Polymer Vesicles Formed by Mixing of Amphiphilic Polymer and Hydrophobic Cholesterol-containing Homopolymer in Water

Sayaka Ohno and Shin-ichi Yusa*
Department of Materials Science and Chemistry, Graduate School of Engineering, University of Hyogo, 2167 Shosha, Himeji, Hyogo 671-2280, Japan
Fax: +81-79-266-8868, e-mail: yusa@eng.u-hyogo.ac.jp

Amphiphilic α-cholesteryl poly(ethylene glycol) (CS-050) consists of hydrophilic poly(ethylene glycol) with a hydrophobic cholesteryl group at the polymer chain end. Hydrophobic poly(cholesteryl 6-methacryloyloxyhexanoate) (PChM) was prepared via reversible addition-fragmentation chain transfer radical polymerization. A mixture of PChM and CS-050 in organic solution was dialyzed against pure water to exchange the solvent. These polymers formed vesicles in pure water, which were characterized using dynamic light scattering, scanning electron microscopy, and transmission electron microscopy techniques. The light scattering intensity for the vesicle solution was increased upon increasing the [PChM]/[CS-050] weight ratio. However, the diameter of the vesicles remained constant, independent of the [PChM]/[CS-050] weight ratio. The thickness of the vesicles increased with increasing amounts of the cholesteryl group content in the vesicle.

Key words: cholesteryl group, amphiphilic polymer, aggregation, RAFT, vesicle

1. Introduction
Nanometer-sized self-assemblies are formed by intermolecular interactions such as hydrophobic, electrostatic, hydrogen bonding, and van der Waals interactions [1-4]. Self-assemblies formed by hydrophobic interactions in water have been widely studied. Amphiphilic copolymers can form various kinds of aggregates such as spherical and rod-like micelles, vesicles, and so on, by hydrophobic interactions in water above a critical aggregation concentration [5]. The structure of the aggregates strongly depends on the balance of the molecular weights between hydrophobic and hydrophilic parts [6].

Hydrophobic cholesteryl (Chol) groups tend to align due to their rigid planar steroid backbone [7]. The pendant Chol groups in polymer chains can align with the lamellae structure [8]. Amphiphilic block copolymers that include Chol groups in the hydrophobic block form various self-assembled shapes other than spherical micelles [9,10]. For example, amphiphilic diblock copolymers composed of hydrophilic poly(N,N-diethylacrylamide) and hydrophobic pendant Chol group-containing blocks form fiber structures in water [11]. Amphiphilic block copolymers composed of hydrophilic poly(ethylene glycol) (PEG) and pendant Chol group-containing blocks form ellipsoidal vesicles due to interaction between Chol groups [12]. Moreover, amphiphilic block copolymers composed of hydrophilic PEG and pendant Chol group-containing blocks form disk-like micelles and stacked disks in water depending on the Chol group composition in the polymer [13]. The structure of the self-assemblies can be controlled by the Chol composition in the polymer. With this strategy, it is necessary to synthesize the block polymers in each case for changing the Chol composition. However, it is easy to control the hydrophobic composition in the system by changing the mixing ratio of the amphiphilic and hydrophobic copolymers [14,15]. Chen et al. [16] have reported that the self-assembled structure of a mixture of poly(acrylic acid)-graft-poly(γ-benzyl-L-glutamate) graft copolymers and poly(γ-benzyl-L-glutamate) can be controlled from rod-like to toroidal micelles by changing the mixing ratio of the graft copolymer and homopolymer.

In the present work, we prepared a hydrophobic polymer (PChM) with pendant Chol groups via reversible addition-fragmentation chain transfer (RAFT) controlled/living radical polymerization [17]. Amphiphilic α-Chol PEG (CS-050) composed of hydrophilic PEG with a hydrophobic Chol group at the polymer chain end was mixed with hydrophobic PChM to form PChM/CS-050 complexes in water (Figure 1). The mixing weight ratio of [PChM]/[CS-050] was changed to control the Chol composition in the...
PChM/CS-050 complex system without changing the chemical structure of the polymer. We studied the effect of the mixing ratio on the size and shape of the PChM/CS-050 complexes in water.

2. Experimental

2.1. Materials

α-Cholesteryl poly(ethylene glycol) (CS-050, molecular weight: 5,000) was obtained as a gift from NOF Corporation and used as received without further purification. 2,2’-Azobisisobutyronitrile (AIBN) was purified by recrystallization from methanol. 4-Cyanopentanoic acid dithiobenzoate (CPD) was synthesized according to the method reported by McCormick and co-workers [18]. Cholesteryl 6-methacryloyloxyhexanoate (ChM) was synthesized according to the method reported by Shannon [19]. Pyrene (> 98.0%) from Kanto Chemical was used as received without further purification. Tetrahydrofuran (THF) was purified by distillation. Water was purified with a Millipore Milli-Q system. Other reagents were used as received.

2.2. Preparation of PChM

ChM (1.00 g, 1.76 mmol), CPD (9.85 mg, 3.35 × 10⁻² mmol), and AIBN (1.17 mg, 7.13 × 10⁻³ mmol) were dissolved in THF (1.76 mL). The solution was deoxygenated by purging with Ar gas for 30 min. Polymerization was carried out at 60 °C for 15 h. 1H NMR analysis indicated that the conversion of PChM was 93.0%. The reaction mixture was dialyzed against THF for three days. The THF solution was poured into a large excess of methanol. The obtained polymer (PChM) was dried in a vacuum oven at 60 °C (0.430 g, 42.9%). Number-average molecular weight (Mn(GPC)) and molecular weight distribution (Mw/Mn) estimated from gel-permeation chromatography (GPC) were 2.06 × 10⁶ and 1.12, respectively. Mw(GPC) and degree of polymerization (DP) were determined from the 1H NMR peak integral intensity ratio between the vinyl protons and the pendant methine proton at 5.3 ppm and terminal phenyl protons at 8.1-8.7 ppm. Mw(GPC) and DP were 2.67 × 10⁶ and 50, respectively.

To investigate the relationship between polymerization time and monomer conversion, the following experiments were performed. Predetermined amounts of ChM, CPD, and AIBN were dissolved in THF. The solution was deoxygenated by purging with Ar gas for 30 min. Polymerization was carried out at 60 °C. Monomer conversion was determined from the 1H NMR peak integral intensity ratio of the vinyl protons at 6.2-6.7 ppm and the pendant methine proton at 5.3 ppm.

2.3. Measurements

1H NMR spectra were obtained with a Bruker DRX-500 spectrometer.GPC measurements were performed using a refractive index (RI) detector equipped with three Shodex KF-803L columns and a Shodex KF-805L column at 40 °C under a flow rate of 1.0 mL/min. THF was used as the eluent. The values of Mw(GPC) and Mn(GPC) were calibrated with standard polystyrene samples. Static light scattering (SLS) measurements were performed with an Otsuka Electronics Photol DLS-7000HL light scattering spectrometer at 25 °C. A He-Ne laser (10 mW at 633 nm) was used as a light source. Sample solutions for light scattering measurements were filtered using a membrane filter with 0.2 µm pores. The weight-average molecular weight (Mw(SLS)) and z-average radius of gyration (Rg) were estimated with DLS and SLS data. The values of do/dc, at 633 nm were determined with an Otsuka Electronics Photol DRM-3000 differential refractometer at 25 °C. Dynamic light scattering (DLS) measurements were performed using a Malvern Instruments Zetasizer Nano ZS equipped with a He-Ne laser (4 mW at 633 nm). Measurements were taken at 173° scattering angle. Scanning electron microscopy (SEM) analyses were carried out on a Keyence VE-9800 with an accelerating voltage of 10 kV. A sample for TEM observation was prepared by dropping a sample solution onto a glass slide sputtered with Pt and completely dried at room temperature. The sample was sputtered with Pt using a Quick Coater SC-701MK II. Transmission electron microscopy (TEM) measurements were performed with a JEOL TEM-1200 electron microscope operated at an accelerating voltage of 200 kV. Samples for TEM were prepared by placing one drop of the aqueous solution on a copper grid coated with thin films of Formvar. Excess water was blotted using filter paper. The samples were stained by sodium phosphotungstate and dried under vacuum for one day.

2.4. Critical Micelle Concentration (CMC) of CS-050

Fluorescence spectra were recorded on a Hitachi F-2500 fluorescence spectrophotometer. Pyrene was used as the fluorescence probe. The pyrene-saturated aqueous stock solution was prepared as previously [21]. CS-050 was dissolved in pyrene-saturated aqueous solution, which was then diluted with a pyrene-saturated aqueous solution to measure fluorescence emission and excitation spectra. For emission spectra, the solutions were excited at 325 nm, and the excitation and emission slit widths were maintained at 20 and 2.5 nm, respectively. For excitation spectra, the solutions were monitored at 390 nm, and the excitation and emission slit widths were maintained at 2.5 and 10 nm, respectively.

2.5. Preparation of PChM/CS-050 Complex

PChM was dissolved in THF at a concentration of 0.3 g/L. The CS-050 THF solutions were added to the PChM solution to reach weight ratios of [PChM]/[CS-050] = 1/1, 2/1, and 5/1. The mixed solution was stirred at 30 °C for 3 h. The mixed solution was then transferred to a dialysis bag, which was dialyzed against pure water for 24 h at room temperature. The final polymer concentration (Cn) of the PChM/CS-050 complex after dialysis was adjusted to 0.05 g/L by diluting with pure water.

3. Results and Discussion

3.1. Preparation of PChM

To investigate the relationship between polymerization time and conversion of ChM, 1H NMR measurements were performed to determine the conversion at varying polymerization times. The conversion of ChM was estimated from the 1H NMR
Figure 2. Time-conversion (○) and first-order kinetic plots (△) for the polymerization of ChM in THF in the presence of chain transfer agent at 60 °C.

Figure 3. 1H NMR spectra for (a) PChM in CDCl₃ at 20 °C and (b) CS-050 in DMSO-d₆ at 100 °C.

molecular weight of CTA. The Rₛ value for PChM in THF was 3.8 nm. This small Rₛ value indicates that PChM can dissolve in THF as a single polymer chain, i.e., unimer state.

3.2 Characterization of CS-050

The Mₙ(GPC) and Mₖ/Mₙ values for CS-050 estimated from GPC using THF as an eluent were 9.0 × 10³ and 1.02, respectively (Table 1). Figure 3b shows the 1H NMR spectrum for CS-050 in DMSO-d₆ at 100 °C. The resonance peaks observed at 0.6, 0.9, and 1.0 ppm were attributed to the methyl protons in the terminal Chol group. The resonance peaks at 2.3 and 5.3 ppm were also attributed to methylene and methine protons in the terminal Chol group. The resonance peak at 3.4-3.7 ppm was attributed to methylene protons in the PEG chain. The DP and Mₙ(NMR) values for CS-050 were calculated to be 121 and 5.71 × 10³ on the basis of the integral intensity ratio of the resonance peaks due to the methine and methylene protons in the terminal Chol group at 5.3 ppm and in PEG at 3.4-3.7 ppm. The Rₛ values for CS-050 in THF and water were 2.0 and 10 nm, respectively. This observation suggests that CS-050 can dissolve in THF as a unimer state; however, CS-050 may form micelle-like aggregates in water due to hydrophobic interactions between the terminal hydrophobic Chol groups.

Table 2. Dynamic and Static Light Scattering Data and Critical Micelle Concentration (CMC) for CS-050 in Water

We studied the association behavior of CS-050 in water using a SLS technique. DLS and SLS data for CS-050 in water are summarized in Table 2. The Rₛ/R₆
ratio is useful for characterizing the shape of molecular assemblies. The theoretical values of $R_g/R_h$ for a homogeneous hard sphere is 0.778 and is substantially higher for less dense structures or polydisperse mixtures, e.g., 1.0 for a sphere, 1.3-1.5 for a random coil, and >1.5 for rod-like structures [23-25]. The $R_g/R_h$ for CS-050 in water is 0.94 suggesting that the CS-050 micelle is a homogeneous spherical shape. The aggregation number ($N_{agg}$) for the micelle, defined as the total number of polymer chains per one polymer micelle, could be calculated from the ratio of $M_w$(SLS) for the polymer micelle to that of the single polymer chain (unimer) estimated from NMR and GPC. $N_{agg}$ for the CS-050 micelle was found to be 53.

The CMC value for the CS-050 micelle in water was 0.014 g/L estimated from the NMR and GPC. The CMC value for the CS-050 micelle in water was 0.94 suggesting that the CS-050 micelle is a spherical CS-050 micelle with microenvironmental polarity around the pyrene nature. First we prepared THF solutions of a mixture of $[\text{PChM}] / [\text{CS-050}]$ were adjusted to be 1/1, 2/1, and 5/1. In this case the molar ratios of $[\text{PChM}]/[\text{CS-050}]$ were 0.013/1, 0.026/1, and 0.065/1, respectively. PChM and CS-050 can dissolve in THF homogeneously without aggregation, because THF is a good solvent for the polymers. The THF solutions of the PChM and CS-050 mixtures of various $[\text{PChM}] / [\text{CS-050}]$ ratios were dialyzed against pure water to exchange the solvent. After the dialysis, PChM and CS-050 formed PChM/CS-050 complexes in water. The $C_p$ values were adjusted to be 0.05 g/L to dilute the aqueous solution using pure water. Immediately after dilution to adjust the PChM/CS-050 complex concentration, $R_h$ and scattering intensity were measured (Figure 4). At $[\text{PChM}]/[\text{CS-050}] = 0$ in water, CS-050 without PChM forms spherical micelles with $R_h = 10$ nm. The $R_h$ values for the PChM/CS-050 complex were about 260 nm. This observation indicates that the shape of the PChM/CS-050 complexes is different from that of the spherical CS-050 micelle with $R_h = 10$ nm. When the weight ratio of $[\text{PChM}]/[\text{CS-050}]$ increased from 1/1 to 5/1, the $R_h$ value remained almost constant at ca. 260 nm. On the other hand, the scattering intensity for PChM/CS-050 complex increased with increasing $[\text{PChM}]/[\text{CS-050}]$ weight ratio. These observations suggest that the size of the PChM/CS-050 complex was almost constant, however the density of the complex increased. The number of the polymer vesicles may keep constant with increasing $[\text{PChM}]/[\text{CS-050}]$ ratio, because the concentration of amphiphilic CS-050 was fixed at a constant value. Turbidity of the PChM/CS-050 complex solution increased with increasing content of PChM.

![Figure 4](image_url)

**Figure 4.** (a) $R_h$ (○) and scattering intensity (△) for PChM/CS-050 complexes as a function of weight ratio of $[\text{PChM}]/[\text{CS-050}]$ in water at $C_p = 0.05$ g/L. (b) Digital photographs of PChM/CS-050 complexes with various $[\text{PChM}]/[\text{CS-050}]$ ratios in water at $C_p = 0.05$ g/L.

3.3 PChM/CS-050 Complex

Generally self-assemblies of amphiphilic molecules in water tend to change from micelles to vesicles with increasing hydrophobic part in the molecules due to stronger hydrophobic interactions. When CS-050 was dissolved in water, the micelles were formed. To control the hydrophobic composition in the system, we changed the mixing ratio of $[\text{PChM}]/[\text{CS-050}]$. PChM cannot dissolve in water directly because of its hydrophobic nature. First we prepared THF solutions of a mixture of PChM and CS-050. The weight ratios of $[\text{PChM}]/[\text{CS-050}]$ were adjusted to be 1/1, 2/1, and 5/1. In this case the molar ratios of $[\text{PChM}]/[\text{CS-050}]$ were 0.013/1, 0.026/1, and 0.065/1, respectively. PChM and CS-050 can dissolve in THF homogeneously without aggregation, because THF is a good solvent for the polymers. The THF solutions of the PChM and CS-050 mixtures of various $[\text{PChM}]/[\text{CS-050}]$ ratios were dialyzed against pure water to exchange the solvent. After the dialysis, PChM and CS-050 formed PChM/CS-050 complexes in water. The $C_p$ values were adjusted to be 0.05 g/L to dilute the aqueous solution using pure water. Immediately after dilution to adjust the PChM/CS-050 complex concentration, $R_h$ and scattering intensity were measured (Figure 4). At $[\text{PChM}]/[\text{CS-050}] = 0$ in water, CS-050 without PChM forms spherical micelles with $R_h = 10$ nm. The $R_h$ values for the PChM/CS-050 complex were about 260 nm. This observation indicates that the shape of the PChM/CS-050 complexes is different from that of the spherical CS-050 micelle with $R_h = 10$ nm. When the weight ratio of $[\text{PChM}]/[\text{CS-050}]$ increased from 1/1 to 5/1, the $R_h$ value remained almost constant at ca. 260 nm. On the other hand, the scattering intensity for PChM/CS-050 complex increased with increasing $[\text{PChM}]/[\text{CS-050}]$ weight ratio. These observations suggest that the size of the PChM/CS-050 complex was almost constant, however the density of the complex increased. The number of the polymer vesicles may keep constant with increasing $[\text{PChM}]/[\text{CS-050}]$ ratio, because the concentration of amphiphilic CS-050 was fixed at a constant value. Turbidity of the PChM/CS-050 complex solution increased with increasing content of PChM.

![Figure 5](image_url)

**Figure 5.** $R_h$ (○) and scattering intensity (△) for PChM/CS-050 complexes with $[\text{PChM}]/[\text{CS-050}] = 5/1$ (w/w) as a function of time in water at $C_p = 0.05$ g/L.

Figure 5 shows the time dependence on $R_h$ and scattering intensity for the PChM/CS-050 complex with $[\text{PChM}]/[\text{CS-050}] = 5/1$ in water. The time is zero in Figure 5, which means $C_p$ was adjusted to be 0.05 g/L after the dialysis. $R_h$ for the PChM/CS-050 complex was almost constant and independent of time. On the other hand, the scattering intensity slightly decreased with increasing time. A small amount of precipitate was formed as time passed. The scattering intensity decreased with increasing time, because the amount of the polymers dissolved in water decreased.
Figure 6. $R_h$ (○) and scattering intensity (△) for PChM/CS-050 complexes with [PChM]/[CS-050] = 5/1 (w/w) as a function of $C_p$ in water.

Figure 6 shows $R_h$ and scattering intensity for the PChM/CS-050 complex with [PChM]/[CS-050] = 5/1 as a function of $C_p$ ranging from 0.05 to 0.00125 g/L. The $R_h$ value for the PChM/CS-050 complex was almost constant at ca. 250 independent of $C_p$ ranging from 0.05 to 0.00125 g/L. The complex was stable against dilution.

Figure 7. SEM and TEM images for PChM/CS-050 complexes with [PChM]/[CS-050] = (a) 1/1, (b) 2/1, and (c) 5/1 (w/w).

From SEM images, the shape of the PChM/CS-050 complex was spherical with a radius of 300 nm, independent of the weight ratio of [PChM]/[CS-050] (Figure 7). The size of the complex estimated from SEM was close to that estimated from DLS. For further confirmation of the PchM/CS-050 complex structure, we carried out TEM measurements (Figure 7). The structures of the PChM/CS-050 complexes were vesicles independent of the weight ratios of [PChM]/[CS-050] ranging from 1/1 to 5/1. The average radii of the PChM/CS-050 complexes estimated from TEM were 283, 262, and 376 nm, with increasing [PChM]/[CS-050] ratios from 1/1, 2/1, and 5/1, respectively. The average thickness values of the vesicle membranes with [PChM]/[CS-050] ratios of 1/1, 2/1, and 5/1 were 18, 29, and 118 nm, respectively, estimated from TEM data. The thickness of the vesicle membrane increased in water with increasing content of Chol groups in the system. The density of the vesicles formed from PChM/CS-050 complexes increased with increasing [PChM]/[CS-050] ratio, because the membrane thickness increased with small vesicle size changes.

4. Conclusions

Hydrophobic PChM containing pendant Chol groups was prepared via RAFT controlled/living radical polymerization. PChM/CS-050 complexes in water produced by a dialysis method formed vesicle structures independent of the [PChM]/[CS-050] mixing weight ratio. The thickness of the vesicle membrane increased with increasing [PChM]/[CS-050] mixing ratio, however the vesicle size remained constant, suggesting that the vesicle density increased with increasing [PChM]/[CS-050] ratio. Hydrophobic and hydrophilic guest molecules could be incorporated in the membrane inside of the vesicle and internal hollow core, respectively. Both the interior and exterior of the vesicle membrane were covered with biocompatible PEG layers. It is expected that the vesicle size can be controlled by adjusting the chain length of the CS-050 and PChM. Therefore, these vesicles are expected to serve as highly versatile self-assembled drug carrier systems.

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References

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