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Temperature-controlled interaction of thermosensitive polymer-modified cationic liposomes with negatively charged phospholipid membranes

Kenji Kono [a, b,*], Akiko Henmi [a], Toru Takagishi [a,b]

[a] *Department of Applied Materials Science, College of Engineering, Osaka Prefecture University, 1-1 Gakuen-cho, Sakai, Osaka 599-8531, Japan*

[b] *Department of Applied Bioscience, Research Institute for Advanced Science and Technology, Osaka Prefecture University, 1-2 Gakuen-cho, Sakai, Osaka 599-8570, Japan*

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Abstract

To obtain cationic liposomes of which affinity to negatively charged membranes can be controlled by temperature, cationic liposomes consisting of 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl]cholesterol and dioleoylphosphatidylethanolamine were modified with poly(*N*-acryloylpyrrolidine), which is a thermosensitive polymer exhibiting a lower critical solution temperature (LCST) at ca. 52°C. The unmodified cationic liposomes did not change its zeta potential between 20–60°C. The polymer-modified cationic liposomes revealed much lower zeta potential values below the LCST of the polymer than the unmodified cationic liposomes. However, their zeta potential increased significantly above this temperature. The unmodified cationic liposomes formed aggregates and fused intensively with anionic liposomes consisting of egg yolk phosphatidylcholine and phosphatidic acid in the region of 20–60°C, due to the electrostatic interaction. In contrast, aggregation and fusion of the polymer-modified cationic liposomes with the anionic liposomes were strongly suppressed below the LCST. However, these interactions were enhanced remarkably above the LCST. In addition, the polymer-modified cationic liposomes did not cause leakage of calcein from the anionic liposomes below the LCST, but promoted the leakage above this temperature as the unmodified cationic liposomes did. Temperature-induced conformational change of the polymer chains from a hydrated coil to a dehydrated globule might affect the affinity of the polymer-modified cationic liposomes to the anionic liposomes.

Keywords: Temperature-sensitive liposome; Cationic liposome; Lower critical solution temperature; Poly(*N*-acryloylpyrrolidine); Membrane fusion

Abbreviations: LCST, lower critical solution temperature; DC-Chol, 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl]cholesterol; DOPE, dioleoylphosphatidylethanolamine; APr, *N*-acryloylpyrrolidine; NDDAM, *N*,*N*-didodecylacrylamide; poly(APr)-2C_{12}, poly(APr) having two dodecyl groups at the terminal; PA, phosphatidic acid; EYPC, egg yolk phosphatidylcholine; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; Rh-PE, lissamine rhodamine B-sulfonyl phosphatidylethanolamine; EDTA, ethylenediaminetetraacetic acid; *R*, fluorescence intensity ratio of NBD-PE to Rh-PE; NMR, nuclear magnetic resonance

* Corresponding author. Fax: +81-722-54-9913; E-mail: kono@ams.osakafu-u.ac.jp

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1. Introduction

In order to obtain liposomes with various functionalities, conjugation of polymers to liposome surfaces has been attempted [1,2]. Fixation of pH-sensitive polymers, such as poly(2-ethylacrylic acid) and poly(acrylic acid), has been reported to provide liposomes with pH-sensitive release property [3,4]. Also, modification of liposomes with succinylated poly(glycidol), which is a polyethylene glycol derivative with pH-sensitivity, has been shown to give liposomes with pH-sensitive fusion property [5,6]. In addition, attachment of various hydrophilic polymers, such as polysaccharides and polyethylene glycol, to liposome surface is known to increase its stability [7,8].

Recently, temperature sensitization of liposomes has been attempted using thermosensitive polymers such as poly(*N*-isopropylacrylamide) [9–12]. It is known that poly(*N*-isopropylacrylamide) has a lower critical solution temperature (LCST) at 30–35°C [13,14]. The polymer is soluble in water and takes on a hydrated coil below the LCST. However, the polymer becomes insoluble in water and exhibits a dehydrated globule above this temperature [15]. Therefore, when poly(*N*-isopropylacrylamide) chains are fixed on liposome surfaces, these polymer chains cover the liposome surface below LCST. However, collapse of the polymer chains above this temperature causes exposure of the bare liposome surface [12].

Cationic liposomes can interact strongly with cell surface and deliver DNA into the cell [16–19]. Thus, they are widely used as vehicles for gene transfer into a variety of cells. Their ability is based on nonspecific electrostatic interactions with negatively charged cell membranes. Therefore, if cationic liposomes are coated with thermosensitive polymers, the liposomes will be covered with a highly hydrated layer of the polymer chains which may suppress their interaction with cell membranes below the LCST. However, the positively charged surface of the liposomes will be exposed by shrinkage of the polymer chains above this temperature. Thus, their affinity to cell surfaces is expected to be controlled by ambient temperature.

The object of this study is to examine temperature-induced control of affinity of cationic liposomes to negatively charged membranes using thermosensitive polymers. In this study, liposomes consisting of 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl]cholesterol (DC-Chol) and dioleoylphosphatidylethanolamine (DOPE) were used as a cationic liposome, because liposomes of this formulation are known to have a high potential for gene delivery [17,20,21]. We designed the cationic liposomes modified with poly(*N*-acryloylpyrrolidine) [poly(APr)], which is a thermosensitive polymer exhibiting a LCST at ca. 52°C [22]. Two kinds of polymers, a copolymer of *N*-acryloylpyrrolidine (APr) and *N*,*N*-didodecylacrylamide (NDDAM) and poly(APr) having two dodecyl groups at the terminal [poly(APr)-2C_{12}], were synthesized as poly(APr) having anchoring groups (Fig. 1). DC-Chol/DOPE liposomes modified with these polymers were prepared and their interaction with anionic liposomes consisting of phosphatidic acid (PA) and egg yolk phosphatidylcholine (EYPC) was investigated. Temperature-controlled interaction of the polymer-modified cationic liposomes with the anionic liposomes has been described.

2. Materials and methods

2.1. Materials

DOPE, EYPC, and calcein were purchased from Sigma (St. Louis, MO, USA). PA from egg lecithin, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (NBD-PE) and lissamine rhodamine B-sulfonyl phosphatidylethanolamine (Rh-PE) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). 2-Aminoethanethiol was from Tokyo Kasei (Tokyo, Japan). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide was supplied from Wako Pure Chemical Industries (Osaka, Japan). Azobis(isobutyronitrile) (AIBN) and ethylenediaminetetraacetic acid (EDTA) were from Kishida Chemical (Osaka, Japan). AIBN was purified by recrystallization from methanol before use. NDDAM and APr were prepared as described elsewhere [22,23]. *N*,*N*-Didodecylsuccinamic acid was prepared according to the method of Okahata et al. [24]. DC-Chol was synthesized according to the method of Gao and Huang [20].

2.2. Synthesis of polymers

Two kinds of polymers were prepared according to

the method previously reported [22]. For the synthesis of poly(APr-co-NDDAM), APr (44 mmol), NDDAM (0.66 mmol) and AIBN (0.22 mmol) were dissolved in freshly distilled dioxane (88 ml) and then, the solution was heated at 60°C for 15 h in N_2 atmosphere. The polymer was recovered by precipitation with diethylether. The polymer was dissolved in dioxane again, reprecipitated with diethylether and then dried under vacuum. For the synthesis of poly(APr)-2C_{12}, APr (34 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 polymer was recovered by precipitation with diethylether and then purified using a LH-20 column eluting with methanol. The polymer having an amino group at the terminal (1.0 g) was reacted with *N*,*N*-didodecylsuccinamic acid (0.6 mmol) by using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (0.6 mmol) in *N*,*N*-dimethylformamide (10 ml) at 4°C for 2 days. The polymer was purified by gel permeation chromatography on a LH-20 column eluting with methanol.

2.3. Estimation of LCST of polymers

The LCST of the polymers was detected by cloud point [14,22]. Transmittance of aqueous polymer 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 versus temperature curves.

2.4. Liposome preparation

Liposomes were prepared by reverse phase evaporation [25]. To DOPE (8.9 mg), DC-Chol (6.0 mg) and polymer (17.8 mg) in chloroform solution (3 ml) was added 10 mM Tris–HCl, 140 mM NaCl and 1 mM EDTA solution (pH 7.4, 1 ml) and the mixed solution was sonicated using a bath-type sonicator for 5 min, forming a homogeneous emulsion. Chloroform was removed from the emulsion by evaporation. The liposome suspension was extruded through a polycarbonate membrane with a pore size of 100 nm. Free polymer was removed by gel permeation chromatography on a Sepharose 4B column using ice-cooled 10 mM Tris–HCl, 140 mM NaCl and 1 mM EDTA solution (pH 7.4). EYPC/PA liposomes were obtained via the same method using EYPC and PA (3:1, mol/mol, 9.4 mg) in chloroform solution. Liposomes encapsulating calcein were prepared via the same method using an aqueous calcein solution (63 mM, pH 7.4) instead of Tris–HCl buffered solution. Liposomes were kept at 4°C until measurements.

2.5. Size of liposome

Size of liposome was measured at 20°C by dynamic light scattering using a laser particle analyzing system (Nicomp, 380 ZLS). For the measurement of cationic liposome-anionic liposome aggregates, cationic liposome and anionic liposome suspensions were mixed and incubated at a given temperature for 5 min at concentrations of 50 μM (DC-Chol/DOPE) and 100 μM (EYPC/PA). Then size of the aggregates formed in the suspension was measured at 20°C by the same method.

2.6. Fusion of liposomes

Fusion of liposomes was detected by resonance energy transfer between NBD-PE and Rh-PE as reported by Struck et al. [26]. Cationic liposomes containing NBD-PE (0.6 mol%) and Rh-PE (0.6 mol%) were prepared according to the above method. An aliquot of dispersion of the cationic liposome containing these fluorescent probes was added to 2 ml of 10 mM Tris–HCl, 140 mM NaCl and 1 mM EDTA solution (pH 7.4) in a quartz cell at a given temperature (final concentration of DC-Chol/DOPE, 50 μM). Then, PA/EYPC liposomes were added to the cell (final concentration of EYPC/PA, 100 μM) and the fluorescence intensities of NBD-PE and Rh-PE in the liposome suspension were monitored using the spectrofluorometer (Shimadzu RF-5000). Fusion of these liposomes was followed by monitoring the fluorescence intensity ratio of NBD-PE to Rh-PE (*R*) [27]. The excitation wavelength for NBD-PE was 485 nm and monitoring wavelengths for NBD-PE and Rh-PE were 520 nm and 580 nm, respectively.

2.7. Electron microscopy

DC-Chol/DOPE liposome suspension (2 mM) and EYPC/PA liposome suspension (4 mM) of the same volume were mixed and incubated for 5 min at 10°C or 60°C with gentle agitation. A small drop of the liposome sample was placed on a collodion-coated grid and drawn off with filter paper. A drop of 2% (w/v) phosphotungstic acid was applied to the grid, drawn off with filter paper, and then allowed to dry. The grid was viewed under an electron microscope (JEOL, JEM-2000FEX II).

2.8. Calcein leakage from liposomes

Calcein leakage from liposomes was measured according to the method previously reported [5]. An aliquot of dispersion of the calcein-loaded EYPC/PA liposome was added to 2 ml of 10 mM Tris–HCl, 140 mM NaCl and 1 mM EDTA solution (pH 7.4) in a quartz cell (final concentration 5.0 μM) at a given temperature. Then an aliquot of dispersion of the cationic liposome (final concentration 5.0 μM) was added to the quartz cell and the fluorescence intensity of the solution was monitored using a spectrofluorometer. The excitation and monitoring wavelengths were 490 nm and 520 nm, respectively. The percent leakage of calcein from the liposome was defined as

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

where F^i and F^t are 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%).

2.9. Other methods

Nuclear magnetic resonance (NMR) spectra were taken with a JEOL JNM-GX 270 MHz instrument. The weight and the number average molecular weights of polymers were estimated by gel permeation chromatography on a system equipped with a Shodex KD-803 columns (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 to 1000 g/mol) were used to calibrate the gel permeation chromatography. The amount of polymer bound to liposome was estimated by high performance liquid chromatography analysis on a SB-803 column (Shodex) as reported previously [22]. Zeta potential of cationic liposomes was measured using a 380 ZLS instrument (Nicomp).

3. Results

3.1. Characterization of polymers

As a thermosensitive polymer having anchoring groups to liposome membranes, two kinds of polymers were prepared in this study (Fig. 1). Poly(APr)-$2C_{12}$ possesses the anchoring group at the terminal of the polymer chain and, hence, the polymer chain is fixed on the liposome at the terminal point. In contrast, the anchoring groups distribute randomly in

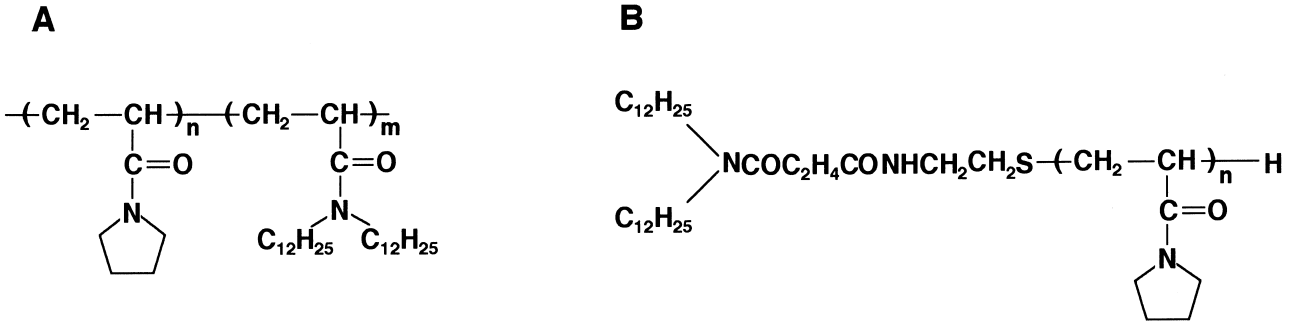

Fig. 1. Structures of poly(APr-co-NDDAM) (A) and poly(APr)-$2C_{12}$ (B).

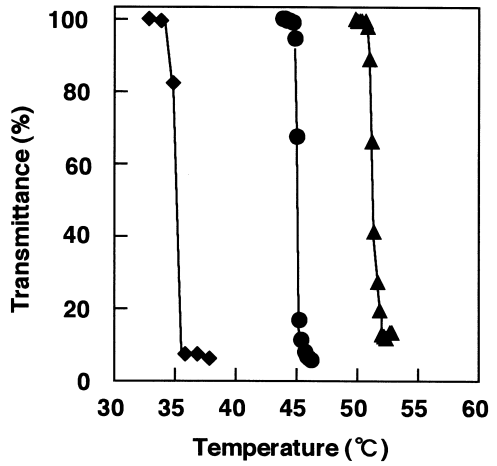

Fig. 2. Cloud point curves for aqueous solutions of poly(APr) (▲), poly(APr)-2C_{12} (●), and poly(APr-co-NDDAM) (♦).

the polymer chain of poly(APr-co-NDDAM). Thus, this polymer is attached to the liposome surface at arbitrary and plural points in the polymer chain [22]. The weight and the number average molecular weights of poly(APr) used for the preparation of poly(APr)-2C_{12} were estimated to be 11 500 and 5500, respectively. The weight and the number average molecular weights of poly(APr-co-NDDAM) were evaluated to be 13 800 and 4800, respectively. The NDDAM unit content of poly(APr-co-NDDAM) was estimated to be 2.1 mol% from ^{1}H-NMR spectrum of the polymer.

The LCSTs of these polymers were determined by measuring cloud point of aqueous polymer solutions. Fig. 2 represents typical cloud point curves for aqueous solutions of poly(APr-co-NDDAM), poly(APr)-2C_{12} and poly(APr) which was used for the preparation of poly(APr)-2C_{12}. The poly(APr) solution exhibited a sharp decrease in transmittance at ca. 51°C. Similarly, transmittances of the poly(APr)-2C_{12} and the poly(APr-co-NDDAM) solutions decreased drastically at ca. 45°C and ca. 35°C, respectively. It has been shown that LCST of thermosensitive polymers is lowered by the incorporation of hydrophobic moieties in the polymer chain [22,28–30]. It is likely that existence of hydrophobic anchoring groups in these polymer chains decreased their LCST from that of poly(APr).

3.2. Characterization of liposomes

The diameter of liposomes prepared in this study was estimated by dynamic light scattering and is listed in Table 1. The diameter of these liposomes is in the range of ca. 200–300 and larger than the pore size of the carbonate membrane (100 nm) used for their preparation. Because DOPE is a lipid with overall cone shape [31], DOPE liposomes with a small diameter should be unstable due to high curvature of their membranes. It is likely that liposomes containing DOPE fused to some extent, forming a larger liposomes. In fact, we have observed that the diameter of poly(*N*-isopropylacrylamide-co-NDDAM)-modified liposomes increases with increasing DOPE content in the membrane [23]. The amount of polymer fixed on the liposomes is also shown in Table 1. About 54% and 65% of polymer were adsorbed on the liposomes for the poly(APr-co-NDDAM)- and the poly(APr)-2C_{12}-modified liposomes, respectively.

3.3. Zeta potential

The electrostatic force plays an important role in cationic liposome-anionic liposome interaction. Thus, we investigated the influence of temperature on charge density of the polymer-modified cationic liposomes. Fig. 3 represents zeta potential of various cationic liposomes at varying temperatures. The un-

Table 1
Characterization of liposomes

Liposome	Polymer in feed (g/g lipid)	Polymer bound (g/g lipid)	Diameter (nm)
Unmodified DC-Chol/DOPE	0	0	205
Poly(APr)-2C_{12}-modified DC-Chol/DOPE	1.20	0.78	184
Poly(APr-co-NDDAM)-modified DC-Chol/DOPE	1.20	0.65	275
EYPC/PA	0	0	144

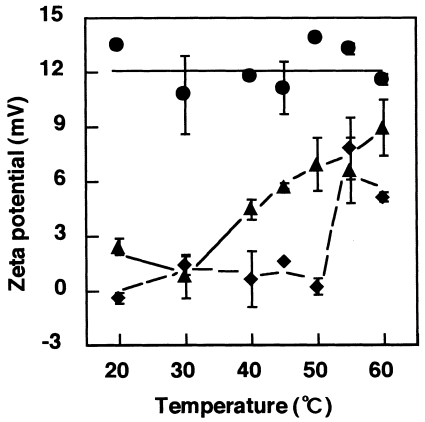

Fig. 3. Zeta potentials of the unmodified (●), the poly(APr)-$2C_{12}$-modified (◆) and the poly(APr-co-NDDAM)-modified (▲) DC-Chol/DOPE (1:1, mol/mol) liposomes as a function of temperature. The bars represent the standard deviations ($n = 3$).

modified cationic liposome showed roughly a constant value, ca. 12 mV, in the range of 20–60°C. The poly(APr)-$2C_{12}$-modified liposome exhibited much lower zeta potential below 50°C, where the polymer chain is hydrated. However, a sudden increase in zeta potential is seen between 50°C and 55°C, which is near the LCST of poly(APr). Similarly, the poly(APr-co-NDDAM)-modified cationic liposome exhibited a low zeta potential below 30°C, but its zeta potential increased gradually above 40°C with raising temperature.

3.4. Association of cationic liposomes with anionic liposomes

Association of the cationic liposomes with EYPC/PA liposomes was investigated by following their particle size. Fig. 4 depicts the weight average diameter of particles existing in the mixed suspensions of the polymer-modified cationic liposomes and EYPC/PA liposomes at various temperatures. When the unmodified cationic liposomes were mixed with the anionic liposomes in the range of 20–60°C, large aggregates precipitated. Their size could not be estimated by dynamic light scattering. In contrast, mixed suspensions of the polymer-modified cationic liposomes and the anionic liposomes exhibited small diameters at low temperatures. In the case of poly-(APr)-$2C_{12}$-modified liposome, the particle size did not change from 20°C to 45°C (Fig. 4A). However, its size increased significantly above 50°C, which is near the LCST of poly(APr). Because the diameter of the same cationic liposome did not change in the absence of the anionic liposome in this temperature region, this result shows that the polymer-modified cationic liposomes associated with the anionic liposomes above the LCST of poly(APr). Similarly, an intensive increase in diameter is seen in the case of poly(APr-co-NDDAM)-modified cationic liposome above 50°C (Fig. 4B), while the diameter started to

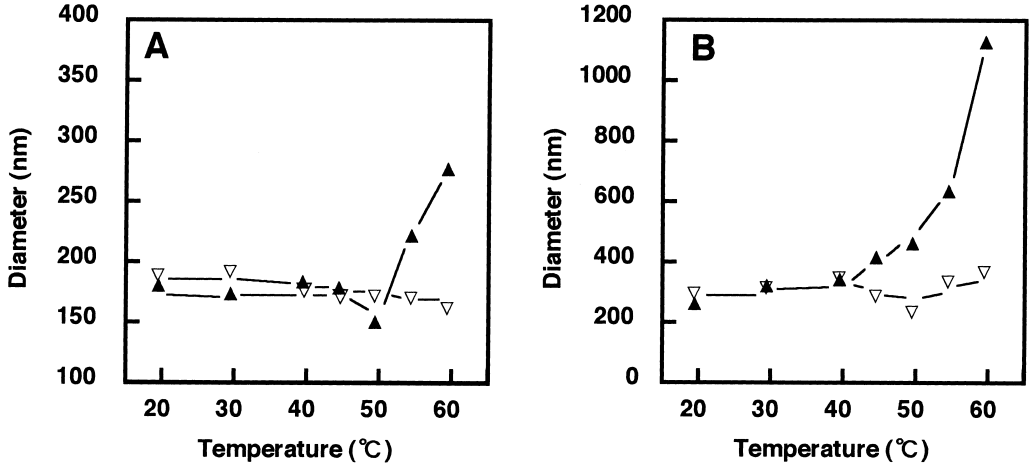

Fig. 4. Diameters of the poly(APr)-$2C_{12}$-modified (A) and the poly(APr-co-NDDAM)-modified (B) DC-Chol/DOPE (1:1, mol/mol) liposomes in the absence (▽) or presence (▲) of EYPC/PA (3:1, mol/mol) liposomes as a function of temperature. Concentrations of DC-Chol/DOPE and EYPC/PA were 50 μM and 100 μM, respectively.

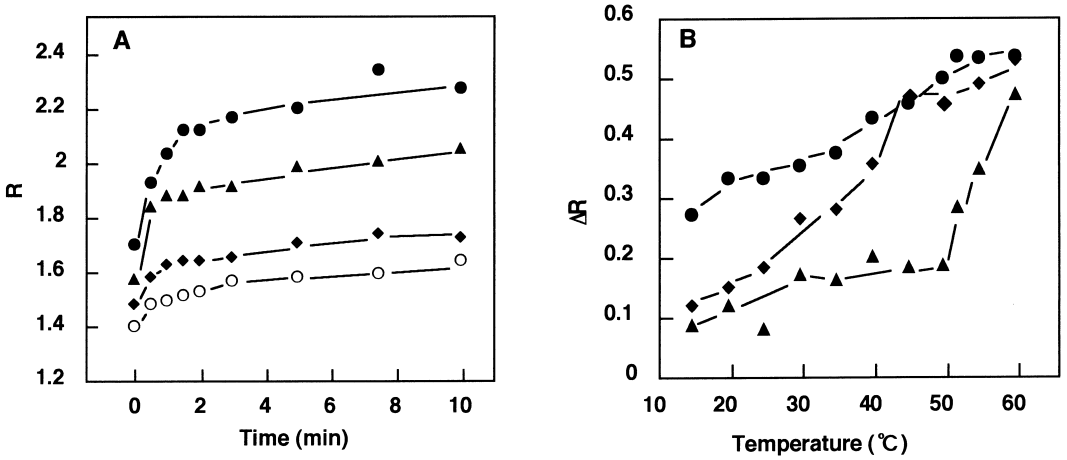

Fig. 5. (A) Time-courses of fusion of the poly(APr-co-NDDAM)-modified DC-Chol/DOPE (1:1, mol/mol) liposomes with EYPC/PA liposomes at 30 (○), 50 (♦), 55 (▲), and 60°C (●). Concentrations of DC-Chol/DOPE and EYPC/PA were 50 μM and 100 μM, respectively. (B) Fusion of the poly(APr-co-NDDAM)-modified (▲), the poly(APr)-$2C_{12}$-modified (♦), and the unmodified (●) DC-Chol/DOPE (1:1, mol/mol) liposomes with EYPC/PA liposomes as a function of temperature. The ordinate represents the increase in R in the initial 3 min after the addition of EYPC/PA (3:1, mol/mol) liposomes. Concentrations of DC-Chol/DOPE and EYPC/PA were 50 μM and 100 μM, respectively.

increase slightly around 45°C, possibly because of partial dehydration of the polymer chain which might induce alteration of polymer chain conformation [22].

3.5. Fusion of cationic liposomes with anionic liposomes

The effect of temperature on fusion of the polymer-modified cationic liposomes with the anionic liposomes was examined. Liposome fusion was detected by the change in resonance energy transfer efficiency from NBD-PE to Rh-PE due to dilution of these fluorescent lipids in the liposome membrane [26]. We have already shown that the fluorescence intensity ratio of NBD to Rh (R) is useful for following membrane fusion [5,27].

Fig. 5A shows the time course of R for the poly(APr-co-NDDAM)-modified cationic liposomes containing NBD-PE and Rh-PE after addition of the fluorescent lipid-free EYPC/PA liposomes. The R value increased immediately after addition of the anionic liposomes. While R increased slightly at 30°C, the increase was more significant at 60°C, indicating that the fusion occurs more intensively at this temperature. Generally, the fusion proceeded fast in the initial 2 min and then became much slower. Fig. 5B depicts the increase in R (ΔR) for various cationic liposomes in the initial 3 min after the addition of the anionic liposomes at varying temperatures. For the unmodified cationic liposome, ΔR increases monotonously from 0.27 to 0.52 with raising temperature in the region of 15–60°C, indicating that the cationic liposome fuses with the anionic liposome more intensively with temperature. For the poly(APr-co-NDDAM)-modified cationic liposome, ΔR is kept at a much lower level below 50°C, compared to the case of the unmodified liposome. However, ΔR increases drastically above 50°C. This result shows that the hydrated polymer chains covering the cationic liposome surface suppress fusion of the cationic liposome with the anionic liposome below the LCST, whereas above the LCST collapse of the polymer chain enables the cationic liposome to fuse with the anionic liposome as the unmodified cationic liposome does.

Similarly, fusion of the poly(APr)-$2C_{12}$-modified cationic liposome with the anionic liposome is suppressed to some extent below 40°C and enhanced from 45°C, which is near the LCST of poly(APr) (Fig. 5B). However, suppression of the fusion is relatively weak in this case, compared to the case of poly(APr-co-NDDAM)-modified liposome, probably due to larger conformational freedom of the polymer chain fixed to the liposome membrane at its terminal point [22].

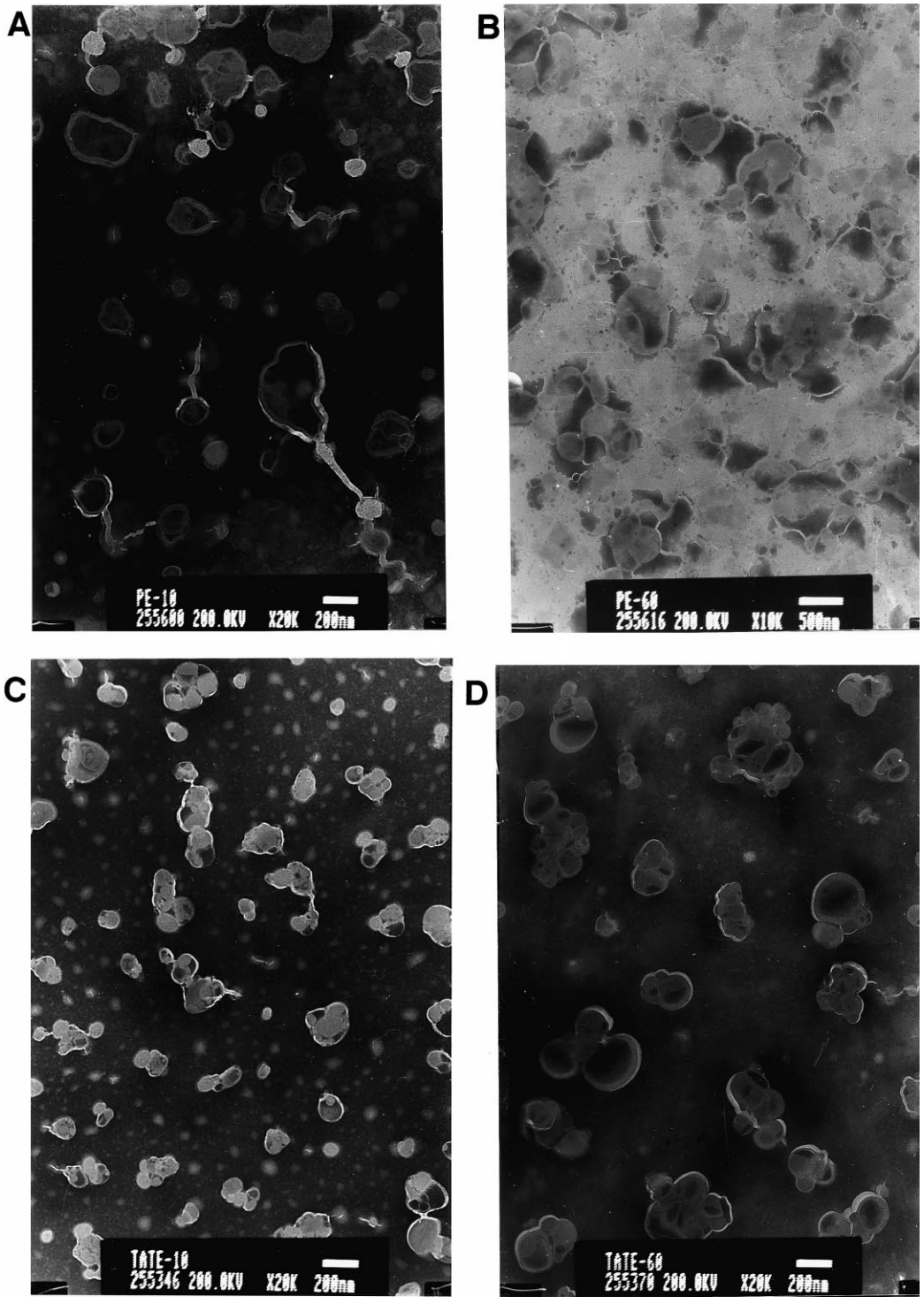

Fig. 6. Negative stain electron micrographs of liposome preparations: the unmodified DC-Chol/DOPE (1:1, mol/mol) liposomes at 10°C (A) and 60°C (B); the poly(APr)-2C_{12}-modified DC-Chol/DOPE (1:1, mol/mol) liposomes at 10°C (C) and 60°C (D); the unmodified DC-Chol/DOPE (1:1, mol/mol) liposomes mixed with EYPC/PA (3:1, mol/mol) liposomes at 10°C (E) and 60°C (F); the poly(APr)-2C_{12}-modified DC-Chol/DOPE (1:1, mol/mol) liposomes mixed with EYPC/PA (3:1, mol/mol) liposomes at 10°C (G) and 60°C (H). The bars shown in A and C–H represent 200 nm. The bar in B represents 500 nm.

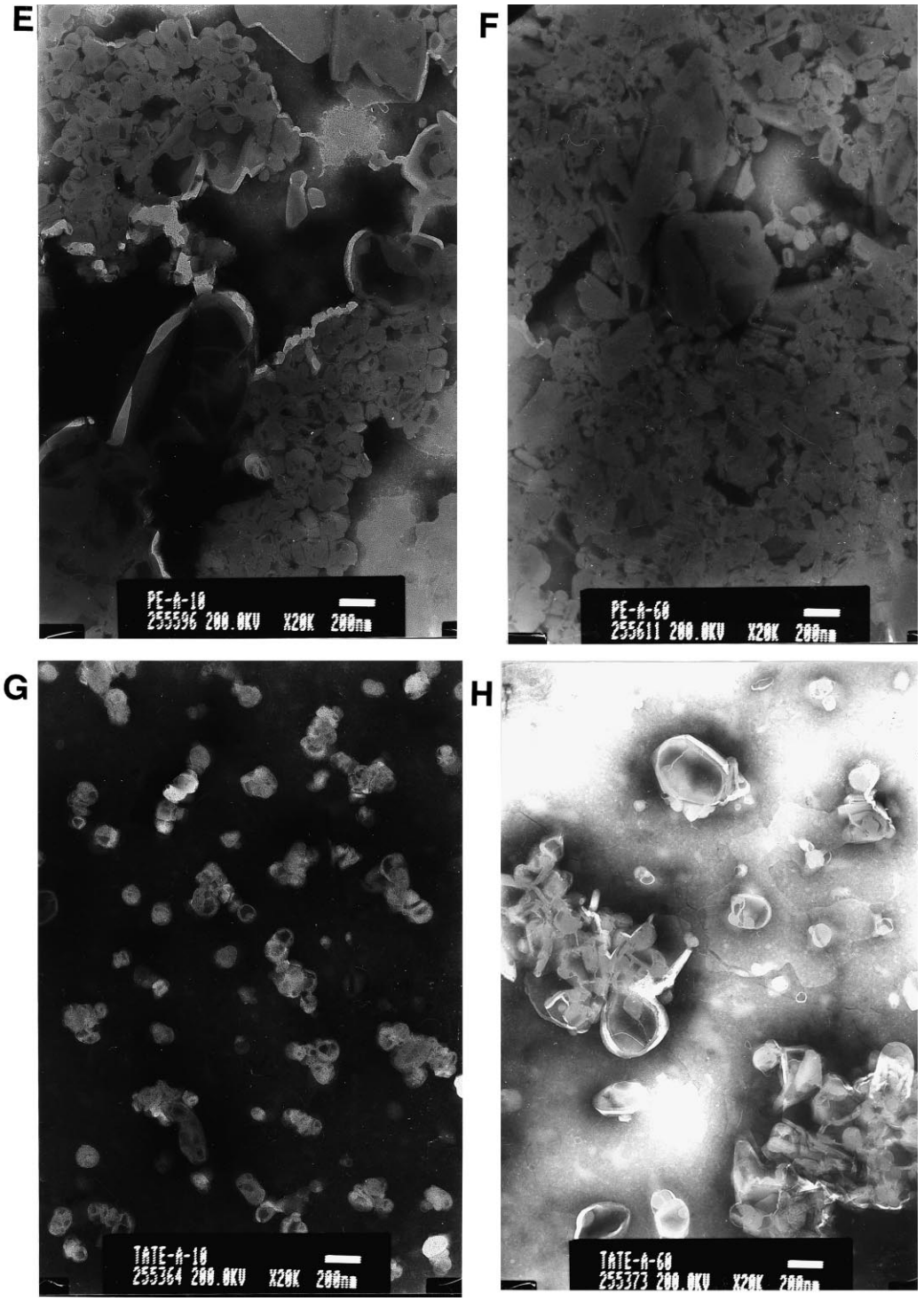

Fig. 6 (*continued*).

3.6. Electron microscopy

Fig. 6A,C show typical images of the unmodified and the poly(APr)-2C_{12}-modified cationic liposomes incubated at 10°C for 5 min. Vesicle sizes of these liposomes are roughly in the range of 50–200 nm and seem to be consistent with those estimated by dynamic light scattering (Fig. 4A). Typical images of

the same liposomes incubated at 60°C for 5 min are shown in Fig. 6B,D. For the unmodified liposomes, intensive aggregation of small vesicles as well as vesicles with large diameters can be seen (Fig. 6B), indicating that these liposomes are not stable at the high temperature. Aggregates consisting of several small liposomes are seen in the image of the polymer-modified cationic liposomes incubated at 60°C (Fig. 6D). Also, the average vesicle size may be slightly larger than that at 10°C, suggesting that liposome fusion occurred to some extent during the incubation at this temperature, although the increase in diameter was not detected by dynamic light scattering (Fig. 4A). However, the polymer-modified cationic liposomes are clearly much more stable than the unmodified liposomes at 60°C. Probably, the polymer chains are still effective to some extent to shield the liposome surface from liposome contact and prevent their aggregation and fusion even after the transition above their LCST.

When the unmodified cationic liposomes were mixed with the anionic liposomes and incubated for 5 min at 10°C or 60°C, intensive aggregation and fusion of these liposomes took place at both temperatures due to the electrostatic interaction (Fig. 6E,F). However, when the anionic liposomes were added to the cationic liposomes modified with poly(APr)-$2C_{12}$, such intensive aggregation and fusion did not occur but the liposomes exhibited their original sizes at 10°C (Fig. 6G). Although small aggregates formed by several liposomes are seen in Fig. 6G, it is apparent that interaction of the polymer-modified liposomes with the anionic liposomes is strongly suppressed at this temperature, in comparison with the case of the unmodified liposomes (Fig. 6E). At 60°C, however, large aggregates and liposomes with large diameters were formed (Fig. 6H). The interaction between the poly(APr-co-NDDAM)-modified cationic liposomes and the anionic liposomes was also

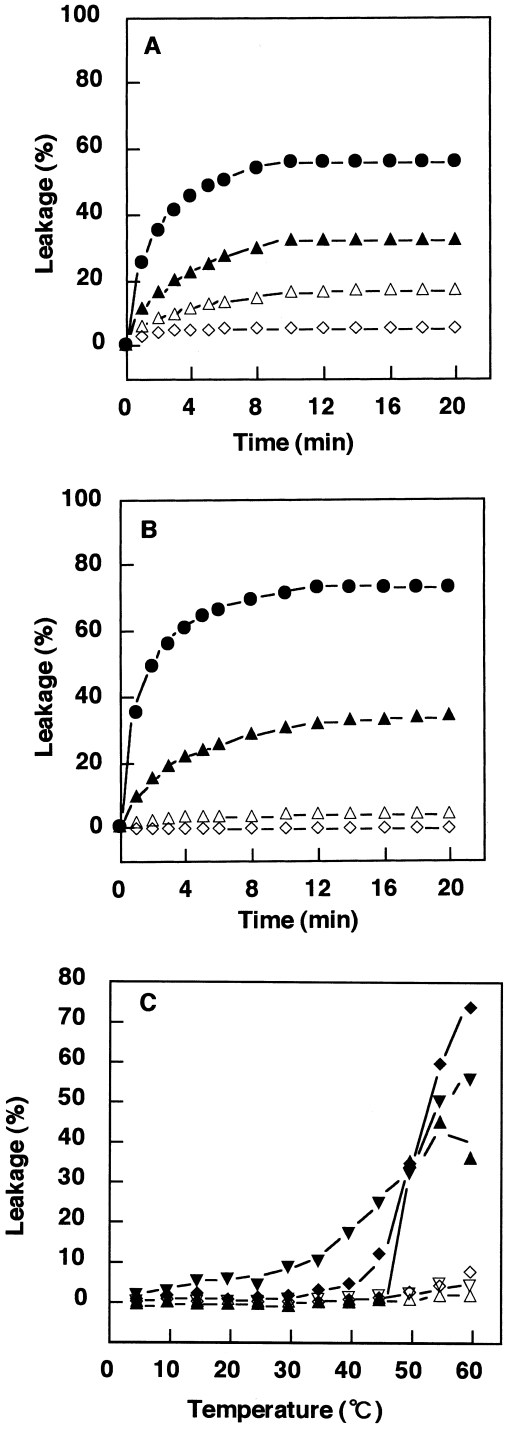

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Fig. 7. Time courses of calcein leakage from EYPC/PA (3:1, mol/mol) liposomes induced by the unmodified (A) and the poly(APr)-$2C_{12}$-modified (B) DC-Chol/DOPE (1:1, mol/mol) liposomes at varying temperatures: 20°C (◇), 40°C (△), 50°C (▲), 60°C (●). (C) Percent leakage of calcein from EYPC/PA (3:1, mol/mol) (closed symbols) and EYPC (open symbols) liposomes induced by the unmodified (▼, ▽), the poly(APr-co-NDDAM)-modified (▲, △), and the poly(APr)-$2C_{12}$-modified (◆, ◇) DC-Chol/DOPE (1:1, mol/mol) liposomes as a function of temperature. Concentrations of DC-Chol/DOPE, EYPC/PA, and EYPC were 5 μM, 5 μM, and 5 μM, respectively.

investigated and similar images of liposomes were obtained (results not shown). The observation using the electron microscopy provided the direct evidence for the temperature-controlled interaction of the polymer-modified cationic liposomes with the anionic liposomes.

3.7. Calcein leakage

Fig. 7A,B show time courses of calcein leakage from EYPC/PA (3/1) liposome induced by the unmodified and the poly(APr)-2C_{12}-modified cationic liposomes at varying temperatures. While the unmodified cationic liposome hardly induced the contents leakage at 20°C, the leakage increased with raising temperature (Fig. 7A). A similar temperature-dependent enhancement of the contents leakage was seen in the case of the poly(APr)-2C_{12}-modified cationic liposome, although the contents leakage was hardly enhanced below 40°C (Fig. 7B). Generally, the calcein leakage was fast immediately after the addition of the cationic liposomes, but it became slower with time and finally reached to a constant level in a steady state. The contents leakage induced by the poly(APr-co-NDDAM)-modified cationic liposome was also examined and similar profiles of the leakage were obtained (results not shown).

Fig. 7C depicts percent leakage of calcein in the steady state induced by various cationic liposomes as a function of temperature. For the unmodified liposome, the contents leakage is only limited below 30°C, but the leakage increases remarkably with raising temperature. In the case of the poly(APr-co-NDDAM)-modified cationic liposome, the release is completely prevented below 45°C, indicating that the interaction of this cationic liposome with the anionic liposome is strongly suppressed by the hydrated polymer chains. However, this cationic liposome induces the leakage above 50°C which corresponds to the LCST of poly(APr). A very similar temperature-dependence of the leakage is seen in the case of poly(APr)-2C_{12}-modified cationic liposomes, while the enhancement of the leakage occurs from 45°C. It was confirmed that in the absence of these cationic liposomes the contents leakage from EYPC/PA liposome was negligible in the experimental temperature region.

It may be possible that this temperature-dependent leakage of calcein from the EYPC/PA liposome is induced by its hydrophobic interaction with the dehydrated polymer chains above the polymer's LCST. Thus, calcein leakage from EYPC liposomes was also examined (Fig. 7C). However, leakage of their contents was not induced by these polymer-modified cationic liposomes in the experimental temperature region, suggesting that the electrostatic interaction between the cationic liposomes and the anionic liposome is crucial for the contents leakage.

4. Discussion

In this study, we designed poly(APr)-modified DC-Chol/DOPE liposomes as a cationic liposome of which affinity to negatively charged membranes can be controlled by temperature. We prepared two types of poly(APr) having anchoring groups to liposome membranes, poly(APr)-2C_{12} and poly(APr-co-NDDAM), which are fixed on the liposome at the chain end and at arbitrary and plural points of the polymer chain, respectively. We have already reported that a copolymer of APr and *N*-isopropylacrylamide having the same anchor at the terminal point undergoes the conformational transition more efficiently than a random copolymer of APr, *N*-isopropylacrylamide and NDDAM [22].

As shown in Table 1, poly(APr)-2C_{12} was fixed on the cationic liposome more efficiently than poly(APr-co-NDDAM), although the latter has on the average ca. 2.2 NDDAM units per polymer chain, as calculated from the weight average molecular weight. In the case of the poly(APr-co-NDDAM)-modified liposome, the whole polymer chain was tightly bound near the liposome surface at several points of the polymer chains. Thus, the liposome surface might be more crowded with the polymer chains, compared with the poly(APr)-2C_{12}-modified liposome. This may explain the difference in efficiency of the polymer fixation.

The polymer-modified liposome is calculated to possess the polymer chain with the polymer chain/lipid (mol/mol) ratio of 1:24 [poly(APr)-2C_{12}] or 1/34 [poly(APr-co-NDDAM)] on the basis of the weight average molecular weight. The poly(APr)-2C_{12}-modified liposomes had a diameter roughly same to that of the unmodified liposome, whereas the diameter of

the poly(APr-co-NDDAM)-modified liposome was larger than these liposomes, suggesting that attachment of poly(APr-co-NDDAM) may affect stability of cationic liposome. A similar increase in vesicle size has been observed for EYPC/DOPE liposomes modified with a copolymer of *N*-isopropylacrylamide and NDDAM [23].

Influence of temperature on charge density of the cationic liposomes was investigated by measuring zeta potential (Fig. 3). Zeta potential of the unmodified cationic liposome did not change between 20°C and 60°C and was ca. 12 mV. However, the poly(APr)-$2C_{12}$-modified cationic liposome revealed much lower zeta potential below 50°C than the unmodified one. The LCST of poly(APr)-$2C_{12}$ was 45°C and was lower than the LCST of poly(APr). It is known that incorporation of hydrophobic groups in thermosensitive polymers causes a decrease in their LCST [22,28–30]. However, when hydrophobic groups are inserted in lipid membranes, their effect on the transition of the polymer chain becomes less significant [30]. Thus, the highly hydrated polymer chains taking on a extended conformation covered the liposome surface below 50°C and shielded positive charges on the liposome surface. A similar shielding effect induced by hydrated polymer chains has been reported for polyethylene glycol-modified cationic liposome-oligonucleotide complexes by Meyer et al. [32].

However, the temperature was elevated above 50°C, the liposome's zeta potential increased suddenly. Under this condition, the polymer chain takes on a dehydrated globule. Thus, the hydrated layer formed by the polymer chains disappears and then the positively charged surface of the liposome is exposed, resulting in increase in the zeta potential.

Temperature dependence of zeta potential was also seen for the poly(APr-co-NDDAM)-modified cationic liposome. Its zeta potential was low below 40°C, as was the case of the poly(APr)-$2C_{12}$-modified liposome, but it increased linearly above 40°C. It has been reported that partial dehydration of the polymer chain occurs even below its LCST [22]. As mentioned above, the whole chain of poly(APr-co-NDDAM) is tethered to the membrane surface at random points in the chain. Thus, dehydrated segments in this polymer chain might be adsorbed to the liposome membrane through hydrophobic interactions more easily than poly(APr)-$2C_{12}$ which is bound to the membrane at its terminal point. Therefore, zeta potential of the cationic liposome modified with poly(APr-co-NDDAM) might be more readily influenced by temperature than that modified with poly(APr)-$2C_{12}$.

Association between the cationic liposomes and the anionic liposome was investigated using dynamic light scattering. As is apparent in Fig. 4, the poly(APr)-$2C_{12}$-modified liposome maintained its original diameter in the presence of EYPC/PA liposome below 50°C. Since the unmodified cationic liposomes formed visible aggregates with the anionic liposomes under the same condition, it is obvious that the polymer chains attached to the liposome strongly suppressed its interaction with the anionic liposome. As already described, the hydrated layer formed by the tethered polymer chains prevents close contact between the cationic liposome and the anionic liposome. In addition, this hydrated layer shields positive charges on the liposome surface (Fig. 3), reducing the electrostatic force between these liposomes.

When the ambient temperature was raised above 50°C, a sudden increase in diameter occurred for liposomes existing in the mixed suspension of the poly(APr)-$2C_{12}$-modified cationic liposome and the anionic liposome, indicating that association of these liposomes took place in this temperature region. Because collapse of the tethered polymer chain causes exposure of the positively charged surface of the liposome, the intensive interaction between these liposome occurs.

The mixed suspension of the poly(APr-co-NDDAM)-modified cationic liposome and the anionic liposome exhibited similar temperature-dependence of vesicle size. However, the diameter started to increase at ca. 40°C which is somewhat lower than the LCST of poly(APr), while more significant increase in diameter was seen above 50°C. Because zeta potential of the poly(APr-co-NDDAM)-modified liposome was elevated gradually above 40°C (Fig. 3), aggregation of the liposomes due to the electrostatic interaction might be enhanced from this temperature.

It has been thought that membrane fusion plays an important role in cationic liposome-mediated transfection [33–35]: DNA is considered to transfer into cytoplasm via direct fusion between cationic lipo-

somes and plasma membrane [16,34] and/or fusion between cationic liposomes and endosomal membrane [18,33,35].

As is seen in Fig. 5, fusion between the unmodified cationic liposome and EYPC/PA liposome was promoted with raising temperature, possibly because mobility of lipid molecules increases with temperature. However, in the case of the poly(APr-co-NDDAM)-modified cationic liposome, liposome fusion was kept at much lower level below 50°C than was the case of the unmodified liposome. This result indicates that the hydrated polymer chains bound to the liposome suppress fusion between the cationic liposome and the anionic liposome effectively. However, the liposome fusion was accelerated above 50°C. Collapse of the tethered polymer chain enables the cationic liposome to fuse with the anionic liposome.

In contrast, fusion between the poly(APr)-$2C_{12}$-modified cationic liposome and EYPC/PA liposome was enhanced with temperature, while a very intensive enhancement of the fusion was seen around 45°C, which is close to the LCST of poly(APr). The polymer chain is attached to the liposome membrane at its terminal in this liposome and, hence, has higher conformational freedom than the polymer chain in the poly(APr-co-NDDAM)-modified liposome. Also, density of the polymer chain in the vicinity of surface of the former liposome might be lower than that of the latter liposome, as already discussed. The differences in mobility and density of the polymer chain might result in the different temperature-dependence of liposome fusion.

The temperature-dependent aggregation and fusion of the polymer-modified liposomes with the anionic liposomes were clearly seen in the images of mixed suspensions of these liposomes taken using an electron microscopy (Fig. 6). In contrast to the unmodified liposomes which form aggregates and large vesicles by the interaction with the anionic liposomes, the polymer-modified liposomes seem to maintain their original vesicle size in the presence of the anionic liposomes below the LCST of poly(APr). However, the polymer-modified liposomes interacted with the anionic liposomes to form aggregates and large vesicles above the LCST.

The temperature-induced control of interaction of the polymer-modified liposomes with the anionic liposome can be further confirmed from the result of calcein leakage (Fig. 7). The unmodified cationic liposome induced calcein leakage from EYPC/PA liposome above 30°C. The leakage became more significant with temperature. However, both types of the polymer-modified liposomes caused the contents leakage only around and above the LCST of poly(APr). In comparison with the unmodified cationic liposome, the poly(APr)-$2C_{12}$-modified cationic liposome induced more intensive leakage at 55°C and 60°C. This result suggests that the hydrophobic polymer chain interacts with and destabilizes the anionic liposome through the hydrophobic interaction. However, the hydrophobic interaction alone is not strong enough to induce the calcein leakage, as is apparent from the result of calcein-loaded EYPC liposome (Fig. 7).

In this study, we prepared two types of polymer-modified cationic liposomes, namely poly(APr)-$2C_{12}$-modified and poly(APr-co-NDDAM)-modified cationic liposomes. While both types of liposomes exhibited similar temperature-dependence in their interaction with the anionic liposomes, the latter seems to achieve a more efficient control of the interaction, as is shown in the results of liposome fusion and calcein leakage. As described above, in the case of the poly(APr-co-NDDAM)-modified cationic liposome, the whole polymer chain might exist near surface of the liposome and, hence, this liposome has the polymer coat with higher polymer density than the poly(APr)-$2C_{12}$-modified liposome. In addition, conformational freedom of poly(APr-co-NDDAM) chain is restricted more severely than poly(APr)-$2C_{12}$, because the former is fixed to the liposome by the anchors at plural points. Such differences in density and mobility of the tethered polymer chain might result in difference in their interaction with the anionic liposome.

In conclusion, it was found that aggregation and fusion of the thermosensitive polymer-modified cationic liposomes with the anionic liposomes were controlled by temperature: these interactions were prevented below the LCST of the polymer, but promoted above this temperature. In this study, the polymer has the LCST at ca. 51°C and hence the interactions were switched on near this temperature. We have recently obtained thermosensitive polymers which exhibit a LCST around the physiological tem-

perature by the copolymerization of *N*-isopropylacrylamide with acrylamide or APr [22,30]. Therefore, it is possible to prepare cationic liposomes that reveal the temperature-response around the physiological temperature by using such thermosensitive polymers. The information obtained in this study may be useful for the design of delivery systems with target specificity for biologically active molecules such as DNA and oligonucleotides.

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