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NOTES

Aggregation Number of Cholesterol Moieties in Hydrophobic Microdomains Formed by Self-Association of a Cholesterol-Bearing Polyelectrolyte in Water

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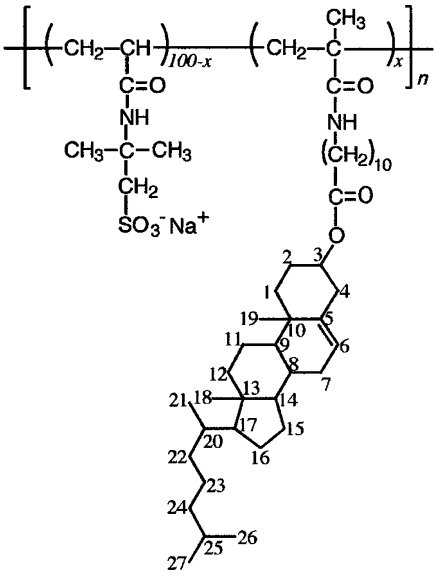
Self-associating hydrophobically modified water-soluble polymers are of current scientific and technological interest because of their relevance to biological macromolecular systems and also because of a variety of practical applications.[1] Hydrophobic interactions are a major driving force for the self-association of such polymers in water.

Previously, we reported the self-association of a random copolymer of sodium 2-(acrylamido)-2-methylpropanesulfonate (NaAMPS) and cholesteryl 6-methacryloyloxyhexanoate (Chol–C_5–MA) (5 mol%) in water.[2,3] The type of self-association of this copolymer was found to be rather unique in that the copolymer forms a "closed" type[4] multipolymer micelle consisting of a number of hydrophobic domains (cores) in one polymer micelle and that the mean aggregation number (N_{agg}) of the cholesteryl groups in one hydrophobic microdomain is very small (*i.e.*, N_{agg} = 17–19). These findings with the NaAMPS/Chol–C_5–MA copolymer are strikingly different from those with a random copolymer of NaAMPS and dodecyl methacrylate (DMA) (9 mol%).[5] Namely, the NaAMPS/DMA copolymer forms a multipolymer micelle consisting of a single hydrophobic microdomain with N_{agg} of dodecyl groups of *ca.* 195. These two NaAMPS copolymers are similar in structure except a cholesterol moiety is attached to each hydrophobic monomer unit *via* a C_5 aliphatic chain in NaAMPS/Chol–C_5–MA whereas a C_{12} aliphatic chain is attached to each hydrophobic monomer unit in NaAMPS/DMA. The small N_{agg} observed for NaAMPS/Chol–C_5–MA may be attributed in part to the intrinsic nature of cholesterol moieties comprised of rigid steroid rings and in part to steric constraints exerted to the rigid hydrophobes by polymer main chain.

To examine the effect of steric constraints by polymer backbone on the aggregation number of cholesterol moieties, we synthesized cholesterol-bearing NaAMPS copolymers where cholesteryl groups are less restricted by polymer chain, *i.e.*, cholesteryl groups are linked to the polymer backbone *via* a longer alkyl chain (*i.e.*, C_{10} chain). In these copolymers, cholesteryl groups are expected to undergo self-association under less constraining conditions than in the case of NaAMPS/Chol–C_5–MA.

In this paper, we have reported the synthesis of the copolymers of NaAMPS and cholesteryl 11-methacrylamidoundecanoate (Chol–C_{10}–MAm) with varying compositions (Figure 1) and their self-associa-



x (f_{Chol}) = 0, 1, 5, 10, 20, and 30 mol%

Figure 1. Structures of NaAMPS/Chol-C_{10}-MAm copolymers.

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tion in water focusing on N_{agg} of cholesteryl groups.

EXPERIMENTAL

Chol–C_{10}–MAm was synthesized according to the method reported by Shibaev.[6] Pyrene, and 3,4-dimethylbenzophenone (DMBP) were recrystalized from methanol. Copolymers of NaAMPS and Chol–C_{10}–MAm were prepared by ordinary free-radical copolymerization in homogeneous solution using *N*, *N*-dimethylformamide as a solvent.[7] The copolymer compositions, determined by ^{1}H NMR spectroscopy, were the same as those in the monomer feed. From GPC with use of a mixture of DMSO/DMF (60/40, v/v) containing 50 mM of LiBr as eluent, weight-average molecular weight (M_w) of the copolymers and NaAMPS homopolymer (polyNaAMPS) were roughly estimated as listed in Table I.

Fluorescence spectra were recorded on a Shimadzu RF-5000 fluorescence spectrophotometer. Emission spectra of pyrene were measured with excitation at 334 nm. Excitation spectra of pyrene were monitored at 390 nm. Sample solutions were prepared by dissolving a known amount of polymer in pyrene containing water (5×10^{-7} M), and the solutions were allowed to stand for a day. Pyrene containing water was prepared as reported previously.[2]

Time-resolved fluorescence quenching (TRFQ) experiments were carried out by a single photon counting technique using a Horiba NAES-550 system. Sample solutions were prepared as follows. Predetermined amounts of pyrene and DMBP were dissolved together in aqueous solution containing the copolymers and the sample solution was deaerated by purging with Ar gas for 30 min prior to measurement. Pyrene was excited at 334 nm and its fluorescence was monitored at 400 nm. In the absence of DMBP, pyrene fluorescence decays are single-exponential with a decay rate constant $k = 1/\tau_0$, where τ_0 is the fluorescence lifetime of pyrene solubilized in cholesteryl aggregates of the copolymer. Fluorescence decay data in the presence of DMBP was fitted to the Infelta–Tachiya equation derived for fluorescence quenching in micelles assuming the distribution of fluorescence probe molecules over the micelle is frozen on the time scale of the fluorescence lifetime[8,9]

$$I(t) = I(0) \exp\left[-\frac{t}{\tau_0} - R\{1 - \exp(-k_Q t)\}\right] \quad (1)$$

where $I(t)$ and $I(0)$ are the fluorescence intensities at time t and zero, respectively, following the excitation, R is the average number of quenchers in the cholesterol microdomain, and k_Q is the first-order quenching rate constant. N_{agg} is obtained from

Table I. Characteristics of the polymers

f_{Chol} (mol%)	$M_w \times 10^5$ [a]	N_{Chol} [b]	N_{agg} [c]
0	1.1		
1	1.2	1	17
5	1.4	5	12
10	1.5	10	14

[a]Determined by GPC using a 50-mM LiBr DMSO/DMF (60/40, v/v) solution as an eluent. [b]Number of cholesteryl groups per one polymer chain. [c]Average aggregation number of cholesteryl groups in one hydrophobic microdomain.

$$N_{agg} = R\frac{[Chol]}{[Q]} \quad (2)$$

where [Chol] and [Q] are the concentrations of the cholesteryl group and quencher, respectively.

RESULTS AND DISCUSSION

The copolymers of NaAMPS and Chol–C_{10}–MAm with Chol–C_{10}–MAm contents (f_{Chol}) of 0, 1, 5, 10, 20, and 30 mol% were prepared. The copolymers were soluble in water when $f_{Chol} \leq 10$ mol% but they were insoluble when $f_{Chol} = 20$ and 30 mol%. Therefore, we employed only the copolymers with $f_{Chol} \leq 10$ mol% for studies of their self-association in water. Since hydrophobes are connected to the main chain *via* amide bonds, the NaAMPS/Chol–C_{10}–MAm copolymers are more soluble in water[5] than the NaAMPS/Chol–C_5–MA copolymers in the previous work, the latter being soluble in water only when $f_{Chol} \leq 5$ mol%. Average numbers of the Chol–C_{10}–MAm units per one polymer chain (N_{Chol}) were roughly calculated from the number-average molecular weights estimated from GPC and Chol–C_{10}–MAm contents for the copolymers with f_{Chol} = 1, 5, and 10 mol% (Table I).

Figure 2 a shows the intensity ratio of the third to first vibronic peaks (I_3/I_1) for pyrene in the presence of polyNaAMPS and the copolymers with f_{Chol} = 1, 5, and 10 mol% as a function of polymer concentrations (C_p). It has been established that I_3/I_1 decreases with increasing the micropolarity.[10–12] For low C_p, I_3/I_1 for all the polymers are estimated to be 0.57–0.60, similar values as that in water. I_3/I_1 in the presence of polyNaAMPS is almost constant at 0.57–0.63, independent of C_p over the whole range examined. In the case of the NaAMPS/Chol–C_{10}–MAm copolymers, I_3/I_1 commences to increase significantly at a certain C_p as C_p is increased. The polymer concentration for the commencement of the increase in I_3/I_1 upon an increase in C_p depends strongly on f_{Chol}, and this commencement of C_p is much lower for higher f_{Chol}. For the copolymer with f_{Chol} = 1 mol%, the commencement of C_p is

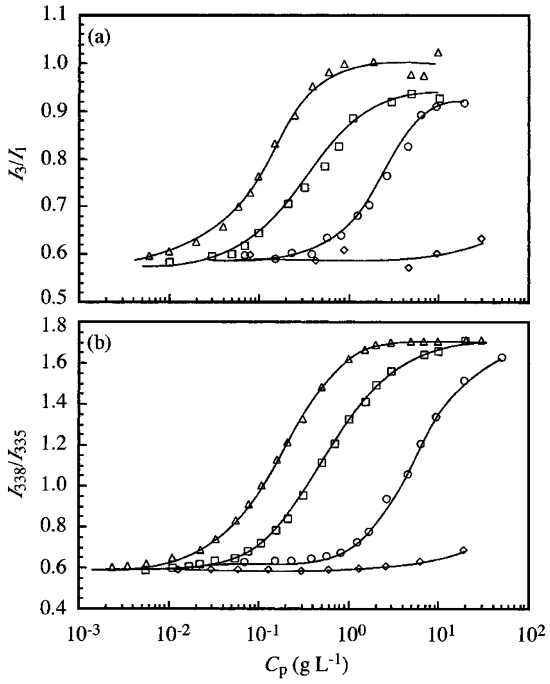

Figure 2. Plots of I_3/I_1 (a) and I_{338}/I_{335} (b) for pyrene (5×10^{-7} M) as a function of C_p for the copolymers with f_{Chol} = 1 (○), 5 (□), and 10 mol% (△). Plots for polyNaAMPS (◇) are presented for comparison.

more than one order of magnitude higher than that for the copolymer with f_{Chol} = 10 mol%.

The 0-0 band for pyrene excitation spectra in water shows a peak at 335 nm whereas it is observed at 338 nm when pyrene is solubilized in hydrophobic microdomains.[7, 13–15] Therefore, it is established that a critical aggregation concentration for amphiphilic molecules can be determined by I_{338}/I_{335} for the 0-0 band in pyrene excitation spectra, where I_{335} and I_{338} are the pyrene emission intensity at 335 and 338 nm, respectively. In Figure 2 b, I_{338}/I_{335} is plotted as a function of C_p. I_{338}/I_{335} for polyNaAMPS shows a value of pyrene in water over the whole range of C_p examined. I_{338}/I_{335} for each polymer exhibits a significant increase at a certain C_p that is close to C_p for the onset of the increase in I_3/I_1. These observations indicated that the cholesteryl groups formed a hydrophobic microdomain where pyrene is solubilized.

Figure 3 shows decay profiles for pyrene fluorescence with and without DMBP in the presence of the copolymer with f_{Chol} = 5 mol% in water at C_p = 40 g L^{-1}. The decay data in the absence of DMBP were reasonably well-fitted to a single-exponential function. On the other hand, the decays in the presence of the quencher were well-fitted to eq 1. A value of τ_0 for pyrene solubilized in cholesterol microdomains was estimated using a conventional deconvolution method to be 312 ns. Therefore, in the fitting procedure for fluorescence decay data in the presence of DMBP, τ_0 in

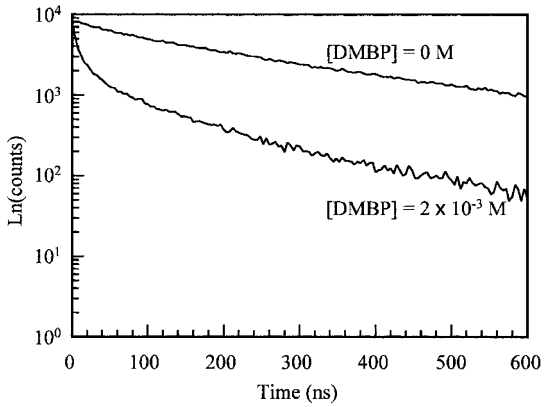

Figure 3. Fluorescence decays for pyrene (5×10^{-7} M) solubilized in aqueous solutions of the copolymer with f_{Chol} = 5 mol% at C_p = 40 g L^{-1} in the absence and presence of DMBP (2×10^{-3} M).

eq 1 was constrained to 312 ns. The N_{agg} value calculated from eq 2 for the copolymer with f_{Chol} = 5 mol% at C_p = 40 g L^{-1} was 12. In the case of the copolymers with f_{Chol} = 1 and 10 mol%, N_{agg} values were estimated to be 17 and 14, respectively. As described earlier, the N_{Chol} values are 1, 5, and 10 for the copolymers with f_{Chol} = 1, 5, and 10 mol%, respectively (Table I). Assuming all pendent cholesteryl groups participate in the event of their aggregation, the copolymer with f_{Chol} = 1 mol%, for example, may form a unicore micelle composed of about 17 polymer chains.

The values of N_{agg} for the copolymers with f_{Chol} = 1–10 mol% were found to be in the range 12–17. These values are similar to those observed for NaAMPS/Chol–C_5–MA (5 mol%) copolymer (N_{agg} = 17–19). Thus, we can conclude that cholesterol moieties in the NaAMPS copolymers form hydrophobic microdomains with small aggregation numbers independent of the spacer length and copolymer composition (*i.e.*, N_{chol}). The conclusion from this study suggests that the similarly small N_{agg} values observed for the cholesterol-bearing NaAMPS copolymers are attributed to the inherent nature of cholesterol moieties.

Akiyoshi and co-workers[16–19] have reported that pullulan covalently modified with a few cholesteryl groups (CHP) forms stable nanoparticles consisting of cholesterol aggregates with N_{agg} ranging from 3.5 to 5.7. These values are essentially the same independent of the molecular weight of the parent pullulan and the degree of substitution with the cholesteryl group.

Cholesterols and bile salts resemble each other in rigidity and chemical structure of steroid rings. Bile salts, such as sodium cholate and sodium deoxycholate possessing hydrophobic and dydrophilic groups, form micelle-like aggregates in water. For example, sodium cholate forms aggregates with N_{agg} ranging from 12 to 19.[20, 21] It has been also reported that the aggregate of

sodium deoxycholate bearing a sulfonate ion in water contains about 11 bile salt molecules.[22]

The small aggregation numbers of cholesterol groups observed in the present study are consistent with these reported values of the aggregation numbers for cholesterols and bile salts, arising from the inherent nature of steroid compounds with rigid ring structures.

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