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Temperature-controlled release property of phospholipid vesicles bearing a thermo-sensitive polymer

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Abstract

As a novel temperature-sensitive liposome, dioleoylphosphatidylethanolamine vesicles bearing poly(*N*-isopropylacrylamide), which shows a lower critical solution temperature (LCST) near 32°C, were designed. Poly(*N*-isopropylacrylamide) having long alkyl chains which are anchors to the lipid membranes was prepared by radical copolymerization of *N*-isopropylacrylamide and octadecyl acrylate using azobisisobutyronitrile as the initiator. The copolymer obtained revealed the LCST at about 30°C in an aqueous solution. Dioleoylphosphatidylethanolamine vesicles coated with the copolymer was prepared and release property of the copolymer-coated vesicles was investigated. While release of calcein encapsulated in the copolymer-coated vesicles was limited below 30°C, the release was drastically enhanced between 30 nad 35°C. Complete release from the vesicles was achieved within several seconds at 40°C. This temperature-controlled release property of the vesicles can be attributable to stabilization and destabilization of the vesicle membranes induced by the copolymer fixed on the vesicles below and above the LCST, respectively. Moreover, the fluorometric measurement using dioleoyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine suggested that the extensive release of calcein observed above the LCST is resulted from the bilayer to H_{II} phase transition of the vesicle membranes. Since LCST of the copolymer is controllable, these vesicles might have potential usefulness as a drug delivery system with high temperature-sensitivity.

Keywords: Liposome; Temperature-sensitivity; Dioleoylphosphatidylethanolamine; Poly(*N*-isopropylacrylamide); Lower critical solution temperature; Lamellar-hexagonal transition; Drug delivery system

1. Introduction

With respect to site specific delivery of drugs, a number of attempts have been made to develop liposomes which can regulate release of drugs responding to various external stimuli such as pH [1–3], light [4–6], and temperature [7]. In particular, temperature-sensitive liposomes have been shown to enhance the efficacy of drugs [8–11] and may be the best for medical application among these stimuli-responsive liposomes. So far, temperature-sensitive liposomes have been prepared using phospholipids with the liquid-crystalline phase transition temperature a few degrees above physiological [7]. However, temperature-sensitive liposomes of this type have some disadvantages. For example, temperature-sensitivity of these liposomes with diameter less than 200 nm is relatively low [12–14]. Also, available lipids for these liposomes are quite limited.

Recently, as another approach to prepare temperature-sensitive liposomes, modification of liposomes with a thermo-sensitive polymer such as poly(*N*-isopropylacrylamide) has been proposed [15–18]. Poly(*N*-isopropylacrylamide) shows a lower critical solution temperature (LCST) at 30–35°C [19,20]. The polymer is hydrophilic and soluble in water below the LCST, but becomes hydrophobic and forms insoluble aggregates above that temperature due to hydrophobic interactions and formation of intermolecular hydrogen bondings [15,19].

Two strategies for preparation of temperature-sensitive liposomes may be possible using the thermo-sensitive polymer. One is to use the polymer to destabilize liposomal membranes above the LCST of the polymer. However, in a previous study, we showed that release of encapsulated materials from phosphatidylcholine liposomes can

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not be controlled completely [17] because interaction of the polymer with the lipid membranes is not strong enough to destroy the phosphatidylcholine membrane [15,17,21]. The other is to use the polymer to stabilize the liposomal membranes. It is well known that phosphatidylethanolamine (PE) with unsaturated acyl chains does not form vesicles by itself under neutral conditions, but the vesicles can be prepared by adding another component which stabilizes PE membranes [22] such as fatty acids [23,24] and cholesterol derivatives [25,26]. If the thermo-sensitive polymer is used as such a stabilizer, it is expected that the polymer highly hydrated below the LCST stabilizes PE vesicle, but stabilizing ability of the polymer diminishes above the LCST, resulting in degradation of the vesicles.

In this study, dioleoylphosphatidylethanolamine vesicles coated with poly(*N*-isopropylacrylamide) having long alkyl chains as anchors to the lipid membrane, namely a copolymer of *N*-isopropylacrylamide and octadecyl acrylate, were prepared and temperature-controlled release property of the copolymer-modified vesicles was studied.

2. Materials and methods

2.1. Materials

L-α-Dioleoylphosphatidylethanolamine (DOPE), egg yolk phosphatidylcholine (EYPC) and calcein were purchased from Sigma (St. Louis, MO, USA). Dioleoyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (NBD-PE) was supplied by Avanti Polar Lipids (Birmingham, AL, USA). *N*-Isopropylacrylamide (NIPAM), octadecyl acrylate (ODA) and azobisisobutyronitrile (AIBN) were obtained from Tokyo Kasei (Tokyo, Japan). Tris(hydroxymethyl)aminomethane (Tris), *N*,*N*-dimethylformamide (DMF), tetrahydrofuran (THF) and ethylenediaminetetraacetic acid (EDTA) were supplied by Kishida Chemical (Osaka, Japan). NIPAM was purified by recrystallization from cyclohexane-*n*-hexane (1:1) before use.

2.2. Synthesis of copoly(NIPAM-ODA)

A copolymer of NIPAM and ODA shown in Fig. 1 was prepared via the method previously reported [17]. In brief, NIPAM (9.8 mmol), ODA (0.2 mmol) and AIBN ($3.0 \cdot 10^{-2}$ mmol) were dissolved in THF (10 ml) and then the solution was heated at 60°C for 4 h in N_2 atmosphere. The copolymer was recovered by precipitation with diethylether. The copolymer was dissolved in THF and reprecipitated with diethylether. The dried copolymer was dissolved in water. The solution was filtered. The copolymer was isolated by lyophilization of the solution.

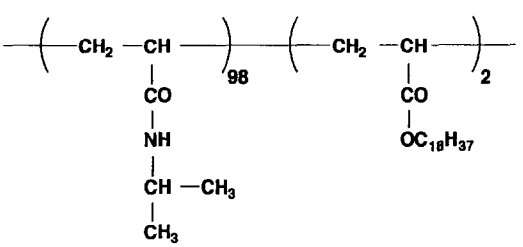

Fig. 1. Structure of copolymer of *N*-isopropylacrylamide and octadecyl acrylate.

2.3. Preparation of DOPE vesicles coated with copoly(NIPAM-ODA)

DOPE vesicles were prepared as follows: a dry thin membrane of 10 mg of DOPE was dispersed in 1 ml of aqueous 20 mM calcein solution of pH 9.0. The solution was sonicated for 15 min by using a ultrasonic disruptor (Tomy Seiko, UD-201). Varying amounts of aqueous solution of the copolymer (10 mg/ml, pH 9.0) containing 20 mM calcein were added to the liposome suspension and incubated at 5.0°C for 4 h with stirring. Free calcein and the free copolymer were removed by gel permeation chromatography on a Sephacryl S-400 column at 10°C using 10 mM Tris-HCl-buffered solution containing 40 mM NaCl and 0.1 mM EDTA at pH 9.0.

2.4. Estimation of amount of copoly(NIPAM-ODA) bound to vesicles

Amount of the copolymer bound to the vesicles was determined by high performance liquid chromatography analysis on Asahipak GF-310HQ using mixture of THF and water (2:1, v/v) as an effluent. The copolymer-modified vesicles were dissolved in the THF/water (2:1, v/v) mixture by adding THF into aqueous dispersion of the vesicles, whose DOPE concentration was determined by the method of Bartlett [27] (typically ca. $4 \cdot 10^{-4}$ M). The solution of the copolymer-modified vesicles in THF/water mixture (20 ml) was injected into the column and the effluent was monitored by absorbance at 210 nm using a UV detector (Jasco, UV-970). The flow rate was 0.6 ml/min. The void volume of the column was 4.0 ml. The elution volumes of the copolymer and DOPE were 5.0 ml and 9.7 ml, respectively. From the absorbance of the copolymer separated, the amount of the copolymer bound on the vesicle was determined.

2.5. Calcein release from vesicles

The release measurements were performed according to the method previously reported [17,28]. The vesicles containing 20 mM calcein in the inside was dispersed in 10 mM Tris-HCl-buffered solution containing 40 mM NaCl and 0.1 mM EDTA of a given pH at various temperatures and the fluorescence intensity was monitored. The excitation and monitoring wavelengths were 493 and 515 nm,

respectively. The percent release of calcein from the vesicles was defined as

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

where F^i and F^t mean the initial and intermediate fluorescence intensities of the vesicle suspension, respectively. F^f is the fluorescence intensity after the addition of Triton X-100 (final concentration 0.15%). Because release of calcein from the copolymer-modified vesicles was very fast at temperatures above 35°C under neutral and weak acidic conditions, it is impossible to determine the F^i value directly. Under these conditions, the F^i value was obtained in the following manner. Since the release of calcein was quite slow at pH 9.0, fluorescence intensity of calcein entrapped in the vesicles could be determined accurately by eliminating F^f from F^i. Therefore, in the measurement above 35°C under neutral and weak acidic conditions the F^i value was obtained by eliminating fluorescence of calcein entrapped in the vesicles, namely the value of $F^f - F^i$ measured at pH 9.0 at a given temperature, from the F^f value with correction for pH-dependent change of the fluorescence intensity although the change of fluorescent intensity was not significant under the experimental conditions.

2.6. Fluorescence measurement using NBD-PE

Fluorescence measurement using NBD-PE was performed by the method of Hong et al. [29]. NBD-PE-labeled vesicles were prepared according to the above procedure using mixture of the lipids and NBD-PE (0.3 mol%). An aqueous solution of the copolymer (10 mg copolymer/ml, pH 9.0, 0.5 ml) was added to the labeled vesicles dispersed in 10 mM Tris-HCl-buffered solution containing 40 mM NaCl and 0.1 mM EDTA (10 mg lipid/ml, pH 9.0, 1 ml) and incubated at 5.0°C for 4 h with stirring.

The vesicle suspension diluted to a given concentration was put in a quartz cuvette at a given temperature and then small volumes of HCl solution was added to change the pH of the solution from 9.0 to 7.3. Fluorescence intensity of the vesicle was monitored continuously during the above procedure by using a fluorescence spectrophotometer (Hitachi, F-3010). The excitation and the emission wavelengths were 475 and 530 nm, respectively.

2.7. Other methods

Phospholipid concentration was measured by the method of Bartlett [27]. Molecular weight of the copolymer was estimated by gel permeation chromatography analysis on Shodex KD-803 column (Showa Denko Co.) using DMF. The size of the vesicles was determined by quasielastic light scattering using a laser particle analyzing system (Otsuka Electronics, LPA 3100S). Nuclear magnetic resonance (NMR) spectra were taken with a JEOL JNM-GX-270 MHz instrument. Differential scanning calorimetry was performed with a Perkin Elmer DSC7 microcalorimeter. The samples were analyzed at a heating rate of 1.0°C /min in the temperature range of 20–35°C.

3. Results and discussion

3.1. Characterization of copolymer

In order to obtain poly(NIPAM) with anchoring groups to the lipid membrane, we chose the copolymer of NIPAM and ODA because many studies indicate that hydrophilic polymers can be fixed on lipid membranes by conjugation of hydrophobic molecules to the polymers [17,30–33]. Ringsdorf and his collaborators have already shown that hydrophobically-modified poly(NIPAM)s bind onto liposomal membranes and that the polymers bound on the liposomal membranes undergo a phase transition at the temperature corresponding to the LCST of the polymers [21,32].

The chemical composition of the copolymer prepared here was determined by ^{1}H-NMR spectra as shown in Fig. 1. The weight average molecular weight (Mw), the number average molecular weight (Mn), and the heterogeneity index, Mw/Mn, were determined to be 9500, 5300, and 1.8, respectively, using GPC analysis.

The LCST of poly(NIPAM) can be determined by microcalorimetric methods [19,20]. Fig. 2 represents DSC curves of the copolymer in an aqueous solution and in the presence of a phospholipid. The copolymer exhibits a broad endotherm centered at about 30°C. This LCST value is somewhat lower than poly(NIPAM) itself. Since LCST of poly(NIPAM) as well as that of other thermo-sensitive polymers has already been shown to decrease when hydrophobic residues are incorporated into the polymer [34–37], it is likely that hydrophobic octadecyl groups on the copolymer make the LCST of the copolymer lower than

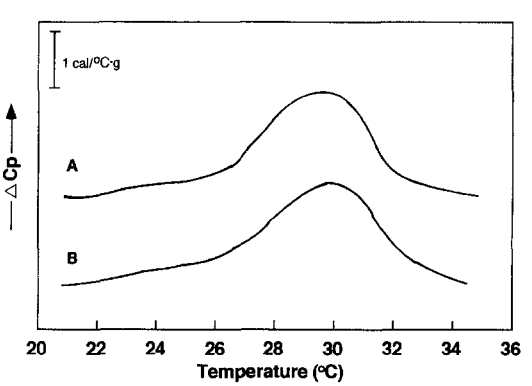

Fig. 2. Microcalorimetric endotherms for aqueous copolymer samples in the absence (A) and in the presence (B) of EYPC vesicles. (A) Copoly(NIPAM-ODA) solution (5.0 wt%); (B) copoly(NIPAM-ODA) (2.5 wt%) solution containing EYPC vesicle (2.5 wt%). The samples were heated at 1°C/min. ΔH for curves A and B are 4.1 and 3.8 cal/g of the copolymer, respectively.

the LCST of poly(NIPAM) itself. On the other hand, the LCST value of the copolymer elevated slightly in the presence of EYPC liposome. Since the copolymer possesses hydrophobic moieties, it might form a polymeric micelle in an aqueous solution [34,38], whereas in the presence of the lipid membrane the polymeric micelle should be disrupted as is observed in the presence of a surfactant by Winnik et al. [38]. Such a change of state of the copolymer may affect the aggregate formation of NIPAM units of the copolymer, resulting in the slight difference of LCST.

Calorimetric enthalpy of endotherm (ΔH) associated with the LCST of the copolymer was ca. 4 cal/g of the copolymer. This value is approximately consistent with the result reported by Schild and Tirrell [20].

3.2. Characterization of copolymer-modified DOPE vesicles

Although PE with unsaturated acyl chains does not form vesicles by itself under the physiological condition, it is known that unilamellar PE vesicles can be produced at high pH, where the ammonium group is deprotonated and the head group is negatively charged [22,39,40]. Allen et al. reported that stable PE liposomes form above pH 8 and lamellar-hexagonal (H_{II}) transition of PE does not occur above pH 8.4 [41]. Since it is known that DOPE vesicle can be obtained above pH 8.6 [42], in this study preparation of DOPE vesicle and incubation of the vesicle with the copolymer were carried out at pH 9.0.

We prepared DOPE vesicles bearing varying amounts of copoly(NIPAM-ODA) by controlling the amount of the copolymer added to DOPE vesicles. The amount of the copolymer added to DOPE vesicles were 0, 0.05, 0.1, 0.25 and 0.5 mg/mg lipid. The diameter of the copolymer-modified vesicles obtained by adding 0, 0.1 and 0.5 mg of the copolymer per 1 mg of the lipid was estimated to be 105, 94 and 97 nm, respectively, by quasielastic light scattering. It is apparent that the diameter of the vesicles was hardly affected by coating with the copolymer, although the copolymer-modified vesicles showed a somewhat smaller diameter compared with the bare DOPE vesicle. Since the copolymer bound to the vesicles is highly hydrated below its LCST, the polymer may suppress contact between the vesicles and hence aggregation and/or fusion of the vesicles. The amounts of the copolymer bound to the vesicles obtained by adding 0.1, 0.25 and 0.5 mg of the copolymer were estimated to be 0.042, 0.113 and 0.113 mg/mg DOPE, respectively, by high performance liquid chromatography. This result indicates that the amount of the copolymer adsorbed on the vesicle reaches to a constant value in the presence of excess of the copolymer. This value corresponds to ca. 1.5 NIPAM units per one DOPE molecule in the outer leaflet of the vesicle membrane, assuming that the vesicle consists of single bilayer membrane and the copolymer adsorbed only on the outer leaflet of the membrane.

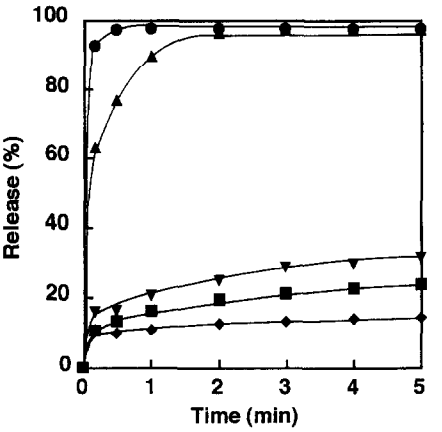

Fig. 3. Typical profiles of calcein release from the copolymer-modified vesicle suspended in 10 mM Tris-HCl-buffered solution containing 40 mM NaCl and 0.1 mM EDTA at pH 7.4 at various temperatures: ◆, 20°C; ■, 25°C; ▼, 30°C; ▲, 35°C; ●, 40°C. The concentration of lipid was $1 \cdot 10^{-5}$ mol/l. The vesicle was prepared by incubation in the aqueous solution containing 5 mg of the copolymer (see Section 2 in the text).

3.3. Release behavior of copolymer-modified DOPE vesicles

Temperature-dependence of release of calcein from the copolymer-modified DOPE vesicles was investigated. The copolymer-modified vesicles and the unmodified vesicle containing calcein released essentially the same amount of calcein after the addition of Triton X-100. Moreover, when the vesicles which did not contain calcein in the inner aqueous phase were incubated in an aqueous calcein solution for 1 h at 5°C and subsequently separated from free calcein by gel permeation chromatography, the release of calcein from the resultant vesicles of either modified and unmodified types was not observed by adding Triton X-100. These facts indicate that calcein is entrapped in the inner aqueous space of the vesicles and the modification of the vesicles with the copolymer does not affect the encapsulation of calcein in the vesicles.

Fig. 3 shows profiles of release of calcein entrapped in the copolymer-modified vesicle. While release of calcein is limited below 30°C, rapid and extensive release was induced above 35°C. The release was completed within several seconds at 40°C. Fig. 4 represents percent release of calcein from the copolymer-modified and -unmodified DOPE vesicles after 30 s as a function of temperature. In the case of unmodified vesicle, a monotonous increase in percent release was observed. As is seen in Fig. 4, the data of the unmodified vesicle have large deviations, because the unmodified vesicle was highly unstable at pH 7.4. Nevertheless, complete disintegration of the vesicle did not occur immediately after addition into the buffer of pH 7.4. Since destabilization of PE vesicles requires contact between the bilayers [25,43,44], prompt rupture of the vesicle might not be induced even above the lamellar-hexagonal II (H_{II}) phase transition temperature (T_H) of DOPE mem-

brane. In fact, it was reported that an increase in temperature to above the T_H does not induce the phase transition of PE membrane at physiological pH when the vesicles are unaggregated [41].

By contrast, release of calcein from the copolymer-modified vesicle changes drastically between 30 and 35°C. This temperature region agrees well with LCST of the copolymer. By comparison of curves in Fig. 4, it is apparent that coating of the copolymer on the vesicle surface results in reduction of the release below 30°C and enhancement of the release above 35°C.

In order to clarify the effect of the copolymer on release property of DOPE vesicle, release of calcein from the vesicles coated with various amounts of the copolymer was investigated. Fig. 5 depicts percent release of calcein from DOPE vesicles prepared by the incubation with various amounts of the copolymer. The number of abscissa indicates the weight ratio of the copolymer supplied to DOPE vesicle during the incubation. As mentioned above, the copolymer-modified vesicles prepared by the incubation with the ratio copolymer/DOPE of 0.25 and 0.5 revealed the same composition. The extent of release from the vesicles increases with increasing amounts of the copolymer at 40°C. By contrast, below 30°C, the release was suppressed with increasing amounts of the copolymer adsorbed on the vesicle. The effect of the copolymer at 35°C is complicated compared with those at 30 and 40°C. When the weight ratio of copolymer to lipid is below 0.1, percent release decreases with increasing amounts of the copolymer bound on the vesicle. However, the vesicles bearing larger amounts of the copolymer reveal significant release.

It is known that PE liposomes, under nonaggregating conditions, exist in the bilayer state at temperatures well

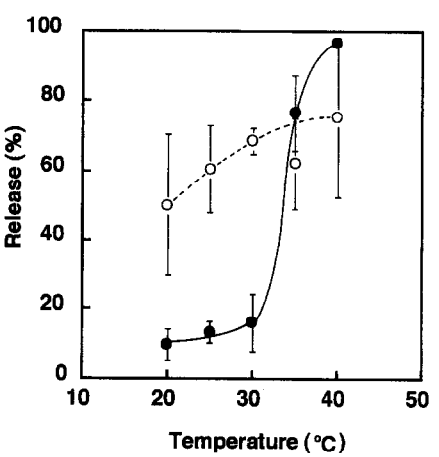

Fig. 4. Percent release of calcein from the copolymer-modified DOPE vesicle (●) and the unmodified DOPE vesicle (○) as a function of temperature in 10 mM Tris-HCl-buffered solution containing 40 mM NaCl and 0.1 mM EDTA at pH 7.4. Percent release after 30 s is shown in the figure. The bars represent the standard deviation ($n = 3$). The copolymer-modified vesicle was prepared by incubation in the aqueous solution containing 5 mg of the copolymer (see Section 2 in the text).

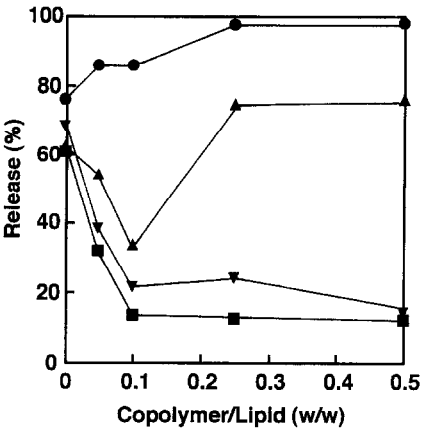

Fig. 5. Effect of the amount of copolymer fixed on the vesicle on the release property of the vesicle in 10 mM Tris-HCl-buffered solution containing 40 mM NaCl and 0.1 mM EDTA at pH 7.4 at various temperatures: ■, 25°C; ▼, 30°C; ▲, 35°C; ●, 40°C. Percent release after 30 s is shown in the figure. The number of abscissa indicates the weight ratio of the copolymer supplied to DOPE vesicle during the incubation (see Section 2 in the text).

above the H_{II} transition temperature and do not release their contents [41]. Therefore, PE liposomes can be obtained under the physiological condition by preventing the interbilayer contact, which induces H_{II} phase formation [22,25]. A number of PE bilayer stabilizers have been reported. For example, highly hydrophilic proteins such as immunoglobulin G [45] and glycophorin A [46] can stabilize DOPE vesicles. Similarly, the copolymer adsorbed on the vesicle reduces the bilayer contact below the LCST because the copolymer becomes highly hydrated and extended under the condition. With increasing amounts of the copolymer adsorbed on the vesicle, the bilayer contact should be inhibited and hence, the vesicle is stabilized more strongly, resulting in decrease in the extent of release. On the other hand, the copolymer shrinks and forms aggregate above the LCST. This shrinkage induces generation of the bare lipid membrane surface, which triggers destabilization of the vesicle. However, only the exposure of bare surface is not sufficient to bring about destabilization of the vesicle, as is indicated in Fig. 5, in which remarkable enhancement of release of calcein from the vesicles bearing small amount of the copolymer is not observed at 35°C. The copolymer becoming hydrophobic above the LCST should interact with DOPE membrane and also facilitate aggregation and contact of the vesicles. In fact, increase in turbidity of the copolymer-coated vesicles of DOPE as well as other lipids such as dipalmitoylphosphatidylcholine was observed (results not shown). Such interactions should promote transition from lamellar phase to H_{II} phase and then significant and rapid release from the vesicles takes place [25,43,44]. In addition, it is known that polar solutes such as poly(ethylene glycol) increase the bilayer-to-H_{II} transition temperature of PE [47], whereas non-polar solutes such as *n*-alkanes promote

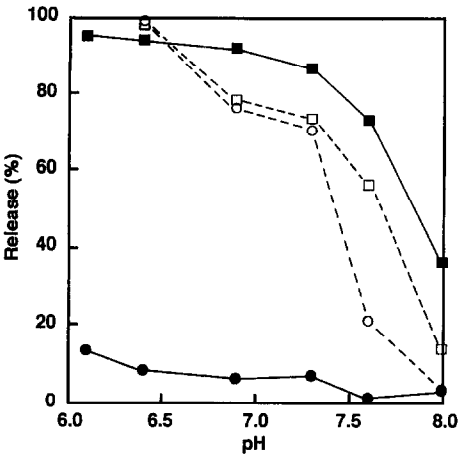

Fig. 6. Percent release of calcein from the copolymer-modified (closed symbols) and the unmodified (open symbols) DOPE vesicles as a function of pH in 10 mM Tris-HCl-buffered solution containing 40 mM NaCl and 0.1 mM EDTA at 25°C (○,●) and 35°C (□,■). Percent release after 60 s is shown in the figure. The copolymer-modified vesicle was prepared by incubation in the aqueous solution containing 5 mg of the copolymer (see Section 2 in the text).

the formation of H_{II} phase [48,49]. Therefore, it seems possible that the interaction of the hydrated or dehydrated copolymer with the membrane affects the phase transition temperature differently.

Since the bilayer-to-H_{II} transition of PE is affected by pH [41], release property of the copolymer-modified DOPE vesicles is expected to reveal pH-dependence in the pH region where the ionization state of amino group of the lipid changes. Fig. 6 shows percent release of calcein from the modified and unmodified DOPE vesicles after 60 s at varying pH values. The release from the unmodified vesicle increases as pH decreases at 35°C as well as at 25°C, probably because protonation of amino group of the lipid enhances the bilayer-to-H_{II} transition. A similar increase of the percent release with decreasing pH is observed for the copolymer-modified vesicle at 35°C, suggesting the common mechanism of destabilization with the unmodified vesicle. By contrast, enhancement of release from the copolymer-modified vesicle was hardly seen at 25°C even when pH was decreased to 6.1. This result indicates that the membrane of the vesicle takes the lamellar phase and the bilayer-to-H_{II} transition of the membrane is strongly suppressed at least above pH 6.1.

3.4. Fluorometric detection of the phase transition of DOPE vesicle in the presence of the copolymer

If the extensive calcein release from the copolymer-modified vesicles observed above 35°C is resulted from the bilayer-to-H_{II} phase transition of the vesicle membranes and subsequent rapture of the vesicles, the phase transition is expected to occur at the temperature. The bilayer-to-H_{II} phase transition of lipid membranes can be detected by various techniques such as ^{31}P-NMR, DSC, freeze-fracture electron microscopy and fluorometry [49]. Among them the fluorometric technique using NBD-PE is well established and is suitable to determine the bilayer-to-H_{II} phase transition in dilute membrane suspensions [29,50]. When NBD-PE is incorporated in a lipid membrane which undergo the bilayer-to-H_{II} phase transition, NBD-PE fluorescence increases during the bilayer-to-H_{II} phase transition.

We prepared the DOPE vesicles containing NBD-PE and fluorescence intensity of NBD-PE was investigated at varying temperatures (Fig. 7). When pH of the vesicle suspension was changed from 9.0 to 7.3 at a given temperature by adding an aqueous HCl solution, the fluorescence intensity increased and reached to the maximum value after about 30 s (data not shown), as reported by Hong et al. [29]. The maximum value F_{max} is considered to represent the fluorescence of the equilibrium state under the new condition [29]. Fig. 7A shows F_{max} of NBD-PE incorporated in the DOPE vesicle membrane in the presence or absence of the copolymer as a function of temperature at pH 7.3. In the absence of the copolymer F_{max} decreases continuously. Although hydrated DOPE has the bilayer-to-H_{II} phase transition temperature of 5–10°C [49], the transition can not be detected possibly because the transition temperature is shifted and lowered a few degrees in the presence of NBD-PE [29]. By contrast, in the presence of the copolymer an increase of F_{max} is apparently seen between 30 and 35°C, suggesting occurrence of the phase transition. In order to confirm the appearance of the transition, we performed the fluorescence measurement at pH 9.0, where DOPE takes the lamellar phase. As is seen in Fig. 7B, a continuous decrease of F_{max} with temperature is observed in the presence of the copolymer as well as in the absence of the copolymer and hence, dehydration of the copolymer does not affect F_{max}.

Moreover, NBD-PE-labeled vesicle of EYPC which does not undergo the bilayer-to-H_{II} phase transition was also prepared and F_{max} of NBD-PE in the EYPC vesicle membrane in the presence and in the absence of the copolymer was investigated (Fig. 7C). It has already been shown that the copolymer reveals a similar affinity for EYPC vesicle as well as for DOPE vesicle [17]. A continuous decrease of F_{max} with temperature is observed in the absence of the copolymer and also in the presence of the copolymer as is observed in the case of DOPE vesicles at pH 9.0. These results indicate that (1) at pH 9.0 DOPE vesicle membrane takes lamellar phase in the presence of the copolymer as well as in the absence of the copolymer and (2) at pH 7.3 in the presence of the copolymer the DOPE vesicle membrane exhibits the lamellar-to-hexagonal (H_{II}) transition at the LCST of the copolymer.

3.5. Concluding remarks

Since liposomes injected into the blood circulate in the body, a rapid release property is important for effective

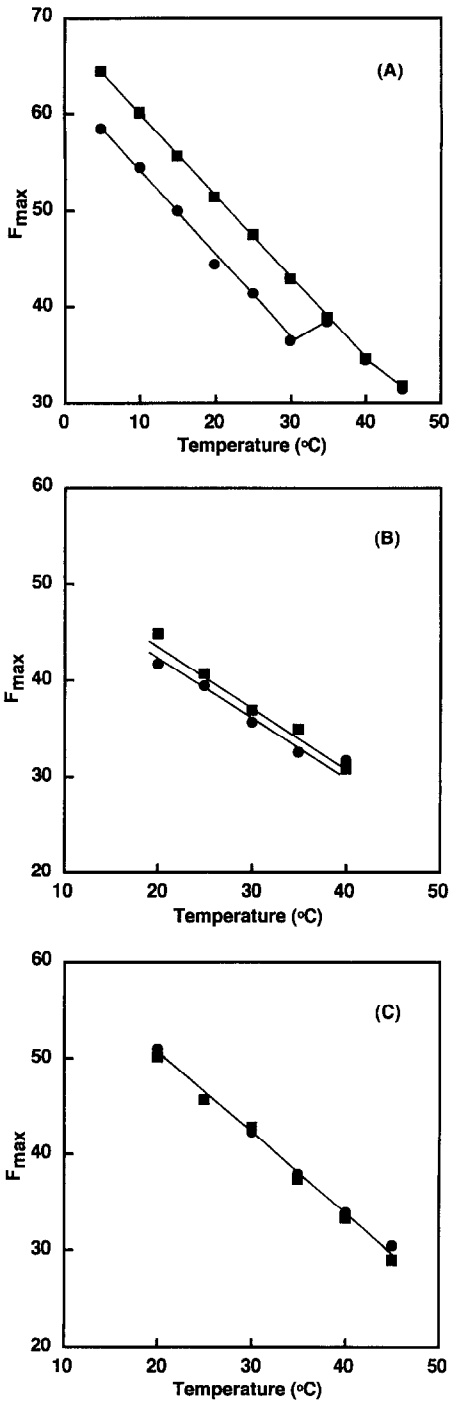

Fig. 7. Fluorescence intensity of NBD-PE-labeled DOPE (A, B) and EYPC (C) vesicles as a function of temperature in 10 mM Tris-HCl-buffered solution containing 40 mM NaCl and 0.1 mM EDTA at pH 7.3 (A,C) and at pH 9.0 (B) in the absence (■) and in the presence (●) of the copolymer. The concentration of the lipids was $6.3 \cdot 10^{-5}$ M.

site-specific delivery of drugs by temperature-sensitive liposomes. Therefore, it is noteworthy that the copolymer-coated DOPE vesicles reveal a fast and extensive release of their content responding to environmental temperature change. Because the LCST of this type of polymers can be controlled by copolymerization with monomers with varying hydrophobicity or hydrophilicity [35–37], it is possible to prepare vesicles which reveal critical temperature for the destabilization a few degrees above the physiological temperature. At present, this new technology is indeed immature. For example, toxicity of these vesicles in the body is unknown. However, by using thermo-sensitive polymers with low toxicity, their application for temperature-sensitive drug delivery system with high sensitivity might be possible.

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