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Temperature-Sensitivity of Liposomal Lipid Bilayers Mixed with Poly(*N*-Isopropylacrylamide-co-acrylic Acid)

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Temperature-sensitive drug release was examined using liposomes mixed with a copolymer of *N*-isopropylacrylamide (NIPAM) and acrylic acid [P(NIPAM-AA)] *i.e.*, thermally responsive liposomes. P(NIPAM-AA) copolymers with transition temperatures of about 30, 33, 37, and 43°C were synthesized by copolymerizing NIPAM and acrylic acid. Thermally responsive liposomes were prepared by mixing hydrophobically modified PNIPAM, or P(NIPAM-AA) with various liposomes, composed of egg phosphatidylcholine (PC), dimyristoylphosphatidylcholine (DMPC)/dipalmitoylphosphatidylcholine (DPPC) mixture (5 : 5, w/w), DPPC, or distearoylphosphatidylcholine (DSPC). The release of a fluorescent marker, calcein, from liposomes was monitored by injecting the liposomal suspension at 17°C into phosphate-buffered saline (PBS, pH 7.4) preadjusted to a temperature ranging from 20 to 46°C. For liposomes of egg PC and DSPC, which do not undergo a phase transition during the temperature jump (17→20-46°C), the release temperature of the liposomes increased as the content of acrylic acid in the copolymers increased. The interaction between copolymer and lipid may induce the release of calcein at LCST of the copolymer. For DPPC liposomes, the release patterns were similar to those of egg PC and DSPC liposomes at 20-36°C, where the phase transition of the liposomal membrane did not occur, while at 36-46°C, where the phase transition of liposomal membrane occurred, the degree of release was almost the same. For DMPC/DPPC (5 : 5, w/w) liposomes, where the transition occurred below those of PNIPAMs, equally enhanced releases were observed as compared with PNIPAMs, even below the LCSTs of PNIPAMs. Thus, regardless of the occurrence of the transition of PNIPAMs, phase transition of DMPC/DPPC liposomes controlled the release of calcein.

Key words: liposome, poly(*N*-isopropylacrylamide), temperature-sensitive release.

Much attention has been paid to liposomes which are sensitive to certain stimuli, such as target (*1, 2*), pH (*3, 4*), and temperature (*5-7*), as potential drug carriers. The sensitivity of liposomes to stimuli can be controlled by either altering the composition of the membrane or modifying the surface. For example, temperature-sensitive liposomes may release active ingredients readily at the phase transition temperature of their membrane with the aid of certain plasma proteins such as high density lipoprotein (*8*).

Recently, it has been proposed to prepare temperature-sensitive liposomes by coating liposomes with poly(*N*-isopropylacrylamide) (*9*). The polymer maintains an expanded form below its LCST of around 32°C, and takes a contracted form above it (*10, 11*). In this system, release of materials entrapped in the inner aqueous phase of liposomes is controlled by changing the temperature, because of the change in the interaction between the polymer and lipid membranes (Fig. 1).

Further, it was reported that the LCST of PNIPAM increases with the content of acrylic acid in the copolymer of NIPAM and acrylic acid (*12, 13*). Thus, the temperature-dependent release behavior of liposomes mixed with copolymer should be quite different from that of liposomes containing homopolymers. In this study, sonicated liposomes of egg PC, DMPC/DPPC (5 : 5, w/w), DPPC, and DSPC mixed with copolymers with different contents of acrylic acid were investigated.

MATERIALS AND METHODS

Materials—Phospholipids of egg phosphatidylcholine, dimyristoylphosphatidylcholine, dipalmitoylphosphatidylcholine, and distearoylphosphatidylcholine, and calcein, a fluorescence marker for the release test, were purchased from Sigma Chemical. A hydrophobic anchor for water-soluble PNIPAM, octadecylacrylate (ODA), and a fluorescent marker for the measurement of membrane fluidity, diphenylhexatriene (DPH), were purchased from Aldrich Chemical. Monomers of NIPAM and acrylic acid (AA) were purchased from TCI and Junsei Chemical, respectively. All

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Abbreviations: PNIPAM, poly(*N*-isopropylacrylamide); P(NIPAM-AA), copolymer of *N*-isopropylacrylamide and acrylic acid; egg PC, egg phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine.

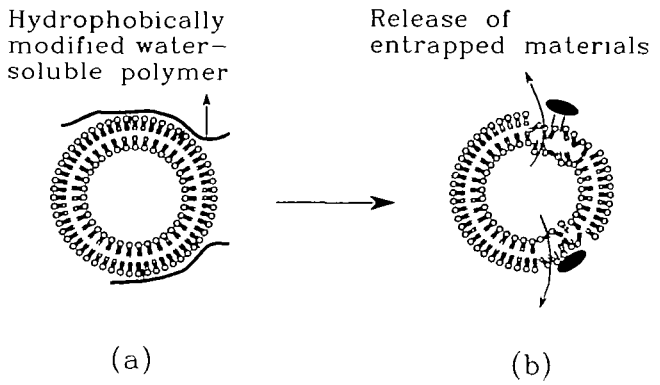

Fig. 1. **Liposomes mixed with poly(*N*-isopropylacrylamide) below (a) and above (b) the LCST of the polymer.** Thermal contraction of the polymer may cause membrane defects, leading to release of entrapped materials.

other reagents were of analytical grade.

Synthesis of Copolymer of N-Isopropylacrylamide and Acrylic Acid—P(NIPAM-AA) was prepared as previously described (*14*). NIPAM (9.9 mmol), ODA (0.1 mmol), acrylic acid (0, 0.11, 0.22, and 0.33 mmol), and azobisisobutyronitrile (AIBN) were dissolved in 20 mmol of freshly distilled dioxane. The solution was degassed by bubbling N_2 for 20 min and then heated to 65°C for 12 h. The copolymer was precipitated upon the addition of diethyl ether. For purification, the precipitated polymer was dissolved in dioxane and reprecipitated with diethyl ether. We prepared the copolymer with NIPAM-to-acrylic acid ratios of 0 : 9.9, 0.11 : 9.9, 0.22 : 9.9, and 0.33 : 9.9 (PNIPAM 1, 2, 3, and 4, respectively)

Determination of Phase Transition Temperature of P(NIPAM-AA)—The phase transition temperatures of P(NIPAM-AA) in aqueous solution were determined by observing light transmission through the polymer solution in PBS (pH 7.4, 5 mg/ml). The cell consists of two parallel cover glasses spaced by a 2 mm thick O-ring. The heating rate was 1°C/min. The percentage of light which did not pass through the polymer solution was determined.

Fluidity of Liposomal Membrane—A 2 mM solution of DPH in tetrahydrofuran (3.41 μl) was added to 1 ml of liposomal suspension so that the molar ratio of DPH/lipid was 1 : 400. The liposomes of egg PC were incubated for 1 h at 30°C or 40°C for DMPC/DPPC (5 : 5, w/w), 50°C for DPPC and 60°C for DSPC and then the liposomes were cooled to room temperature. The fluorescence polarization of DPH was determined based on the following equation

$$\text{Polarization} = (I_{\text{II}} - I_{\text{L}})/(I_{\text{II}} + I_{\text{L}})$$

where I_{II} and I_{L} are emission intensities when the emission polarizer is oriented parallel and perpendicular to the excitation polarizer, respectively. The emission intensities were recorded at 430 nm with excitation at 366 nm, at suspension temperatures from 20 to 46°C.

Preparation of Liposomes—A dry film of 20 mg of phospholipid was dispersed into 2 ml of 50 mM calcein in PBS (pH 7.4) and was sonicated for 10 min with a tip-type sonicator (Sonics & Materials). To remove unentrapped calcein, the suspension was chromatographed through Bio-Gel A-0.5m. Fractions of liposomes were mixed with P(NIPAM-AA) so that the ratios of polymer to phospholipid varied from 0.0125 to 0.5. The final concentration of lipid was adjusted to 2 mg/ml. The suspensions were incubated at 4°C for 12 h.

In the case of egg PC, liposomes were prepared by two different procedures to investigate whether hydrophobically modified PNIPAM was anchored to the liposomes or not. In the first procedure, as described previously, liposomes suspended in calcein solution were first chromatographed through Bio-Gel A-0.5m to remove unentrapped calcein and then mixed with PNIPAM 1 so that the ratio of polymer to lipid was 0.1. In the second experiment, the liposomes suspended in calcein solution were first mixed with PNIPAM 1 in the same ratio, and then chromatographed through Sephacryl S-400 to remove unentrapped calcein and free polymer. The separation of polymers from liposomes was confirmed using a UV detector (Pharmacia) in the absence of calcein.

Calcein Release—The liposomes containing calcein, suspended in 0.15 ml of PBS at 17°C, were injected into a fluorescence cell containing 2.6 ml of PBS preadjusted at temperatures ranging from 20 to 46°C. The change in fluorescence was monitored at 520 nm with excitation at 490 nm. The percent release of calcein was determined as follows

$$\%\ \text{release} = (F^{\text{t}} - F^{\text{i}})/(F^{\text{f}} - F^{\text{i}}) \times 100$$

where F^{t} is the intensity of fluorescence at a given temperature in 80 s and F^{i} is the initial intensity at 17°C. F^{f} is the total fluorescence after adding deoxycholate so that the final concentration is 0.12%.

RESULTS AND DISCUSSION

Phase Transition Temperature of P(NIPAM-AA)—Figure 2 shows the turbidity change of PNIPAM 1, 2, 3, and 4 in solution as the temperature was increased. The temperatures at which the turbidity of polymers 1, 2, 3, and 4 started to increase were 29, 33, 37, and 43°C, respectively. This is consistent with the LCST of PNIPAM (*12, 13*).

Phase Transition of Liposomal Membrane—Figure 3 shows the change of fluorescence polarization of DPH embedded in liposomal membrane. For egg PC liposomes, the polarization remained almost unchanged with temperature and was slightly less than 0.15. Such a low polarization may indicate that the membrane of egg PC bilayer is a fluid liquid crystal in the temperature region from 20 to 46°C. The polarization of DPPC liposomes was about 0.4 below 36°C, and decreased sharply above 40°C. This is due to the phase transition of the bilayer from solid gel to fluid liquid crystal (*15*). In Fig. 3, DMPC was added to DPPC liposomes up to 50 wt%, and a sharp decrease in polarization was observed at around 28°C. Finally, the polarization of DSPC liposomes was almost constant, approximately 0.4, over the whole temperature range. The bilayer may form a solid gel from 20 to 46°C.

Evidence for Anchoring of Hydrophobically Modified PNIPAM to Liposomes—Figure 4 shows the release of calcein at 40°C from egg PC liposomes prepared by two different procedures which, as described in the "MATERIALS AND METHODS" section, involved chromatography before and after mixing of PNIPAM 1 with liposomes. No significant difference in the degree of release was observed between the two methods. Both showed approximately 20%

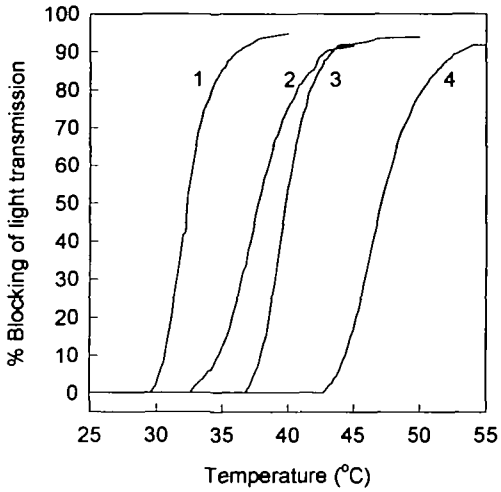

Fig. 2. **Change of turbidity of hydrophobically modified PNIPAM and P(NIPAM-co-AA) in PBS (pH 7.4) with temperature.** Plots 1, 2, 3, and 4 correspond to the turbidity changes of polymer solution of which the AA contents are 0, 1.09, 2.15, and 3.19 mol%, respectively. The concentration of polymers was 5 mg/ml in each, and the heating rate was 1°C/min. The cell consists of two parallel cover glasses spaced by a 2 mm thick O-ring.

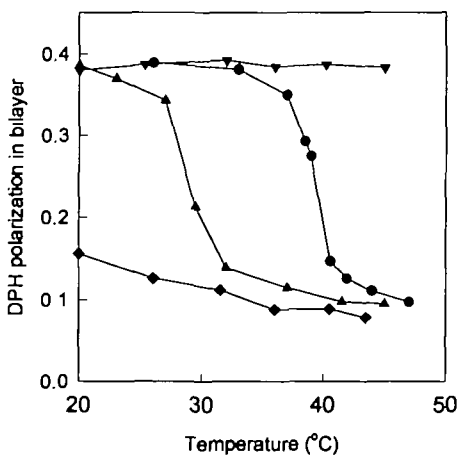

Fig. 3. **Change of polarization of DPH embedded in egg PC (◆), DPPC/DMPC (5 : 5, w/w) (▲), DPPC (●), and DSPC (▼) liposomal membranes with temperature.**

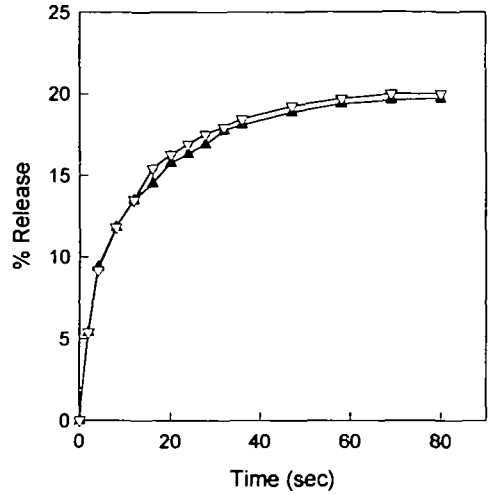

Fig. 4. **Calcein release from egg PC liposomes at 40°C before (▽) and after (▲) removal of free PNIPAM 1.** The ratio of polymer to lipid was 0.1.

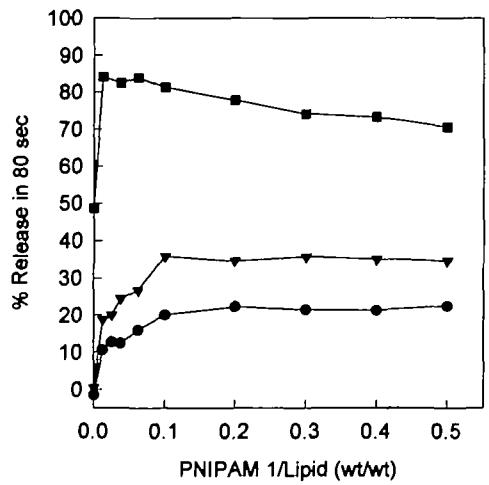

Fig. 5. **Calcein release from egg PC (●), DPPC (■), and DSPC (▼) liposomes with various ratios of PNIPAM 1/lipid at 40°C.**

release in 80 s. This indicates that PNIPAM 1 is anchored to the liposomes. Thus, in all the release tests, PC liposomes containing calcein were simply mixed with PNIPAMs without further separation of free polymers.

Calcein Release vs. PNIPAM/Lipid Ratio—Figure 5 shows the % release of calcein in 80 s from egg PC, DPPC, and DSPC liposomes at 40°C in relation to the ratio of PNIPAM 1/lipid. In the absence of copolymer, egg PC and DSPC exhibit almost no release. However, the degree of release from DPPC liposomes was about 50%. This is probably because DPPC undergoes phase transition at that temperature (see Fig. 3). When polymer was included, the degree of release from egg PC and DSPC liposomes increased, showing a saturation pattern. Clearly the polymer enhances the release of calcein, probably because the thermal contraction of PNIPAM may lead to a packing defect of the liposomal membrane (*9*). In both PCs, saturation of release was obtained near the PNIPAM 1/lipid ratio of 0.1. Therefore, the ratio of polymer to lipid was set at 0.1 for the release experiment. For DPPC liposomes, a ratio lower than 0.1 was sufficient to obtain fully developed release.

Temperature-Dependent Release from Egg PC Liposomes—Figure 6 shows the degree of release from egg PC liposomes mixed with PNIPAM 1, 2, 3, and 4. In the absence of polymer, no significant release was observed at high temperature. In the temperature range from 20 to 46°C, no significant change in polarization of DPH was observed in egg PC membrane (see Fig. 3). In fact, the phase transition temperature of egg PC is less than 0°C, and thus in this temperature range, the liposomal membrane remains as a fluid liquid crystal and does not undergo a phase transition. For egg PC liposomes, therefore, there is no contribution of phase transition-induced release. If PNIPAM 1 is included, an appreciable amount of calcein

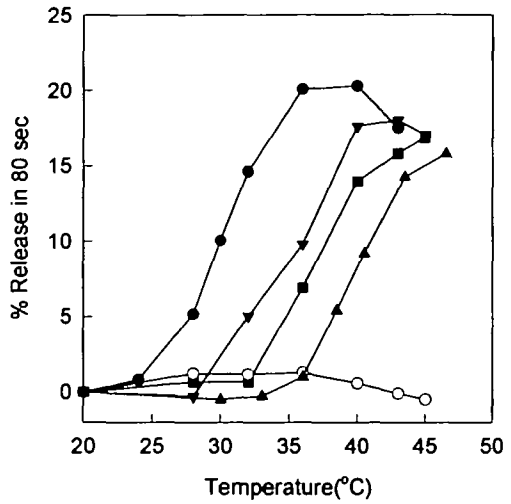

Fig. 6. **Temperature-dependent calcein release from egg PC liposomes mixed with no polymer (○), or PNIPAM 1 (●), 2 (▼) 3 (■), or 4 (▲).** The ratio of the copolymer to lipid was 0.1.

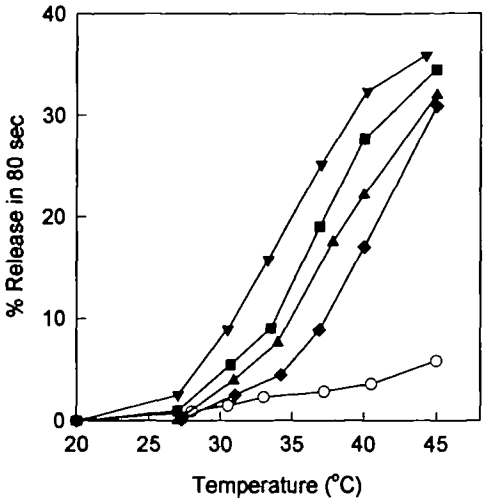

Fig. 8. **Temperature-dependent calcein release from DSPC liposomes mixed with no polymer (○), or PNIPAM 1 (▼), 2 (■) 3 (▲), or 4 (◆).** The ratio of copolymer to lipid was 0.1

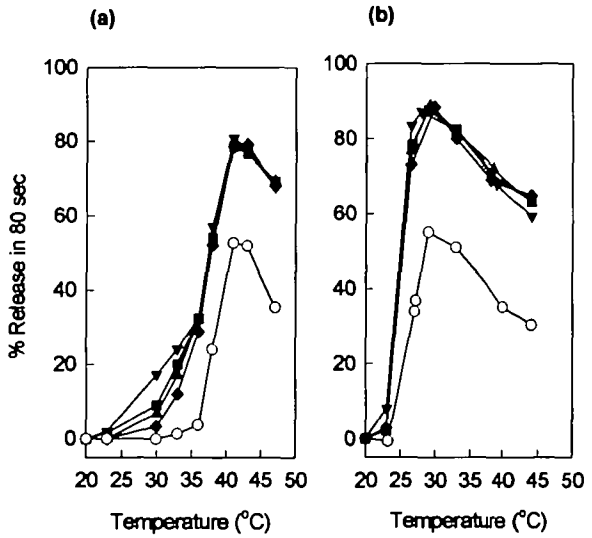

Fig. 7. **Temperature-dependent calcein release from DPPC (panel a) and DMPC/DPPC (5 : 5, w/w) (panel b) liposomes.** In each panel, ▼, ■, ▲, and ◆ represent the degree of release from the liposomes mixed with PNIPAM 1, 2, 3, or 4, respectively. The ratio of copolymer to lipid was 0.1. The degree of release from liposomes without polymers (○) is also shown.

release was observed at 28°C and the degree of release increased up to approximately 20% when the temperature was 35°C. Since there was no significant release from liposomes free of polymer, the release may be attributed to the interaction between PNIPAM 1 and the membrane. A temperature jump through 31°C, the LCST, causes the copolymer to undergo transition from an expanded form to a contracted form, and probably results in membrane defects. For PNIPAM 2, 3, and 4, appreciable amounts of calcein were released at elevated temperature. The release temperature of entrapped calcein decreased in the order of PNIPAM 4, PNIPAM 3, PNIPAM 2, and PNIPAM 1. This is also the order of the LCSTs (see Fig. 2). For egg PC liposomes, however, the release temperature does not exactly agree with the transition temperature of the polymers, but polymer with a higher transition temperature causes the liposomes to leak at a higher temperature.

Temperature-Dependent Release from DPPC and DMPC/DPPC Liposomes—Figure 7a shows the degree of release from DPPC liposomes. In the absence of polymer, liposomes started to release calcein at around 36°C and approximately 50% leakage was observed around 41°C. From 36 to 41°C, a sharp decrease of the polarization of DPH was observed (see Fig. 3). Therefore, the release may be a consequence of the phase transition of the liposomal membrane. In the presence of polymer, however, the pattern of the release was rather complicated. In the temperature region of 20 to 36°C, where phase transition of the liposomal membrane does not occur, the degree of release decreased in the order of PNIPAM 1, PNIPAM 2, PNIPAM 3, and PNIPAM 4. Conversely, liposomes mixed with PNIPAM 4 require the highest temperature to afford a certain degree of release. This may reflect the fact that PNIPAM 4 has the highest LCST (see Fig. 2). On the other hand, in the temperature region of 36-46°C where phase transition of the liposomal membrane occurred, the degree of release was almost the same for all polymers. At this temperature, phase transition of both polymer and liposomal membrane contributes to the release. The release from DMPC/DPPC liposomes (5 : 5, w/w) is shown in Fig. 7b. In this case, phase transition of the liposomal membrane occurred below the transition temperature of PNIPAMs (see Figs. 2 and 3). As shown in Fig. 7b, equally enhanced releases were observed with PNIPAMs, even below the LCSTs of PNIPAMs, with no differentiation in polymer type. Thus, whether the transition of PNIPAMs occurs or not, the polymers aid in destabilizing the liposomal membrane undergoing a phase transition.

Temperature-Dependent Release from DSPC Liposomes—Figure 8 shows the degree of release from DSPC liposomes with PNIPAMs 1, 2, 3, and 4. The degree of release from liposomes free of polymer slightly increased with temperature, but the change was not significant. As

shown in Fig. 3, the fluorescence polarization of DPH embedded in DSPC liposomal membrane remained almost constant with temperature from 20 to 46°C. Thus, for DSPC liposomes there is no liposomal phase transition-induced release. In the presence of polymer, the liposomes of PNIPAM 1 start to release at the lowest temperature and the liposomes of PNIPAM 4 start to release at the highest temperature. This may reflect the LCSTs of PNIPAM 4, PNIPAM 3, PNIPAM 2, and PNIPAM 1 (see Fig. 2). In fact, the release from DSPC liposomes is higher than that from egg PC liposomes. This is probably because the polymer is adsorbed more efficiently on DSPC liposomes than on egg PC liposomes. The polymer may be adsorbed more efficiently on gel state membrane than fluid state membrane (*9*).

In summary, if a liposomal membrane does not undergo phase transition during a temperature jump (17→20–46°C), liposomes of polymer with higher LCST start to release at higher temperature, as in the cases of egg PC and DSPC liposomes. Below 36°C the DPPC membrane remained in the solid gel state, and the patterns of release from DPPC liposomes were similar to those of egg PC and DSPC liposomes. As in the case of DMPC/DPPC (5 : 5, w/w) liposomes, if phase transition occurred in the temperature region and transition of PNIPAMs did not occur, release was equally enhanced by all the PNIPAMs. In the temperature range from 36 to 46°C, where the phase transition of DPPC and the polymers takes place, there was no difference of release between liposomes with different PNIPAMs. Thus, if the phase transition of liposomes occurs, the polymers destabilize the liposomal membrane at its phase transition, even if there is no phase transition of the polymers.

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