Introduction

AB-type block copolymers having both hydrophilic and hydrophobic segments are known to form micellar structures in aqueous media because of their amphiphilic character. Highly hydrated outer shells can inhibit intermicellar aggregation of hydrophobic inner cores. Polymeric amphiphiles consisting of hydrophilic and hydrophobic segments have become attractive building blocks in the growing field of molecular self-assembly in aqueous media because of their unique solution properties and technical applications in various fields. In analogy with low-molecular-weight amphiphiles and lipids, they undergo intermolecular association by the hydrophobic segments leading to the construction of micelles or nano-aggregates, which have various morphological characteristics. In addition, the hydrophobic core is surrounded by a hydrophilic outer shell so that the inner core can serve as a microcontainer for various substances. Therefore, there has been a growing interest in the design and characterization of novel amphiphilic copolymers and hydrophobically modified water-soluble polymers, which can self-assemble to form micelles or micelle-like aggregates in an aqueous phase.

Compared with amphiphilic small molecules, such as phospholipids, polymeric micelles maintain their satisfactory aqueous stability irrespective of the high content of hydrophobic drug bound within the micelle inner...
core. Furthermore, the size range of polymeric micelles (<100 nm) reduce nonselective reticuloendothelial system (RES) scavenging and enhance permeability and retention effects at tumor tissue sites.\[22\] Recently, considerable attention has been paid to phospholipids because it is known that they consist of hydrophilic and hydrophobic groups and form a lipid bilayer and they are important building units of plasma membranes.\[23,24\] It is believed that polymers containing the phospholipid moiety provide biomembrane mimicry and should be more compatible with the human body.\[25\] On the basis of this “bioinspired” approach, the phospholipid-analogous vinyl polymer containing 2-(methacryloyloxy)ethyl 2-aminoethyl hydrogen phosphate and a very useful vinyl monomer, 2-(methacryloyloxy)ethyl phosphorylcholine (MPC), was successfully synthesized.\[26,27\] Since then a vast amount of work has extended the use of MPC, all of which has shown it to suppress protein adsorption,\[28,29\] platelet adhesion,\[30\] thrombus formation,\[31\] and cell adhesion.\[32\] Currently, solutions, hydrogels,\[33\] and nanoparticles\[34\] composed of PC-polymer are being evaluated as delivery vehicles in cosmetics\[35,36\] and pharmaceutical formulations.\[37\]

However, there are few studies on the self-assembling behavior of the well-defined polymeric amphiphiles that consist of hydrophilic poly[2-(methacryloyloxy)ethyl phosphorylcholine] (pMPC) and hydrophobic low-molecular-weight natural components. In this study, we describe the synthesis and the micellar characteristic of a novel well-defined polymeric amphiphile based on pMPC as a hydrophilic segment and cholesterol (Chol) as a hydrophobic segment. It is well known that sterols are widely present in membranes of most eukaryotic cells. Cholesterol (Chol) is one of the most common membrane sterols in animals that regulates membrane fluidity and plays an important role in the self-association of molecules in biological systems. Yusa et al.\[39\] found that Chol-containing amphiphilic polyelectrolytes form an intermolecularly bridged flower-type micelle in aqueous solution and suggested that Chol had a strong tendency for self-association even if the contents of Chol in polymers were very low. Hence it was possible to design strongly self-associative polymers by utilizing cholesteryl moiety in a very small amount.\[40\] In this study, it is expected that the introduction of cholesterol as a hydrophobic segment of pMPC can induce the self-association of Chlo-pMPC polymeric amphiphiles leading to the formation of micelles in an aqueous phase. The synthetic strategy for well-defined Chlo-pMPC polymeric amphiphiles in this study may allow the preparation of a biomimetic surfactant, which can be applied in the area of drug delivery. The preparation of well-defined Chlo-pMPC polymeric amphiphiles was carried out by the atom transfer radical polymerization (ATRP) of MPC using 2-bromoiso- butyryl cholesteryloxydecanol as the macroinitiator. Micellar characteristics of this novel polymeric amphiphile in an aqueous phase were investigated using ²H NMR spectroscopy, fluorescence probe techniques, and atomic force microscopy (AFM). The anti-cancer drug adriamycin (ADR) was chosen as a hydrophobic drug to be incorporated into the inner cores of the micelles. The drug-loading behavior of the novel biomimetic micelles was then investigated by AFM.

**Experimental Part**

**Materials**

The MPC monomer was synthesized according to the literature.\[26,27,41\] 10-Cholesteryloxydecanol was also synthesized according to the literature.\[42\] 2,2'-bipyridine (bpy) (AR, Hangzhou Chemical Reagent Factory) and 2-bromoiso-butyryl bromide (Aldrich, 98%) were used as received. CuBr (AR, Shanghai No. 1 Chemical Reagent Factory) was purified by washing with glacial acetic acid, followed by absolute ethanol and ethyl ether, and then dried under vacuum. Cation exchange resin 732 was from Hangzhou Shuanglin Chemical Factory. Ethanol and propan-2-ol were purchased from Huadong Medicine Co. Pyrene was purchased from Aldrich Co. and used as received. Dichloromethane and triethylamine were refluxed over calcium hydride for 24 h before use. Other reagents were purified by conventional methods.

The conversion of 10-cholesteryloxydecanol into the ATRP macrorinitiator (CholBr) by reaction with 2-bromoiso-butyryl bromide was performed as follows: Into a three-neck flask equipped with a constant pressure dropping funnel and thermometer, 2-bromoiso-butyryl bromide (4.029 g, 17.8 mmol) and triethylamine (1.801 g, 17.8 mmol) were mixed in CH₂Cl₂ at 0 °C (ice-water bath). A 15 mL aliquot of a 19% (w/v) CH₂Cl₂ solution of 10-cholesteryloxydecanol (2.848 g, 5.2 mmol) was added dropwise over 1 h. After the addition was complete, the reaction solution was allowed to warm to room temperature, and the reaction mixture was stirred for another 12 h. The reaction mixture was then filtered, and the CH₂Cl₂ was removed using the rotary evaporator. The resulting yellow crude product was precipitated into cold ethanol. The precipitate was filtered off and dried under vacuum.

**Polymerization Procedures**

A typical protocol for the controlled polymerization of MPC using the CholBr, in an ethanol/propan-2-ol (v:v = 1:1) mixture is as follows: MPC (1.00 g, 3.365 mmol) and CholBr (49.7 mg, 0.0675 mmol, 1 equiv.) were dissolved in ethanol/propan-2-ol (v:v = 1:1) mixture (50 mL). The flask, containing a stirrer bar, was degassed and back-filled with argon three times. The CuBr catalyst (9.5 mg, 0.0675 mmol, 1 equiv.) and bpy ligand (21.0 mg, 0.135 mmol, 2 equiv.) were added to the stirred solution and the reaction mixture immediately became dark brown. The flask was then placed into an oil bath thermostat at 50 °C. After 20 h, the reaction mixture was cooled down and the flask was opened. On exposure to air, the reaction solution turned blue, indicating aerial oxidation of the Cu catalyst. The resulting Chol-pMPC diblock copolymer was precipitated into tetrahydrofuran (THF), and then redissolved in ethanol. Cation exchange resins were added and the reaction...
Incorporation of Adriamycin (ADR)

Chol-pMPC polymer (CMPC10, 10 mg) was dissolved in 5 mL of water. ADR hydrochloride (1.0 mg) was solubilized in 2 mL of a mixture of chloroform and triethylamine (20 mL of chloroform and 2.4 µL of triethylamine). This solution was added dropwise to the aqueous micelle solution under vigorous stirring. The mixture was then vigorously stirred at room temperature overnight open to the atmosphere. Then micelle solution was then centrifuged at 12 000 rpm to eliminate unloaded adriamycin and aggregated particles. The drug loaded micellar solution was then used in the following experiments.

Measurements

1H NMR Spectroscopy

All 1H NMR spectra were recorded using a 500 MHz Bruker instrument. The polymers were analyzed in pure CD3OD, pure D2O, and CD3OD/D2O (v/v = 1:1) solvent mixtures with reference to tetramethylsilane (TMS).

Fluorescence Measurements

Fluorescence spectra were recorded on a spectrofluorometer (FP-770, Japan Spectroscopic) at room temperature. Pyrene dissolved (FP-770, Japan Spectroscopic) at room temperature. Pyrene fluorescence spectra were recorded on a spectrofluorometer in acetone (4.8 M) was used as a hydrophobic fluorescent probe. Pyrene dissolved (FP-770, Japan Spectroscopic) at room temperature. Pyrene fluorescence measurements were conducted relative to tetramethylsilane (TMS).

Atomic Force Microscopy

All measurements were performed in the tapping mode under ambient conditions using a commercial scanning probe microscope, Seiko SPI3800N, equipped with a silicon cantilever, Nanosensors, typical spring constant 40 N/m. The polymer micelle solution (ca. 2 or 0.05 mg·mL⁻¹) was cast onto a freshly cleaved mica surface. After the evaporation of water, the samples for AFM were dried at atmospheric pressure and ambient temperature for ca. 5 h.

Results and Discussion

Synthesis of Biomimetic Block Copolymers Chol-pMPC (CMPC)

Atom transfer radical polymerization (ATRP) can offer synthesized polymers with well-defined compositions, architectures, and functionalities. [44–46] Armes and coworkers recently reported that ATRP is particularly effective for a wide range of hydrophilic monomers in protic media such as water and/or lower alcohols under mild conditions [47,48] and was the first to report the controlled homopolymerization of MPC [48] using aqueous or methanolic ATRP. The MPC-based block copolymers were also synthesized through the macroinitiator route or the sequential monomer addition route [49].

Novel biomimetic surfactants based on cholesterol as the hydrophobic segment and poly[2-(methacryloyloxy)ethyl phosphorylcholine] (pMPC) as the hydrophilic segment (Figure 1) were synthesized in the present study by the atom transfer radical polymerization (ATRP) of 2-(methacryloyloxy)ethyl phosphorylcholine (MPC) using a cholesterol-based macroinitiator (Figure 2).

The 1H NMR spectrum of the cholesterol-based macroinitiator is shown in Figure 3. The assignment of the 1H NMR signals are as follows: δ = 0.65 (H of CH3 from cholesterol (a)); 0.85 (H of CH3 from cholesterol (b)); 0.95 (H of CH3 from cholesterol (c)); 1.10 (H of CH3 from cholesterol (d)); 0.70–2.50 (H from CH2–CH2 and CHCH2 from cholesterol (e)); H of (CH2)k from the decamethylene (i); H of –C(CH3)2COO– (k)); 5.30 (H from =CH– from cholesterol (f)); 3.20 (H from –CH–O– from cholesterol (g)); 3.48 (H of CH2–O from decamethylene (h)); 4.11 (H of COOCH2 from the decamethylene (j)).

(C17H27O3Br) Calcd. C 71.17, H 10.34; Found C 71.01, H 10.61.

All the polymerizations were carried out in a solvent mixture of ethanol and propan-2-ol (v/v = 1:1) at 50 °C. The degree of polymerization, Dp, was controlled by the initial monomer/initiator molar ratio. A summary of the various composition and molecular-weight data are given in Table 1.
Figure 4 shows the $^1$H NMR spectrum of the copolymer CMPC10 in CD$_3$OD: $\delta = 0.65$ (H of CH$_3$ from cholesterol (a)); 0.85 (H of (CH$_3$)$_2$ from cholesterol (b)); 1.10 (H of CH$_3$ from cholesterol (d)); 0.70–2.50 (H from CH$_2$–CH$_2$ and CHCH$_2$ from cholesterol (e)); H of --(CH$_2$)$_9$-- from the decamethylene (i); H of --C(CH$_3$)$_2$COO-- (k); H of CH$_2$ (l) and CH$_3$ (m) from the main chain; 5.30 (H from C–H from cholesterol (f)); 3.20 (H from --C–O– from cholesterol (g)); 3.30 (H of (CH$_3$)$_3$ from phosphorylcholine group (r)); 3.48 (H of CH$_2$–O from decamethylene (h)); 3.76 (H of --C–H-- from phosphorylcholine group (q)); 4.11 (H of PO–C–CH$_2$–CH$_2$–N from phosphorylcholine group (p)); H of --OOC-- from the decamethylene (j)); 4.23 (H of PO–CH$_2$–CH$_2$–O from phosphorylcholine group (o)); 4.36 (H of PO–CH$_2$–CH$_2$–O from phosphorylcholine group (n)).

The molar ratio of the Chol terminal group to the MPC monomeric unit was calculated from the intensity ratio of the resonance band associated with the Chol methyl protons on C18, arising at 0.66 ppm, to the resonance band associated with the methylene protons in the phosphorylcholine side chain in the region 3.76–4.36 ppm. This molar ratio was used to calculate the molecular weight of the polymers. The results were shown in Table 1.

**Micelle Formation of Chol-pMPC**

Figure 5 shows the $^1$H NMR spectra of the selected copolymer, CMPC10, measured in pure CD$_3$OD, pure D$_2$O, and a D$_2$O/CD$_3$OD (v/v = 1:1) solvent mixture to observe the influenced of the solvent polarity on the structure in solution. In D$_2$O, the resonance peaks arising from Chol protons are not observable because of considerable line broadening, suggesting the association of Chol groups. In CD$_3$OD, however, resonance peaks associated with the terminal Chol group are observed in the region 0.6–1.0 ppm. Apparently,

### Table 1. Summary of synthesis, molecular weight data, and the critical micelle concentration (cmc) of copolymers.

<table>
<thead>
<tr>
<th>Sample</th>
<th>CholBr</th>
<th>MPC</th>
<th>CuBr</th>
<th>bpy</th>
<th>$\overline{M}_{n,\text{theor}}^{a)}$</th>
<th>$\overline{M}_{n,\text{exp}}^{b)}$</th>
<th>cmc$^{c)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>g</td>
<td>mg</td>
<td>mg</td>
<td>g</td>
<td>g</td>
<td>g mL$^{-1}$</td>
</tr>
<tr>
<td>CMPC10</td>
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<td>1.0</td>
<td>47.5</td>
<td>105</td>
<td>3557</td>
<td>2970</td>
<td>7.27 $\times$ 10$^{-3}$</td>
</tr>
<tr>
<td>CMPC20</td>
<td>124.3</td>
<td>1.0</td>
<td>23.8</td>
<td>53.6</td>
<td>6507</td>
<td>6350</td>
<td>13.47 $\times$ 10$^{-3}$</td>
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<tr>
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<td>1.0</td>
<td>11.9</td>
<td>26.3</td>
<td>12407</td>
<td>–</td>
<td>20.77 $\times$ 10$^{-3}$</td>
</tr>
</tbody>
</table>

$^{a)}$ Theoretical molecular weight calculated from the feed ratio based on 100% conversion.

$^{b)}$ Experimental molecular weight calculated from the $^1$H NMR spectroscopy.

$^{c)}$ Calculated from the excitation spectra of pyrene as a function of polymer concentrations in water.
along with the change of the solvent from CD$_3$OD to CD$_3$OD/D$_2$O = 1:1 and to D$_2$O, the mobility of the hydrophobic groups is lowered. This is indicative of a structural rearrangement of the amphiphilic copolymer in solution and the hydrophobic groups interacting with each other. Hence, in solution with a low affinity solvent, the hydrophobic groups may organize into a micellar structure. This phenomenon is attributed to the increase in the repulsive forces between the hydrophobic group and their poor solvent. In a good solvent (CD$_3$OD) for both the hydrophilic and hydrophobic part, both parts stick out into the solution, whereas in a poor solvent (D$_2$O) for the hydrophobic part, a “flip-flop” in morphology would prompt these groups to agglomerate into micellar domains.

**Fluorescence Studies**

Pyrene is commonly used as a fluorescence probe to monitor micropolarity.\(^{[50]}\) Pyrene is a condensed aromatic hydrocarbon that is highly hydrophobic and sensitive to the polarity of the surrounding environment. When the pyrene experiences a change from a polar environment to a non-polar environment, a red shift of the (0,0) band in the excitation spectra is observed. Hence the $I_{339}/I_{334}$ ratio, where $I_{334}$ and $I_{339}$ are the pyrene fluorescence intensities excited at 334 and 339 nm, respectively, is larger in a less polar environment.\(^{[51]}\) Figure 6a shows the excitation spectra of pyrene as a function of CMPC10 concentration in water. A plot of $I_{339}/I_{334}$ versus the logarithm of the block copolymer concentration for CMPC10 is shown in Figure 6b. It is believed that the major change in the slope indicates the onset of micellization.\(^{[51]}\) Below the critical micelle concentration (cmc), pyrene is solubilized in water, a medium of high polarity. When micelles are formed, pyrene partitions preferentially toward the hydrophobic domain afforded by the micellar core and thus, experiences a non-polar environment. The cmc was obtained from the intersection of two straight lines: the base line and the rapidly rising $I_{339}/I_{334}$ in Figure 6b. The cmc of the polymers in an aqueous phase were determined from the excitation

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**Figure 4.** $^1$H NMR spectrum of the copolymer CMPC10 in CD$_3$OD.

**Figure 5.** $^1$H NMR spectra of the copolymer CMPC10 in pure CD$_3$OD, pure D$_2$O, and CD$_3$OD/D$_2$O = 1:1 (v/v) mixture solvent.

**Figure 6.** (a) Excitation spectra of pyrene as a function of CMPC10 concentrations in water and (b) a plot of $I_{339}/I_{334}$ versus the logarithm of the block copolymer concentration for CMPC10.

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spectra of pyrene at various concentrations of the polymers and the values were in the range of ca. \(10^{-2}\) mg · mL\(^{-1}\) in water (Table 1), which were much smaller than those of low-molecular-weight surfactants and comparable with other micelle-like polymer aggregates.

**AFM Studies**

To further characterize the micelles and obtain direct visualization of the size and morphology of the micelles, we investigated the micellar systems by atomic force microscopy (AFM). Tapping-mode (TM-AFM) AFM was performed on mica.

The samples for AFM study were prepared by drop-casting the aqueous micellar solution in pure water onto mica. Figure 7 shows the situation observed when the initial micellar aqueous solution with a concentration of 2 mg · mL\(^{-1}\) of CMPC10 was deposited on mica. Obviously, the mica surface is uniformly covered by CMPC10 micelles (Figure 7B). The height and diameter of micelles have been measured to be approximately 1.32 ± 0.06 nm and 39.15 ± 1.06 nm, respectively (Figure 7C). In addition, there exists a worm-like morphology of the micelles in Figure 7B. It is believed that when the concentration of the micelles in a solution is too high, the micelles will accumulate as a result of the evaporation of water as depicted in Figure 8. Consequently, the accumulation of the micelles will result in a worm-like morphology. The very small height of the micellar layer compared to the size of the solvated individual micelles suggests that the pMPC blocks collapse as depicted in Figure 8. This scheme also explains some rod structures in the AFM image in Figure 7B. It is deduced that at a sufficiently low concentration of the micelle solution, individual micelles will appear. Indeed, individual CMPC micelles have been observed together with aggregates on mica when the initial micellar solution was diluted to 0.05 mg · mL\(^{-1}\) (Figure 9).

**Morphology of Drug (ADR)-Loaded Polymeric Micelles**

Figure 10 shows an AFM photograph of drug-loaded CMPC10 micelles. The shape of the particles was mostly spherical and the sizes ranged from ca. 60 to 100 nm in diameter.

This dimension compares with the dimension of viruses and, thus, they may be able to penetrate the sinusoidal and fenestrated capillaries that have pores of approximately 100 nm in size. This suggested that this novel biomimetic surfactant could be used as an effective drug delivery
system. A more detailed investigation into the release of ADR from the drug-loaded system and the cell-uptake behavior of the drug-loaded system is underway.

**Conclusion**

The novel biomimetic surfactants, Chol-pMPC, were prepared and their micellar behavior in an aqueous phase was investigated. The $^1$H NMR spectrum of the polymer in CD$_3$OD showed both the cholesterol group and the phosphorylcholine group, while the cholesterol group did not appear in the $^1$H NMR spectrum of the polymer in D$_2$O, which implied the formation of a micelle structure. The critical micelle concentrations of the polymers, CMPC10, CMPC20, and CMPC40 were $7.27 \times 10^{-3}$, $13.47 \times 10^{-3}$, and $20.77 \times 10^{-3}$ mg·mL$^{-1}$, respectively. The AFM images showed micelles with a spherical shape and the mean diameters of the micelles were in the range of ca. 40 nm. After loading the ADR into the micelles, the diameter increased to the range of about 60–100 nm. Since the polymers contain both a phospholipid moiety and a cholesterol moiety and provide biomembrane mimicry, they are expected to express low toxicity in the body. They might be suitable as a carrier vehicle for delivering bioactive agents.

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