Microscopic Structure and Thermoresponsiveness of a Hydrogel Nanoparticle by Self-Assembly of a Hydrophobized Polysaccharide

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ABSTRACT: Various cholesterol-bearing pullulans (CHPs) with different molecular weights of the parent pullulan and degrees of substitution (DS) of the cholesteryl moiety were synthesized. The structural characteristics of CHPs in water were studied by static (SLS) and dynamic light scattering (DLS) and the fluorescence probe method. Irrespective of the molecular weight of the parent pullulan and the DS, all of CHPs provided unimodal and monodisperse self-aggregates in water. The size of the self-aggregate decreased with an increase in the DS of the cholesteryl moiety (hydrodynamic radius, 8.4−13.7 nm). However, the aggregation number of CHP in one nanoparticle was almost independent of the DS. The polysaccharide density within the self-aggregate (0.13−0.50 g/mL) was affected by both the molecular weight and the DS of CHPs. The mean aggregation number of the cholesteryl moiety (3.5−5.7), which was estimated by the fluorescence quenching method using pyrene and cetylpyridinium chloride, was almost same for all the CHP self-aggregates. The CHP self-aggregate is regarded as a hydrogel nanoparticle, in which pullulan chains are cross-linked noncovalently by associating cholesteryl moieties. The microenvironment inside or the structural characteristic of the self-aggregate was spectrometrically studied using a fluorescence probe, ANS. The characteristic temperature to cause a structural change of the nanoparticle (T^*) decreased with an increase in the DS of CHP and the ionic strength of the medium. The thermoresponsiveness of the nanoparticle hydrogel is related to the partial dehydration of the hydrophobized pullulan upon heating.

Introduction

Self-assembly of amphiphilic molecules is emerging as a new strategy to develop nanosize materials.[1] Small amphiphiles such as surfactants or lipids spontaneously self-assemble in water and form various types of self-aggregates such as micelles, bilayer membranes, and vesicles. The morphology of the self-aggregate is controlled by the chemical structure and hydrophobicity of the amphiphile.[2] Recently, amphiphilic polymers have attracted much interest with respect to biotechnological and pharmaceutical applications.[3−8] Solution properties of water-soluble polymers drastically change by partial modification with hydrophobes. Association of the hydrophobes controls the polymer conformation, which eventually affects the rheological properties in aqueous fluid. Various hydrophobized polymers were synthesized, and their aggregation or rheological behavior has been extensively studied.[3,4,9−15] The structure of the parent polymer or hydrophobe significantly affects polymer dynamics.[16−19] However, the microscopic structural characteristics of the hydrophobized polymers in water still are not established well.

The cholesterol-bearing polysaccharide, especially cholesterol-bearing pullulan (CHP), forms stable and monodisperse self-aggregates (20−30 nm) by intra- and/or intermolecular self-aggregation in a dilute aqueous solution.[20] The fluorescent probe method suggests that the CHP self-aggregate provides two domains consisting of the hydrophobic cholesteryl moiety and the hydrophilic polysaccharide skeleton. The self-aggregate complexes with various hydrophobic substances[21] and even with various soluble proteins.[22] For example, one α-chymotrypsin dimer[23] or one bovine serum albumin[24] spontaneously complexes with one CHP self-aggregate in water. The thermal stability of the protein increases drastically upon the complexation.[23] Such behavior would be due to a unique microscopic structure of the self-aggregate.[20−24]

Therefore, in this work, we investigated in detail the effect of molecular weight of the parent pullulan and the substitution degree (DS) of the cholesteryl moiety of CHP on the microscopic structure of the nanoparticle hydrogel. Using the fluorescence quenching technique,[25] we could clarify that the association of cholesteryl moieties provides noncovalent cross-linking points of the gel and distributes in the particle to form a polycore structure. We describe also the thermoresponsiveness of the nanoparticle hydrogel in this article.

Experimental Section

Materials. Cholesterol-bearing pullulans (CHPs) were synthesized according to the same method as reported previously.[20] Pullulans with three different molecular weights (M_w = 35 × 10^3, 55 × 10^3, and 108 × 10^3 by size exclusion chromatography (SEC)) were used in this study. CHPs were coded according to the following convention. Thus, CHP-*X*-*Y* indicates that the M_w of the parent pullulan is $X \times 10^3$ and Y is the number of cholesteryl moieties introduced per 100 glucose units.

Aqueous suspensions of the CHP self-aggregates were prepared by using the sonication method. Details of the procedure were described elsewhere.[20] The size distribution of the CHP self-aggregate was checked by SEC using TSK-Gel G4000SW_{XL} and G3000SW_{XL} (Tosoh Ltd., Tokyo, Japan) which were connected in series. SEC measurements were performed on the same apparatus as used in the previous study.[20] Pyrene was purchased from Wako Pure Chemicals Co., Ltd., Osaka, Japan, and purified by silica gel column chromatography with cyclohexane as a mobile phase. Cetylpyridinium chloride (CPC) was purchased from Wako Pure Chemicals Co., Ltd. and recrystallized twice from ethanol. Magnesium 1-anilinonaphthalene-8-sulfonate (ANS) (Nacalai

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Tesque, Inc., Kyoto) was commercially available and used as received. Milli-Q water (Millipore, Bedford, MA) was used throughout the study. All other reagents were commercially available and used without any further treatment.

Static Light Scattering (SLS) Measurements. SLS measurements were performed on a DLS-700 (Otsuka Electronics, Osaka, Japan), equipped with a vertically polarized 5-mW He−Ne laser (633 nm) and a thermoregulated bath (RTE-110, Neslab). An optically clear sample suspension was obtained by centrifugation at 30000*g* for 2 h. The resulting suspension was then directly placed into a cylindrical cell through filtration with a membrane filter (nominal pore size, 0.22 μm, Millex-HV, Millipore). The scattering angle was varied from 30 to 130°. Though the specific refractive index increment of CHP-55-1.3 (0.150 mL/g) was also measured,[20] this was almost identical within experimental error with that of the parent pullulan (0.148 mL/g).[26] This means that the effect of chemical modification of pullulan with cholesterol could be negligible on the specific refractive index increment. Therefore, it was also considered that all CHPs employed in this work had almost the same specific refractive index increment value. The weight-average molecular weight and the radius of gyration (R_G) of the CHP self-aggregate were obtained by the Zimm plot. The R_G values were around 10 nm for all the samples measured. Because it was impossible to precisely measure R_G of these samples by SLS with incident light of 633 nm, R_G values were not given.

Dynamic Light Scattering (DLS) Measurements. The particle size of the CHP self-aggregate was measured by DLS. The instrument consisted of a Spectra-Physics Series 2000 argon ion laser, which was operated at 488 nm and 200 mW, with a Brookhaven BI-2030 256 channel digital correlator. Optically clear sample suspensions were obtained by exactly the same procedure as used for the SLS measurement. The total sample number was kept constant at 10^9 in each measurement. The measuring temperature was 25 °C. The concentration of the sample was kept constant at 1 mg/mL. The measured autocorrelation function was analyzed by the cumulant method.[27] The hydrodynamic radius of the CHP self-aggregate was calculated by the Stokes−Einstein equation.

Fluorometric Measurements. Fluorescence spectra were recorded on a Hitachi F-3010 fluorescence spectrophotometer equipped with a thermoregulated cell compartment. The temperature of the sample was controlled by a thermoregulated bath (Advantec LCH).

The aggregation number of an associating cholesteryl domain was estimated by using the steady-state fluorescence quenching technique.[25] The steady-state quenching data in a microheterogeneous system such as an aqueous micellar solution do not fit in the simple Stern−Volmer kinetics, but fit in the quenching kinetics:[25,28]

$$\ln\left(\frac{I_0}{I}\right) = \frac{[\mathrm{Q}]}{[\mathrm{M}]} \tag{1}$$

where I and I_0 are fluorescence intensity in the presence or absence of a quencher, [Q] is the bulk concentration of the quencher, and [M] is the concentration of polymer self-aggregate. The plot of $\ln(I_0/I)$ against the quencher concentration gives a straight line, the slope of which corresponds to $[\mathrm{M}]^{-1}$, and thus the aggregation number, N_{CHP}, can be given by eq 2.

$$N_{\mathrm{CHP}} = \frac{[\text{cholesterol}]}{[\mathrm{M}]} \tag{2}$$

A stock solution of pyrene in ethanol (1×10^{-4} M) was added to a vial, and ethanol was evaporated by flushing gaseous nitrogen to form a sample thin film at the bottom of the vial. To the thin film was added a CHP suspension, and the resulting mixture was stirred at 50 °C overnight. The final concentration of pyrene in the vial was 1×10^{-6} M. An aqueous solution of CPC was added just before the measurement. Pyrene was excited at 339 nm. The slit width was set at 5 nm for the excitation and 1.5 nm for the emission.

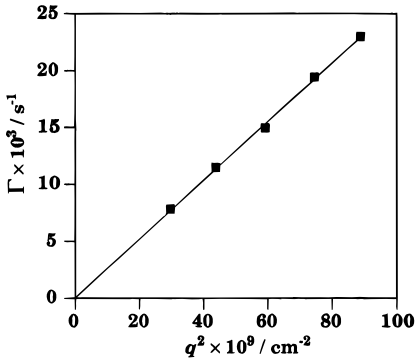

Figure 1. Γ *vs* q^2 for the diffusive mode of a 1.1 mg/mL aqueous suspension of CHP-55-2.1 at 25 °C.

Table 1. Structural Characteristics of CHP Self-Aggregates Determined by Light Scattering Measurements

sample	M_w[a] ($\times 10^5$)	agg no. (N_{CHP})[a]	A_2[a] $\times 10^{-4}$ (mol cm^3 g^{-2})	R_H[b] (nm)	Φ (g/mL)
CHP-35-2.1	5.8	16	3.01	9.2	0.30
CHP-55-1.1	6.2	11	0.68	11.6	0.16
CHP-55-1.7	5.6	10	1.21	10.2	0.21
CHP-55-2.1	5.8	10	4.20	9.5	0.27
CHP-55-3.4	7.4	12	2.72	8.4	0.50
CHP-108-0.9	8.2	7.4	2.46	13.7	0.13
CHP-108-1.5	7.3	6.4	1.26	12.9	0.13

[a] Determined by static light scattering. [b] Determined by dynamic light scattering.

The effect of temperature for binding of ANS was also investigated by fluorescence measurements. A new stock solution of ANS in water was prepared daily. A 10 μL stock solution of ANS (1×10^{-3} M) was mixed with 1 mL of a CHP suspension. The mixture was kept 60 °C for 1 h before the measurement to ensure complete mixing. ANS was excited at 380 nm. The slit width was set at 10 nm for the excitation and 3 nm for the emission. The fluorescence emission spectra were recorded at the scan rate of 60 nm/min.

Results and Discussion

Association Behavior of CHP. All the sonicated samples of dilute suspensions of CHP (0.5−1 mg/mL) gave a single peak on a high-performance SEC after enough sonication as reported previously.[20] The apparent polydispersity of the CHP self-aggregate was calculated using a calibration curve made in advance using a set of standard pullulans, and the M_w/M_n value was below 1.1 for all the aggregates studied. All CHPs formed unimodal and monodisperse self-aggregates in water. The hydrodynamic radius (R_H) of the CHP self-aggregate was determined by DLS. A plot of the inverse of the relaxation time (Γ) *vs* the square of the magnitude of the scattering vector (q^2) is shown for CHP-55-2.1 in Figure 1. A linear relationship through the origin was obtained, and thus the measured relaxation time was predominantly attributed to a diffusive mode. DLS measurements for other samples were performed by fixing the scattering angle at 45°. Diffusion coefficients measured were converted to the hydrodynamic radius (R_H) by using the Stokes−Einstein equation, and they are listed in Table 1. The R_H tends to decrease with an increase in the degree of substitution (DS) of the cholesteryl moiety. The apparent molecular weight of the CHP self-aggregate was determined by SLS measurements (Table 1). The aggregation number (N_{CHP}) of the self-aggregate was calculated from the apparent molecular weight. The self-aggregate consisted of ap-

proximately ten CHP molecules.[20,21] For all the CHPs studied in this work, the aggregation number did not change much with the DS value and decreased with an increase in the molecular weight of CHP.

Assuming nondraining hydrodynamic behavior, an average polymer density within the self-aggregate (Φ_H) can be calculated from R_H and M_w according to eq 3.

$$\Phi_H = \frac{M_w}{N_A\left(\frac{4}{3}\pi R_H^3\right)}^{-1} \quad (3)$$

where N_A is Avogadro's number. The results are also listed in Table 1. For example, the self-aggregate of CHP-55-1.7 is composed of 21% (by weight) polysaccharide and 79% (by weight) water. Hence, the self-aggregate is regarded as a nanosize hydrogel. The density of the self-aggregate changed depending on the DS of the cholesteryl moiety. The more condensed and smaller self-aggregate was formed with CHP bearing the higher DS. The parent pullulan behaves as an expanded flexible coil.[26,29−33] For example, the R_H of the pullulan with a molecular weight of 5.7×10^5 is reported to be 21.8 nm.[30] This value is almost double the value for the CHP self-aggregate (10.2 nm) that has almost the same molecular weight. The Φ_H value of pullulan is calculated by eq 3 to be 0.02 g/mL, which is 10 times less than that of the CHP self-aggregate. The polysaccharide skeleton of CHP would be more densely packed than that of the parent pullulan. The polymer density of the CHP self-aggregate is very similar to that of agarose or the alginic acid hydrogel.[34]

The reason why a monodisperse nanoparticle is formed by self-aggregation of CHP is not yet clear. The chemical structure of hydrophobes generally influences the association mode of the hydrophobized polyelectrolyte in water.[13,19] Morishima *et al.* reported that polyelectrolytes bearing a bulky hydrophobic group such as cyclododecyl or adamantyl show a stronger tendency for intramolecular self-association than does the usual long alkyl hydrocarbon chain.[19] The cholesterol molecule has a rigid and highly hydrophobic sterol skeleton. Its derivatives show interesting properties such as formation of liquid crystals[35] and gelation of organic fluids.[36] The unique solution property of CHP must be due to a strong association of the cholesteryl moiety in water. Inter- or intramolecular hydrogen bondings among tightly packed pullulan skeletons in the aggregate would also promote the self-aggregation of CHP.

Microscopic Structure of the Self-Aggregate. In order to investigate the intraparticle distribution of hydrophobic domains and estimate the number of associating cholesteryl moieties in one self-aggregate particle, a fluorescence quenching experiment was carried out. The fluorescence quenching technique has been successfully applied to determine the aggregation number of surfactant micelles or microdomains of polysoaps.[25,37,38] The value of I_3/I_1, the ratio of the fluorescence intensity at 384 nm to that at 377 nm, can be used to know the microenvironment around excited pyrene.[39] The I_3/I_1 value of pyrene with the CHP self-aggregate (0.80) was similar to that in an aqueous micelle such as Triton X-100 (0.76), cetyltrimethylammonium bromide (0.77), and sodium dodecyl sulfate (0.88).[39] These results indicate that pyrene is certainly entrapped in the hydrophobic microdomain of associated cholesteryl groups. Figure 2 shows the plot of $\ln(I_0/I)$ *vs* [CPC] in the presence of CHP at various concentrations. The data fit well to eq 1, and a good liner relationship was

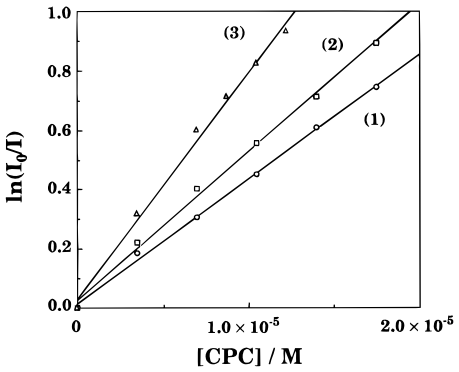

Figure 2. $\ln(I_0/I)$ of pyrene fluorescence as a function of the CPC concentration in the presence of CHP-55-2.1: (1) 0.989, (2) 0.742, and (3) 0.495 mg/mL of CHP, [Py] = 1×10^{-6} M. Lines are best fits of eq 1 to the data.

Table 2. Results of Fluorescence Quenching Experiments in CHP Self-Aggregates

sample	agg no. of cholesteryl groups in one hydrophobic domain (N_{chol})	total no. of cholesteryl groups in one nanoparticle	no. of cholesteric domains in one nanoparticle (n_{domain})
CHP-35-2.1	5.7 ± 0.5	74	13
CHP-55-1.1	4.4 ± 0.5	41	9
CHP-55-1.7	4.2 ± 0.5	59	14
CHP-55-2.1	5.0 ± 0.3	70	14
CHP-55-3.4	3.5 ± 0.3	140	40
CHP-108-0.9	3.7 ± 0.5	44	12

obtained with all the cases (correlation factor, $r = 0.99$). Hence, the mean aggregation number of the cholesteryl moiety, N_{chol}, could be estimated from the slope of the straight line. Table 2 shows the N_{chol} values of the self-aggregates of various CHPs thus obtained. The total number of cholesteryl moieties associated and that of cholesteryl domains in one self-aggregate are also listed. For example, the CHP-55-1.7 self-aggregate consists of approximately 10 polymers. Because the CHP-55-1.7 molecule carries 5.9 cholesteryl moieties on average, one nanoparticle contains 59 cholesteryl moieties, which corresponds to 14 times the N_{chol} value (4.2). This suggests that approximately 14 independent domains distribute in one nanoparticle.[21] The number of cross-linking points in the nanoparticle increased with an increase in the DS of the cholesteryl moiety (Table 2). Taking all into account, a polycore model (Figure 3) seems more plausible as preliminarily proposed,[21] at least under the present conditions. The microdomain of the cholesterol association provides noncovalent cross-links in the hydrogel network, and so the cross-linked network is highly stable.

Assuming a homogeneous distribution of the hydrophobic domain in the matrix of the CHP hydrogel nanoparticle, we can roughly estimate the average distance, d, between the hydrophobic domains using eq 4.

$$d = \left[\frac{4}{3}\pi(R_H)^3/n_{domain}\right]^{1/3} \quad (4)$$

where n_{domain} is the number of cholesteryl domains in one nanoparticle. For example, the distance was calculated to be 9.0 nm for CHP-55-1.1, 6.8 nm for CHP-55-1.7, and 4.0 nm for CHP-55-3.4. The distance between cross-linked domains in the hydrogel matrix was altered by changing the DS of the cholesteryl moiety.

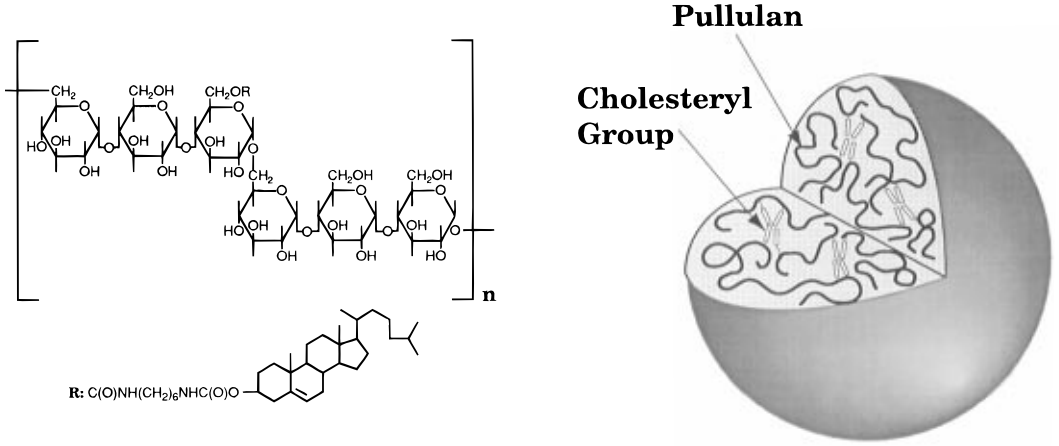

Figure 3. Schematic representation of hydrogel nanoparticle of CHP.

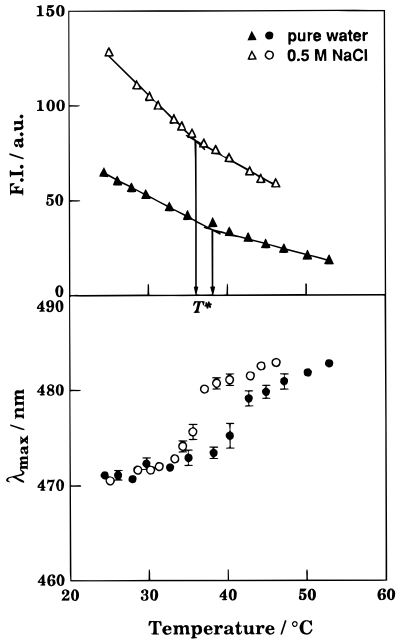

Figure 4. Thermoresponsive binding of ANS to the CHP-55-2.1 self-aggregate in the presence (open symbols) or absence (closed symbols) of 0.5 M NaCl. [CHP-55-2.1] = 1.0 mg/mL and [ANS] = 1×10^{-5} M.

Effect of Temperature. The CHP nanoparticles were colloidally stable upon heating. For example, both R_H and N_{CHP} were virtually independent of temperature; R_H = 9.6 nm and N_{CHP} = 9.7 at 25 °C, while R_H = 9.7 nm and N_{CHP} = 8.7 at 55 °C. In addition, the N_{chol} value did not change at all over this temperature range. No precipitation was observed even after heating at 90 °C for 1 h. The colloidal stability must be due to the strong association between the cholesteryl moieties. The temperature effect on the microenvironment within the CHP nanoparticle was investigated by the fluorescence probe method. Results for CHP-55-2.1 using ANS are shown in Figure 4. To compare the thermoresponsiveness of various CHPs, its characteristic temperature, T^*, was defined by the intersection of the two slopes (Figure 4, top). The T^* corresponds to the temperature that the emission maximum shift begins. The emission maxima of ANS changed sigmoidally between 38 and 43 °C. The fluorescence intensity inflected at around 38 °C (T^*), and the temperature effect was more significant below T^* than above T^*. The change of the microscopic polarity around the probe from a less polar environment to a more polar one causes a red shift of the emission maximum of the probe.[40] The energy of the emission correlates with the solvent polarity around the probe.[41] The micropolarity around ANS in the CHP self-aggregate below T^* is comparable to the polarity of absolute ethanol, while above T^* it becomes comparable to that of the more polar microenvironment corresponding to 80% (v/v) aqueous ethanol. T^* with various CHP self-aggregates in water are summarized in Table 3. T^* decreased not only with an increase in the DS of the cholesteryl moiety but also with an increase in the ionic strength (Figure 4).

Table 3. Characteristic Temperature of Various CHPs Determined from Fluorescence Intensity

sample	characteristic temp (T^*)/°C
CHP-55-1.1	44
CHP-55-1.7	43
CHP-55-2.1	38
CHP-55-2.1 (0.1 M NaCl)	35
CHP-55-2.1 (0.5 M NaCl)	34
CHP-55-3.4	35

Water-soluble polymers such as poly(*N*-isopropylacrylamide) (PNIPAM),[42] ethyl(hydroxyethyl)cellulose (EHEC),[43] and triblock copolymers of poly(ethylene oxide)−poly(propylene oxide)−poly(ethylene oxide) (Pluronic)[44] exhibit a lower critical solution temperature (LCST) in an aqueous solution. When the polymer solution is heated above the LCST, the polymer precipitates accompanied by dehydration. This comes from a change in the subtle balance between hydrogen bondings and hydrophobic associations. The LCST is very sensitive to the intrinsic structure of the polymer or additive such as salt or surfactant.[43] The effect of temperature on the property of the CHP self-aggregate was very similar to that observed with other polymers showing a LCST. A similar spectral change of ANS, the red shift upon heating, was observed with the Pluronic-conjugated CHP.[45] The thermoresponsiveness of the CHP nanoparticle could be ascribed to the dehydration of CHP similar to that of usual water-soluble polymers above the LCST. If the dehydration mainly occurs at a very local site, the global size of the self-aggregate may not significantly change even before and after the dehydration.

Conclusion

The partial modification of a polysaccharide by a very hydrophobic group such as cholesterol leads to the formation of a monodisperse hydrogel nanoparticle in

water. The global size and the polymer density of the hydrogel nanoparticle are controllable by changing the DS of the cholesteryl group of CHP. However, the aggregation number of the polymer in one nanoparticle is almost independent of the DS. The thermoresponsiveness of the nanoparticle hydrogel is related to partial hydration upon heating.

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