3062 *Macromolecules* **1993,** *26,* 3062-3068

Self-Aggregates of Hydrophobized Polysaccharides in Water. Formation and Characteristics of Nanoparticles

Kazunari Akiyoshi, Shigeru Deguchi, Nobuhiro Moriguchi, Shigehiko Yamaguchi,[†] and Junzo Sunamoto*

Department of Polymer Chemistry, Faculty of Engineering, Kyoto University, Sakyo-Ku, Yoshida Hommachi, Kyoto 606, Japan

Received December 14, 1992; Revised Manuscript Received March 9, 1993

ABSTRACT: Solution properties in water of hydrophobized pullulan containing 1.6 cholesterol groups per 100 glucose units (CHP-55-1.6) were studied by size exclusion column chromatography (SEC), dynamic (DLS) and static light scattering (SLS) methods, electron microscopy, ^{1}H NMR, and fluorescence spectroscopy. SEC measurements show that CHP (1.0 mg/mL, 0.10 wt %) intermolecularly aggregates and provides relatively monodispersive particles upon ultrasonication. Spherical particles with relatively uniform size (the diameter, 25 ± 5 nm) were observed in the negatively stained electron microscopy of the aqueous CHP solution. The hydrodynamic radius of the CHP self-aggregate determined by DLS was approximately 13 nm, and the aggregation number determined by SLS was approximately 13; the weight averaged molecular weight of the self-aggregate was 7.6×10^5, the root mean-square radius of gyration (R_G) was 16.8 nm, and the second virial coefficient (A_2) was 2.60×10^{-4} (mol mL)/g^2. The critical concentration of the self-aggregate formation determined fluorometrically was 0.01 mg/mL. In addition, they showed no surface activity at all up to the concentration of 0.145 mg/mL. Existence of microdomains which consist of both the rigid core of hydrophobic cholesterol and the relatively hydrophilic polysaccharide shell was suggested on the basis of both the line broadening of the proton signal of the cholesterol moiety of CHP(δ = 0.6-2.4 ppm) in the ^{1}H NMR spectrum and the incorporation of several hydrophobic fluorescent probes in the CHP self-aggregates. The CHP self-aggregates strongly complexed with hydrophobic and less hydrophilic fluorescent probes similarly to the case of cyclodextrin.

Introduction

Self-assembly or aggregation of polymer amphiphiles is of growing interest with respect to biological importance and pharmaceutical or biotechnological application.[1] Recently, solution properties of block copolymer micelles[2] or self-aggregates of hydrophobized water soluble polymers[3] have been extensively studied. Biopolymers and various synthetic charged or nonionic polymers have been used as the hydrophilic backbone of the polymer amphiphile.[1a] Among these water soluble polymers, naturally occurring polysaccharides which are biodegradable polyhydroxyl compounds show unique properties such as formation of a hydrogel or liquid crystal.[4a] Therefore, they have been widely utilized in biomedical and cosmetic usages.[4] Especially, the hydrophobized polysaccharide derivatives which have long alkyl chains have been prepared for a variety of applications, such as a carrier gel for hydrophobic chromatography,[5] a material for immobilizing enzymes,[6] a biologically active polymer[7] and a coating reagent for an erythrocyte[8] or a cytoplasma membrane.[9] We also have prepared hydrophobized polysaccharide derivatives which bear long alkyl or cholesterol moieties for coating liposomes[10] and oil droplets in water.[11] In spite of these many applications, studies of the solution behavior of such hydrophobized polysaccharide derivatives in an aqueous medium are very rare.[12-14] Nonionic cellulose ethers bearing long alkyl chains[12] or pyrene-labeled (hydroxypropyl)cellulose[14] take part in interchain or intramolecular aggregation in an aqueous medium. 3-*O*-Octadecylated (1-6)-α-D-glucopyranan forms a micellar structure and binds an organic solute in its hydrophobic domain.[13] We have preliminarily reported that palmitoyl- or cholesterol-substituted derivatives of various naturally occurring polysaccharides such as pullulan, amylopectin, mannan, and dextran form self-aggregates in water and, in addition, the cholesterol-bearing polysaccharides form more stable nanoparticles compared with palmitoyl-bearing polysaccharide.[15] In this paper, we describe the synthesis and the solution properties of a nonionic cholesterol-modified pullulan derivative (CHP-55-1.6, Figure 1) in water. The formation of monodispersive nanoparticles by the self-aggregation of CHP and their complexation with several hydrophobic and less hydrophilic compounds were studied by size exclusion column chromatography, dynamic and static light scattering methods, electron microscopy, ^{1}H NMR, and fluorescence spectroscopy.

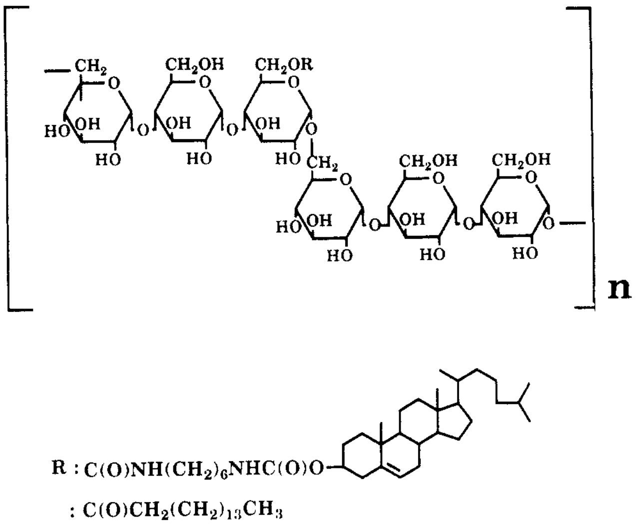


Figure 1. Structures of hydrophobized pullulan.

Experimental Section

Materials. Pullulan was purchased from Hayashibara Biochemical Laboratory, Inc., Okayama, Japan. *N*-Phenyl-1-naphthylamine (PNA) (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan), magnesium 1-anilinonaphthalene-8-sulfonate (ANS) (Nacalai

---

* To whom correspondence should be addressed.

[†] On leave from Tsukuba Research Laboratory, Nippon Oil & Fats Co., Ltd., Tokodai 5-10, Tsukuba, Ibaraki 300-26, Japan.

0024-9297/93/2226-3062$04.00/0

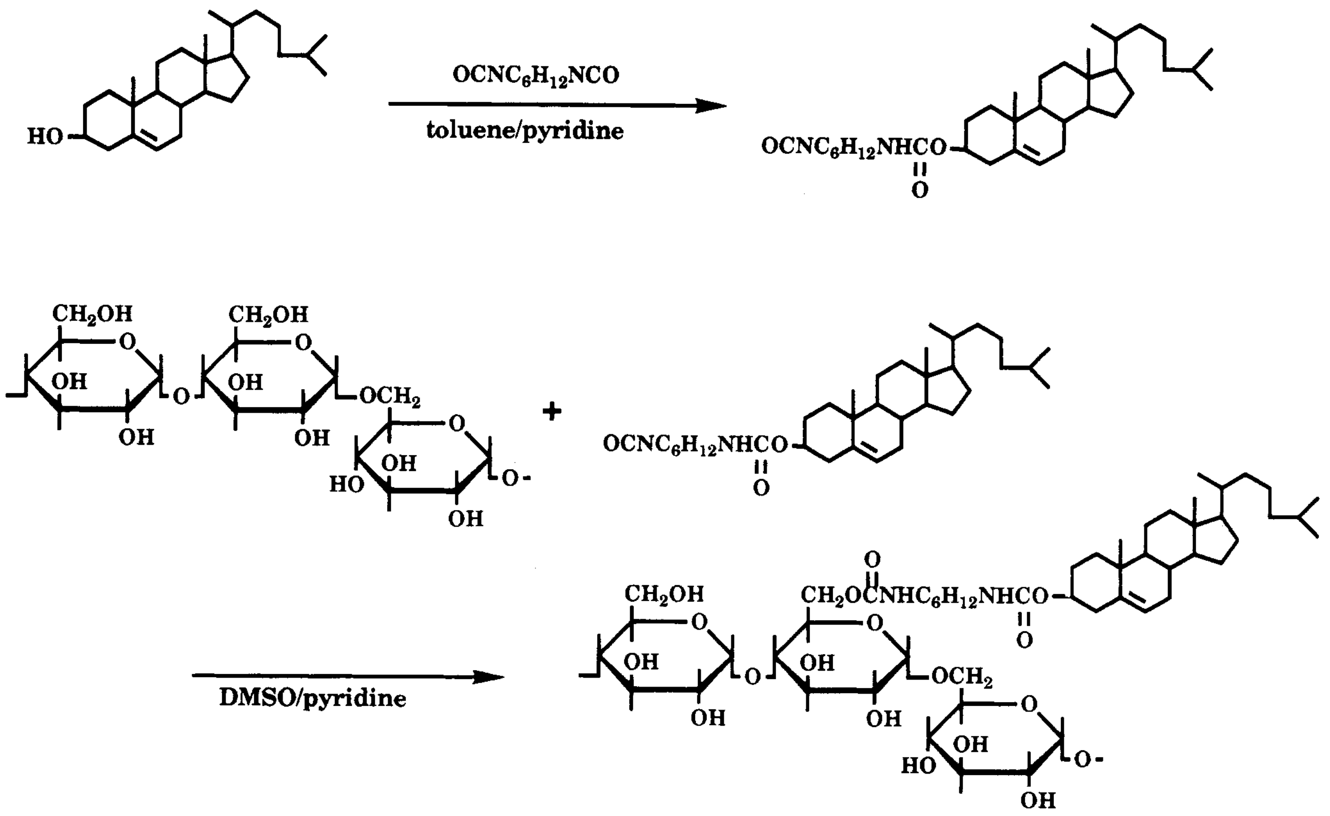


Figure 2. Synthetic route of CHP.

Tesque, Inc., Kyoto, Japan), potassium 2-*p*-toluidinonaphthalene-6-sulfonate (TNS) (Nacalai Tesque, Inc.), and 1-(dimethylamino)naphthalene-6-sulfonamide ethyltrimethylammonium perchlorate (DASP) (Nacalai Tesque, Inc.) were commercially available and used without further purification. HPLC grade DMF (Wako Pure Chemical Co., Ltd., Osaka, Japan) was used for SEC measurements. Other solvents were distilled once before use. Other organic and inorganic chemicals were commercially available and used without further purification.

Synthesis of Polysaccharide Derivatives. In our previous work[10g] cholesterol-bearing pullulans were synthesized *via* the aminoethyl carboxymethyl derivative of pullulan followed by the condensation with cholesteryl chloroformate. In this procedure, however, we could not escape from a problem that a trace, but excess, amount of unreacted carboxylic and amino groups remain in the final product. Therefore, in this work, we decided to employ the following procedures in order to completely eliminate any ionic impurity in the final product (Figure 2). The present procedure involves the synthesis of cholesteryl *N*-(6-isocyanatohexyl)carbamate (1) and the subsequent condensation of 1 with pullulan.

Cholesterol (7.80 g, 20.2 mmol) was reacted with 1,6-hexyl diisocyanate (48 mL, 0.30 mol) in 200 mL of dry toluene containing 4.0 mL of pyridine at 80 °C for 48 h. The proceeding of the reaction was monitored by TLC. After the solvent was removed *in vacuo*, 600 mL of petroleum ether was added to the residue and it was stored overnight at −10 °C. The precipitates obtained were separated and dried *in vacuo* to give 1 as a white powder; yield, 4.97 g (44%). ^{1}H NMR ($CDCl_3$ with TMS): δ 0.69 (3H, *s*, cholesterol 18-H_3), 0.7–2.40 (28H, cholesterol 1-H_2, 2-H_2, 4-H_2, 7-H_2, 8-H_1, 9-H_1, 11-H_2, 12-H_2, 14-H_1, 15-H_2, 16-H_2, 17-H_1, 20-H_1, 22-H_2, 23-H_2, 24-H_2, and 25-H_1), 0.87 (6H, *d*, $J = 9$ *Hz*, cholesterol 26-H_3 and 27-H_3), 0.97 (3H, *d*, $J = 7$ *Hz*, cholesterol 21-H_3), 1.02 (3H, *s*, cholesterol 19-H_3), 1.30–1.55 (8H, *m*, C_4H_8), 3.10 (2H, *m*, CH_2N), 3.20 (2H, *t*, $J = 9$ *Hz*, CH_2CNO), 3.50 (1H, *s*, cholesterol 3-H_1), 5.36 (1H, *m*, cholesterol 6-H_1). IR (KBr, cm^{-1}): 3260 (ν_{NH}), 2320 ($\nu_{NC=O}$), 1680 ($\nu_{C=O}$), 1130 (ν_{OCO}). Anal. Calcd for $C_{35}H_{58}O_3N_2$: H, 10.5; C, 75.8; N, 5.1. Found: H, 10.7; C, 75.5; N, 5.2.

The cholesterol derivative 1 (0.344 g, 0.62 mmol) was reacted with pullulan (M_w 55 000, $M_w/M_n = 1.54$) (4.00 g, 24.8 mmol equiv as the anhydroglucoside unit) in 100 mL of dry DMSO containing 8.0 mL of pyridine at 80 °C for 8 h. The disappearance of cholesteryl *N*-(6-isocynatohexyl)carbamate was checked by TLC ($R_f = 0.50$, developed by chloroform). Ethanol (500 mL) was added to the reaction mixture, and the suspension so obtained was stored overnight at 4 °C. The precipitates were separated, purified by dialysis against water using a seamless cellulose tube (VISKASE SALES Corp.), and lyophilized to give white powder; yield, 3.81 g (90.0%). The degree of substitution of the cholesterol group was determined by ^{1}H NMR and elemental analysis. In this work, pullulan (M_w 55 000) was substituted by 1.6 cholesterol groups per 100 anhydroglucoside units and it was coded as CHP-55-1.6. OPP-55-5.4 also was synthesized by exactly the same method as that used in our previous work.[10a,g] No degradation of the polymer during the chemical modification was ascertained by size exclusion column chromatography (SEC) in DMF. ^{1}H NMR (DMSO-d_6/D_2O with TMS = 20/1, by vol): δ 0.60–2.40 (cholesterol *H*), 2.60–4.60 (glucose 2*H*, 3*H*, 4*H*, 5*H*, and 6*H*), 4.60 (glucose 1*H*(1→6)), 5.05 (glucose 1*H*(1→4)). IR (KBr, cm^{-1}): 1680 ($\nu_{C=O}$), 1180–900 (ν_{COC}).

Sample Preparation. The parent pullulan easily dissolves in water without sonication and gives a clear solution. Hydrophobized pullulan dissolves in DMF and DMSO, but not so easily in water. The following two methods were therefore carried out to prepare an optically clear solution. The CHP concentration was determined by the phenol–sulfuric acid method.[16]

Method I (Sonication Method). CHP (1.0–5.0 mg/mL) was suspended and swelled in water or an aqueous buffered solution under stirring for 12–24 h at 50–60 °C to give a milky suspension. This was further sonicated using a probe type sonifier (TOMY, UR-200P) at 40 W and room temperature for 5 min. This procedure was repeated several times until an optically clear solution of CHP was obtained. No significant degradation of the polymer was observed during the sonication for at least 20 min.

Method II (DMSO Dissolution Method). CHP (10.0 mg) was dissolved in 0.1 mL of DMSO and then diluted to 10 mL with water under vigorous stirring. The resulting solution was ultrafiltered until the concentration of DMSO in the final solution becomes less than 1 ppm on HPLC.

Size Exclusion Chromatography (SEC) Measurements. The SEC system (Tosoh Ltd., Tokyo, Japan) employed in this work was composed of a CCPD dual pump, a CO-8010 column oven, a RI-8010 refractive index detector, and a Chromatocorder 12 data procession system with a GPC extension module. SEC was made using TSK-Gel G4000SW$_{XL}$ and G3000SW$_{XL}$ (Tosoh Ltd.), which were connected in series, with water or by TSK-Gel GMH$_{XL}$-L with DMF. A sample was eluted by water containing 0.02 wt % NaN_3 at the flow rate of 1.0 mL/min or by DMF at 0.5 mL/min. The measuring temperature was kept constant at 40 °C for water or 60 °C for DMF. The SEC system was calibrated using a standard sample of pullulan (P-82, Showa Denko).

Dynamic Light Scattering (DLS) Measurements. The particle size of CHP in water was measured by DLS on a DLS-700 (Otsuka Electronics, Osaka, Japan), equipped with a 5-mW He–Ne laser (vertically polarized, and the wavelength, 633 nm) and a thermoregulated bath (RTE-110, Neslab) at 25.0 °C. An optically clear sample solution was obtained by centrifugation at 30000g for 2 h. The resulting sample solution was directly filtered into a measuring cell with a membrane filter (nominal pore size, 0.45 μm, Millex-HV, Millipore). Measurements were carried out by fixing the scattering angle at 25°. The sample concentration of CHP also was kept constant at 4.15 mg/mL in all the runs. It took several hours to accumulate data because of the very weakness of the scattered light intensity. The diffusion coefficient was evaluated from the time autocorrelation function, $g^2(\tau)$, using the forced single-exponential fit (eq 1):

$$g^2(\tau) = Ae^{-2\Gamma\tau} + B \qquad (1)$$

$$\Gamma = Dq^2 \qquad (2)$$

$$q = (4\pi n/\lambda_0)\sin(\theta/2) \qquad (3)$$

where τ is the delay time, both A and B are constants, D is the translational diffusion coefficient, q is the scattering vector, n is the refractive index of pure solvent, λ_0 is the wavelength of incident light *in vacuo*, and θ is the scattering angle. The hydrodynamic radius, R_H, was calcuated using the Einstein–Stokes equation

$$R_H = k_B T/6\pi\eta D_0 \qquad (4)$$

where k_B, T, and η are the Boltzmann constant, the absolute temperature, and the solvent viscosity, respectively.

Static Light Scattering (SLS) Measurements. In order to determine the molecular weight and the aggregation number of the polysaccharide self-aggregates, the SLS method was adopted. Measurements were carried out over the angular range from 30 to 130°. The concentration was changed from 0.72 to 1.93 mg/mL.

The refractive index increment of the CHP solution was 1.50 mL/g, which was determined on a RM-102 differential refractometer (Otsuka Electronics) as calibrated with an aqueous sucrose solution. The molecular weight of the CHP self-aggregates was estimated by the Zimm plot.[2e]

Transmission Electron Microscopic (TEM) Observation. A 5-μL solution of the CHP self-aggregate (1.0 mg/mL) was mixed with 5 μL of a saturated aqueous solution of uranyl acetate (approximately 2.0 wt %) on a 200 mesh copper grid deposited by carbon, dried *in vacuo*, and washed by a small amount of water. TEM observation was carried out on a JEOL JEM-100SX (JEOL Ltd., Tokyo, Japan).

^{1}H NMR Measurements. ^{1}H NMR measurements were performed on a JEOL FX-90Q FT-NMR (JEOL Ltd.). To 1.0 mL of D_2O (>99.8%, Commissariat a L'Energie Atomique) was added 10.0 mg of CHP. After stirring overnight at 50 °C, the resulting suspension was sonicated at 40 W and 25 °C for 2.5 min under a N_2 atmosphere. The final concentration of CHP in a D_2O–DMSO-d_6 mixed solvent was kept constant at 50.0 mg/mL in all the runs.

Fluorometric Measurements. Complexation between the polysaccharide self-aggregates and a hydrophobic guest molecule such as PNA, ANS, TNS, or DASP was fluorometrically investigated. The fluorescence intensity change of these guest molecules was determined as a function of the CHP concentration. From the results obtained, both the critical concentration of the self-aggregation of the polymers and the binding constant of the hydrophobic substrates to the polymers were estimated. The concentration of the guest was 1.0 × 10^{-6} M in water. For water soluble guest molecules, 0.01 mL of the stock solution of the guest molecule (1.0 × 10^{-4} M) was mixed with 0.99 mL of the CHP solution. PNA was dissolved in acetone because of its low solubility in water. A 0.01-mL acetone solution of PNA was added to a vial and the solvent was evaporated by flushing with gaseous nitrogen to form a thin film at the bottom of the vial. To the thin film obtained was added a CHP stock solution (0.99 mL) (9.3 × 10^{-4}–1.7 × 10^{-1} mg/mL), and the resulting mixture was kept at 40 °C for 1 h. Fluorescence spectra were measured by excitation at 340 nm for PNA, at 380 nm for ANS, at 330 nm for TNS, and at 350 nm for DASP on a Hitachi 650-10S fluorescence spectrophotometer equipped with a thermoregulated cell compartment. The binding constant (K) between a fluorescent guest molecule and the CHP self-aggregates was calculated by the Benesi–Hildebrand relationship[17]

$$\frac{I_0}{I} = \frac{1}{[\mathrm{CHP}]}\frac{I_0}{KI_\infty} + \frac{I_0}{I_\infty} \qquad (5)$$

where K is the binding constant, I/I_0 is the relative fluorescence intensity in the presence (I) and absence (I_0) of a given amount of CHP, I_∞ is the fluorescence intensity when it was considered that all fluorescent probes were complexed with the polymer self-aggregates. Both I and I_0 were measured at 418 nm for PNA, 475 nm for ANS, and 434 nm for PNA, that correspond to the respective emission maximum of each probe in the presence of CHP. The plot of I_0/I *vs* 1/[CHP] yields a straight line for each polysaccharide derivative. From the slope of the straight line, the binding constant K, is obtained, while I_∞ is obtained from the intercept on the vertical axis.

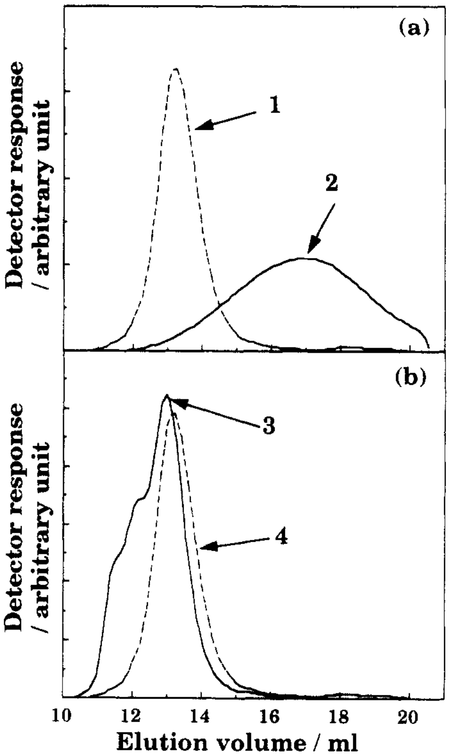


Figure 3. SEC chromatograms of parent pullulan and its derivatives detected by RI in 0.02 wt % aqueous NaN_3 solution at 40 °C; flow rate 1.0 mL/min. (a) 1, 1.0 mg/mL CHP-55-1.6 prepared by method I (under sonication, twice for 5 min); 2, 1.0 mg/mL pullulan (M_w = 55 000). (b) 3, 1.0 mg/mL CHP-55-1.6 prepared by method II; 4, 1.0 mg/mL CHP-55-1.6 prepared by further sonication of sample 3 for 5 min.

Surface Tension Measurements. The surface tension of an aqueous solution of CHP-55-1.6 or OPP-55-5.4 was measured over a concentration up to 0.145 mg/mL at room temperature on a DIGI-O-MATIC ESB-IV (Kyowa Scientific Co.) by the Wilhelmy plate method.

Results and Discussion

Self-Aggregation of Hydrophobized Pullulan in Water. The property of CHP-55-1.6 in water was investigated by SEC. Figure 3a shows the chromatogram of the CHP self-aggregates (1.0 mg/mL) in water prepared by method I (after sonication twice for 5 min) and its

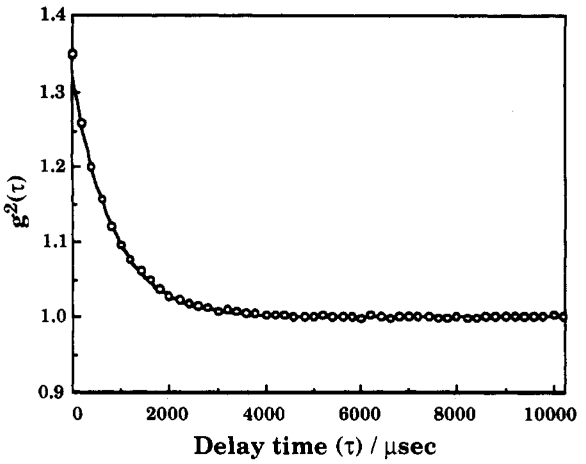


Figure 4. Plot of the autocorrelation function, $g^2(\tau)$, of CHP-55-1.6 in water at 25.0 °C against the delay time (τ). [CHP] = 4.15 mg/mL; θ = 25°. The solid line is the single exponential curve computationally fitted.

parent pullulan (1.0 mg/mL). The elution curve of CHP was different from that of the parent pullulan. Even further sonication did not change the elution pattern of CHP. The CHP self-aggregates eluted earlier than the parent pullulan. This means that the apparent molecular weight of the polymer aggregates is higher than that of the parent pullulan. The polydispersity of the CHP self-aggregate was calculated using the calibration curve obtained in advance with a standard sample of pullulan. The M_w/M_n of the CHP self-aggregate in water was 1.17, while that of CHP in DMF was 1.65. This suggests that the aggregate in water is relatively monodispersive. Figure 3b shows the chromatogram of the aggregates prepared by method II (DMSO dissolution method). Compared with the case of method I, the sample prepared by method II showed additional peaks in the region corresponding to the higher molecular weight. However, when the sample prepared by method II was further sonicated for 5 min at 40 W and room temperature, only a single peak appeared (Figure 3b, curve 4). In addition, the peak position was completely identical with that seen for the sample prepared by method I (Figure 3a, curve 1). In any event, it was revealed that enough ultrasonication of the polymer suspension gives reliable and monodispersive particles of the self-aggregate of the polymer. The radius of gyration (R_G) for the CHP self-aggregates was estimated using the calibration curve obtained in advance for the standard pullulan based on the relationship between the radius given by SLS and the retention time in SEC.[18] The R_G of the CHP self-aggregates was 16.5 nm in average. That the aggregate was colloidally very stable was revealed from evidence that the size of the CHP self-aggregates did not change at all even after keeping for 1 month at 25 °C. When the aggregate of the palmitoyl group-bearing pullulan (OPP-55-5.4, 1.0 mg/mL) prepared by method I was kept for 2 h at 25 °C, an increase in the turbidity of the sample suspension was observed and precipitation took place after 24 h. The OPP self-aggregate was colloidally less stable than the CHP self-aggregate,[15] and the structure of hydrophobic moiety of the polysaccharides influences the colloidal stability of the aggregates.

An average hydrodynamic radius (R_H) of the CHP self-aggregates was directly measured by DLS. The radius was 13.3 nm (D = 1.85 × 10^{-7} cm^2/s), and the formation of relatively monodispersive particles was shown also from the data as analyzed by single exponential fitting (Figure 4). The molecular weight and the aggregation number of the self-aggregate were obtained by SLS. Figure 5 shows the Zimm plot for the CHP suspension over the range 0.72–1.93 mg/mL. Over the whole concentration range studied, the aggregate was still monodispersive enough in water and this was confirmed by SEC. The molecular weight (M_w), the root mean-square radius of gyration (R_G = $\langle S^2\rangle^{1/2}$), and the second virial coefficient (A_2) of the CHP self-aggregates were found to be 7.6 × 10^5, 16.8 nm, and 2.60 × 10^{-4} (mol mL)/g^2, respectively. The value of R_G estimated by SEC was almost identical with that obtained by SLS. The result suggested that one CHP self-aggregate (M_w = 7.6 × 10^5) consists of approximately 13 CHP molecules (M_w = 5.8 × 10^4). It is known that the parent pullulan molecule is significantly flexible and behaves as an expanded flexible coil.[18] Kato et al. investigated the size of pullulan having different molecular weights by SLS and proposed an empirical equation of R_G and A_2 as a function of the molecular weight (M):[18]

$$R_G = 1.47 \times 10^{-2} M^{0.58} \tag{6}$$

$$A_2 = 5.42 \times 10^{-3} M^{-0.26} \tag{7}$$

where M stands for the weight averaged molecular weight of the pullulan under consideration. If R_G and A_2 were calculated by using eqs 6 and 7 on the assumption that the M of pullulan is now 7.6 × 10^5, the following values are obtained: R_G = 37.9 nm and A_2 = 1.60 × 10^{-4} (mol mL)/g^2. They are almost double those observed for the CHP self-aggregates. This means that the polysaccharide chain of the CHP self-aggregates must be more compact in water compared with that of the parent pullulan. Spherical particles with relatively uniform size (the diameter, 25 ± 5 nm) were confirmed also by negatively stained electron microscopic observation (Figure 6).

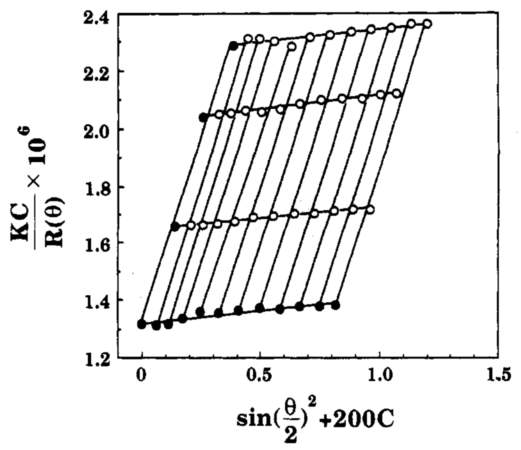


Figure 5. Double reciprocal Zimm plot for a sample solution of CHP-55-1.6 at 25.0 °C.

Critical Aggregation Concentration of Hydrophobized Pullulan. The structural change upon the dilution of the CHP self-aggregates in water was investigated by fluorometry in the presence of PNA as the fluorescent probe. PNA strongly emits in a nonpolar solvent or within a hydrophobic environment, while it is fairly quenched in polar media.[19] When PNA was with the CHP self-aggregates, however, the emission maximum of PNA (1.0 × 10^{-6} M) shifted to the lower wavelength and the intensity drastically increased as a function of the concentration of CHP. Such a spectral change was not brought about at all by the parent pullulan. The emission maximum and the relative fluorescence intensity of PNA in the presence and absence of the polysaccharide derivatives were plotted as a function of the polymer concentration (Figure 7). A clear break point was observed for changes in both the emission maximum and the intensity at the

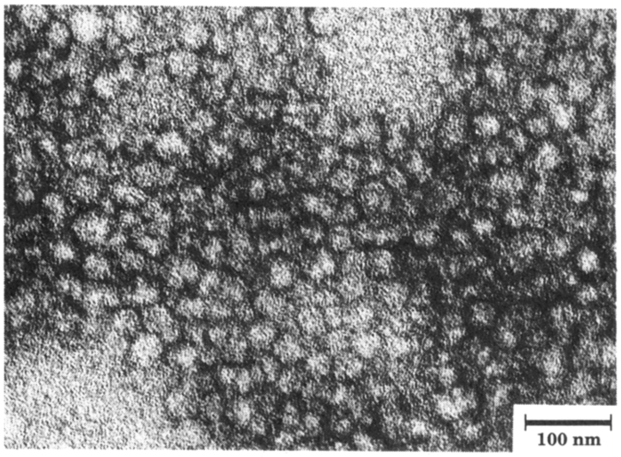


Figure 6. Electron micrograph of 1.0 mg/mL CHP-55-1.6 negatively stained by uranyl acetate solution (approximately 2 wt %).

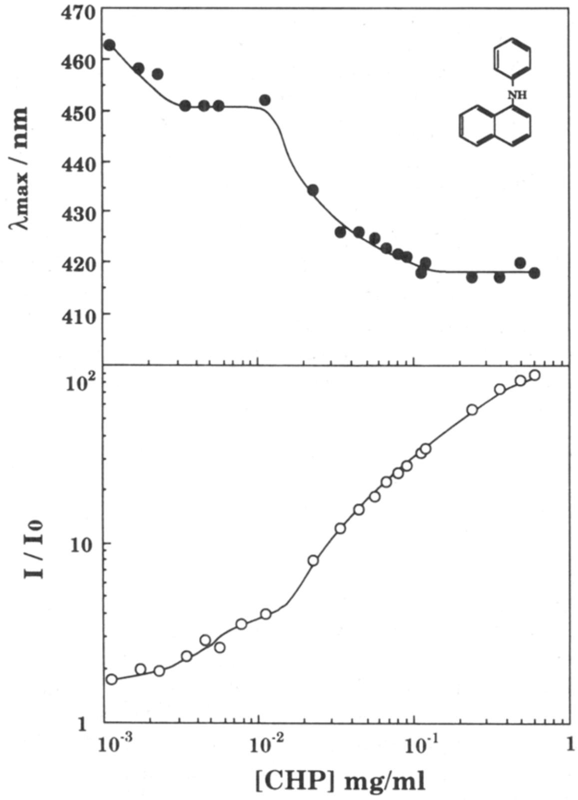


Figure 7. Emission maximum and relative fluorescence intensity (I/I_0) of PNA as a function of the CHP concentration at 25.0 °C. [PNA] = 1.0×10^{-6} M.

concentration around 0.01 mg/mL (0.001 wt %). Such a phenomenon is usually common in systems of various aqueous surfactant micelles,[20] polysoaps,[3e,f] and hydrophilic–hydrophobic block copolymers.[2d] In the case of CHP, the break point observed should correspond to the critical concentration where the intermolecular aggregation of CHP occurs. It is reasonable to consider that the CHP molecule undergoes a certain intramolecular aggregation even below the critical concentration because the binding of the probe accompanied by changes in the emission maximum and the intensity is somewhat, but obviously, observed even below the concentration.

Landoll has reported the critical concentration of the intermolecular association of (hydroxyethyl)cellulose ethers bearing a long alkyl chain (C12–C24).[12] In order to compare the critical concentration of various nonionic surfactants from the same view point, he employed a parameter H_0 (g of hydrocarbon/dL) which is the concentration of the hydrocarbon moiety of the polymer in solution. The smaller H_0 value means the formation of a self-aggregate at the lower concentration. The H_0 values of various nonionic surfactants are summarized in Table I. The H_0 value of (hydroxyethyl)cellulose ether derivatives (compound **6** in Table I) is close to those of poly(ethylene glycol) derivatives (**1** and **2**). On the other hand, the H_0 value of CHP is 10 or 100 times smaller than those of cholesterol derivatives of poly(ethylene glycol) (**4** and **5**).[21] Its value of CHP was closer to that of poly(ethylene glycol) bearing two alkyl chains (**3**) which forms rather bilayer structures than micelles.[22] These data apparently suggest that the cholesterol moiety is a more powerful hydrophobic pendant for forming the self-aggregates than the palmitoyl moiety. The H_0 value of OPP also was smaller than those of (hydroxyethyl)celluloses bearing long alkyl chain (**6**). Therefore, the rather flexible skeleton of pullulan may associate and form more easily the compact aggregate compared with the relatively rigid skeleton of cellulose.[18]

Table I. Critical Micelle or Aggregate Concentration (H_0) of Various Nonionic Amphiphiles[a]

nonionic amphiphile	H_0 (g of hydrocarbon/dL)	ref
1. $CH_3(CH_2)_{11}O(CH_2CH_2O)_8H$	1.8×10^{-2}	12
2. $CH_3(CH_2)_{11}O(CH_2CH_2O)_{18}H$	1.4×10^{-2}	12
3. $(CH_3(CH_2)_{11}O(CH_2)_2)_2CHO(CH_2CH_2O)_{15}H$	4.0×10^{-5}	22
4. Chol-$O(CH_2CH_2O)_8H$	4.7×10^{-4}	21
5. Chol-$O(CH_2CH_2O)_{25}H$	1.4×10^{-3}	21
6. (hydroxyethyl)cellulose derivatives	2.0×10^{-2}–4.0×10^{-2}	12
7. OPP-55-5.4	1.5×10^{-4}	this work
8. CHP-55-1.6	5.0×10^{-5}	this work

[a] The H_0 values for compounds **1** through **6** were calculated by using Landoll's equation from data given in the literature. For compounds **7** and **8**, the H_0 values were calculated from data obtained by using PNA.

Most of amphiphilic polymers such as (hydroxyethyl)cellulose derivatives usually decrease largely the surface tension of an aqueous solution with an increase in the polymer concentration.[12] When the OPP concentration was increased to 0.5 mg/mL, in fact, the surface tension of the solution significantly decreased to 57.0 dyn/cm^2 at 23.0 °C. However, the addition of CHP in water did not decrease the surface tension of water at all. In addition, the surface tension was kept unchanged even at the higher concentration: 74.0 dyn/cm^2 at 0.145 mg/mL CHP and 11.0 °C (for pure water, 73.8 dyn/cm^2 at 11.0 °C). This means that the hydrophobic core of the CHP aggregates is completely and stably covered by the hydrophilic shell of the polysaccharide skeleton. The very low critical aggregation concentration and the surface inactivity of CHP indicate that colloidally stable nanoparticles are certainly formed above the critical concentration.

Microstructure of CHP in an Aqueous Solution. The ^1H NMR spectra of CHP in DMSO-d_6 and D_2O are shown in Figure 8. The proton signals of the cholesterol moiety of CHP (δ = 0.6–2.4 ppm) obviously appeared in DMSO-d_6, but completely disappeared in D_2O. Changes of the half-width of the two independent peaks are shown in Figure 9 as a function of the D_2O content in DMSO-d_6. The half-width of the peak (at $\delta = 0.92$), which corresponds to the C21 proton of the cholesterol group, gradually broadened with an increase in the D_2O content in DMSO-d_6, and the peak completely disappeared in 50% (v/v) D_2O–DMSO-d_6 solution. On the other hand, the half-width of the peak at $\delta = 5.05$ (1H at C1 ($\alpha1\rightarrow4$) of pullulan) did not change at all even in the same solvent. Line broadening of the proton signals of the cholesterol group

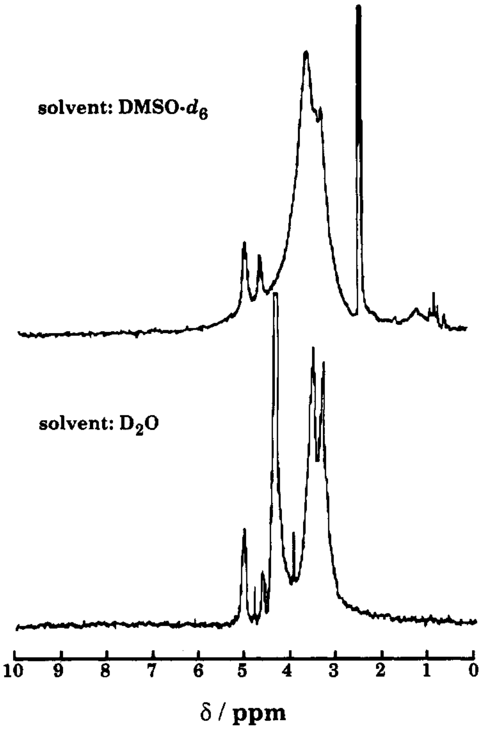


Figure 8. ^{1}H NMR spectra of CHP-55-1.6 (50.0 mg/mL) in D_2O and DMSO-d_6 at 30 °C.

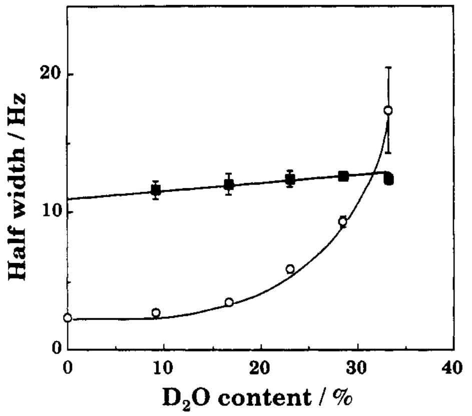
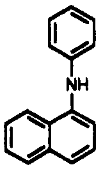
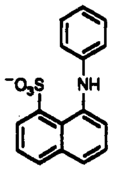
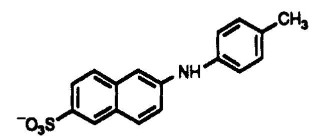
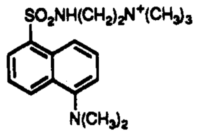


Figure 9. Change of the half-width of the ^{1}H NMR proton signals (at δ 0.92 (○) and 5.05 ppm (■)) for CHP-50-1.6 (50.0 mg/mL) as a function of D_2O content in DMSO-d_6 at 30 °C.

of CHP in an aqueous medium is ascribed to the restricted molecular motion of the cholesterol moieties upon self-aggregation. On the other hand, the mobility of the polysaccharide skeleton of CHP was held even if the solvent polarity increased. These data suggest that the CHP self-aggregate has microdomains as provided by both a rigid core of hydrophobic cholesterol moieties and a relatively mobile shell of a hydrophilic polysaccharide skeleton.

Complexation with Various Fluorescent Substances. Binding of various fluorescent probes such as hydrophobic PNA, less hydrophilic TNS and ANS, and hydrophilic DASP to the CHP self-aggregate above the critical concentration was studied, and their binding constants (K) were obtained by the Benesi-Hildebrand relationship (eq 5).[17] Results are summarized in Table II. ANS, TNS, and PNA rather strongly complexed with the CHP self-aggregate, while DASP did not complex with it at all. The binding constant increased with an increase in the hydrophobicity of the probe. This means that the main driving force for the complexation is primarily a hydrophobic interaction. The micropolarity around these probes in the CHP self-aggregate can be estimated from their emission maxima. Less hydrophilic ANS and TNS were considered to locate in a domain of the polysaccharide skeleton comparable to the polarity of methanol[23] and ethanol,[24] respectively. Hydrophobic PNA was in a domain comparable to the polarity of propanol.[19]

Table II. Emission Maxima of Four Fluorescent Probes in Water with or without CHP-55-1.6 and the Binding Constant of Them to the CHP Self-Aggregate at 25.0 °C

fluorescent probe		λ_{em} (nm in water)		K
		with CHP	without CHP	(M^{-1})
PNA		420	459	1.7×10^5
ANS		474	515	3.8×10^4
TNS		434	500	1.3×10^5
DASP		555	555	~0

Polysaccharides show a unique binding property even to hydrophobic substances because of their amphiphilic property in water.[25] For example, amylose binds lipids in the cavity, as provided by its helical structure.[26] Even parent pullulan also weakly interacts with a hydrophobic fluorescent probe.[25] Cyclodextrins, which possess a structural unit similar to that of amylose, can incorporate various hydrophobic substances into the cavity[27] and have been extensively studied in the field of host–guest chemistry.[28] We compared the binding properties of ANS and TNS with other nonionic oligo- and polysaccharides. Kobayashi et al. reported[13] the binding of ANS to 3-*O*-octadecylated (1–6)-α-D-glucopyranans (DS = 0.07), and Harada et al.[29] compared the interaction of ANS or TNS with poly(acryloyl-β-cyclodextrin) (poly-β-CD-A) to that with β-cyclodextrin (β-CD). In the study of Klotz et al.,[30] the K^* value, which is the binding constant expressed in the weight concentration of the host molecule (10^5 g/L), was used in order to compare various different systems from the same viewpoint.[30] Table III summarizes K^* values and emission maxima of TNS and ANS in various systems. Of those, the largest blue shift of the emission maxima for both probes was observed in the case of CHP. CHP also showed a high affinity to TNS comparable to those of β-CD and poly-β-CD-A. The stronger binding of TNS and the larger blue shift of the emission maximum of TNS in the case of poly-β-CD-A were ascribed to a cooperative effect where two cyclodextrin units of the polymer chain participate in binding one TNS molecule.[29] In such a manner, the TNS molecule is completely encapsulated in the cavity of cyclodextrin.[29] The emission maxima of TNS and ANS incorporated in the CHP self-aggregate were almost identical to those observed in the case of poly-β-CD-A. This suggests that the micropolarity of the polysaccharide shell of the CHP self-aggregate is

Table III. Binding Constants (K^*) and Emission Maxima (λ_{max}) of ANS and TNS Complexed with β-Cyclodextrin or Polysaccharides[a]

	ANS		TNS		
	λ_{max} (nm)	$10^{-5}K^*$ (g/L)	λ_{max} (nm)	$10^{-5}K^*$ (g/L)	ref
β-CD	495	4.9×10^3	460[b] 446[c]	3.5×10^5 1.8×10^3	29
poly-β-CD-A	475		437	8.8×10^5	29
amylose			450	3.9×10^3	25
pullulan	505		450		this work
3-*O*-octadecylated-(1–6)-α-glycan[d]	480	2.8×10^2			13
CHP-55-1.6	474	6.9×10^4	434	2.4×10^5	this work

[a] Binding constants (K^*) were fluorometrically determined. [b] CD:TNS = 1:1 by mol. [c] CD:TNS = 2:1 by mol. [d] DS = 0.07.

comparable to that of the cyclodextrin cavity. The polysaccharide shell of the CHP self-aggregate is considered to be compact enough (upon the association of cholesterol moieties), as judged from the SLS and SEC measurements. Similarly to cyclodextrins, the compact domain of the polysaccharide shell of CHP self-aggregates offers effective binding sites for various hydrophobic or less hydrophilic substances.

Conclusion

Cholesterol-bearing pullulans aggregate by themselves in water and provide colloidally stable and monodispersive nanoparticles above the critical concentration. The CHP self-aggregate is potent for making a stable complex with various hydrophobic and less hydrophilic substances. The main driving force of the complexation is the hydrophobic interaction.

Recently, we have found that the CHPs self-aggregate complexes also with various globular soluble proteins.[31] Since it is possible to bring cell specificity to the hydrophobized polysaccharides, as already reported by ourselves,[10,32] they are expected to behave as a novel carrier for various lipophilic drugs, proteins, and so forth.

Acknowledgment. The authors are grateful to Professor Koji Kano, Department of Industrial Chemistry, Doshisha University, for his help in the measurement of the surface tension and to Mr. Minoru Yamamoto, this laboratory, for his skillful electron microscopic observation.

References and Notes

(1) (a) *Water-Soluble Polymers*; Shalaby, S. W., McCormick, C. L., Butler, G. B., Eds.; ACS Symposium Series 467; American Chemical Society: Washington, DC, 1991. (b) Ringsdorf, H. *J. Polym. Sci., Polym. Symp.* **1975**, *51*, 135. (c) Ulbrich, K.; Konak, C.; Tuzar, Z.; Kopecek, J. *Makromol. Chem.* **1987**, *188*, 1261. (d) Yokoyama, M.; Inoue, S.; Kataoka, K.; Yui, N.; Okano, T.; Sakurai, Y. *Makromol. Chem.* **1989**, *190*, 2041. (e) Naka, K.; Ohki, A.; Maeda, S. *Chem. Lett.* **1991**, 1303.

(2) (a) Tuzar, Z.; Kratochvil, P. *Adv. Colloid Interfac. Sci.* **1976**, *6*, 201. (b) Nagarajan, R.; Ganesh, C. *Macromolecules* **1989**, *22*, 4312. (c) Tontisakis, A.; Hilfiker, R.; Chu, B. *J. Colloid Interface Sci.* **1990**, *135*, 427. (d) Wilhelm, M.; Zhao, Ch-L.; Wang, Y.; Xu, R.; Winnik, M. A.; Mura, J.-I.; Riess, G.; Croucher, M. D. *Macromolecules* **1991**, *24*, 1033. (e) Xu, R.; Winnik, M. A.; Hallett, F. R.; Riess, G.; Croucher, M. D. *Macromolecules* **1991**, *24*, 87. (f) Kiserow, D.; Prochazka, K.; Ramireddy, C.; Tuzar, Z.; Munk, P.; Webber, S. E. *Macromolecules* **1992**, *25*, 461.

(3) (a) Klotz, I. M.; Royer, G. P.; Sloniewsky, A. R. *Biochemistry* **1969**, *8*, 4752. (b) Chu, D-Y.; Thomas, J. K. *Macromolecules* **1987**, *20*, 2133. (c) Dualeh, A. J.; Steiner, C. A. *Macromolecules* **1990**, *23*, 251. (d) Winnik, F. M.; Winnik, M. A.; Ringsdorf, H.; Venzmer, J. *Langmuir* **1991**, *7*, 2583. (e) Seo, T.; Take, S.; Miwa, K.; Hamada, K.; Iijima, T. *Macromolecules* **1991**, *24*, 4255. (f) Ezzell, S. A.; McCormick, C. L. *Macromolecules* **1992**, *25*, 1881. (g) Schild, H. G.; Tirrell, D. A. *Macromulecules* **1992**, *25*, 4553. (h) Minoda, M.; Sawamoto, M.; Higashimura, T. *Macromolecules* **1992**, *25*, 2796.

(4) (a) Yalpani, M. *Polysaccharides Studies in Organic Chemistry*; Elsevier Science Publishers B. V.: New York, 1988; Vol. 36. (b) Schacht, E.; Vercauteren, R.; Vansteekiste, S. *J. Bioact. Compat. Polym.* **1988**, *3*, 72. (c) Sezaki, H.; Takakura, Y.; Hashida, M. *J. Bioact. Compat. Polym.* **1988**, *3*, 81.

(5) Butler, L. G. *Arch. Biochem. Biophys.* **1975**, *171*, 645.

(6) (a) Sandberg, M.; Lundahl, P.; Greijer, E.; Belew, M. *Biochim. Biophys. Acta* **1987**, *924*, 185. (b) Caldwell, K. D.; Axen, R.; Porath, J. *Biotechnol. Bioeng.* **1976**, *18*, 433.

(7) Suzuki, M.; Mikami, T.; Matsumoto, T.; Suzuki, S. *Carbohydr. Res.* **1977**, *53*, 223.

(8) Tsumita, T.; Ohashi, M. *J. Exp. Med.* **1964**, *119*, 1017.

(9) (a) Hämmerling, U.; Westphal, O. *Eur. J. Biochem.* **1967**, *1*, 46. (b) Wolf, D. E.; Henkart, P.; Webb, W. W. *Biochemistry* **1980**, *19*, 3893.

(10) (a) Takada, M.; Yuzuriha, T.; Katayama, K.; Iwamoto, K.; Sunamoto, J. *Biochim. Biophys. Acta* **1984**, *802*, 237. (b) Sunamoto, J.; Iwamoto, K.; Takada, M.; Yuzuriha, T.; Katayama, K. In *Polymers in Medicine*; Chillini, E., Giusti, P. A., Eds.; Plenum Publishing Co.: New York, 1984; p 157. (c) Baszkin, A.; Rosilio, V.; Albrecht, G.; Sunamoto, J. *Chem. Lett.* **1990**, 299. (d) Baszkin, A.; Rosilio, V.; Albrecht, G.; Sunamoto, J. *J. Colloid Interface Sci.* **1991**, *145*, 502. (e) Moellerfeld, J.; Prass, W.; Ringsdorf, H.; Hamazaki, H.; Sunamoto, J. *Biochim. Biophys. Acta* **1986**, *857*, 265. (f) Akiyoshi, K.; Sunamoto, J. In *Organized Solutions*; Friberg, S. E., Lindman, B., Eds.; Marcel Dekker, Inc.: New York, 1991; Chapter 20. (g) Sunamoto, J.; Sato, T.; Taguchi, T.; Hamazaki, H. *Macromolecules* **1992**, *25*, 5567.

(11) (a) Carlsson, A.; Sato, T.; Sunamoto, J. *Bull. Chem. Soc. Jpn* **1989**, *62*, 91. (b) Iwamoto, K.; Kato, T.; Kawahara, M.; Koyama, N.; Watanabe, S.; Miyake, Y.; Sunamoto, J. *J. Pharm. Soc.* **1991**, *80*, 219. (c) Yamaguchi, S.; Fukui, H.; Akiyoshi, K.; Sato, T.; Sunamoto, J. *Nippon Kagaku Kaishi* **1992**, 186.

(12) Landoll, L. M. *J. Polym. Sci., Polym. Chem. Ed.* **1982**, *20*, 443.

(13) Kobayashi, K.; Sumitomo, H.; Ichikawa, H. *Macromolecules* **1986**, *19*, 529.

(14) (a) Winnik, F. M.; Winnik, M. A.; Tazuke, S.; Ober, C. K. *Macromolecules* **1987**, *20*, 529. (b) Winnik, F. M. *Macromolecules* **1989**, *22*, 734. (c) Winnik, F. M. *J. Phys. Chem.* **1989**, *93*, 7452.

(15) Akiyoshi, K.; Yamaguchi, S.; Sunamoto, J. *Chem. Lett.* **1991**, 1263.

(16) Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. *Anal. Chem.* **1956**, *28*, 350.

(17) Benesi, H. A.; Hildebrand, J. H. *J. Am. Chem. Soc.* **1949**, *71*, 2703.

(18) Kato, T.; Katsuki, T.; Takahashi, A. *Macromolecules* **1984**, *17*, 1726.

(19) Mcclure, W. O.; Edelman, G. M. *Biochemistry* **1966**, *5*, 1908.

(20) Fendler, J. H.; Fendler, E. J. *Catalysis in Micellar and Macromolecular Systems*: Academic Press: New York, 1975.

(21) Miyajima, K.; Baba, T.; Nakagaki, M. *Colloid Polym. Sci.* **1987**, *265*, 943.

(22) Kuwamura, T. *Kougyo Kagaku Zasshi* **1961**, *64*, 1958.

(23) Stryer, L. *J. Mol. Biol.* **1965**, *13*, 482.

(24) Traube, H.; Overath, P. *Biochim. Biophys. Acta* **1973**, *307*, 491.

(25) Nakatani, H.; Shibata, K.; Kondo, H.; Hiromi, K. *Biopolymers* **1977**, *16*, 2363.

(26) Bulpin, P. V.; Cutler, A. N.; Lips, A. *Macromolecules* **1987**, *20*, 44.

(27) Cramer, F.; Saenger, W.; Spatz, H. *J. Am. Chem. Soc.* **1976**, *89*, 14.

(28) Tabushi, I. *Acc. Chem. Res.* **1982**, *15*, 66.

(29) Harada, A.; Furue, M.; Nozakura, S. *Macromolecules* **1977**, *10*, 677.

(30) Klotz, I. M.; Shikama, K. *Arch. Biochem. Biophys.* **1968**, *123*, 551.

(31) Akiyoshi, K.; Nagai, K.; Nishikawa, T.; Sunamoto, J. *Chem. Lett.* **1992**, 1727.

(32) (a) Sunamoto, J.; Sakai, K.; Sato, T.; Kondo, H. *Chem. Lett.* **1988**, 1781. (b) Akiyoshi, K.; Takanabe, H.; Sato, T.; Sato, T.; Kondo, H.; Sunamoto, J. *Chem. Lett.* **1990**, 473.