Reconsideration of Drug Release from Temperature-Sensitive Liposomes

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The liposomal phase transition temperature was monitored in unstirred suspensions using a differential scanning calorimeter. The main and pre-transition temperatures under conditions of stirring were measured by the change in 90° light scattering using a fluorescence spectrophotometer. Both methods show the same main transition temperature either with or without stirring. Temperature sensitive liposomes were made of DPPC (dipalmitoylphosphatidylcholine), DMPC (dimyristoylphosphatidylcholine) or DSPC (distearoylphosphatidylcholine). The calcein release profile from the liposomes depends on the stirring time of the liposome suspension at the main transition temperature. For 1 h incubation, the leakage profile with and without stirring is similar. It had been hypothesized that temperature sensitive liposomes released drug at the main-transition temperature. However, calcein leakage from liposomes is observed also at the pre-transition temperature. Thus, a liposomal encapsulated drug will likely leak from DPPC liposomes at body temperature (37°C), even if the liposomes were designed to have a higher main transition temperature.

Key words liposome; pre-transition; phase transition; drug release; temperature-sensitive

Liposomes have been studied extensively as a drug delivery system and for the introduction of DNA into cells.1,2 There are also many reports of applications of liposomes in the fields of immunology, including induction of cell immunity,3,4 reinforcement of immunity,5 vaccine development6 and at light stimulated drug release.5

Yatvin et al.7 and Weinstein et al.8 reported a method for the medical application of liposomes which allows for the control of drug release from liposomes according to the physico-chemical nature of the phospholipid membrane. The barrier efficiency of the membrane abruptly decreases near the phase transition temperature.9 Using vesicles formed by the reverse phase evaporation method, Szoka et al.10 showed it is possible to make liposomes with a high drug encapsulation efficiency.

Liposomes cannot exist long in the bloodstream, because the reticuloendothelial system eliminates liposomes with a large particle diameter. Therefore, the target of liposome development was the following: liposomes with a small particle diameter11; liposomes with a high drug encapsulation efficiency12; and liposomes which are not taken up by the reticuloendothelial system requiring, for example, a modification of the membrane surface.13 Many investigators14 have studied the temperature-dependent change of membrane structure in lipid bilayers. According to Ruocco et al.,15 the structure of the lipid bilayer makes a change at both the pre-transition and the main-transition temperatures. We have studied the biopharmaceutical characteristics of temperature sensitive liposomes16–18 and have reported the characteristics of calcein release from these liposomes.19 We have measured both the main-transition and pre-transition temperatures from changes in the 90° light scattering at 400 nm using a fluorescence spectrophotometer. Using this method, the transition temperature under conditions of stirring could be measured. The results indicate that the drug release characteristics of temperature sensitive liposomes should be reconsidered.

MATERIALS AND METHODS

Materials 1,2-Diacyl-3-sn-phosphatidylcholines [dipalmitoylphosphatidylcholine (DPPC), dimyristoylphosphatidylcholine (DMPC), distearoylphosphatidylcholine (DSPC)] were purchased from Nippon Oil and Fats Co., Ltd., Tokyo (no less than 98% purity). All other reagents were of reagent grade from Wako, Osaka or Nakalai Tesque, Kyoto.

Liposome Preparation Liposomes, in a reverse-phase evaporation vesicle (REV), were prepared according to the methods of Szoka et al.,10 and calcein at 100 mM was encapsulated. DPPC (29.4 mg) was added to a 50 ml round-bottom flask, and 12 ml of organic solvent (chloroform: isopropyl ether=1:1) was added to the flask. Two milliliters water or calcein solution (100 mM) was added to the organic solvent. The resulting two-phase system was sonicated briefly (5 min) in a bath-type sonicator (Sonorex Super RK156BH, Bandelin electronic) until the mixture became a clear one-phase dispersion that did not separate for at least 30 min after sonication. The mixture was then placed on a rotary evaporator and the organic solvent was removed under reduced pressure (with water aspirator) at 42°C rotating at approximately 200 rpm. As the solvent was removed, the mixture first formed a viscous gel and subsequently became an aqueous suspension. The aqueous suspension was evaporated for an additional 30 min at 47°C to remove traces of the organic solvent. Calcein, which was not encapsulated, was separated by centrifugation as follows: the REV suspension was centrifuged at 30000×g at 4°C for 20 min. The precipitate was resuspended in water or TES buffer (pH 7.0, it was same osmolarity as 100 mM calcein solution) after being washed with a cold buffer 3 times. Liposomal inner and outer osmolarity were same. The liposome size was measured by quasi-elastic light scattering using a laser particle analyzing system (Otsuka Electronics Co., LPA 3000/3100). The mean diameter of the liposomes was approximately 1000 nm.

Turbidity Measurement Phase transitions of the liposome dispersions were detected by the change of the 90° light scattering intensity which was recorded with an X–Y...
recorder (WX4421 WATANABE) at 400 nm using an fluorophotometer (RF-5000 SHIMADZU). The liposome dispersions were heated from 20 to 60 °C in approximately 20 min by circulating water through the sample compartment. Temperature was measured directly in the sample using a thermocouple microprobe connected to a Digital Thermometer (TAKARA THERMISTOR).

**Calcein Release Measurement** The temperature dependence of calcein release from liposomes was determined under continuous temperature scanning. The extra-liposomal calcein concentration was evaluated by fluorescence self-quenching according to thermostatic bath. The fluorescence intensity of calcein at 520 nm (excitation at 490 nm) was measured using a fluorophotometer (RF-5000 SHIMADZU). Experiments both with and without stirring were performed using aliquots of the same stock of liposome suspension. Calcein release measurement was started by adding calcein-containing temperature-sensitive liposomes (10 μl) into the fluorophotometer’s chamber, and this point was zero time. For calcein release measurement from liposomes under stirring conditions, the sample was continuously stirred for 5 min (or 1 h), and the scattering intensity was measured. For calcein release measurement from liposomes without stirring, the samples were kept at the indicated temperature for 5 min (or 1 h), and the scattering intensity was measured. After incubation, 10 μl of TritonX-100 (10%) was added to the sample to disrupt the liposomes. The total scattering intensity was defined as $f_{\text{totalT}}$. The influence of extra-liposomal calcein, which was not removed by the ultracentrifugation, was taken into account. Scattering intensity after 10 s was defined as $f_{\text{ext}}$ with negligible calcein release from the liposomes at room temperature. $f_{\text{totalT}}$ at this temperature was equal to $f_{\text{totalrt}}$. Calcein release efficiency from the liposome was calculated according to the following equations:

$$\text{calcein release (\\%)} = \frac{f_{\text{t}} - f_{\text{ext}}}{f_{\text{totalT}} - f_{\text{ext}}} \times 100$$  

$$f_{\text{t}} = f_{\text{total}} \times \frac{f_{\text{ext}}}{f_{\text{total}}}$$

**RESULTS**

**Liposomal Phase Transition Profile under Stirring**

![Fig. 1a. Dependence of DSC (Upper) and Light Scattering Intensity (Lower) on Temperature Using DPPC Liposomes](image1)

![Fig. 1b. Dependence of DSC (Upper) and Light Scattering Intensity (Lower) on Temperature Using DSPC Liposomes](image2)

![Fig. 1c. Dependence of DSC (Upper) and Light Scattering Intensity (Lower) on Temperature Using DMPC Liposomes](image3)
Conditions  In the 90° light scattering measurement at 400 nm, liposome membranes with DPPC or DSPC showed a gradual decrease of scattering intensity near the pre-transition temperature and a sharp increase near the main-transition temperature (Figs. 1a, b). The decrease of scattering intensity near the pre-transition temperature was not observed using the DMPC liposomal membranes (Fig. 1c). Figure 2 illustrates the measurement of the pre- and main-phase transition temperature using the 90° light scattering. The three parallel lines A, B and C and the two auxiliary lines D and E are drawn. The points of intersection of lines A, B, C and D, E decided points a, b, c and d. The pre-transition temperature and the main-transition temperature are calculated as the midpoint of lines a, b and c, d respectively. Table 1 shows that the same phase transition temperature was observed with the 90° light scattering method under conditions of both slow and rapid stirring. The phase transition temperatures which were observed by differential scanning calorimetry and by the 90° light scattering measurement under conditions of stirring were compared (Table 2). The main and pre-transition temperatures measured by the two methods agreed closely with each other. Using the DMPC bilayer, a pre-transition temperature was not observed using either method. It was confirmed that a change in stirring speed did not alter the phase transition temperature.

Calcein Release from Liposomes  The calcein release profiles at each temperature from liposomes with or without

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<tr>
<th>Table 1. Comparison of the Phase Transition Temperature with Stirring at Maximum and Minimum Speeds Using DPPC Liposomes</th>
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<td>Stirrer speed</td>
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<td>Average±S.D. (°C)</td>
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<td>Data represent the mean±S.D. of 8 experiments.</td>
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<th>Table 2. Comparison of the Phase Transition Temperatures Using DSC and 90° Light Scattering with Stirring on DPPC, DSPC and DMPC Liposomes</th>
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<td>Differential scanning calorimetry</td>
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<tr>
<td>Pre-transition temp. (°C)</td>
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<td>DPPC REV</td>
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<td>DSPC REV</td>
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<td>DMPC REV</td>
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<td>Fig. 3a. Temperature Dependence of Calcein Release from DPPC REV for 5 min; □ with Stirring, ● without Stirring</td>
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<td>Values represent the mean±S.D. of 3 experiments.</td>
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<td>Fig. 3b. Temperature Dependence of Calcein Release from DPPC REV for 1 h; □ with Stirring, ● without Stirring</td>
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<td>Values represent the mean±S.D. of 3 experiments.</td>
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<td>Fig. 3c. Temperature Dependence of Calcein Release from DPPC REV for 8 h; □ with Stirring, ● without Stirring</td>
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stirring are shown in Figs. 3a—c. As seen in Fig. 3a, calcein leakage from liposomes was observed near the pre-transition temperature, with the maximum calcein release observed at the main-transition temperature with stirring. As seen in Fig. 3b, the calcein release efficiency was 85% at 37 °C with or without stirring. Figure 4 shows the time dependence of light scattering intensity for DPPC liposomes after 8 h with stirring. Calcein was released from liposomes continuously at 34.6 °C, the pre-transition temperature. On the other hand, calcein was released rapidly over the first 1 h when the temperature was above the pre-transition point.

DISCUSSION

In this study, we observed the liposomal main- and pre-transition temperatures by measuring 90° light scattering at 400 nm. In DMPC liposomes, the decrease of light scattering intensity was not observed near the pre-transition temperature. The pre-transition was also not observed using differential scanning calorimetry. Using MLVs (multilamellar vesicles) made of DMPC, an endothermic shift was observed at the pre-transition temperature by differential scanning calorimetry. However, in the 90° light scattering measurement at 400 nm, no decrease of intensity at the pre-transition was observed. With differential scanning calorimetry, the absorption energy of multiple membranes is higher than for single membranes. Therefore, a pre-transition endotherm was observed by differential scanning calorimetry for MLVs made of DMPC. On the other hand, these MLVs did not show a pre-transition intensity shift indicating a membrane surface change by 90° light. For REV liposomes made with DPPC and DSPC, the pre- and main-transition intensity shifts were observed at each experiment.

The change of the membrane surface structure at the pre-transition temperature was gradual (Fig. 1a). Ruocco et al. reported that the structural change of the membrane began at about 30 °C and caused a rippled structure of the DPPC liposomal bilayer. The structure was changed again to the original liquid-crystalline phase at the main-transition temperature. This change appeared as an increase in intensity of light scattering. The structural change of the membrane has an influence on the barrier properties of the bilayer. Drug release is observed at about 30 °C from DPPC liposomes as shown in Fig. 3a. This release temperature was the same as the temperature found by light scattering in Fig. 1a. Thus, with stirring, it was found that calcein release from liposomes began just in the transition point of the membrane surface structure.

We thought that the energy associated with stirring might change the transition temperature of the bilayer. However, the measured temperatures of phase transitions by both differential scanning calorimetry and 90° light scattering at 400 nm with stirring were the same (Table 2), suggesting that stirring did not affect the transition temperature. Moreover, calcein release occurred near the pre-transition temperature after 1 h incubation (Fig. 3b). The change in membrane structure to a rippled structure appeared to lower the barrier efficiency of the liposomal membrane. The calcein concentration around the liposomes decreased with stirring, thus promoting calcein release. On the other hand, the calcein concentration near the outer membrane must have been kept higher without stirring. The calcein concentration gradient across the liposomal membrane must therefore have been diminished. It was thought that the barrier efficiency controlled calcein release from the liposomes. For incubation times equal to or greater than an hour, the calcein concentration around the liposome presumably had sufficient time to equilibrate with the surrounding medium even without stirring. Although there was a difference in calcein release from liposomes with or without stirring in the 8 h incubation experiments, this difference was not observed at 1 h. Moreover, at the pre-transition temperature, a higher release was found with stirring at 8 h than at 1 h as shown in Figs. 3b and c. The time dependence of the light scattering intensity with stirring of DPPC liposomes is shown in Fig. 4. Calcein release occurred continuously over 8 h at 34.6 °C. However, the light scattering intensity did not increase over 8 h at 31.6 and 33.6 °C. As the pre-transition temperature was about 34 °C, the temperature which can suppress release must be below the pre-transition temperature. For example, if the temperature sensitive liposome suspension was injected in systemic circulation, the liposomal encapsulated drug will leak out at body temperature (37 °C). In this work, calcein was used as a model drug for characterizing the difference of liposomal membrane permeability at different temperatures and different conditions of stirring.

Our results run counter to the idea that drug release from temperature-sensitive liposomes occurs only at the main transition temperature. It was concluded that drug encapsulated temperature-sensitive liposomes begin to release drug at the pre-transition temperature and not at the main-transition temperature. These results should help in the development of clinical applications for liposomes in the future.

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REFERENCES