Biochimica et Biophysica Acta, 978 (1989) 185–190
Elsevier

BBA 74283

Temperature-sensitive release of adriamycin, an amphiphilic antitumor agent, from dipalmitoylphosphatidylcholine-cholesterol liposomes

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(Received 28 July 1988)

Key words: Targeting chemotherapy; Antitumor agent; Adriamycin; Liposome stability; Cholesterol; Dipalmitoylphosphatidylcholine

Drugs for temperature-sensitive liposomes have been limited to the hydrophilic drugs, such as methotrexate and *cis*-dichlorodiammineplatinum, with a low affinity for the lipid bilayer. It was, however, of importance to investigate whether the concept of temperature-sensitive liposomes can be extended to amphiphilic or lipophilic compounds, because some useful drugs are amphiphilic or lipophilic. In this study we tried to use adriamycin, an amphiphilic antitumor agent, as a drug for temperature-sensitive liposomes. In the absence of serum, the liposomes prepared from dipalmitoylphosphatidylcholine released adriamycin in a temperature-sensitive manner, i.e., they retained the major portion of entrapped adriamycin at a lower temperature 32°C, and released around 70% of the drug at 42°C, a temperature higher than the phase-transition temperature of the phospholipid. However, when serum was present, the liposomes were leaky even at 32°C. To raise the stability of the liposomes, we included various mol% of cholesterol in the liposomal membrane and examined temperature sensitivity and stability of the liposomes in the presence of serum. Our results indicated that the liposomes including 20 mol% cholesterol were considerably stable and exhibited the maximal temperature-sensitive release of adriamycin in the presence of serum.

Introduction

Liposomes are considered to be potentially useful drug carriers. They are nontoxic, biodegradable and capable of entrapping various compounds. However, the potential use of liposomes for chemotherapy is limited by several factors, including lack of the target specificity of liposomes. To circumvent this disadvantage, a number of studies have been performed by modifying the surface of liposomes with biological-recognition molecules, such as antibodies, lectins and polysaccharides [1–8].

Yatvin and his co-workers have used a unique alternative approach for controlled release of drugs by using temperature-sensitive liposomes in conjunction with local hyperthermia [9–12]. They encapsulated methotrexate and *cis*-dichlorodiammineplatinum into either small unilamellar vesicles composed of DPPC and DSPC or reverse-phase evaporation vesicles composed of DPPC and dipalmitoylphosphatidylglycerol. In in vitro experiments, they observed temperature-sensitive release of the drugs upon brief exposure to heating at 42°C, a few degrees higher than the physiological temperature. In mouse model experiments with mouse leukemia L1210 or Sarcoma 180, they showed that the drugs were accumulated 4-fold more efficiently in the tumor heated at 42°C than in the unheated contralateral control tumors in the same animals. They also observed that growth of L1210 or Sarcoma 180 tumor was significantly delayed by the treatment of the animals with the temperature-sensitive liposomes in conjunction with the local hyperthermia.

So far as the drugs for temperature-sensitive liposomes are concerned, only hydrophilic agents such as methotrexate and *cis*-dichlorodiammineplatinum have been used by Yatvin and his co-workers [9–11]. The

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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principle of temperature-sensitive drug release may, however, be extended to amphiphilic and lipophilic agents, although such systems have not yet been developed. Adriamycin, an anthracycline aminoglycoside, is a potent antitumor chemotherapy agent with a broad spectrum of antitumor activity. It is an amphiphilic drug and may act on cell membranes besides intercalating into DNA molecules [13]. Goldman et al. [14] reported that various derivatives of adriamycin, such as daunomycin, *N*-trifluoroacetyladriamycin-14-valerate, adriamycin-14-octanoate and adriamycin-14-acetate, profoundly affected the thermotrophic behavior of DPPC liposomes. In the case of adriamycin, however, only slight changes were observed in the temperature of gel-liquid crystalline phase transition (T_m) and in the enthalpy of melting. Therefore, adriamycin is a possible candidate for the drugs which can be encapsulated into temperature-sensitive liposomes. In this study we attempted to prepare adriamycin-entrapped, temperature-sensitive liposomes which are stable in the presence of serum.

Materials and Methods

Chemicals

Dipalmitoyl-L-α-phosphatidylcholine was purchased from Nippon Fine Chemicals (Osaka). Di-*O*-hexadecyl-DL-α-phosphatidylcholine and cholesterol were from Sigma (St. Louis, MO). The purity of the lipids was checked by thin-layer chromatography on silica gel plates (Merck, Darmstadt), and all of them exhibited single spots on the plates. Adriamycin was purchased from Kyowa Hakkou (Tokyo), and it was dissolved in distilled water at 20 mg/ml before use. Inosine was from Sigma and was dissolved in distilled water. Fetal bovine serum was from JR Scientific (Woodland). Mouse serum was obtained from C3H/He mice (Institute of Medical Science, University of Tokyo).

Preparation of adriamycin-entrapped liposomes

This was performed essentially as described previously [15]. 20 μmol of DPPC or DPPC plus cholesterol in chloroform were evaporated under reduced pressure to form a lipid film on the wall of a 10 ml conical-bottomed flask. After drying for 30 min under reduced pressure, the lipid film was dispersed by vortexing at 45–50°C in 0.25 ml of the aqueous solution of adriamycin (20 mg/ml). The lipid dispersion formed was submerged in a 45–50°C water-bath, and sonicated with a probe-type sonicator Bio Ultra Sonic 150 (Chiyoda Riken, Tokyo) at 100 mA for five 1-min intervals, with cooling periods in between. The sonicated lipid dispersion was passed through a column of Sephadex G-50 (1 × 30 cm) equilibrated with 10 mM Hepes buffer (pH 7.4) containing 0.85% NaCl. The column was equipped with a water jacket to maintain the temperature at 32°C by circulating water. The liposome fractions, which were eluted at the void volume of the column, were collected and then used within a few hours.

The amount of adriamycin entrapped in the liposomes was assayed as described below. The mean value obtained from three to five independent experiments was 21, 18, or 30 mmol of adriamycin entrapped per mol of lipid for liposomes containing 0, 20 or 50 mol% cholesterol, respectively. The size of the adriamycin-entrapped liposomes was measured by the dynamic light scattering method with Coulter Model N4 (Coulter Electronics, Hialeah, FL). Mean diameters of all liposome preparations were within the range 90–110 nm (data not shown).

Measurement of adriamycin release from liposomes

150 μl of the adriamycin-entrapped liposomes and 150 μl of fetal bovine serum or 10 mM Hepes buffer (pH 7.4) containing 0.85% NaCl were mixed and incubated at the indicated temperature for 30 min. After the incubation period, 200 μl of the mixture were withdrawn and applied to a Sephadex G-50 column (1 × 10 cm), which was equipped with water jacket to keep the column at a given temperature. The column was eluted with 10 mM Hepes (pH 7.4)/0.85% NaCl, and 1 ml fractions were collected. 200 μl of each fraction were withdrawn and mixed with 1800 μl of methanol to disintegrate the vesicle structure. Fluorescence intensity of the mixture was measured with a Hitachi 650-40 fluorescence spectrophotometer (Hitachi, Tokyo) at an excitation of 490 nm and emission of 590 nm.

Assay of temperature-sensitive release of inosine from DPPC liposomes

Inosine-loaded DPPC liposomes were prepared as described above but using 0.1 M inosine. 300 μl of the liposome suspension were incubated at 42°C for a given period and then applied to a Sephadex G-50 column (1 × 10 cm), which was maintained at 42°C. The column was eluted with 10 mM Hepes (pH 7.4)/0.85% NaCl, and 1 ml fractions were collected. After receiving 50 μl of chloroform, each fraction was vigorously mixed to disintegrate the membrane structure and then centrifuged at 3000 r.p.m. for 15 min. Absorbance of the water layer obtained was measured at 260 nm.

Differential scanning calorimetry

Differential scanning calorimetry of various liposomes was performed by a DSC20 (Seiko E & I, Tokyo) equipped with a SSC/580II Thermal Controller (Seiko E & I, Tokyo). 10 μl of liposome suspensions which contained 0.14 μmol of DPPC were analyzed by using a heating rate of 3 C° per min and water as reference.

Determination of phosphorus and cholesterol

Total phosphorus was determined by the method of Gerlach and Deuticke [16]. Cholesterol was determined enzymatically using the assay kit of Nissui Pharmaceutical (Tokyo).

Results

Temperature-sensitive release of entrapped adriamycin from DPPC liposomes

Since the thermotrophic behavior of DPPC liposomes is not drastically altered by the incorporation of adriamycin [14], we presumed that efflux rate of adriamycin from the liposomes may be markedly increased around the T_m of DPPC, 41°C.

It was reported that smaller liposomes are more slowly taken out of circulation by the reticuloendothelial system than larger liposomes [17,18]. We therefore prepared sonicated small liposomes with mean diameters of approx. 100 nm and examined temperature-sensitive release of adriamycin from the liposomes. Efflux of adriamycin was estimated fluorophotometrically after separation of the released drug from the liposome fractions by Sephadex G-50 column chromatography. Fig. 1A illustrates a representative elution profile of released and liposome-associated adriamycin. The results indicate that the major portion (more than 80%) of adriamycin was retained in the DPPC liposomes when the liposomes were incubated at 32°C for

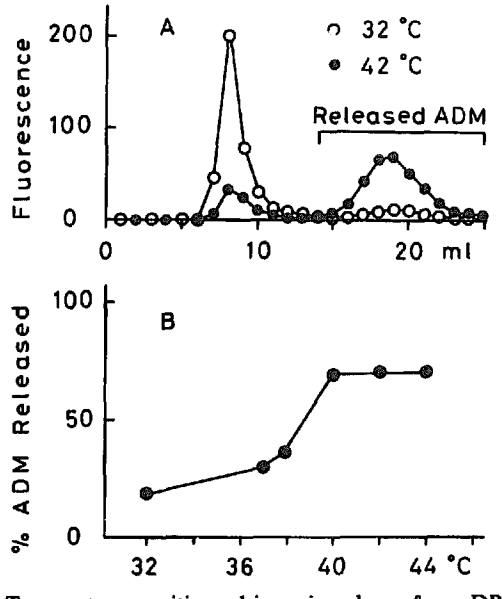

Fig. 1. Temperature-sensitive adriamycin release from DPPC liposomes. Release of adriamycin from DPPC liposomes was measured upon exposure to heating at various temperatures between 32 and 44°C for 30 min, as described in Materials and Methods. (A) Representative elution profiles of adriamycin released from the liposomes heated at 32 (○) and 42°C (●). (B) Percentage release of adriamycin from the liposomes is plotted versus the heating temperatures between 32 and 44°C. Each value represents the mean of three independent experiments. ADM, adriamycin.

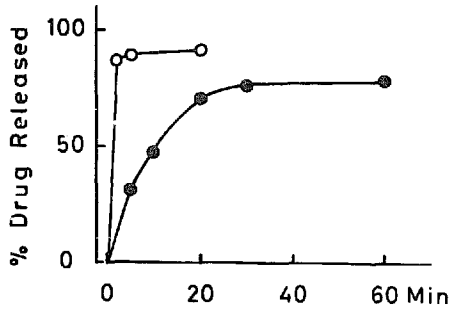

Fig. 2. Time-course of drug release from DPPC liposomes. Drug release from the DPPC liposomes at 42°C was assayed for adriamycin (●) and inosine (○) as described in Materials and Methods. Mean values of three independent experiments are plotted.

30 min. By contrast, around 70% of the drug was released upon exposure of the liposomes to heating at 42°C for 30 min, indicating that the major part, but not all, of the entrapped adriamycin was released at 42°C, i.e., above the T_m of DPPC. We also measured the percentage of drug release from the liposomes at various temperatures between 32 and 44°C. As shown in Fig. 1B, the degree of the drug release was not increased between 32 and 37°C, but it was markedly enhanced around 40°C. These results may suggest that the gel to liquid-crystalline phase transition of the adriamycin-entrapped DPPC liposomes occurrred at approx. 40°C, i.e., at a temperature slightly lower than the T_m of the lipid. Thus, adriamycin, an amphiphilic compound, was released from DPPC liposomes in a temperature-sensitive manner. In the following experiments, we employed 32 and 42°C, respectively, as the temperatures under and above the critical temperature at which adriamycin was markedly released from the DPPC liposomes.

The time-course of drug release at 42°C was followed for 1 h (Fig. 2). The results indicated that the percentage of drug release increased linearly up to 10 min and reached a plateau at 30 min. The rate of adriamycin efflux was very low, compared with that of efflux of inosine (a representative of hydrophilic compounds; Fig. 2) and cytosine arabinoside as described by Magin and Weinstein [12]. This delayed efflux of adriamycin may be due to the amphiphilic nature of the drug.

For the purpose of using liposomes in targeting chemotherapy, we need temperature-sensitive liposomes that are stable in the presence of serum. Therefore, we examined the effect of serum on the release of adriamycin from the DPPC liposomes at 32 and 42°C. As shown in Fig. 3, over 50% of entrapped adriamycin was released by the incubation at 32°C for 30 min with 50% (v/v) fetal bovine serum (Fig. 3), human serum or C3H mouse serum (data not shown). These results indicated that the DPPC liposomes are temperature-sensitive in the absence of serum, but they are much less temperature-sensitive because of their leakiness at lower temperatures in the presence of serum.

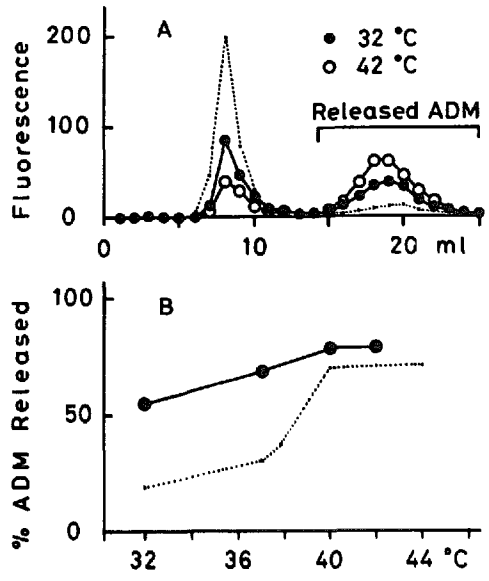

Fig. 3. Effect of serum on the release of adriamycin (ADM) from DPPC liposomes. Release of adriamycin from the liposomes in the presence of serum was measured as described in Materials and Methods. (A) Representative elution profiles of the drug release at temperatures of 32 (●) and 42°C (○) in the presence of serum. Dotted line represents the elution profile of adriamycin at 32°C in the absence of serum (Fig. 1A). (B) Percentage release of adriamycin from the liposomes was measured at various temperatures between 32 and 42°C in the presence of serum. Mean values of two or three independent assays are plotted. Dotted line represents the release of adriamycin at various temperatures in the absence of serum (Fig. 1B).

Efffect of inclusion of cholesterol on the temperature sensitivity and stability of liposomes in the presence of serum

Inclusion of cholesterol in the liposomal membrane generally increases the stability of the liposomes [19], but it abolishes the phase transition of the membrane at contents higher than 20–25 mol% in the case of DPPC [20]. Therefore, we tried to determine the conditions under which cholesterol stabilizes the liposomes in the presence of serum but does not eliminate the temperature-sensitive release of adriamycin.

To optimize the cholesterol content, we included various mol percentages of cholesterol in the liposomal membranes and examined the temperature-sensitive release of adriamycin in the absence or presence of 50% fetal bovine serum. As shown in Fig. 4, the liposomes containing up to 25 mol% cholesterol released the major portion of entrapped-adriamycin in a temperature-sensitive manner when serum was absent. In the presence of serum, however, the maximal temperature-sensitive release of the entrapped drug was observed with the liposomes containing 20 mol% cholesterol. The other liposomes containing less than 20 mol% cholesterol were less temperature-sensitive because of their leakiness at 32°C (Fig. 4). In contrast, the liposomes containing 33 or 50 mol% cholesterol were not temperature-sensitive because the liposomes were so stable that they did not release large amounts of adriamycin even at 42°C (Fig. 4). Similar results were obtained with human or mouse serum instead of fetal bovine serum (data not shown). These results indicated that the liposomes containing 20 mol% cholesterol were most temperature-sensitive in the presence of serum. Furthermore, more than 90% of adriamycin was released at 42°C from the liposomes containing 20 mol% cholesterol, whereas less than 80% of the drug was released from the liposomes devoid of cholesterol, indicating that inclusion of cholesterol enhanced the cumulative percentage release of adriamycin from the liposomes at 42°C (Fig. 4). It was also noteworthy that the stability of the liposomes containing 20 mol% cholesterol at 32°C was comparable to that of the liposomes containing 33 or 50 mol% cholesterol (Fig. 4).

Drug release from the liposomes containing 20 mol% cholesterol was determined in the presence of serum at various temperatures between 32 and 42°C. The results shown in Fig. 5 clearly demonstrated that the liposomes were less leaky at temperatures lower than 37°C but they released more adriamycin at temperatures higher than 40°C, and thus inclusion of 20 mol% cholesterol made DPPC liposomes temperature-sensitive in the presence of serum. However, it was also observed that relatively large efflux of adriamycin occurred at around 37°C (Fig. 5), suggesting that the presence of cholesterol lowered the temperature of onset of drug release.

Differential scanning calorimetry of the adriamycin-loaded liposomes composed of DPPC and cholesterol

Thermotrophic behavior of the adriamycin-loaded liposomes was examined by differential scanning calorimetry. As shown in Fig. 6A and B, incorporation of adriamycin into DPPC liposomes induced only slight

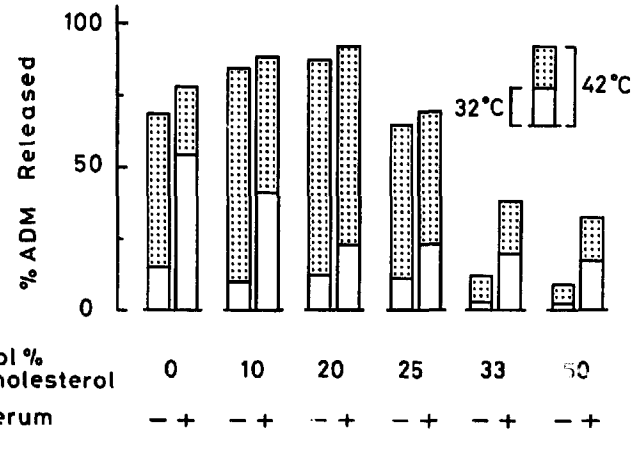

Fig. 4. Effect of cholesterol inclusion on the temperature-sensitive release of adriamycin (ADM) from liposomes. Adriamycin-entrapped liposomes were prepared from DPPC and cholesterol in various molar ratios as described in Materials and Methods, and drug release from these liposomes in the presence or absence of serum was assayed at 32 and 42°C. Height of the bar indicates percentage release of the drug at 32 or 42°C, and thus the dotted sections indicate the amount of temperature-sensitively released drug. Mean values of three to five independent experiments are plotted.

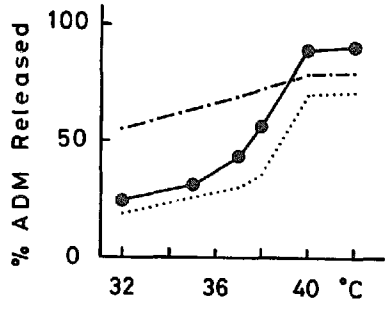

Fig. 5. Release of adriamycin (ADM) at various temperatures from the liposomes containing 20 mol% cholesterol. Drug release from the liposomes containing 20 mol% cholesterol was assayed at various temperatures as described in Materials and Methods, and mean values of three independent assays are plotted (●). For comparison, the results of adriamycin release from the DPPC liposomes in the absence (·····; Fig. 1) or in the presence (·–·–·; Fig. 3) of serum are also illustrated.

changes in the temperature of the main phase transition and in the enthalpy of melting. These results confirmed the data of Goldman et al. [14], and were consistent with the results of drug release described in Fig. 1. We also examined the effect of cholesterol on the thermotrophic behavior of the adriamycin-loaded liposomes. As shown in Fig. 6B–E, the main phase transition was observed with the liposomes including up to 20 mol% cholesterol, although the enthalpy of melting was reduced with increasing cholesterol content. The results of Fig. 6D indicated that inclusion of 20 mol% cholesterol broadened the transition profile but did not significantly shift the temperature of the main phase transition. These results were consistent with the results of temperature-sensitive release of adriamycin from the liposomes (Fig. 4), suggesting that the cooperative phase transition of liposomal membranes is a prerequisite for the temperature-sensitive release of adriamycin from the liposomes.

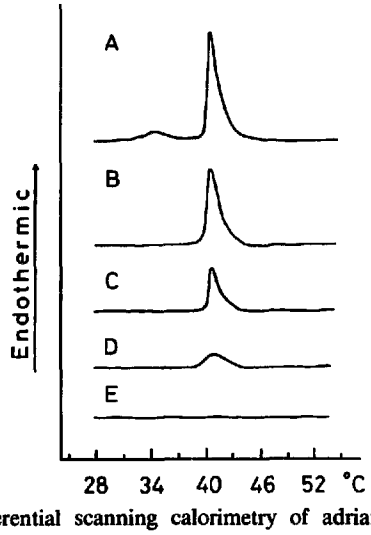

Fig. 6. Differential scanning calorimetry of adriamycin-entrapped liposomes. Differential scanning calorimetry of the liposomes prepared from DPPC and cholesterol in various molar ratios was performed as described in Materials and Methods. The liposome preparations contained (mol% cholesterol) 0, A; 0, B; 10, C; 20, D; and 33, E. All liposomes except A were loaded with adriamycin.

Discussion

In this paper we showed that the adriamycin-loaded DPPC liposomes released the drug in a temperature-sensitive manner, and that inclusion of 20 mol% cholesterol in the liposomal membrane stabilized the liposomes in the presence of serum. As described by Magin and Weinstein [12], it has been considered that the drug for temperature-sensitive liposomes should generally be hydrophilic with a low affinity for the lipid bilayer. However, our results clearly indicated that the concept of temperature-sensitive drug release can be extended to an amphiphilic antitumor agent, adriamycin. Adriamycin may be one of the most appropriate antitumor drugs for temperature-sensitive liposomes because of the following reasons. (1) As shown above, entrapment of adriamycin in liposomes does not drastically change the thermotrophic behavior of the DPPC-cholesterol liposomes. (2) Due to its amphiphilic nature, adriamycin can be entrapped more efficiently in liposomes, compared with hydrophilic drugs [15]. (3) Encapsulation of adriamycin into liposomes markedly reduces the cardiotoxicity of the drug [21–23]. (4) As reported by Hahn et al. [24], adriamycin is one of the drugs which exhibit strikingly enhanced cytotoxicity against tumor cells when combined with 42–43°C hyperthermia.

To stabilize adriamycin-loaded liposomes in the presence of serum, we included various mol percentages of cholesterol in the DPPC liposomal membrane and examined the temperature sensitivity of the liposomes. Our results indicated the following two beneficial effects of cholesterol on the temperature-sensitive drug release from the liposomes. (1) The liposomes containing 20 mol% cholesterol exhibited maximal temperature-sensitivity because of their low leakiness at lower temperatures in the presence of serum (Fig. 4 and 5). (2) Inclusion of cholesterol increased cumulative percentage release of adriamycin at 42°C in the presence of serum, i.e., the liposome containing cholesterol released more than 90% of entrapped adriamycin at 42°C, whereas the percentage drug release from the liposomes composed of DPPC alone did not exceed 80% (Fig. 4). In addition to these beneficial effects, however, inclusion of 20 mol% cholesterol also had a detrimental effect on the temperature-sensitive liposomes, i.e., it broadened the temperature-sensitive release properties of the liposomes by reducing the temperature of onset of drug release, resulting in the drug release of a significant amount at 37–38°C (Fig. 5). To circumvent this effect, we are now attempting to raise the temperature of onset of drug release by including DSPC, whose T_m is 55°C, in the liposomal membrane.

A differential scanning calorimetric study by Estep et al. [20] showed that inclusion of less than 25 mol% cholesterol reduced but not did abolish the highly cooperative phase transition in the DPPC membrane. Similar results were obtained by the differential scanning calorimetry of the adriamycin-entrapped liposomes (Fig. 6), indicating that the encapsulation of adriamycin into DPPC-cholesterol liposomes did not drastically affect their thermotrophic behavior. As shown in Fig. 6D, the adriamycin-loaded liposomes containing 20 mol% cholesterol exhibited a cooperative phase transition, indicating that the temperature-sensitive release of adriamycin from the liposomes is correlated with the thermotrophic properties of the liposomes. The lowered temperature of onset of drug release from the liposomes (Fig. 5) may be explained, at least in part, by the broadening of the phase transition profile (Fig. 6D). However, although the enthalpy of melting was significantly reduced in the liposomal membrane containing 20 mol% cholesterol in comparison with the liposomes devoid of cholesterol (Fig. 6D and 6B), the former liposomes were more temperature-sensitive than the latter, regardless of the presence of serum (Fig. 4). These results suggest that the high temperature sensitivity of the liposomes containing 20 mol% cholesterol should also be due to the interaction between adriamycin and the liposomal membrane, which was not reflected in the thermotrophic behavior of the membrane.

Hermetter and Paltauf [25] reported that the stability of phosphatidylcholine liposomes in the presence of serum was increased by replacement of the ester linkage in the position 2 of glycerol with ether linkage. Therefore, we examined the effect of inclusion of di-*O*-hexadecylphosphatidylcholine, whose T_m is close to that of DPPC [26], on the stability of liposomes. However, our results indicated that the inclusion of the ether-type lipid in bilayers at various mol percentages did not significantly improve stability of the liposomes in the presence of serum (data not shown).

The results of our preliminary experiments using mouse model system indicated that adriamycin-loaded DPPC liposomes, when injected intravenously, delivered 4–5-times more adriamycin to tumors heated to 42°C than to unheated control tumors of the same animals. Such in vivo experiments are now in progress.

Acknowledgement

We thank Ms. Shigemi Yanagihara for her excellent technical assistance.

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