsulation efficiency of DOX was quantified using the ultrafiltration method (Table S1). DOX-loaded liposome was firstly ultrafiltrated by high-speed micro centrifuge (Hitachi, Japan) at 21,500 × g for 30 min at 4°C with Amicon® centrifugal filter unit with MWCO 30 kDa (Merck KGaA, Darmstadt, Germany) to separate DOX-loaded liposome and free DOX. Then, a 10 μL aliquot of the separately filtrated free DOX and DOX-loaded liposome before ultrafiltration

was accurately transferred into each Eppendorf tube. A 10 μL of 0.1% Triton-X was added as an emulsifier of liposome into both solutions and heated at 65°C for 3 min. A 980 μL of 30% acetonitrile solution was added to both emulsified solutions. The total DOX and free DOX concentrations were quantified using ultra-fast liquid chromatography (UFLC). Finally, DOX encapsulation efficiency was calculated by comparing the concentration of total DOX (C_{total}) and filtrated free DOX ($C_{filtrated}$) using equation (1).

$$\text{Encapsulation efficiency (\%)} = 100\times(1-C_{filtrated}/C_{total}) \quad (1)$$

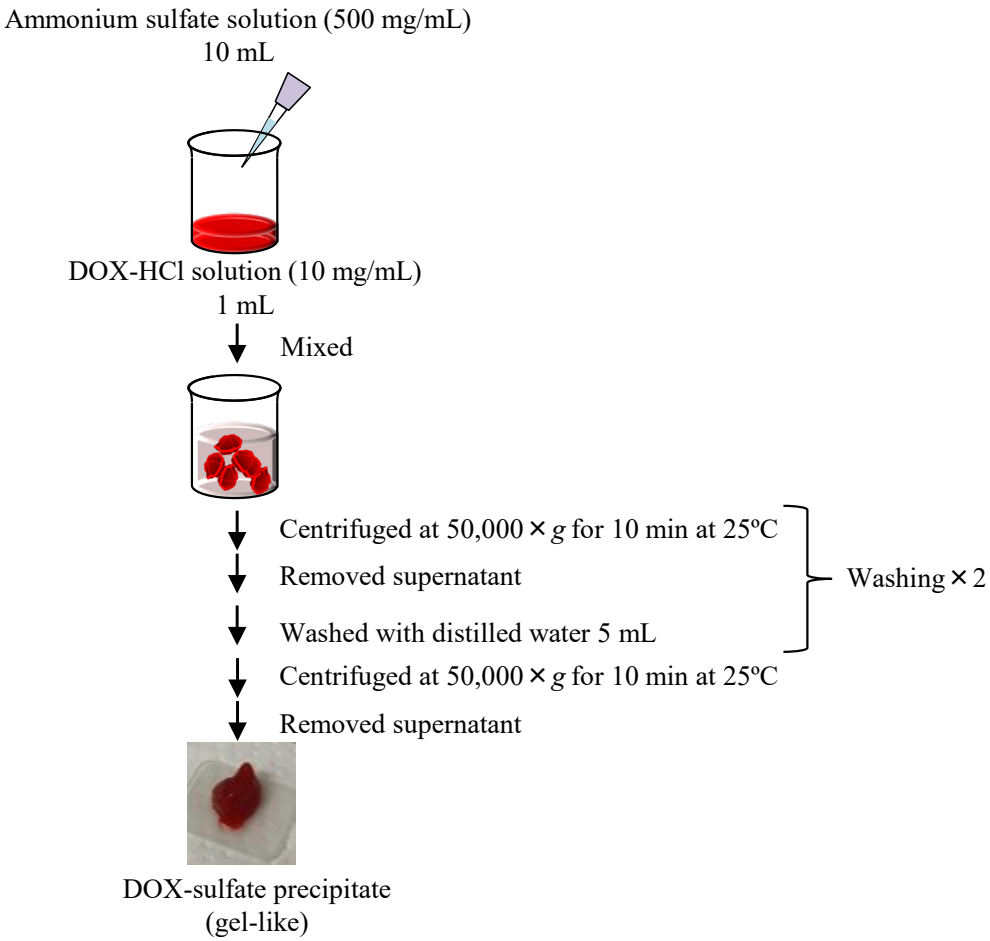
Table S1 shows the DOX encapsulation efficiency for DOX-loaded liposomes prepared with different cooling rates. These results confirmed that over 90% of the dose DOX amount (30 mol% against lipids) was loaded in each liposome independent from the cooling rate.

Table S1. The encapsulation efficiency of DOX-loaded liposomes prepared with different cooling rates (n = 3, mean ± S.D.).

Cooling procedure	Encapsulation efficiency (%)
Slow cooling	92.7 ± 0.3
Fast cooling	92.4 ± 1.0
Rapid freezing	90.7 ± 1.3

Preparation of DOX-sulfate precipitates in bulk water phase

DOX-sulfate precipitates suspended in bulk water were prepared without liposome (Schme S2). 1 mL of DOX-HCl solution (10 mg/mL) was mixed with 10 mL of ammonium sulfate solution (500 mg/mL). The DOX-sulfate precipitate was centrifuged at 50,000 × g for 10 min at 25°C. After removing the supernatant, the sample was washed with 5 mL of distilled water, and this washing cycle was repeated 2 times.



Scheme S2. Preparation method of DOX-sulfate precipitate in bulk water

Analytical methods for evaluating DOX-loaded liposomes

DLS

Dynamic light scattering (DLS) was conducted using a Microtrac 9340-UPA particle analyzer (MicrotracBEL Corp., Osaka, Japan) at 25 ºC. Liposome suspension was diluted 100 times with the same solution used for the liposomal outer phase.

UFLC

UFLC measurement was carried out using UFLC system (Shimadzu Corp., Kyoto, Japan, Column: L-column®2 ODS; 5 μm, 4.6×150 mm) with the following conditions; flow rate: 1 mL/min, column temperature: 40°C, mobile phase: 0.1% TFA in 30% acetonitrile, detector: SPD-M20A HPLC Photodiode Array Detector, analysis wavelength: 495 nm.

Cryo-TEM

Cryo-TEM images were obtained using a JEM-2100F microscope (JEOL Co., Ltd., Tokyo, Japan). A 200-mesh copper grid covered with a perforated polymer film (Nisshin RM Co. Ltd., Tokyo, Japan) was processed with hydrophilic treatment for 60 s using an HDT-400 device (JEOL Co., Ltd.). A 2 μL aliquot of each suspension, whose lipid concentration was adjusted to 1 mM using the outer aqueous phase (270 mM propylene glycol/20 mM HEPES), was applied to the hydrophilized grid. The grid was blotted with a filter paper for 3 seconds and immediately vitrified by plunging into liquid ethane cooled with liquid nitrogen using a Leica EM CPC cryofixation system (Leica Microsystems GmbH, Wetzar, Germany). Frozen samples were maintained at approximately -170°C using a Gatan 626 cryo-holder (Gatan, Inc., Pleasanton, CA). The cryo-TEM instrument was operated at 120 kV.

SAXS with synchrotron radiation

Synchrotron SAXS measurements were conducted at High Energy Accelerator Research Organization using Photon Factory BL-10C, Tsukuba, Japan. The measurement conditions were as follows: X-ray wavelength: 1.00 Å; exposure time: 240 sec; beam size: 0.5 × 0.5 mm^2; camera

distance: 2029 mm; detector: PILATUS 2M; temperature range: 25-65°C. *In situ* SAXS measurement was conducted to monitor the preparation processes of DOX-loaded liposomes, namely the incubation at 65°C for 30 min and the subsequent cooling process. Firstly, the DOX-HCl solution was mixed with the liposome suspension at a ratio of 30 mol% against the total lipid concentration, and the mixture was sealed in a capillary and then subjected to a SAXS measurement at 25°C (Figure 3, profile shown in yellow). After taking out the capillary, the temperature of the SAXS apparatus was raised to 65°C. The capillary was set again on the SAXS apparatus at 65°C, and the cycle of exposure to X-ray for 4 min and non-exposure for 1 min was repeated 6 times (Figure 3, profiles shown in red). Then, the SAXS apparatus was cooled from 65°C to 25°C in 5°C steps. The sample was stabilized for 3 min after the SAXS apparatus reached the target temperature and was exposed to X-ray for 4 min (Figure 3, profiles shown in blue).

^{1}H NMR

^{1}H NMR measurements were performed using a JNM-ECZ600R (JEOL Resonance Co., Ltd., Tokyo, Japan). Ethylene glycol and D_2O were used instead of propylene glycol and H_2O to prevent overlapping NMR peaks. DOX-loaded liposome (DOX amount: 30% against the total lipid concentration, outer aqueous phase: 270 mM ethylene glycol/20 mM HEPES) prepared with the slow cooling procedure was used as an NMR sample. Trimethylsilyl propanoic acid (TSP; 0.0 ppm) was used as standard. The measurement conditions were as follows: temperature range: 30-80°C; relaxation delay: 3 sec; scan: 64 times. *In situ* ^{1}H NMR measurement for 30 mol% DOX-loaded liposome prepared with slow cooling was conducted with heating from 30 ºC to 80º C and cooling from 80 ºC to 30 ºC.

DSC

DSC measurements were conducted using DSC-7000X (Hitachi High-Tech Science Corporation, Tokyo, Japan) under the N_2 atmosphere. DOX-sulfate was enclosed in aluminum pan processed chromate conversion coating known as ALODINED SEALED PAN (Hitachi High-Tech Science Corporation, Tokyo, Japan) to prevent from evaporation of water contained in DOX-

sulfate. The measurement conditions were as follows: N_2 flow rate: 50 mL/min; temperature changes from 25°C to 90°C at the heating rate: 10°C/min and then cooled from 90°C to 25°C at the cooling rate of 5, 10, 20, and 30ºC/min.

Supporting results

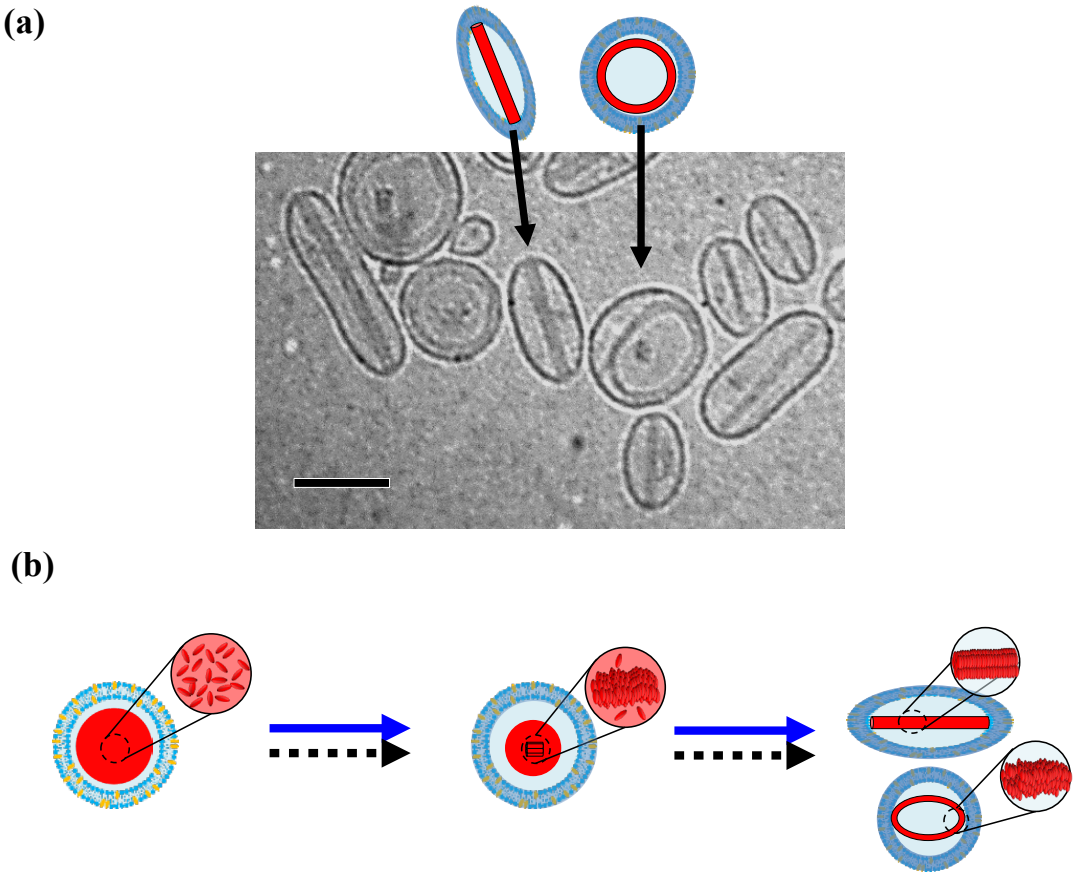


Figure S3. (a) Cryo-TEM image of DOX-loaded liposome prepared with fast cooling (cooled from 65°C to 4°C by water bath at 4 °C). The black bar represents 100 nm. **(b)** A proposed mechanism of morphological change during fast cooling. The solid blue arrow shows the fiber-bundle formation and elongation *via* DOX-sulfate crystallization, while the dotted black arrow indicates stiffness increase by phase transition of the lipid membrane.

The formation of DOX-sulfate fiber bundle and the stiffness increase of liposomal membrane could occur competitively. As a result, spherical liposomes containing a curved fiber-bundle and prolate liposomes containing a linear fiber-bundle are formed.

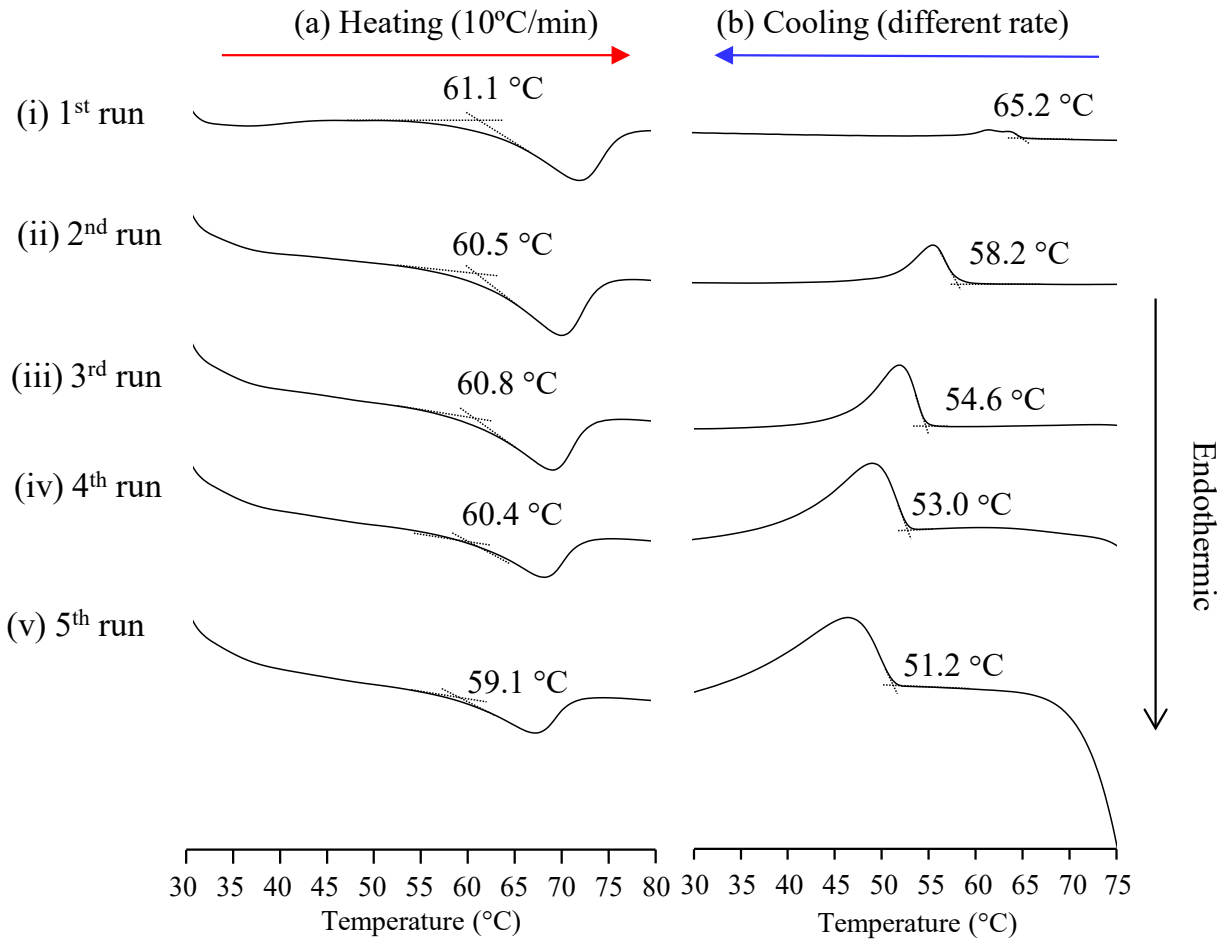


Figure S4. Differential scanning calorimetry (DSC) curves of DOX-sulfate precipitates prepared in the bulk water phase. The heating and cooling cycle was repeated 5 times with (a) heating process from 25 ºC to 90 ºC at the constant rate of 10 °C/min and (b) subsequent cooling process from 90 ºC to 25 ºC at the rate of (i) 1ºC/min, (ii) 5ºC/min, (iii) 10ºC/min, (iv) 20ºC/min, and (v) 30ºC/min.

During heating, the onset peak of endothermic melting in DOX-sulfate was approximately 60ºC across all five cycles, and the offset peak was below 80 °C. In the cooling process at different rates, the onset and offset exothermic peak of DOX-sulfate recrystallization shifted to the lower temperature as the cooling rate increased. This result indicates that the crystallization of DOX-sulfate was dependent on the cooling rate, with the time scale ranging from minutes or even slower. For example, when the cooling rate was high at 30 ºC/min, DOX-sulfate did not crystallize until 51.2 °C (below the transition temperature of HPSC at 53.6 ºC) and remained in a supercooled-liquid state until 30 °C.

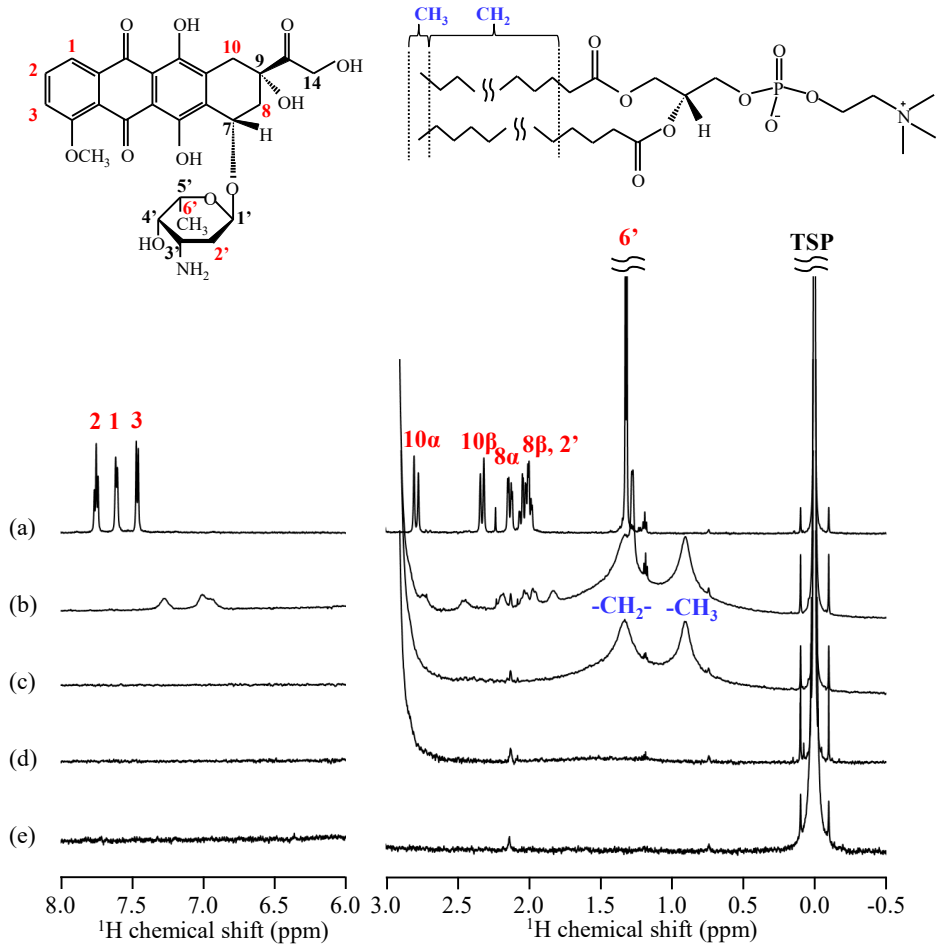


Figure S5. ^{1}H NMR spectra at 70ºC of (a) DOX-HCl solution, (b) DOX-loaded liposome, (c) liposome, (d) outer phase (270 mM ethylene glycol, 20 mM HEPES), and (e) ammonium sulfate.

The peaks of DOX in the liposomal inner phase appeared at a higher magnetic field than those in DOX-HCl solutions. In particular, the chemical shift of aromatic peaks in the inner phase was approximately 0.5 ppm lower than in the DOX-HCl solution. It has been reported that DOX molecules can aggregate in solution through π-π stacking interactions. The much higher concentration of DOX in the liposomal inner phase compared to bulk water could enhance π-π stacking, resulting in a shift to a lower magnetic field. Other factors may also affect the chemical shift, such as the difference in DOX salt composition (DOX-sulfate in the inner phase and DOX-HCl in the outer phase) and the interaction of DOX molecules with the inner leaflet of the lipid membrane. The peak shapes of DOX in the liposomal inner phase were broader than those of DOX-HCl solutions, probably due to lower molecular mobility. The diffusion of DOX molecules confined within limited spatial spaces was lower, and their mobility would be suppressed by intermolecular interactions with surrounding DOX molecules in the DOX-concentrated situation. Other factors, such as the chemical exchange between monomeric DOX molecules and DOX aggregates, can also influence the peak shapes of DOX.