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Thermosensitive polymer-modified liposomes that release contents around physiological temperature

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Abstract

To obtain temperature-sensitive liposomes which release their contents around the physiological temperature, we designed dioleoylphosphatidylethanolamine liposomes modified with copolymers of *N*-isopropylacrylamide and acryloylpyrrolidine. Copolymers of acryloylpyrrolidine and *N*-isopropylacrylamide, which exhibit a lower critical solution temperature around the physiological temperature, were prepared by free radical copolymerization using azobis(isobutyronitrile) as the initiator. The copolymers with anchors to the liposome membrane were obtained by using *N,N*-didodecylacrylamide as an additional comonomer. The copolymer having the anchor group at the terminal of the polymer chain was also synthesized by copolymerization of these monomers in the presence of 2-aminoethanethiol and subsequent conjugation of *N,N*-didodecyl succinamic acid to the terminal amino group of the copolymer. Calcein-loaded dioleoylphosphatidylethanolamine liposomes modified with these copolymers were prepared and release of the contents from these liposomes was investigated. It was found that the release from these copolymer-modified liposomes was promoted around and above the lower critical temperature of the copolymer. Also, the liposomes modified with the terminal anchor-type copolymer released the contents more drastically responding to a small temperature change than the liposomes modified with random copolymers containing *N,N*-didodecylacrylamide units as the anchor.

Keywords: Temperature-sensitive liposome; Poly(*N*-isopropylacrylamide); Poly(acryloylpyrrolidine); Lower critical solution temperature; Dioleoylphosphatidylethanolamine

1. Introduction

Since liposomes made from naturally occurring lipids are biocompatible, their application to drug delivery systems has been extensively attempted [1]. To elevate their usefulness, a number of liposomes with various functionalities have been designed. For example, a variety of stimulus-sensitive liposomes, which release their contents in response to various physical or chemical stimuli, have been designed for

Abbreviations: NIPAM, *N*-isopropylacrylamide; APr, acryloylpyrrolidine; NDDAM, *N,N*-didodecylacrylamide; DOPE, L-α-dioleoylphosphatidylethanolamine; AIBN, azobis(isobutyronitrile); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; LCST, lower critical solution temperature; Mw, weight average molecular weight; Mn, number average molecular weight

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site-specific drug delivery. Temperature- [2], pH- [3–6], target- [7] and light- [8–10] sensitive liposomes have been developed as such liposomes. Also, for cytoplasmic delivery of membrane-impermeable molecules, fusogenic liposomes have been developed [11–14]. The fusogenic liposomes are considered to fuse with the plasma membrane or the endosomal membrane and release their contents into cytoplasm [15–17]. In addition, sterically stabilized liposomes are one of the most important functional liposomes, which reveal significantly long circulation time in the blood stream and enhance uptake by tumors [18,19].

While several approaches to the production of functional liposomes have been attempted, one of the most effective methods is their modification with polymers [20,21]. A number of functional liposomes have been developed by the modification of liposomes with naturally occuring polymers [22] and synthetic polymers [5,6,14].

Recently, it has been shown that conjugation of poly(*N*-isopropylacrylamide) (poly(NIPAM)) onto liposomes provides a temperature-sensitive property to the liposomes [23–27]. It is well-known that poly(NIPAM) demonstrates a lower critical solution temperature (LCST) at ca. 32°C in aqueous solutions [28,29]. The polymer is soluble in water and takes on a hydrated coil state below that temperature. However, it becomes water-insoluble and takes on a dehydrated globule state above that temperature. When liposomes are coated with poly(NIPAM), they are stabilized by the hydrated polymer chains below the LCST [25]. However, the liposomes are destabilized above that temperature due to interaction between the liposome membrane and the hydrophobic polymer chains, resulting in release of the contents [23–26]. Also, it has been shown that aggregation and fusion of poly(NIPAM)-coated liposomes take place above the LCST, indicating that surface property of liposomes can be made temperature-sensitive by the modification of liposomes with poly(NIPAM) [27]. Recently, Meyer et al. reported that a copolymer of NIPAM, methacrylic acid and octadecyl acrylate induces release of contents from egg yolk phosphatidylcholine liposomes under weakly acidic conditions and is useful for the preparation of pH-sensitive liposomes [30]. These findings suggest potential usefulness of poly(NIPAM)-modified liposomes as new drug delivery systems.

Poly(NIPAM)-modified liposomes exhibit temperature response around their LCST, ca. 32°C, which is lower than the physiological temperature. For the use of these liposomes as drug delivery systems, it is essential for the liposomes to reveal temperature response around the physiological temperature. Because the LCST of this type of polymers can be controlled by copolymerization of NIPAM with hydrophilic or hydrophobic comonomers [31], it is possible to synthesize polymers having an LCST around the physiological temperature.

Poly(acryloylpyrrolidine) (poly(APr)) is known to have an LCST at ca. 50°C. Because LCSTs of poly(NIPAM) and poly(APr) are relatively close to the physiological temperature, it is expected that precise control of LCST is possible by copolymerization of NIPAM and APr.

Control of temperature at which the contents release from NIPAM copolymer-coated liposomes is triggered, has been attempted by Kim et al. [26]. They examined calcein release from liposomes of various phosphatidylcholines mixed with copolymers of NIPAM, acrylic acid and octadecyl acrylate having various LCSTs. It was shown that the polymer with a higher LCST enhances release of the contents slightly at a higher temperature, but the extent of release was quite low if the liposome membrane does not undergo the gel-to-liquid crystalline phase transition at the temperature of the measurement. This study as well as our own [23] indicates that interaction between the phosphatidylcholine membranes and the NIPAM copolymers is not strong enough to destroy the membranes.

Thermosensitive polymers can be used as a temperature-sensitive stabilizer for a membrane of non-bilayer forming lipids, such as dioleoylphosphatidylethanolamine (DOPE) which has a strong tendency to take on hexagonal II phase under the physiological condition. We have reported that DOPE liposomes are stabilized by hydrated poly(NIPAM) chains attached to the surface below the LCST [25]. However, the poly(NIPAM)-attached DOPE liposomes disintegrate very quickly above the LCST, because the stabilizing effect of the polymer vanishes above the LCST and the bilayer-to-hexagonal II

transition occurs in the liposome membranes [25]. If a thermosensitive polymer with an LCST around the physiological temperature is used to stabilize DOPE liposomes, temperature-triggered release from the liposomes is expected to be induced around the physiological temperature.

In this study, we synthesized two types of copolymers of APr and NIPAM having anchors to the liposome with an LCST near the physiological temperature: copolymers having anchors in the middle of the copolymer chain and copolymers having an anchor at the terminal of the copolymer chain. Calcein-loaded DOPE liposomes modified with these copolymers were prepared and temperature-dependent release of the contents from these liposomes was investigated. We found that release of calcein from the liposomes was triggered by the hydrophilic-to-hydrophobic change of the copolymer chains. Also, the liposomes modified with the terminal anchor-type copolymer exhibited a more drastic enhancement of the contents release responding to a small temperature change than the liposomes modified with the middle anchor-type copolymers.

2. Materials and methods

2.1. Materials

L-α-Dioleoylphosphatidylethanolamine (DOPE) was purchased from Sigma (St. Louis, MO, USA). *N*-Isopropylacrylamide (NIPAM), *N,N*-didodecylamine and 2,6-di-*tert*-butyl-*p*-cresol, 2-aminoethanethiol were obtained from Tokyo Kasei (Tokyo, Japan). Acryloyl chloride and pyrrolidine were supplied by Wako Pure Chemical Industries (Osaka, Japan). Azobis(isobutyronitrile) (AIBN), tris(hydroxymethyl)aminomethane (Tris), *N,N*-dimethylformamide, and ethylenediaminetetraacetic acid (EDTA) were supplied by Kishida Chemical (Osaka, Japan). NIPAM and AIBN were purified by recrystallization from benzene-*n*-hexane and methanol, respectively, before use.

2.2. Synthesis of N,N-didodecylacrylamide and acryloylpyrrolidine

N,N-Didodecylacrylamide (NDDAM) and acryloylpyrrolidine were prepared by acylation of the amines with acryloyl chloride as previously reported [32,33]. *N,N*-Didodecylamine or pyrrolidine (50 mmol), triethylamine (55 mmol) and 2,6-di-*tert*-butyl-*p*-cresol (0.11 mmol) were dissolved in methylene chloride (150 ml). To the solution, acryloyl chloride (60 mmol) was added dropwise under N_2 atmosphere at 40°C. The reaction mixture was stirred for 2 h and then washed with 0.1 N HCl, water, saturated $NaHCO_3$ solution, and water and dried over $MgSO_4$. The solvent was removed by evaporation and the crude product was purified by recrystallization from acetone (NDDAM) or silica gel column chromatography eluting with 3.4% methanol-chloroform (APr).

2.3. Synthesis of APr-NIPAM copolymers

The synthetic routes for APr-NIPAM copolymers, APr-NIPAM-NDDAM copolymers and an APr-NIPAM copolymer having didodecyl group at the terminal (poly(APr-NIPAM)-$2C_{12}$) are shown in Fig. 1. APr-NIPAM copolymers with various compositions were prepared according to the method previously reported [23,25,27]. In brief, APr and NIPAM (total 44 mmol) and AIBN (0.22 mmol) were dissolved in freshly distilled dioxane (88 ml) and then the solutions were heated at 60°C for 18 h in N_2 atmosphere. The copolymers were recovered by precipitation with diethylether. The copolymers were dissolved in dioxane again, reprecipitated with diethylether and then dried under vacuum. APr-NIPAM copolymers having anchors in the middle of the polymer chain, namely poly(APr-co-NIPAM-co-NDDAM)s, were prepared by the above method except that NDDAM (0.66 mmol) was added as a comonomer. The terminal anchor-type copolymer, namely poly(APr-NIPAM)-$2C_{12}$, was prepared according to the method reported by Okano and coworkers [34]. APr (27.2 mmol), NIPAM (6.8 mmol), 2-aminoethanethiol (1.87 mmol) and AIBN (0.36 mmol) were dissolved in *N,N*-dimethylformamide (15 ml) and heated at 75°C for 15 h in N_2 atmosphere. The copolymer was recovered by precipitation with diethylether and then purified using an LH-20 column eluting with methanol. The copolymer having an amino group at the terminal (1.0 g) was reacted with *N,N*-didodecylsuccinamic acid (6×10^{-4} mol), which was prepared according to the method

of Okahata et al. [35], by using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (6×10^{-4} mol) in *N,N*-dimethylformamide (10 ml) at 4°C for 2 days. The copolymer was purified by an LH-20 column eluting with methanol.

2.4. Estimation of LCST of copolymers

The LCST of the copolymer was detected by cloud point [36]. Transmittance of aqueous copolymer solutions (5 mg/ml) at 500 nm was monitored using a spectrophotometer (Jasco V-520) with a water-jacketed cell holder coupled with a circulating bath. Temperature was raised at 0.3°C/min. Cloud points were taken as the initial break points in the resulting transmittance vs. temperature curves.

2.5. Liposome preparation

The liposomes were prepared as follows: DOPE in chloroform solution (1 mg/ml, 10 ml) and varying volumes of the copolymer in chloroform solution (10 mg/ml) were mixed in a flask and the solvent was removed by evaporation. The thin lipid/copolymer membrane obtained was further dried under vacuum overnight and dispersed in 1.5 ml of aqueous calcein solution (63 mM, pH 9.0) using a bath-type sonicator. The resultant liposome suspension was extruded through a polycarbonate membrane with a pore diameter of 100 nm at 0°C. Free calcein and free copolymer were removed by gel permeation chromatography on a Sepharose 4B column at 4°C using 10 mM Tris-HCl-buffered solution containing 140 mM NaCl and 1 mM EDTA at pH 7.4. The liposomes were kept at 5°C until the measurement.

2.6. Estimation of the amount of copolymer bound to liposome

The amount of the copolymer bound to the liposome was estimated using high performance liquid chromatography analysis as reported previously [27]. The liposomes bearing the copolymer were dried under vacuum and then dissolved in methanol. The solution was filtered through a poly(tetrafluoroethylene) membrane with a pore size of 0.25 μm. The filtrate (20 μl) was injected into an SB-803 column and the effluent was monitored by the absorbance at 210 nm using a UV detector (Jasco, UV-790). From the absorbance of the copolymer separated, the amount of copolymer was estimated. Also, the concentration of the lipid was determined by the method of Bartlett [37].

2.7. Calcein release from liposomes

The release measurements were performed according to the method previously reported [23,25]. An aliquot of dispersion of the calcein-loaded liposomes was added into 2 ml of 10 mM Tris-HCl, 140 mM NaCl and 1 mM EDTA solution (pH 7.4) in a quartz cell (final concentration of the lipid 5.0 μM) at a given temperature and the fluorescence intensity of the solution was monitored using a spectrofluorometer (Hitachi MPF-4). The excitation and monitoring wavelength were 490 nm and 520 nm, respectively. The percent release of calcein from the liposomes was defined as:

$$\%\ \text{release} = (F^{t}-F^{i})/(F^{f}-F^{i})\times 100 \quad (1)$$

where F^{i} and F^{t} mean the initial and intermediary fluorescence intensities of the liposome suspension, respectively. F^{f} is the fluorescence intensity of the liposome suspension after the addition of Triton X-100 (final concentration 0.15%). Since the release of calcein was very fast above the LCST of the copolymers, it was impossible to determine F^{i}. In contrast, the release was slow below the LCST and liposomes hardly release calcein at 5°C. Therefore, we used the initial fluorescence intensity of the liposome suspension at 5°C as F^{i}. The change in fluorescence intensity depending on temperature was corrected although the change of the intensity was not significant under the experimental conditions.

2.8. Other methods

Nuclear magnetic resonance (NMR) spectra were taken with a JEOL JNM-GX 270 MHz instrument. The size of the liposomes was evaluated by dynamic light scattering using a 380 ZLS instrument (Nicomp). The weight average molecular weight (Mw) and the number average molecular weight (Mn) of the copolymers were estimated using gel permeation chromatography on a system equipped with a Sho-

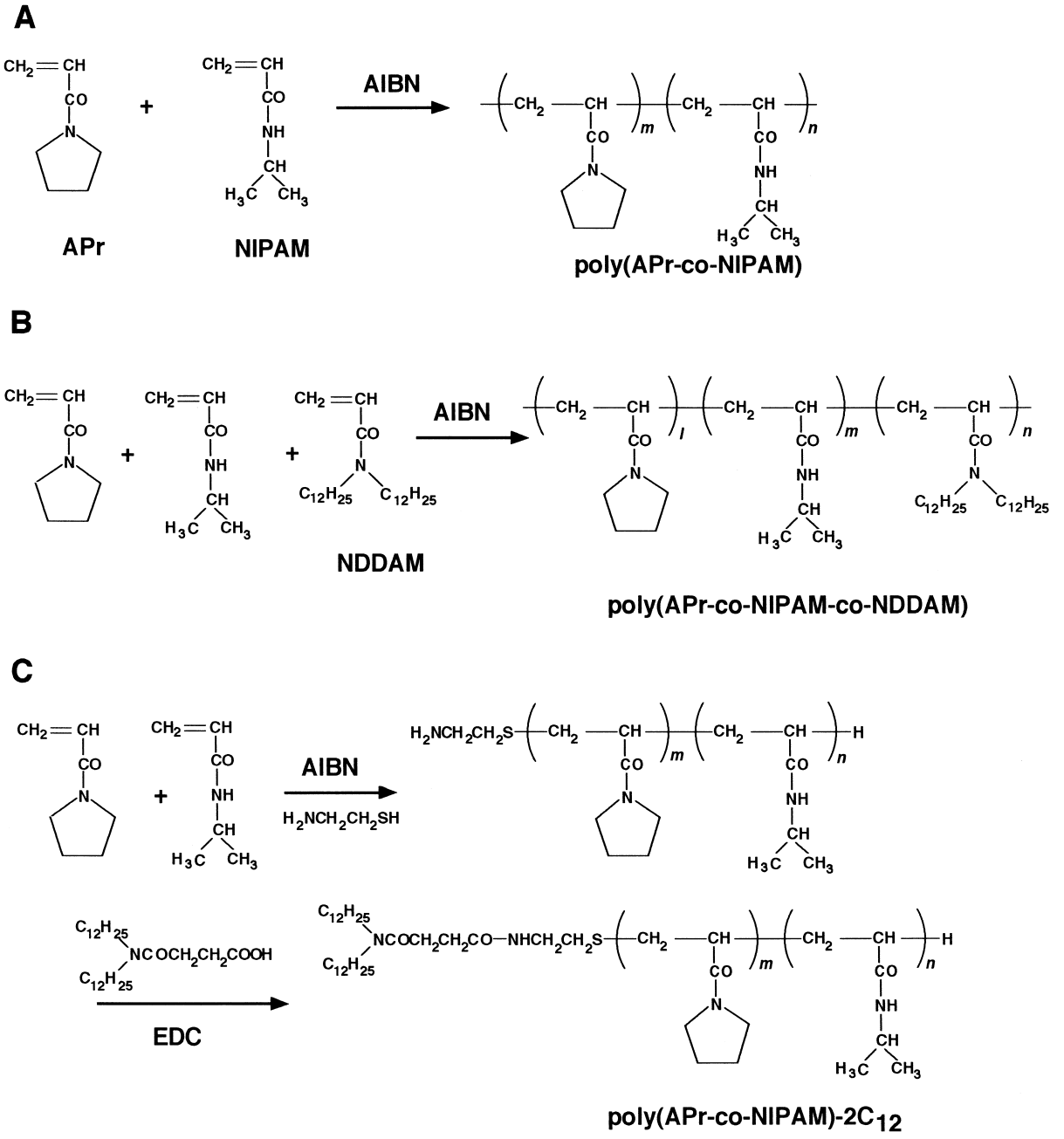

Fig. 1. Synthetic routes for thermosensitive polymers. A: Poly(APr-co-NIPAM); B: poly(APr-co-NIPAM-co-NDDAM); C: poly-(APr-co-NIPAM)-2C_{12}.

dex KD-803 column (Showa Denko) with differential refractive index detection (Jasco, RI-930) using *N,N*-dimethylformamide at 0.3 ml/min as an eluent at 40°C. Polyethylene glycol standards in the range 20 000–1000 g/mol were used to calibrate the gel permeation chromatography.

3. Results and discussion

3.1. LCST of copoly(APr-NIPAM)

Three kinds of poly(APr-co-NIPAM)s and poly-(APr) were synthesized. Composition and molecular

Table 1
Preparation of poly(APr-co-NIPAM)

Polymer	Feed	Composition	Mw	Mn
	APr/NIPAM (mol/mol)	APr/NIPAM (mol/mol)		
1	100/0	100/0	10 800	4800
2	90/10	90.5/ 9.5	11 000	5300
3	80/20	79.8/20.2	17 700	8300
4	70/30	68.2/31.8	18 000	6700

weight of the polymer and copolymers were estimated by ^{1}H-NMR and GPC, respectively, and are listed in Table 1. The LCST of the polymer and copolymers were evaluated from cloud point of the aqueous polymer solutions. Fig. 2 illustrates typical results of cloud point measurement. These polymer and copolymer solutions revealed a drastic decrease in transmittance in narrow temperature regions, indicating the occurrence of the transition of the polymers. Fig. 3 shows a relationship between NIPAM content and LCST of the copolymer. The LCST decreased with increasing NIPAM content in the copolymer chain. Taylor and Cerankowski have shown that the LCST decreases with increasing polymer hydrophobicity [31]. Because hydrophobicity of the NIPAM unit might be higher than that of the APr unit, overall hydrophobicity of the copolymer chain becomes higher with increasing NIPAM unit content, resulting in a decrease in LCST. This result shows that LCST of the copolymer can be controlled by the NIPAM/APr ratio.

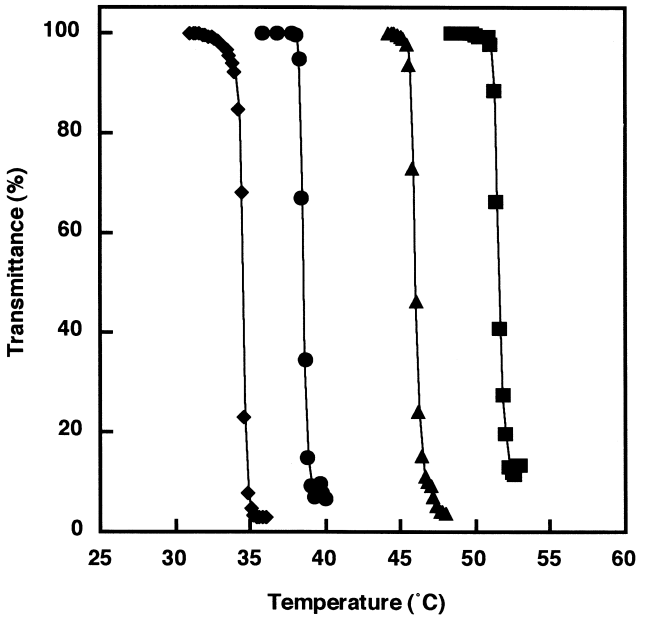

Fig. 2. Cloud point curves for aqueous solutions of poly(APr) (■) and poly(APr-co-NIPAM)s with APr/NIPAM (mol/mol) ratio of 90.5:9.5 (▲), 79.8:20.2 (●), and 68.2:31.8 (♦).

3.2. Release property of copolymer-modified liposomes

We and other groups have already shown that poly(NIPAM) [23,38,39] as well as other hydrophilic polymers [14,40] can be bound to liposomes by the conjugation of hydrophobic groups to the polymers. We prepared in this study poly(APr-co-NDDAM) and two kinds of poly(APr-co-NIPAM-co-NDDAM)s as the thermosensitive polymers having anchor groups. The composition, molecular weight and LCST of these copolymers are summarized in Table 2. The LCSTs of poly(NIPAM-co-APr-co-NDDAM)s were remarkably lower than those of the NIPAM-APr copolymers with the same NIPAM/APr ratio due to the existence of hydrophobic anchor groups.

Three kinds of poly(APr-co-NDDAM) (polymer-5)-modified liposomes with different polymer contents (0.12, 0.25 and 0.51 mg polymer/mg lipid) were made. The liposome with the composition of

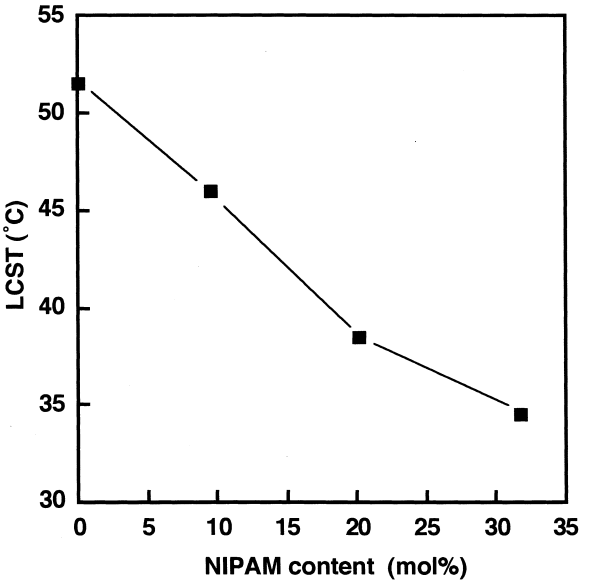

Fig. 3. Lower critical solution temperature of poly(APr-co-NIPAM) as a function of NIPAM content.

Table 2
Preparation of poly(APr-co-NIPAM-co-NDDAM)

Polymer	Feed	Composition	Mw	Mn	LCST (°C)
	APr/NIPAM/NDDAM (mol/mol/mol)	APr/NIPAM/NDDAM (mol/mol/mol)			
5	98.5/0/1.5	97.9/0/2.1	10 800	4800	34.2
6	88.7/9.8/1.5	88.7/8.9/2.4	13 300	5600	33.6
7	78.8/19.7/1.5	79.4/18.5/2.1	18 800	7740	25.6

0.12 mg polymer/mg lipid was not stable even at 5°C, indicating that this amount of the polymer is not sufficient to stabilize DOPE liposomes. Also, the liposome with the composition of 0.51 mg polymer/mg lipid revealed a slightly lower temperature sensitivity than the liposome with the composition of 0.25 mg polymer/mg lipid, suggesting that when too many polymer chains are bound on the liposome surface, not all polymer chains participate in destabilization of the liposome (result not shown). Thus, we chose ca. 0.25 mg polymer/mg lipid as the composition of polymer/modified liposomes. Preparation condition, diameter and amount of polymer of the liposomes used in this study are listed in Table 3. These liposomes had diameters of about 100 nm, which corresponds to the pore diameter of the polycarbonate membrane used for their preparation. Efficiency of fixation of the polymer on the liposome increased in the order of polymer-5 < polymer-6 < polymer-7. While these polymers revealed approximately the same NDDAM unit content, average numbers of the anchor per polymer chain are calculated to be 0.8 (polymer-5), 1.0 (polymer-6) and 1.3 (polymer-7) from the number average molecular weight. Thus, it is considered that the number of the anchor per polymer chain affected the fixation efficiency of the polymer to the liposome.

Fig. 4A represents a typical example of release profiles of poly(APr-co-NDDAM)-modified DOPE liposomes at varying temperatures. Also, typical release profiles for a prolonged period are shown in Fig. 4B. It is apparent that release of calcein from the polymer-modified liposomes is temperature-dependent. The liposomes hardly released the contents at 25°C. However, the extent of the release increased with rising temperature: 41 and 64% of the liposome contents were released in 5 min at 40 and 45°C, respectively. At 50°C, a drastic release occurred immediately after changing temperature. As is seen in Fig. 4B, the rate of the contents release decreased significantly with time. After 10 min, the liposomes have reached a plateau (ca. 94%) for the contents release at 50°C. Similarly, the extent of release became almost constant (ca. 68%) at 45°C after 20 min. A very slow release continued for a prolonged period at 40°C, but the release stabilized around 50% after ca. 60 min. While the final extent of the contents release varied depending on temperature, the most part of the contents release took place within 10–15 min and then the release became very slow or had

Table 3
Preparation of DOPE liposomes modified with thermosensitive polymers

Polymer	Polymer added (mg/mg lipid)	Polymer fixed (mg/mg lipid)	Diameter (nm)
APr/NIPAM/NDDAM			
Polymer-5	0.50	0.25 ± 0.04	87.4
	1.00	0.51 ± 0.02	86.1
Polymer-6	0.30	0.24 ± 0.01	93.5
Polymer-7	0.25	0.23 ± 0.01	107.3
APr/NIPAM-2C_{12}			
Polymer-8	0.50	0.26 ± 0.04	109.0

stopped at all temperatures. Similar release behaviors were observed for all APr-NIPAM-NDDAM-co-polymer-modified DOPE liposomes. We have already shown that disintegration of poly(NIPAM)-coated DOPE liposomes is induced above the transition temperature of the polymer because dehydrated polymer chains do not stabilize DOPE liposomes and promote the bilayer-to-hexagonal II transition of the liposome membrane [25]. Therefore, it is considered that a certain fraction of the liposomes ruptured rapidly due to the bilayer-to-hexagonal II transition induced by the interaction with polymer chains of increased hydrophobicity.

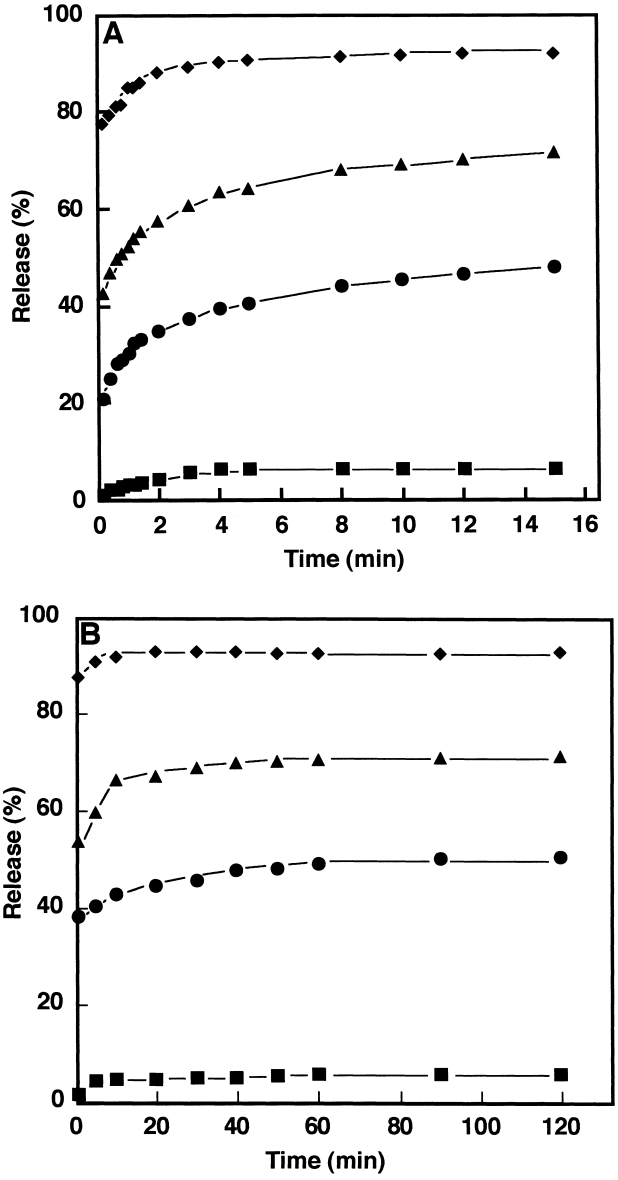

Fig. 4. Release profiles of calcein from DOPE liposomes modified with poly(APr-co-NDDAM) at various temperatures: 25°C (■), 40°C (●), 45°C (▲), 50°C (◆). The APr/NDDAM ratio of the polymer was 97.9:2.1 (mol/mol). A: Short period-release experiment. B: Prolonged period-release experiment.

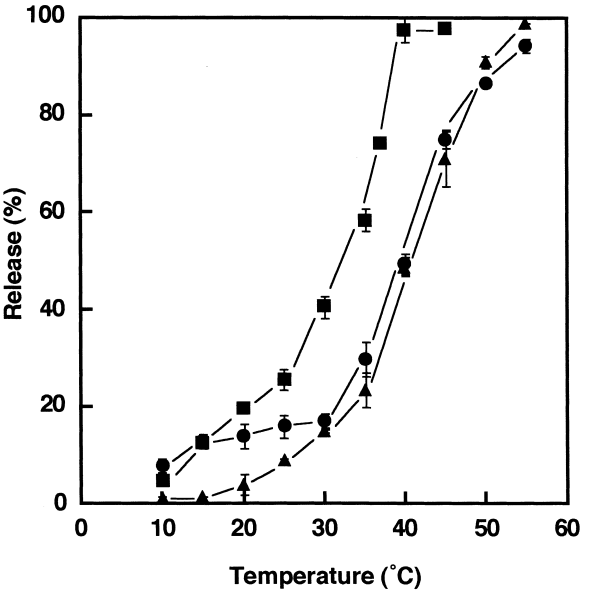

Fig. 5. Percent release of calcein from DOPE liposomes modified with poly(APr-co-NIPAM-co-NDDAM)s with various APr/NIPAM/NDDAM (mol/mol/mol) ratios: 97.9:0:2.1 (▲), 88.7:8.9:2.4 (●), 79.4:18.5:2.1 (■). Percent release after 15 min incubation was shown.

However, a very slow release following the initial fast release was also observed for a prolonged time at 40°C. It is known that interbilayer contact between DOPE liposomes induces destabilization of the liposomes [41]. The contact between the DOPE liposomes with partly dehydrated polymer chains may induce the gradual destabilization of the liposomes. We examined the influence of liposome concentration on the contents release at 40°C. However, the release was not affected by the liposome concentration, indicating that the interbilayer contact was not involved in the contents release (result not shown). Although the LCST of poly(APr) is ca. 52°C, some portions in the polymer chain might be dehydrated at 40°C as mentioned below. It is considered that a weak interaction between slightly dehydrated polymer chains and the liposome membrane caused a slow and transient leakage of the contents from the liposomes and/or gradual disintegration of the liposomes which do not have sufficient amounts

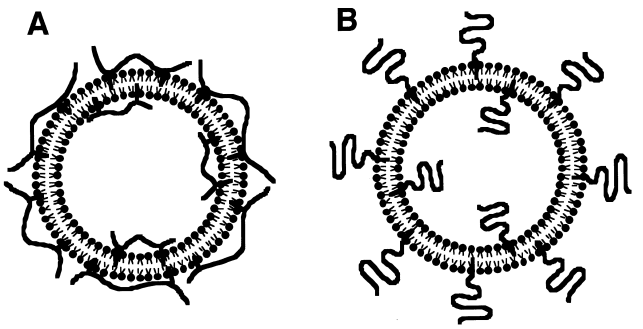

Fig. 6. Schematic illustration of liposomes modified with middle anchor-type polymer (A) and terminal anchor-type polymer (B).

of polymer chains to induce their prompt disintegration.

The temperature dependence of calcein release from APr-NIPAM-NDDAM copolymer-modified DOPE liposomes is shown in Fig. 5. These liposomes hardly released their contents under the low temperature condition but the release was enhanced significantly from particular temperatures as the temperature was raised. While it is difficult to determine precisely the temperatures from which enhancement of the release starts, they are around 35, 30, and 25°C for the liposomes modified with polymer-5, polymer-6 and polymer-7, respectively. Also, temperatures at which 50% release was obtained for these liposomes are 41, 40 and 32°C, respectively.

If the anchor groups on the polymer chain are inserted into the lipid membrane, these groups might not affect the transition of the polymer chain strongly. Thus, the transition temperatures of polymer-5, polymer-6 and polymer-7 may be considered from the relationship between LCST and NIPAM content (Fig. 3) to be around 52, 46, and 39°C, respectively. Apparently, promotion of the release from these polymer-modified liposomes started at lower temperatures than these transition temperatures. This is probably because partial dehydration of the polymer chain takes place below its transition temperature and dehydrated units in the polymer chain interact with the liposome membrane, inducing leakage of the contents to some extent. Although the contents release was promoted from a temperature lower than LCST of the copolymer, the liposomes having polymer chains with a higher LCST revealed enhancement of the release in a higher temperature region. This result implies that the release was triggered by the hydrophilic-to-hydrophobic change of the polymer chains.

3.3. Release property of liposomes modified with copolymers having an anchor at the terminal

As mentioned above, the APr-NIPAM-NDDAM copolymer-modified DOPE liposomes promoted the contents release in specific temperature regions, depending on the composition of the copolymer. However, promotion of the release from the liposomes occurred in a relatively wide temperature region, compared with the region where the water-solubility change of the polymer takes place (Fig. 2). Okano and his coworkers have reported that the poly(NIPAM) chain grafted to a surface at the terminal of the polymer chain undergoes the conformational transition more efficiently than that grafted at arbitrary and plural points in the polymer chain [42]. As shown in Fig. 6A, in the case of the APr-NIPAM-NDDAM copolymer-modified liposomes, many polymer chains are considered to be attached to the liposome surface at arbitrary and plural points in the polymer chain and, hence, its freedom of conformation might be reduced to some extent. In contrast, when the polymer chain is attached to the liposome

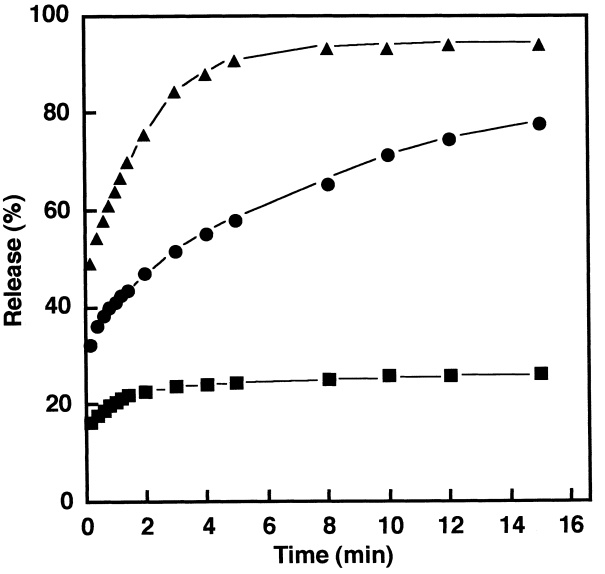

Fig. 7. Release profiles of calcein from DOPE liposomes modified with poly(APr-co-NIPAM)-2C_{12} at various temperatures: 37°C (■), 41°C (●), 42°C (▲). The APr/NIPAM ratio of the polymer was 81.6:18.4 (mol/mol).

surface at its terminal (Fig. 6B), more efficient transition of the copolymer chain is expected to take place. Thus, we prepared poly(APr-co-NIPAM)-$2C_{12}$ (polymer-8) and the release property of the DOPE liposome modified with this copolymer was investigated.

The APr/NIPAM (mol/mol) ratio of polymer-8 was estimated to be 81.6/18.4 by ^{1}H-NMR. The weight and number average molecular weights of the polymer before conjugation of the anchor were estimated by gel permeation chromatography analysis to be 9200 and 4100, respectively. The LCST of the copolymer before and after conjugation of the anchor was 39.6 and 36.4°C, respectively. The calcein-loaded DOPE liposomes modified with this copolymer were prepared. The diameter of the liposome was estimated to be 109 nm by dynamic light scattering (Table 3).

The release profiles of calcein from the poly(APr-co-NIPAM)-$2C_{12}$-modified liposome at various temperatures are shown in Fig. 7. While only a small portion of the contents was released at 37°C, the release became more drastic with raising temperature. At 42°C the liposomes released the contents almost completely in 5 min. Also, a remarkable release took place at 41°C, although the release rate during the first several minutes was slower than at 42°C. The release continued until 15 min and then reached a plateau very slowly in another 25 min at 41°C (results not shown). Because the polymer chain is less hydrophobic at 41°C than at 42°C, prompt destabilization of the liposome could not be induced at 41°C.

Fig. 8 depicts temperature dependence of the calcein release from the poly(APr-co-NIPAM)-$2C_{12}$-modified liposome. For comparison, the result of DOPE liposomes modified with the poly(APr-co-NIPAM-co-NDDAM) with approximately the same APr/NIPAM ratio (polymer-7) is also shown in Fig. 8. As described above, the middle anchor-type polymer-modified liposome enhanced release of the contents in a broad temperature region. In the case of the terminal anchor-type polymer-modified liposome, limited extents of release were observed in the temperature region of 10–35°C. However, an intensive enhancement of the release occurred around 40°C, which is the same as the LCST of the copolymer before the anchor attachment. Because in this

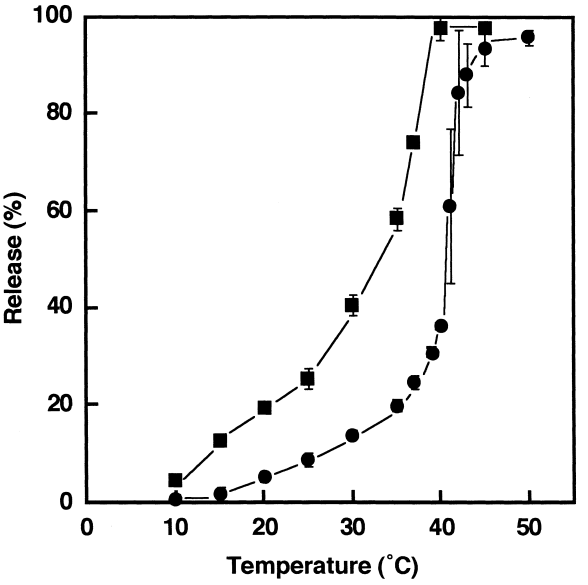

Fig. 8. Percent release of calcein from DOPE liposomes modified with poly(APr-co-NIPAM)-$2C_{12}$ (●) and poly(APr-co-NIPAM-co-NDDAM) (■). The APr/NIPAM (mol/mol) and APr/NIPAM/NDDAM (mol/mol/mol) ratios of these polymers were 81.6:18.4 and 79.4:18.5:2.1, respectively. Percent release after 15 min incubation was shown.

case the polymer chain was bound to the liposome membrane at the terminal, its mobility might not be restricted so strongly as was the APr-NIPAM-NDDAM copolymer chain which was attached onto the liposome membrane at arbitrary and plural points in the polymer chain. Thus, the terminal anchor-type polymer could undergo the conformational transition in the narrow temperature region on the liposome membrane as the polymer chain does in an aqueous solution, resulting in a sharp increase in the contents release.

Because anchor groups in the copolymer chains are considered to be buried in the liposome membrane [38], it is supposed that these groups do not affect the transition of the copolymer chains strongly. The APr/NIPAM ratios of the middle anchor-type and the terminal anchor-type polymers in Fig. 8 are 19:81 and 18:82, respectively, and, hence, these polymer chains are considered to undergo the transition at about 39–40°C (Fig. 3). As is seen in Fig. 8, the contents release from the poly(APr-co-NIPAM-co-NDDAM)-modified liposomes was enhanced strongly in the temperature region between 35 and 40°C. However, enhancement of the release was also

seen below the transition temperature of the polymer. The transition of the polymer chain in aqueous solutions is related to its solubility. The polymer chain becomes insoluble in water at that temperature. However, partial dehydration of the polymer chain might occur below that temperature, although the whole polymer chain is still soluble in water at that temperature. Also, the degree of dehydration of the polymer chain should increase with temperature. It is likely that the dehydrated units in the polymer chain interact with the membrane and induce partial release of the contents or rupture of a small fraction of the liposomes even below the transition temperature of the polymer.

Similarly, such partial dehydration might take place in the polymer chain bound to the liposome surface at the terminal. However, in this case, the dehydrated units should be covered by hydrated units of the polymer chain, due to greater chain mobility resulting from single-point attachment than the middle anchor-type polymer chain. Because interaction between the partly dehydrated polymer chain and the liposome membrane is suppressed below the transition temperature, a drastic and intensive release of the contents was induced for the poly(APr-co-NIPAM)-2C_{12}-modified liposome at the polymer transition temperature, where the whole polymer chain becomes hydrophobic.

4. Conclusion

The APr-NIPAM copolymers with LCSTs around the physiological temperature were prepared by controlling the APr/NIPAM ratio of the copolymer. DOPE liposomes modified with the NIPAM-APr copolymers having an anchor release their contents around the transition temperature of the copolymers. Thus, novel temperature-sensitive liposomes, which release their contents near the physiological temperature, were obtained by the modification of DOPE liposomes with these copolymers. Temperature sensitivity of the copolymer-modified liposomes was affected by the method for the polymer attachment: when the terminal anchor-type copolymer was used for the modification, the polymer-modified liposomes revealed a more drastic release at a narrow temperature region than the liposomes modified with the middle anchor-type polymers.

We showed in this study that the liposomes modified with a particular NIPAM/APr ratio released their contents slightly above the physiological temperature, which is the original concept of the temperature-sensitive liposomes [2]. In addition, liposomes modified with these polymers are considered to change their surface property, depending on temperature. We have already shown that aggregation of liposomes coated with a NIPAM copolymer occurs above the transition temperature of the copolymer, because the hydrophobic copolymer chains make the liposome surface hydrophobic [27]. If liposomes having thermosensitive polymer grafts are injected into the blood stream, the highly hydrated polymer chains may reduce interaction between liposomes and proteins and improve circulation time in the blood stream as polyethylene glycol drafts do [19]. However, such effects of the copolymer chain will disappear above the LCST and interaction of the liposomes with the blood components might be enhanced by the hydrophobic copolymer chains. Therefore, the biodistribution of the liposomes may be controlled by temperature. These features of the thermosensitive polymer-modified liposomes may allow their use as a new drug delivery system with temperature-controlled functionalities which the conventional temperature-sensitive liposomes do not possess.

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