Cholesteryl Moiety Terminated Amphiphilic Polymethacrylates Containing Nucleic Acid Bases for Drug Delivery

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Received 8 August 2006; accepted 31 August 2006
DOI 10.1002/app.25445
Published online in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: Poly[9-(2-methacryloyloxyethyl)adenine] and poly[1-(2-methacryloyloxyethyl)thymine] with one pendant cholesteryl moiety at the polymer end (PMEA-Chol, PMET-Chol) and with two pendant cholesteryl moieties at both polymer ends as terminal groups (PMEA-2Chol, PMET-2Chol) were prepared by radical polymerization of 9-(2-methacryloyl-oxyethyl)adenine (MEA) and 1-(2-methacryloyloxy-ethyl)thymine (MET) initiated with 4,4′-azobis[(3-cholesteryl)-4-cyanopentanoate] in the presence of 2-mercaptoethanol or thiocholesterol as chain transfer reagents, respectively. The copolymers [PNiPAAm-*co*-PMEA-*n*Chol (n = 1,2)] composed of *N*-isopropylacrylamide (NiPAAm) and MEA were also prepared in a similar manner. The self-organization of these polymers and copolymers was confirmed by a fluorescence measurement, and then their critical concentrations of micelle formation (CMC) were determined. The mixture of PMEA-2Chol and cholesterol as a lipophilic drug model formed a lamella type of complex with an interplaner spacing of d = 35.3 Å. The hypochromism based on the formation of a 1 : 1 interaction of adenine and thymine moieties was found to appear in the mixed aqueous solution of PMEA-Chol and PMET-Chol. Complementary interactions were also confirmed in the system of PMET-2Chol and adenosine as well as PMEA-2Chol and uridine. Cis-dichlorodiammine platinum(II) (CDDP) was bound to PNiPAAm-*co*-PMEA-Chol through the adenine moiety by ligand substitution atoms of CDDP. The amount of CDDP loaded on the copolymer was found to be 0.143 g g^{-1}. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 103: 3064–3075, 2007

Key words: adenine; thymine; radical polymerization; polymethacrylates; cholesteryl moiety; self-organization; hydrophobic interaction; hypochromism; CDDP; DDS

INTRODUCTION

In recent decades, there has been a growing interest in self-assembled polymeric micelles, which can be prepared in two ways: One is to self-assemble micelles as random copolymers,[1–5] block copolymers,[6–12] and graft-copolymer[13] made up of hydrophilic and hydrophobic segments. The other one is to self-assemble polymers soluble in water containing a few hydrophobic substituent per chain and hydrophobic groups attached to the polymer ends.[14–20] Amphiphilic polymers modified hydrophobically with one or two pendant lipophilic groups at the chain end were prepared by (1) the free-radical polymerization of water soluble monomers using a lipophilic radical initiator in the presence or in the absence of lipophilic mercaptans as a chain transfer agent,[14–17] (2) the polymer reaction of amine-terminated water soluble polymers and lipophilic acid[18] or acid chloride.[2,19,20] Polyacrylic acid modified with an *N*,*N*-dioctadecyl amide group as a terminal group forms a stable liposome with dipalmitoyl phosphatidylcholine.[14] We have reported that poly[*N*-(2-hydroxypropyl)methacrylamide]s[16] and poly[2-(methacryloyloxy)-ethylphosphorylcholine]s[17] with a single pendant cholesteryl group at the polymer end and two cholesteryl groups at both polymer ends as terminal groups can self-organize in an aqueous solution and exhibit critical micelle concentration (CMC). Poly(*N*-isopropylacrylamide) (PNiPAAm) substituted with two pendant *n*-octadecyl groups at the chain end can form multimolecular micelles, which consist of a rigid core of octadecyl chain and a corona of solvated PNIPAAm chain.[15] On the other hand, amphiphlic polymer obtained from the coupling of amine-terminated PNiPAAm with cholic acid can self-assemble in an aqueous media.[18] A stearoyl group incorporated into one end of PNiPAAm also forms a core–shell micellar structure in an aqueous solution.[2,20] In these polymeric micelles, the hydrophobic inner core is surrounded and stabilized by the hydrophilic outer shell. Generally, it can be said that hydrophobically modified polymers soluble in water can form heterogeneous structures composed of hydrophilic microdomains of water soluble segments and hydrophobic microdomains of the

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lipophilic part incorporated in aqueous solution. These amphiphilic polymers have been investigated by theoretical approaches for use in biomedical and pharmaceutical areas, especially as drug carriers for a drug delivery system (DDS).[2] Since hydrophobically modified poly[*N*-(2-hydroxypropyl)-methacrylate]s and poly[2-(methacryloyloxy)ethyl phosphorylcholine]s form heterogeneous structure composed of a rigid core of cholesteryl group and a solvated corona of hydrophilic segments, drugs with a hydrophobic character can be held in a hydrophobic layer formed by terminated cholesteryl groups.[16,17] On the other hand, considering the ability to form intermolecular complexes of deoxyribonucleic acid (DNA), polymers, which contain nucleic acid bases, e.g., adenine and thymine, are expected to combine uridine and adenosine type of drugs, respectively, by complementary interaction.[21,22] In addition to the complementary intermolecular complex of DNA, an anticancer drug, *cis*-dichlorodiammine platinum(II) [*cis*-Pt(NH_3)2Cl_2: CDDP], can bind specifically to DNA in adenine, guanine, cytosine, and their derivatives.[23,24] In this study, novel cholesteryl moiety-terminated methacrylate polymers having adenine (PMEA-*n*Chol, n = 1,2) and thymine (PMET-*n*Chol, n = 1,2) moieties were obtained from the polymerization of 9-(2-methacryl-oyloxyethyl)adenine (MEA) and 1-(2-methacryloyloxyethyl)thymine (MET) initiated with 4,4′-azobis[(3-cholesteryl)-4-cyanopentanoate] in the presence of 2-mercaptethanol or thiocholesterol. Since these amphiphilic polymethacrylates can self-assemble in water and form core–shell type micelles, they are expected to carry lipophilic drugs in aqueous media. Moreover, they are anticipated to be carrier molecules of nucleic acid base type of drugs based on the complementary interaction the adenine moiety and uridine and CDDP based on the ligand exchanging. The present article describes the self-organization phenomena of cholesteryl moiety-terminated methacrylate polymers having nucleic acid bases in the side chains to obtain basic information on a novel carrier for drug delivery.

EXPERIMENTAL

Reagents

4,4′-Azobis(4-cyanopentanoic acid) was kindly supplied by Wako Pure Chemicals (Japan) and purified by recrystallization from methanol. 4,4′-Azobis (4-cyanopentanoic acid chloride) was obtained from the reaction of 4,4′-azobis(4-cyanopentanoic acid) and phosphorus pentachloride in benzene according to the method of Smith.[25] 9-(2-Methacryloyl-oxyethyl)-adenine (MEA) was prepared by the reaction of 9-(2-hydroxyethyl)-adenine and methacrylic anhydride in 69% yield, according to the method described in the literature.[26,27] mp. 198.8–201.9°C (Ref. 27, mp. 201–203°C), ANAL. Calcd. for $C_{11}H_{13}O_2N_5$ = 247.256: C, 53.43%, H, 5.30%, N, 28.32%, Found: C, 52.93%, H, 5.15%, N, 28.51%. 1-(2-Methacryloyloxyethyl)thymine (MET) was also prepared by the reaction of 1-(2-hydroxyethyl)thymine and methacrylic anhydride in 76% yield, according to the method described in the literatures.[26,28] mp. 169.5–172.0°C (Ref. 28, mp. 170–172°C), ANAL. Calcd. for $C_{11}H_{14}O_4N_2$ = 238.243: C, 55.45%, H, 5.92%, N, 11.75%, Found: C, 55.81%, H, 6.3%, N, 11.50%. Thiocholesterol (HS-Chol; SIGMA) and cholesterol (Chol; Wako Pure Chemicals) were recrystallized from ethanol. *N*-Isopropylacrylamide (NiPAAm; Wako Pure Chemicals) was recrystallized from the mixed solvent of benzene and hexane (1 : 30 v/v). Adenosine (Tokyokasei, Japan), uridine (Acros Organics), *cis*-dichlorodiammine platinum(II) (CDDP; Wako Pure Chemicals), and deoxyribonucleic acid from salmon sperm (DNA; Wako Pure Chemicals) were used as obtained. 2-Mercaptoethanol (2-ME: Tokyokasei) was redistilled before use. 1-(6-Dimethylamino-2-naphthyl)dodecanone (DMAND; Molecular Probes, USA) was used as obtained. Distilled deionized water was used throughout the experiments.

Preparation of azoinitiator

4,4′-Azobis[(3-cholesteryl)-4-cyanopentanoate] (VA-Chol) was prepared from the reaction of 4,4′-azobis(4-cyanopentanoic acid chloride) and cholesterol, according to the method described in the previous paper.[17]

^{1}H-NMR($CDCl_3$) δ (ppm): 0.68(s, 6H, —CH_3), 0.86–0.87(d, 12H, —CH_3), 0.91–0.92(d, 6H, —CH_3), 1.01–1.02(s, 6H, —CH_3), 1.73(s, 6H, —CH_3), 2.30–2.33(m, 4H, —CH_2—), 2.37–2.51(m, 8H, (—CH_2—)$_2$), 4.60–4.64(m, 2H, —O—CH<), 5.35–5.38(d, 2H, —CH<). UV: λ_{max} = 346 (ε 19). Anal Calcd for $C_{66}H_{104}O_4N_4$ = 1017.577: C, 77.90%, H, 10.30%, N, 5.51%. Found: C, 77.59%, H, 10.19%, N, 5.26%. VA-Chol has a mp 118–120°C (dec).

The rate constant of thermal decomposition of VA-Chol[16] at 50°C was found to be $k_d = 0.80 \times 10^{-5}$ s^{-1}.

Preparation of polymethacrylates having one cholesteryl moiety as terminal group

A mixed solution of *N*,*N*-dimethylformamide (DMF) and tetrahydrofuran (THF) (10 mL; 80/20 v/v) containing 400 mg (1.6 mmol) of MEA, 20 mg (0.02 mmol) of VA-Chol as an initiator, and 1.6 mg (0.02 mmol) or 27 mg (0.34 mmol) of mercaptoethanol as a chain transfer agent in a glass tube was degassed by the freeze-thaw technique with a liquid nitrogen bath and was sealed in a vacuum. After polymerization at 60°C for 12 h, the contents of the tube were poured into a large amount of diethyl ether to precipitate the polymethacrylate abbreviated to PMEA-Chol with one cholesteryl moiety as a terminal group. PMEA-Chol was isolated from the crude product by reprecipitation

using the diethyl ether–ethanol system. The polymer was purified with the repeating of precipitation from ethanol solution with diethyl ether twice. The yield was calculated from the dry polymer obtained. The polymerization of MET initiated with VA-Chol was also carried out in the presence of 0.02 mmol of 2-mercaptoethanol in a manner similar to that mentioned earlier, to give polymethacrylate (abbreviated to PMET-Chol) with one cholesteryl moiety as a terminal group.

Preparation of polymethacrylates having two cholesteryl groups as both terminal ends

The polymerization of MEA and MET initiated with VA-Chol was carried out in the presence of 0.02 mmol of thiocholesterol as a chain transfer agent in a manner similar to that mentioned earlier, to give methacrylates (abbreviated to PMEA-2Chol and PMET-2Chol) with two cholesteryl groups as both terminal ends, respectively.

Preparation of copolymers of MEA and NiPAAm

Copolymerization of MEA (110 mg; 0.44 mmol) and NiPAAm (500 mg; 4.42 mmol) initiated with VA-Chol (47 mg; 0.04 mmol) was carried out in the presence of 2-mercaptoethanol (2-ME; 0.048 mmol) or thiochoresterol (HS-Chol; 0.048 mmol) in a mixed solvent of DMF and THF (80 : 20 v/v, 10 mL) at 60°C for 12 h in a manner similar to that mentioned earlier. Copolymers with one pendant cholesteryl group at the polymer end and two pendant groups at both polymer ends (abbreviated to PNiPAAm-*co*-PMEA-Chol and PNiPAAm-*co*-PMEA-2Chol) were obtained in 73.3% and 66.4% yields, respectively. The number–average molecular weight (M_n) calculated by GPC was found to be $M_n = 9.11 \times 10^3$ ($M_w/M_n = 1.28$) and $M_n = 10.2 \times 10^3$ ($M_w/M_n = 1.38$) for PNiPAAm-*co*-PMEA-Chol and PNiPAAm-*co*-PMEA-2Chol, respectively. The molar ratios of NiPAAm : MEA in copolymers were determined from the areas of a methine proton (3.82 ppm) of the isopropyl group in NiPAAm and protons (8.12 ppm) on the purine ring of MEA in ^{1}H-NMR(DMSO-d_6) spectra.

Measurements of critical micelle concentration

The fluorescence spectra of the aqueous solutions of PMEA-*n*Chol and PMET-*n*Chol ($n = 1,2$) were recorded with a Shimadzu RF-1500 fluorophotometer containing DMAND. Since polymethacrylates appeared in this study have no detectable fluorescence activity, DMAND with a 364 nm excitation wavelength was used as a fluorescent probe for hydrophobicity. The concentration of DMAND was adjusted to 1 μmol L^{-1} by the addition of a 20 μL solution containing DMAND with 500 μmol L^{-1} of methanol solution to 10 mL of each aqueous solution of the modified PMEA or PMET. The concentration of the polymethacrylates was changed in the range of 2.4–5.0 $\times 10^3$ mg L^{-1}. The critical micelle concentration (CMC) was determined by the break point in the plot of fluorescence intensity at maximum emission wavelength (λE) = 364 nm and the concentration of each polymethacrylate.

Measurements of phase transition temperature[29]

The optical transmittance of the 0.5 wt % of aqueous PNiPAAm-*co*-PMEA-*n*Chol ($n = 1,2$) solution in the temperature range of 20 to 40°C was monitored at 600 nm using a Shimadzu UV-160A spectrophotometer. The quartz cell was kept in a thermostat maintained at a definite temperature with a circular water jacket equipped with a temperature controller. The lower critical solution temperature (LCST) was defined as the temperature at 50% turbidity.

Interaction of adenine and thymine moieties in the side chain of polymethacrylates

PMEA-*n*Chol and PMET-*n*Chol ($n = 1,2$) have an absorption maximum of 260 nm, based on nucleic acid bases. The continuous variation method was applied to the system of PMEA-Chol and PMET-Chol in aqueous solution, using UV spectra. The concentrations of adenine and thymine units in polymethacrylates were changed from 0 to 0.125 mmol L^{-1} and 0.125 to 0 mmol L^{-1}, respectively.

Drug-delivery system 1: lipophilic type of drug

Thirty milligram of PMEA-2Chol in 10 mL of water and 3 mg (7.7×10^{-3} mmol) of cholesterol as the lipophilic drug model in 10 mL of dimethylsulfoxide were placed into a 50-mL three-necked, round-bottomed flask equipped with a magnetic stirrer. The solution was stirred for 1 h and then vacuum distilled to give a thin membrane as a DDS model compound in a 60% yield. It was dried in vacuum at room temperature for 24 h. The small-angle X-ray scattering (SAXS) measurements were performed with a Rigaku RINT-2500/2203 E1, E5 using powder samples. The scattering intensity was registered at 0.01°s^{-1} for values of 2θ ranging from 0.11° to 10.0°.

Drug-delivery system 2: nucleic acid base type of drug

The interaction of PMET-2Chol and adenosine as a nucleic acid base type drug was confirmed by the continuous variation method. The concentration of thymine units in PMET-2Chol and adenosine was changed from 0 to 0.125 mmol L^{-1} and 0.125 to 0 mmol L^{-1}, respectively. The interaction of PMEA-2Chol and uridine as a model of 5-fluorouracil derivatives was also examined by the addition effect of uridine on the CMC

of PMEA-2Chol in a manner similar to that mentioned earlier in an aqueous solution using fluorescence spectroscopy.

Drug-delivery system 3: CDDP

The interaction of PMEA-Chol and CDDP was examined by the addition effect of CDDP on the CMC of PMEA-Chol in a manner similar to that mentioned earlier using fluorescence spectroscopy. The fluorescence spectra of aqueous solutions of PMEA-Chol (5.0 g L^{-1}) and CDDP were also measured in the presence and in the absence of DNA using DMAND as a probe. The concentration of PMEA-Chol was changed in the range of 0.005–5.0 mg L^{-1} with a constant concentration of 1.0 g L^{-1} of DNA. The optical transmittance of aqueous PNiPAAm-*co*-PMEA-2Chol solution (5.0 g L^{-1}) was measured in 0.5°C increments, in the absence and in the presence of CDDP (1.0 g L^{-1}) at a temperature range from 20°C to 44°C, measuring the absorbance at 600 nm. The LCST was defined as the temperature at 50% turbidity. The optical transmittance of aqueous solution of the mixed system of PNiPAAm-*co*-PMEA-Chol (5.0 g L^{-1}) and CDDP (1.0 g L^{-1}) was also measured in the absence and in the presence of DNA. The amount of CDDP bonded to PNiPAAm-*co*-PMEA-Chol was quantitatively determined by means of ICP emission spectroscopy using Seiko instruments ICP-AES ESPS-4000.

Other measurements

^{1}H-NMR spectra were recorded on a Jeol EX-400 spectrometer. The phase transition temperature of DDS model was determined by differential scanning calorimetry (DSC) using a Rigaku thermo plus DSC 8230. The sample quantity was 10 mg with 5°C min^{-1} rate of heating and cooling. Gel-permeation chromatographic (GPC) analyses were conducted in ammonium acetate buffer (pH 5.5, 40°C) solution with a TSK gel column (TOSOH α-4000, α-3000, and α-2500) or in a DMF (40°C) solution with a TSK gel column (TOSOH HHR 6000, 5000, 4000, 3000, and 2000) using a TOSOH HLC-8020GPC apparatus.

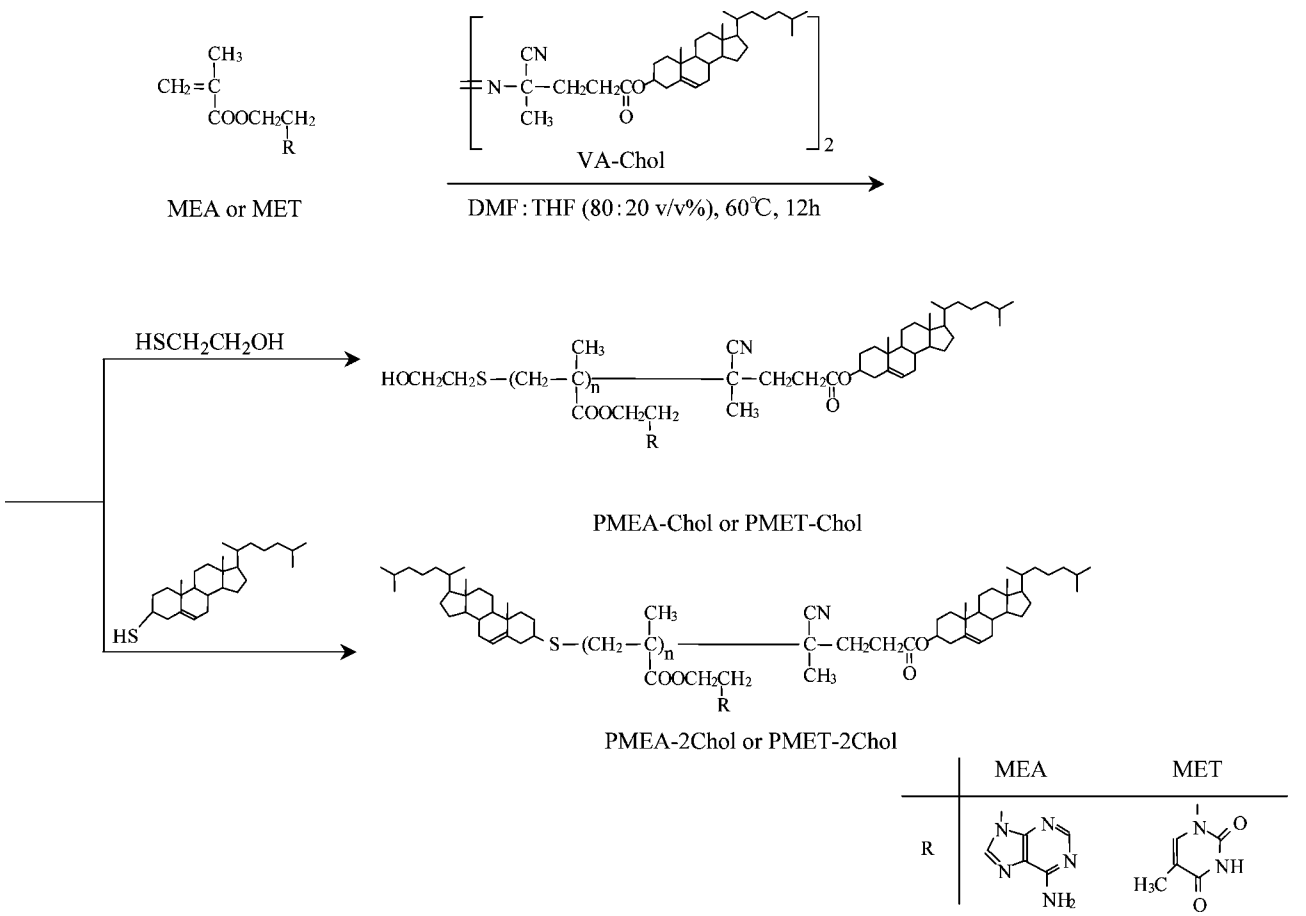
RESULTS AND DISCUSSION

Preparation of cholesteryl groups-terminated polymethacrylates

Amphiphilic polymers with lipophilic groups attached to the end of the water soluble polymers can be prepared several ways, such as by the polymerization of hydrophilic vinyl monomers initiated with a lipophilic radical initiator[14,15] and by the reaction of an amino-terminated water soluble polymer with long alkyl acid[18] or alkyl acid chloride.[2,20] According to the former method, we synthesized self-organizable water soluble poly[*N*-(2-hydroxy-propyl) methacrylamide][16] and poly[2-(methcryloyloxy)ethylphosphorylcholine][17] with lipophilic cholesteryl groups introduced to both polymer ends by the radical polymerization of corresponding vinyl monomers using VA-Chol as an initiator and thiocholesterol as a chain transfer agent. Since these polymers can self-associate in water and form the core–shell type of micelles in aqueous media, the inner core of the polymeric micelles acts as a microcontainer for lipophilic drugs, whereas the outer shell is expected to have the ability to control the release of drugs from these micelles. In this study, two series of cholesteryl-group-terminated polymethacrylates PMEA-*n*Chol and PMET-*n*Chol (n = 1,2), having adenine and thymine moieties as side chains and chlolesteryl groups as terminal ends, were prepared via the radical polymerization of MEA or MET initiated with VA-Chol in the presence of thiocholesterol or 2-mercaptoetanol as a chain transfer agent with a structure as depicted in Scheme 1. For the radical polymerization of MEA or MET, recombination and disproportionation of growing radicals cannot be expected to cause as a bimolecular termination in the presence of an excellent chain-transfer agent. In the case of polymerization of methyl methacrylate, the rate constants of propagation and termination are $k_p = 734$ L mol^{-1} s^{-1} and $k_t = 3.7 \times 10^7$ L mol^{-1} s^{-1}, whereas chain-transfer constants to 2-mercaptoethanol and 2-naphthalenethiol as a model compound of thiocholesterol were found to be 0.62 and 1.3, respectively. PMEA-Chol and PMET-Chol with a single pendant cholesteryl group were, therefore, obtained by the radical polymerization of MEA or MET initiated with VA-Chol in the presence of 2-mercaptoethanol, whereas PMEA-2Chol and PMET-2Chol with two cholesteryl groups at both polymer ends were given through the same polymerization in the presence of thiocholesterol as the chain-transfer agent. Table I shows the results of polymerization and characterization of resulting polymethacrylates. These polymethacrylates having nucleic acid base moieties were obtained in a moderate yield.

Preparation of copolymers of MEA and NiPAAm

To prepare the thermoresponsive drug delivery system using polymeric micelles, radical copolymerization of NiPAAm and MEA was also carried out in the presence of mercaptoethanol or thiochoresterol initiated with VA-Chol in a mixed solvent of dimethylformamide and tetrahydrofuran (80 : 20 v/v) at 60°C for 12 h. The results of polymerization are also summarized in Table I. Scheme 2 shows the structure of the obtained copolymers (abbreviated to PNiPAAm-*co*-PMEA-*n*Chol (n = 1,2)). The monomer ratios of NiPAAm : MEA in copolymers were found to be $m : n = 0.951 : 0.049$ and 0.953 : 0.047 for PNiPAAm-*co*-PMEA-Chol and PNiPAAm-*co*-PMEA-2Chol, respectively, determined by



Scheme 1 Preparation of PMEA-Chol, PMET-Chol, PMEA-2Chol, and PMET-2Chol.

the areas of proton signal of H_a (3.82 ppm) of NiPAAm and H_b (8.12 ppm) of MEA, as shown in Scheme 2.

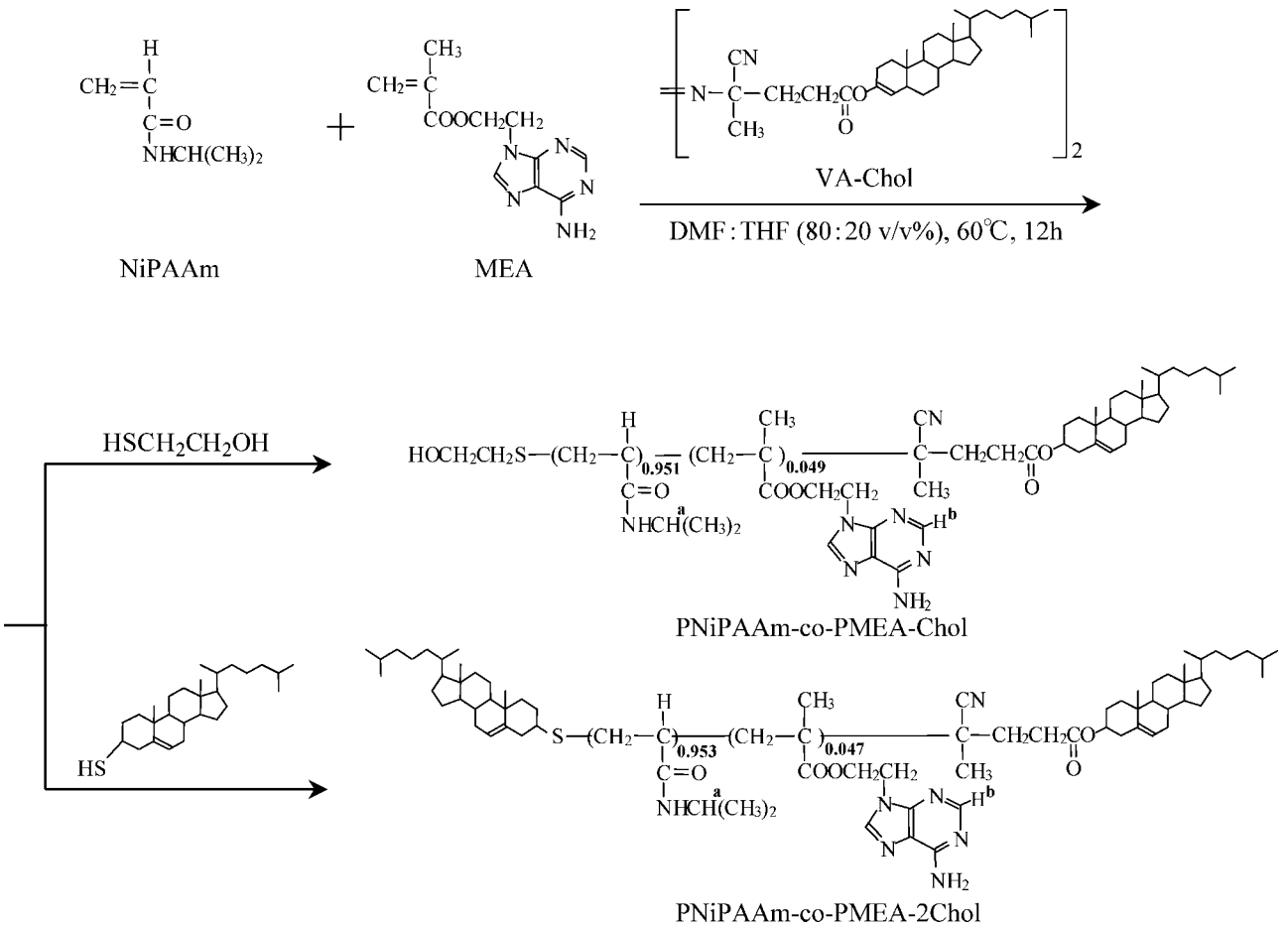
Self-organization

In a previous paper,[17] it was clarified from ^{1}H-NMR measurements that the poly[2-(methacryloyloxy)ethyl phosphorylcholine]s having cholesteryl moieties as their terminal groups are self-organized in aqueous media. The proton signal of the C_{14}-position of the methyl group in the cholesteryl moiety ($\delta = 0.7$ ppm) that appeared in CD3OD disappeared completely in D_2O, whereas the signal of methyl protons ($\delta = 3.8$ ppm) of the trimethyl-ammonium group in the phosphoryl-

TABLE I
Preparation and Characterization of Various Amphiphilic Polymethacrylates Having Nucleic Acid Bases in the Side Chain

Polymers	ME (mmol)	MET (mmol)	VA-Chol (mmol)	2-ME (mmol)	HS-Chol (mmol)	Yield (%)	M_n (P_n)[a] (10^3)	CMC (10^2 g L^{-1})
PMEA-Chol[b]	1.6	–	0.016	0.016	–	88.0	3.2 (11)	9.0
PMEA-2Chol[b]	1.6	–	0.016	–	0.016	78.5	3.5 (10)	2.0
PMEA-Chol[c]	–	2.0	0.020	0.020	–	77.0	77.0 (24)	60
PMEA-2Chol[c]	–	2.0	0.020	–	0.020	57.4	57.4 (26)	30
PNiPAAm-*co*-PMEA-Chol[d]	0.4	–	0.040	0.040	–	73.3	73.3 (70)[e]	30
PNiPAAm-*co*-PMEA-2Chol[d]	0.4	–	0.040	–	0.040	66.4	66.4 (75)[e]	20

[a] Number–average of molecular weight M_n obtained from GPC analysis (columns; TOSOH, Japan: TSKgel α-4000, α-3000, and α-2500, eluent: ammonium acetate buffer at pH 5.5, calibrated with standard polyethyleneglycol).
[b] Condition: DMF:THF (4:1 vol %); 15 mL, 60°C, 12 h.
[c] Condition: DMF:THF (4:1 vol %); 5 mL, 60°C, 12 h.
[d] Condition: DMF:THF (4:1 vol %); NiPAAm(4.4 mmol), 10 mL, 60°C, 12 h.
[e] Number–average of molecular weight M_n obtained from GPC analysis (columns; TOSOH, Japan: TSKgel H_{HR} 6000, 5000, 4000, and 2500, eluent: DMF, calibrated with standard polystyrene).



Scheme 2 Preparation of PNiPAAm-*co*-PMEA-Chol and PNiPAAm-*co*-PMEA-2Chol.

choline side groups did not show any change. The half-width of the proton signal of cholesteryl moiety gradually broadened with an increase in the D_2O content, while the peak completely disappeared in a 50 v/v% D_2O. However, the half-width of the peak of methyl protons of the phosporylcholine group did not change with a change in the D_2O content. Line broadening of the proton signal of the cholesterol group at the polymer ends in an aqueous medium is ascribed to the restricted molecular motion of the terminal located cholesteryl groups upon self-aggregation.[20,30] Nevertheless, the mobility of the hydrophilic main chain of the phosphorylcholine polymer was held even if the solvent polarity increased. This data suggests that the amphiphilic polymethacrylates consisting of a hydrophilic main chain and the lipophilic cholesteryl moiety attached to the polymer ends can self-aggregate and have microdomains, as provided by both the rigid core of the lipophilic cholesteryl groups and the mobile shell of the hydrophilic main chain. This concept is applicable to any other amphiphilic polymers obtained in this study, such as PMEA-*n*Chol and PMET-*n*Chol ($n = 1,2$). Figure 1 shows typical fluorescence spectra at different concentrations for an aqueous solution of PMEA-Chol with the degree of polymerization $P_n = 11$ in the presence of DMAND as a probe. No fluorescence emission was observed in the DMAND aqueous solution without PMEA-Chol or at a low polymer concentration below the 1.9×10^{-2} g L^{-1} level. When PMEA-Chol concentration increased, the fluorescence intensity at 364 nm of the DMAND solution became stronger with a blue shift. This is due to the assembly of lipophilic terminated-cholesteryl moieties of PMEA-Chol and the formation of hydrophobic domains in water by the hydrophobic interaction of cholesteryl groups at the polymer end. Other cholesteryl group-terminated hompolymers and copolymers with NiPAAm gave fluorescence spectra similar to those of PMEA-Chol. The critical micelle concentration (CMC) due to the association of PMEA-Chol in an aqueous solution can be deduced from the concentration at which there is a rapid increase of the fluorescence intensity of DMAND in water with a gradual increase in the concentration of PMEA-Chol. Figures 2–4 illustrate the concentration dependence on fluorescence intensity of the aqueous PMEA-*n*Chol, PMET-*n*Chol, and PNIPAm-*co*-PMEA-*n*Chol ($n = 1,2$) solutions. The breaks at 0.09 and 0.02 g L^{-1} in

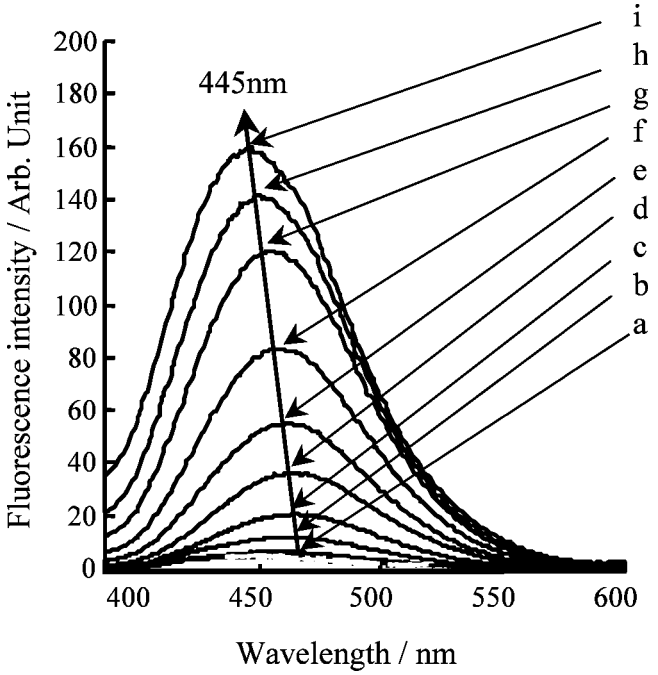


Figure 1 Fluorescence spectra of DMAND in aqueous solution of PMEA-Chol. [PMEA-Chol] was varied: (a) 0.019, (b) 0.039, (c) 0.078, (d) 0.15, (e) 0.31, (f) 0.63, (g) 1.30, (h) 2.50, (i) 5.00 g L^{-1}, [DMAND] = 1.0 μmol L^{-1}, λ EX = 364 nm.

Figure 2, for instance, can be taken as the CMCs for the aqueous solutions of PMEA-Chol and PMEA-2Chol, respectively. The CMCs for other polymers were also obtained in a similar manner and are summarized in Table I. From the effect of the number of cholesteryl

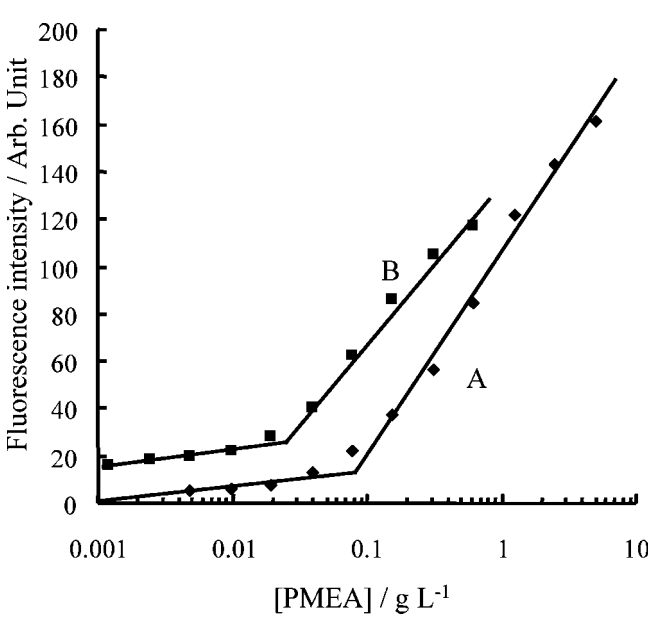


Figure 2 Relationship between fluorescence intensity and the concentration of PMEA-*n*Chol (*n* = 1,2) in aqueous solution. [DMAND] = 1.0 μmol L^{-1}, λ EX = 364 nm. A: PMEA-Chol, CMC = 0.09 g L^{-1}; B: PMEA-2Chol, CMC = 0.02 g L^{-1}.

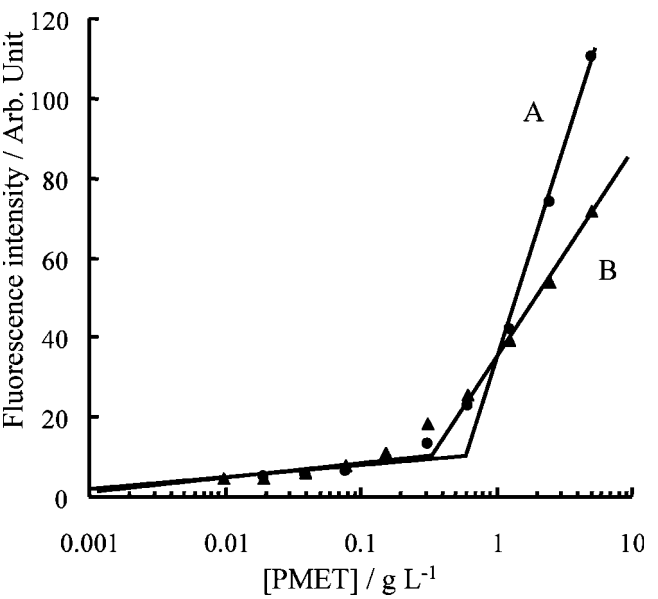


Figure 3 Relationship between fluorescence intensity and the concentration of PMET-*n*Chol (*n* = 1,2) in aqueous solution. [DMAND] = 1.0 μmol L^{-1}, λ EX = 364 nm. A: PMET-Chol, CMC = 0.6 g L^{-1}; B: PMET-2Chol, CMC = 0.3 g L^{-1}.

groups on CMC, those amphiphilic polymethacrylates, which introduced two cholesteryl groups at either the polymer end, can assemble more easily than those with one cholesteryl group at the polymer end. The introduction of two cholesteryl groups in hydrophilic polymers is, therefore, useful for assembling the lipophilic groups and forming a rigid core in a micelle formation.

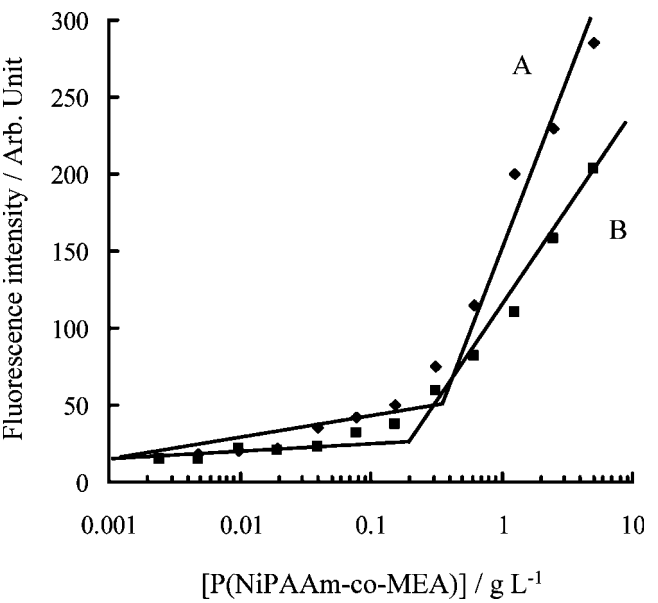


Figure 4 Relationship between fluorescence intensity and the concentration of PNiPAAm-*co*-PMEA-*n*Chol (*n* = 1,2) in aqueous solution. [DMAND] = 1.0 μmol L^{-1}, λ EX = 364 nm. A: PNiPAAm-*co*-PMEA-Chol, CMC = 0.3 g L^{-1}; B: PNiPAAm-*co*-PMEA-2Chol, CMC = 0.2 g L^{-1}.

Phase transition

It is known that the aqueous PNiPAAm solution exhibits phase separation at elevated temperatures, which is called the lower critical solution temperature (LCST). The transmittance of aqueous PNiPAAm-*co*-PMEA-*n*Chol (n = 1,2) (concentration = 5 g L^{-1}) was measured, as shown in Figure 5. Although PNiPAAm has a LCST at 32°C, PNiPAAm-*co*-PMEA-Chol and PNiPAAm-*co*-PMEA-2Chol show transparency below 35°C and 38°C, and suddenly turn opaque when the temperature rises to 37°C and 40°C, respectively, as shown in Figure 5. It is well-known that in the case of the copolymer of NiPAAm the LCST increases with the introduction of hydrophilic comonomer, whereas it decreases with the introduction of hydrophobic ones.[31,32] Thermoresponsible polymers like PNiPAAm-*co*-PMEA-Chol are considered to be useful anticancer drug-carrier molecules for a drug-delivery system (DDS)[11,17] mentioned below.

Carrier for the lipophilic type of drug

Since two cholesteryl groups at either end of PMEA-2Chol can self-organize in water to form the hydrophobic inner core stabilized by hydrophilic PMEA segments as an outer shell, our interest was first directed to the application to a carrier molecule of lipophilic type drugs for a DDS.[17] The DDS model was then prepared from PMEA-2Chol and cholesterol as a substitute of a lipophilic drug, as mentioned in experimental parts. Figure 6 depicts the DSC traces for the

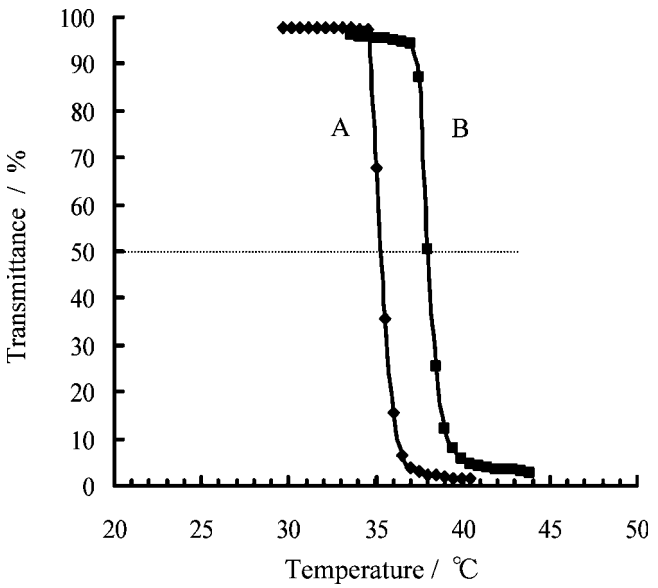


Figure 5 Effect of temperature on the transmittance (T) of aqueous PNiPAAm-*co*-PMEA-*n*Chol (n = 1,2) solution. [PNiPAAm-*co*-PMEA-*n*Chol] = 5.0 g L^{-1}. A: PNiPAAm-*co*-PMEA-Chol, LCST = 35.2°C; B: PNiPAAm-*co*-PMEA-2Chol, LCST = 38.0°C.

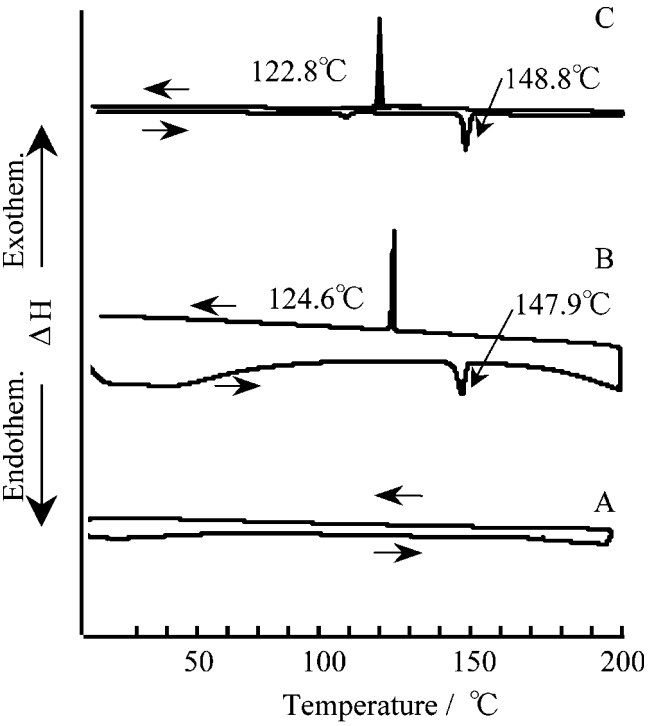


Figure 6 DSC thermograms for A: PMEA-2Chol, B: DDS model compound, C: cholesterol, heating and cooling rate: 5.0°C min^{-1}, sample quantity: 5 mg.

DDS model (PMEA-2Chol and cholesterol system) and cholesterol. During the melting process, the cholesterol and DDS model show one endothermic peak at 147.9°C and 148.8°C, respectively, whereas PMEA-2Chol has no peaks during either the heating or cooling cycles. The DDS model compound shows a slightly lower melting point than that of cholesterol. This means that PMEA-2Chol carrying lipophilic drug is built by the hydrophobic intermolecular interaction of the cholesterol molecule as lipophilic drug with the polymer end groups. Figure 7 shows the SAXS profile of the model, together with the profiles of PMPC-2Chol and cholesterol. The model shows a peak at 2θ = 2.5°C corresponding to an interplaner spacing of d = 35.3 Å, whereas cholesterol shows a peak at 2θ = 7.1° (d = 12.5 Å). The peak for the model is assigned to the layer spacing in a structure analogous to the biomembrane,[17,33] including the cholesterol molecules, as shown in Scheme 3.

Carrier for the nucleic acid base type of drug

Since adenine is the complementary nucleic acid base for uracil moiety, the interaction of PMEA-Chol and PMET-Chol having an absorption maximum of 260 nm based on nucleic acid bases was first confirmed by the continuous variation method using UV spectra. The concentrations of adenine and thymine units in the polymethacrylates were changed from 0 to

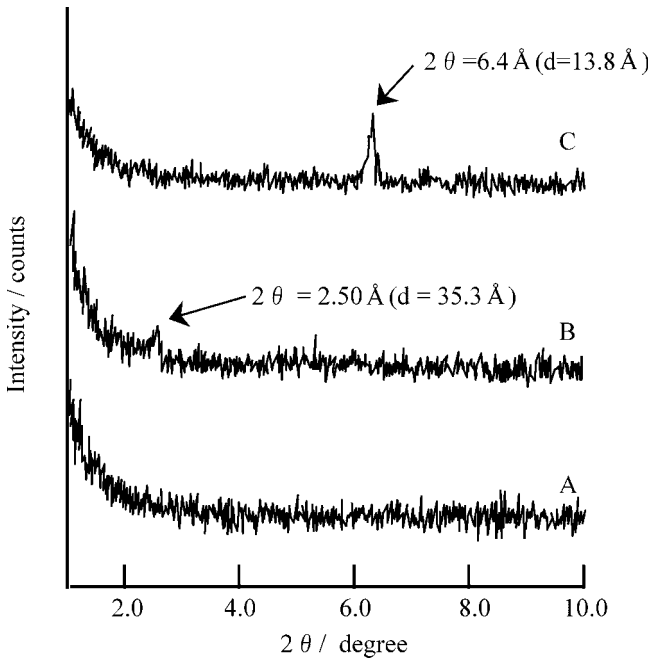


Figure 7 SAXS diffraction scans for powder samples. A: PMEA-2Chol, B: DDS model compound, C: cholesterol.

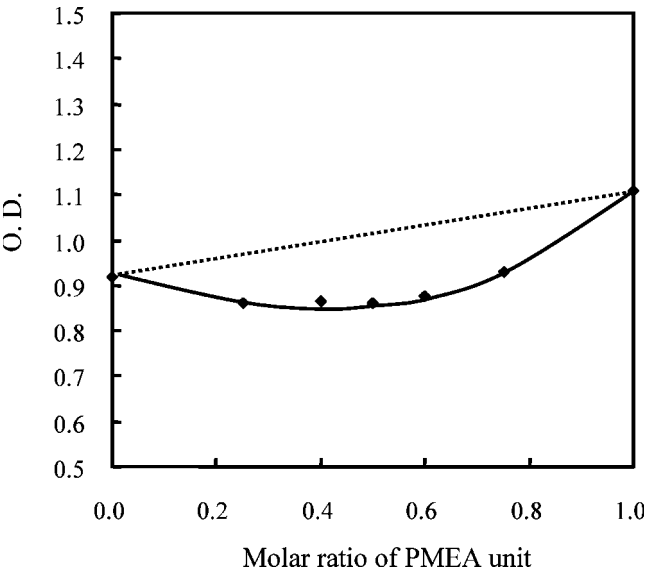
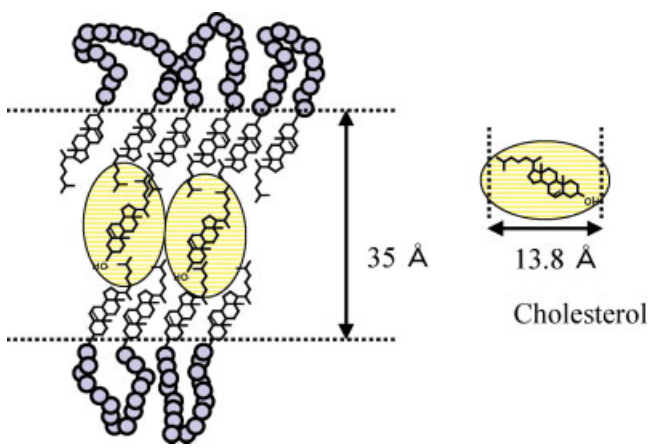


Figure 8 Continuous variation method applied to the system of PMEA-Chol and PMET-Chol. The concentrations of adenine and thymine units in PMEA-Chol and PMET-Chol were changed from 0 to 0.125 mmol L^{-1} and 0.125 to 0 mmol L^{-1}, respectively.

0.125 mmol L^{-1} and 0.125 to 0 mmol L^{-1}, respectively. Figure 8 shows the comparison of observed and Beer's law absorbance (dashed line) at 265 nm for aqueous solution containing different relative amounts of PMEA-Chol and PMET-Chol at 0.125 unit mmol L^{-1} of the total molar solute concentration. A negative deviation from Beer's law was observed and the relatively large deviation occurs in an equimolar solution mixture of them, and therefore, a hypochromism appears to be related to the formation of a 1 : 1 interaction of adenine and thymine moieties.[21] The interaction of PMET-2Chol and adenosine as a nucleic acid base type of drug was then confirmed by the continuous variation method. Figure 9 shows the hypochromism which appeared in the system of PMET-2Chol and adenosine. On the other hand, the interaction of uridine and PMEA-2Chol was confirmed by the addition effect of 0.1 mmol of uridine on the CMC of PMEA-2Chol solution (0.1 unit mmol in 5 mL of water). As can be seen in Figure 10, the CMC of PMEA-2Chol shifts from 2.0×10^{-2} g L^{-1} to 3.4×10^{-2} g L^{-1}. This means that uridine molecules



Scheme 3 Tentative structure of DDS loaded cholesterol. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

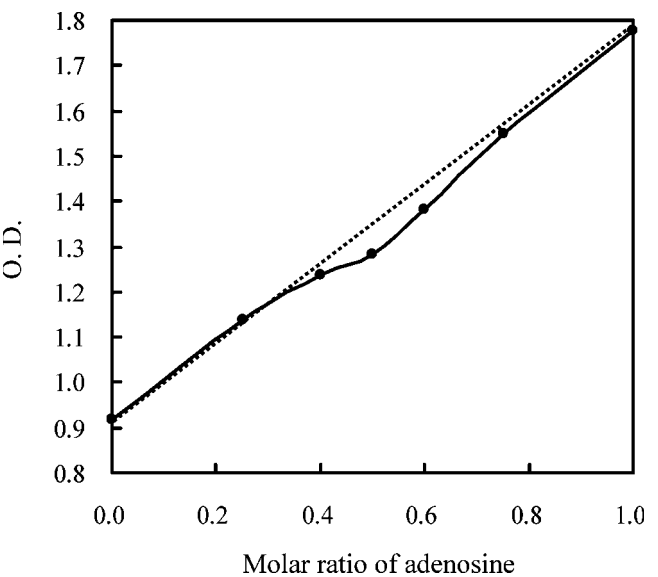


Figure 9 Continuous variation method applied to the system of PMET-Chol and adenosine. The concentrations of thymine unit in PMET-Chol and adenosine were changed from 0 to 0.125 mmol L^{-1} and 0.125 to 0 mmol L^{-1}, respectively.

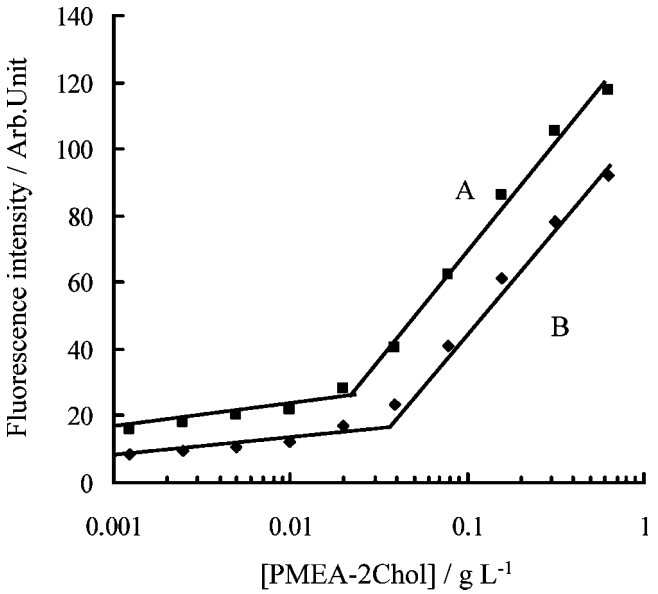


Figure 10 Addition effect of uridine on the CMC of PMEA-2Chol aqueous solution. [uridine] was a equimolar quantity of the adenine unit in PMEA-2Chol, [DMAND] = 1.0 μmol L^{-1}, λ EX = 364 nm. A: PMEA-Chol, [CMC] = 0.02 g L^{-1}; B: PMEA-2Chol + uridine, [CMC] = 0.034 g L^{-1}.

interact complementarily with adenine moiety in hydrophilic PMEA shell parts. The complementary interaction between uridine molecules and adenine moiety in hydrophilic PMEA segments results in increased bulkiness and hydrophilicity of shells, based on the pentose moiety in uridine. Consequently, the PMEA and PMET having cholesteryl groups at polymer ends can be applied to carriers of the complementary nucleic acid base type of drugs, such as 5-fluorouridine (Doxifluridine™) and adenosine (Vidarabine™).

Carrier for CDDP

Cis-dichlorodiammine platinum(II) (CDDP) has a potent antineoplastic activity against a wide spectrum of human tumors.[34] The anticancer activity of CDDP is attributed to intra- or interstranded bifunctional cross-linking of DNA and proteins, resulting in inhibition of DNA replication and of subsequent RNA transcription.[35] However, the clinical use of CDDP is strictly limited due to side effects such as unexpected and severe thrombocytopenia, gastrointestinal, renal, and neurological toxicities.[36,37] To reduce drug toxicity and to maintain high and prolonged drug levels, CDDP is modified to DDS by linking it to macromolecular carriers, such as poly(nucleotide)s,[38] poly(amino acid)s,[38,39] carboxymethyl dextran,[40] poly(L-glutamic acid),[40] and poly(ethylene glycol)-poly(aspartic acid) block copolymer.[41] We prepared the CDDP-incorporated polymeric micelles of PNiPPAm-*co*-PMEA-Chol as a novel DDS for CDDP. The incorporation of CDDP with PNiPPAm-*co*-PMEA-Chol and the release of CDDP from the carrier were confirmed for the addition effect of CDDP or/and DNA on the LCST of aqueous PNiPPAm-*co*-PMEA-Chol solution. The optical transmittance of aqueous PNiPAAm-*co*-PMEA-Chol solution (5.0 g L^{-1}) was measured in 0.5°C increments in the absence and in the presence of CDDP (1.0 g L^{-1}) at temperatures from 20 to 40°C, measuring the absorbance at 600 nm. The LCST was defined as the temperature at 50% turbidity.

The aqueous solution of PNiPAAm-*co*-PMEA-Chol (5.0 g L^{-1}) exhibits the LCST at 35.2°C as shown in Figure 5. It is interesting to say that the LCST decreases to 31.1°C when CDDP added to the aqueous solution and the LCST increases again toward the temperature at 35.2°C when DNA is added to the solution system as shown in Figure 11.

It is well-known that more the hydrophobicity becomes the lower is the LCST.[42] The first shift of the LCST (35.2°C → 31.1°C) is explained by the decreasing in hydrophilicity of PNiPAAm-*co*-PMEA-Chol based on ligand exchanging of CDDP molecules and the hyodrophilic adenine moiety. The second shift (31.1°C → 34.5°C) is explained by the increasing the hydrophilicity of PNiPAAm-*co*-PMEA-Chol based on the ligand exchanging of from the adenine moiety of the polymer to the guanine moiety in DNA (so called the intercalation). According to the Robins report[23], the second order rate constants for the reaction of CDDP with guanosine and adenosine were found to be 106 × 10^{-3} cm^{-1} s^{-1} and 6 × 10^{-3} cm^{-1} s^{-1}, respectively. Taking into account the rate constants of

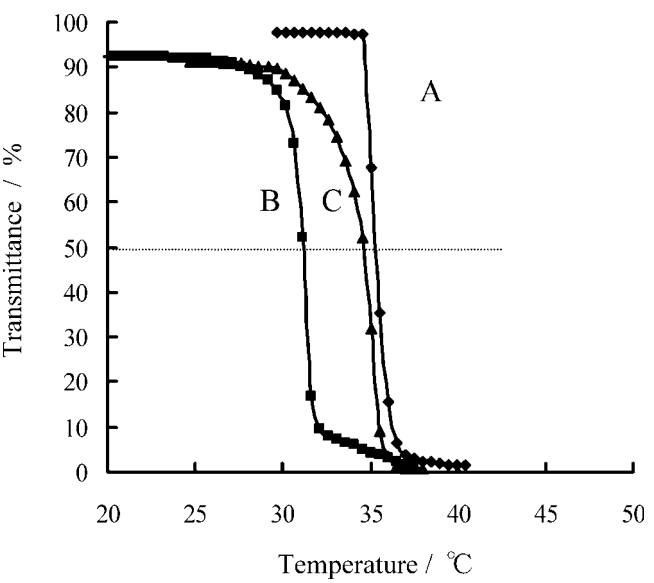
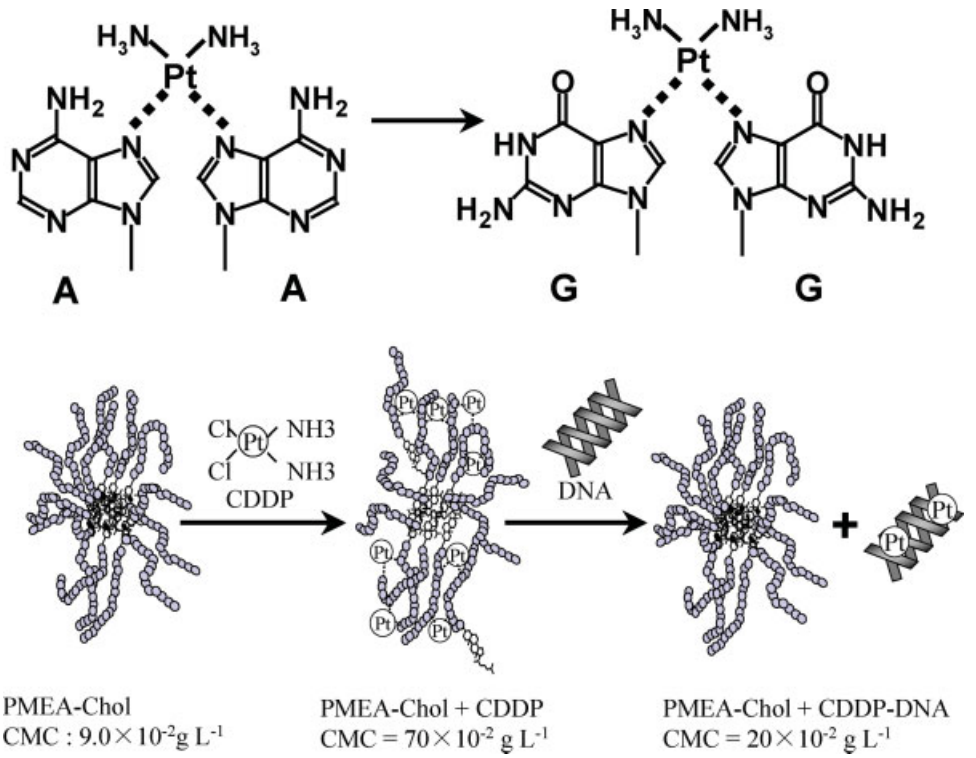


Figure 11 Addition effect of CDDP or/and DNA on the LCST of PNiPAAm-*co*-PMEA-Chol aqueous solution. [PNiPAAm-*co*-PMEA-Chol] = 5.0 g L^{-1}, [CDDP] = 1.0 g L^{-1}, [DNA] = 1.0 g L^{-1}, A: PNiPAAm-*co*-PMEA-Chol, LCST = 35.2°C, B: PNiPAAm-*co*-PMEA-Chol + CDDP, LCST = 31.1°C, C: PNiPAAm-*co*-PMEA-Chol + CDDP + DNA, LCST = 34.5°C.



Scheme 4 Chelation of CDDP with adenine groups in PMEA-Chol and intercalation to DNA. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

nucleic acid constituents, CDDP chelated to the adenine moiety in the MEA moiety is bound to the guanine moiety in DNA by ligand substitution reaction (Scheme 4), and the LCST of the aqueous PNiPAAm-*co*-PMEA-Chol solution increases from 31.1°C to 34.5°C, again very close to the original LCST at 35.2°C. The amount of CDDP bound to PNiPAAm-*co*-PMEA-Chol through the adenine moiety was measured by means of ICP emission spectroscopy of the clear layer of the copolymer solution that contained the free CDDP. The amount of CDDP loaded to the copolymer was found to be 0.143 g L^{-1}.

The following conclusion can be drawn

1. Nucleic acid base polymers, PMEA-*n*Chol, PMET-*n*Chol, and PNiPPAm-*co*-PMEA-*n*Chol (n = 1,2) with the cholesteryl group as terminal ends, can self-organize to form micelles based on the association of hydrophobic cholesteryl groups.
2. Hydrophobic drugs can be incorporated into polymeric micelles by physical entrapment in the inner core of the micelles. PMEA-2Chol includes cholesterol molecules as a lipophilic drug model in the core of a micelle to form a complex with a layer spacing (d = 35.2°) in a structure analogous to the biomembrane.
3. Nucleic acid base type drugs can be incorporated into polymeric micelles by interaction with the complementary nucleic acid base in the shell parts of the micelles.
4. CDDP can be incorporated into micelles by ligand substitution reaction of the adenine moiety in the shell part of the micelles.
5. Since it is conceivable that, the architectural incompleteness of tumor vessels is responsible for the enhanced permeability and retention of macromolecules (EPR effects) in tumors,[43] the polymeric micelles discussed in this study are considered to be a sufficiently novel carrier for drug delivery.

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