Macromolecular Complexation between Bovine Serum Albumin and the Self-Assembled Hydrogel Nanoparticle of Hydrophobized Polysaccharides

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Abstract: Macromolecular complexation between bovine serum albumin (BSA) and self-assembled hydrogel nanoparticle formed by the self-aggregation of cholesterol-bearing pullulan (CHP) was studied by high performance size exclusion column chromatography (HPSEC) and circular dichroism (CD). The CHP self-aggregates complexed with one BSA molecule to give colloidally stable nanoparticles (R_G = 17 nm) at pH 7.0 and 25 °C. This was almost irrespective of the substitution degree of the cholesterol group of CHP. The helical content of BSA decreased upon complexation. Unfolding of BSA by either heating or a denaturant such as urea was largely suppressed upon complexation. BSA would be incorporated inside into the hydrogel matrix of the CHP nanoparticle. Kinetic analysis of the complexation suggested a two-step process: namely, the fast pre-equilibrium of looser binding of BSA to the CHP self-aggregate followed by the slower process of tighter inclusion into the hydrogel network. The substitution degree of the cholesterol group in CHP significantly affected the complexation kinetics.

Introduction

The study of self-organization of macromolecule is essential for understanding many biological phenomena.[1] In living systems, association of various biopolymers such as DNA, polysaccharides, or proteins is well-controlled by noncovalent and relatively weak interactions such as ionic bonding, hydrogen bonding, and hydrophobic association. In an artificial system, however, macromolecular association is still difficult to control.[2] Amphiphilicity of biopolymers is one of the important factors for their self-organization in water. In this sense, various polymer amphiphiles were designed and synthesized, and the structure−function relationship of these self-assemblies has been extensively studied.[3,4] Such assemblies of amphiphilic macromolecules are of interest both for understanding of supramolecular assembly in nature and for designing new materials in biotechnology and medicine. Interaction of proteins or enzymes with water-soluble polymers has been investigated with respect to the application to separation[5] and stabilization of proteins.[3,6] However, a basic study of the complexation between polymer amphiphiles and proteins scarcely has been done.[7−9]

Recently, we reported a new hydrogel nanoparticle (diameter, 20−30 nm), which was formed in water by self-aggregation of a polymer amphiphile such as hydrophobized polysaccharide.[10,11] Especially, cholesterol-bearing pullulan (CHP, Figure 1) self-aggregates to form a monodisperse and stable hydrogel nanoparticle, in which the domains of the associated cholesterol groups of CHP provide cross-linking points in a noncovalent manner. The size and density of the hydrogel nanoparticle was controlled by changing the substitution degree of cholesterol groups of CHP. The CHP self-aggregate binds various hydrophobic substances[11] and various soluble proteins.[9,12,13] For example, α-chymotrypsin dimer (Chy) is spontaneously complexed by one hydrogel nanoparticle of the CHP self-aggregate, and the thermal stability of Chy drastically increases upon complexation.[9] This is a unique macromolecular complex between polymer amphiphile and protein. Chemical modification of enzymes by synthesized polymers has been widely adopted to obtain hybrid materials for their pharmaceutical and biotechnological application. The supramolecular assembly of proteins with polymer amphiphile has attracted interest as a new methodology for functionalization of proteins.[7,13−15]

In order to obtain further information about the CHP−protein complex, we describe herein a kinetic study of the complexation between the CHP self-aggregate and bovine serum albumin (BSA) and, especially, the importance of the hydrogel structure of the CHP self-aggregate for this complexation. We describe also the improved stability of the complexed protein against heat and denaturant such as urea.

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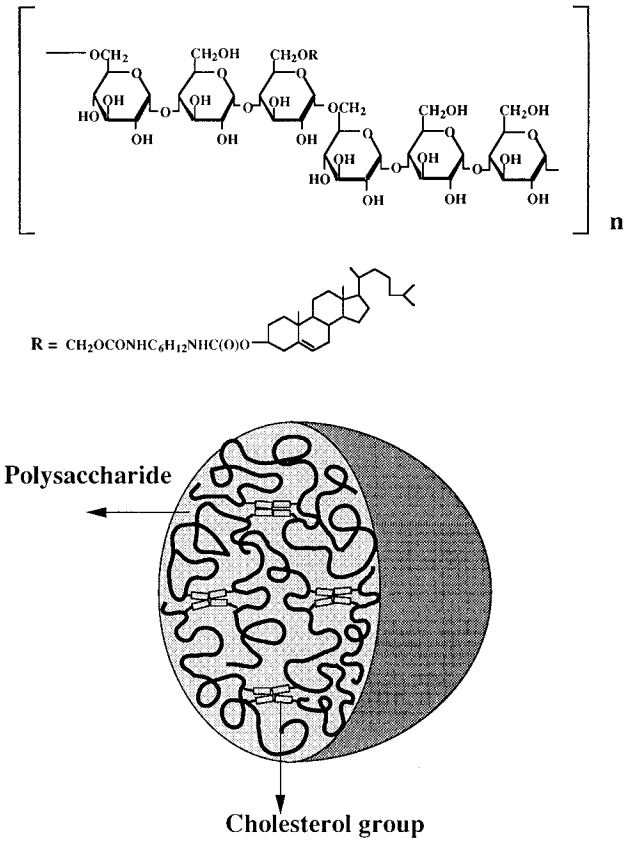

Figure 1. Structure of cholesterol-bearing pullulan and a schematic picture of the hydrogel nanoparticle formed by self-aggregation.

Experimental Section

Materials. Cholesterol-bearing pullulan (CHP) was synthesized according to the method previously reported.[11] Pullulan (MW = 55 × 10^3, M_w/M_n = 1.54) was substituted by 1.0, 1.7, and 3.4 cholesterol moieties per 100 glucose units of pullulan. These CHPs are coded as CHP-55-1.0, CHP-55-1.7, and CHP-55-3.4. Fatty acid-bearing bovine serum albumin (BSA) (Code No. 250010, Miles Inc., Diagnostic Division, Kankakee, IL) was treated by iodoacetamide to prevent formation of intermolecular disulfide bond.[16] Oligomeric BSA was removed by gel chromatography using Sephadex S-200 HR (∅ 2.5 × 100 cm). Only monomeric BSA so obtained was used for the complexation with the CHP self-aggregate. Other organic and inorganic chemicals were commercially available and used without further purification.

High Performance Size Exclusion Column Chromatography (HPSEC). The HPSEC system (Tosoh Ltd., Tokyo) was composed of a CCPD dual pump, a RI-8010 refractive index detector, an UV-8010 UV detector, and a Chromatocorder 12 data procession system with a GPC extension module. A superdex 200 HR 10/30 column (Pharmacia, ∅ 1.0 × 30 cm) was employed. A sample was eluted with 10 mM phosphate buffered saline (PBS, pH 7.0) at a flow rate of 1.0 mL/min and 25 °C. The HPSEC system was calibrated with a set of standard samples of pullulans (P-82, Showa Denko, Tokyo). To estimate radius of gyration (R_G) of the aggregate by HPSEC, an empirical equation for standard pullulan proposed by Kato *et al.*[17] was adopted; $R_G = 1.47 \times 10^{-2}\ M^{0.58}$.

CHP (1.5−9.0 mg / mL) was suspended and swollen in 10 mM PBS under stirring for 12−24 h at 50 °C to give a milky suspension. The resulting suspension was further sonicated using a probe type sonifier (TOMY, UR-200P, Tokyo) at 40 W and 25 °C for 10 min. This procedure was repeated three times. The sample solution so obtained was filtered sequentially through three different membrane filters (Supor Acrodisc 25, Gelman Science, pore size 1.2 μm, 0.45 μm, and then 0.2 μm). The filtration procedure was repeated twice.

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Table 1. Change of Radii of Gyration of CHP Self-Aggregate upon Complexation with BSA

CHP self-aggregate	R_G/nm: CHP self-aggregate	R_G/nm: CHP−BSA complex	BSA/CHP agg
CHP-55-1.0	16.3 ± 0.3	17.4 ± 0.3	0.95 ± 0.1
CHP-55-1.7	13.6 ± 0.3	17.0 ± 0.3	0.90 ± 0.1
CHP-55-3.4	11.8 ± 0.3	16.9 ± 0.3	1.00 ± 0.1

Reproducibility of the sample preparation was carefully checked by HPSEC. A given volume of BSA solution (10.8 or 13.3 mg/mL) was mixed and incubated with a CHP suspension (0.5−4.5 mg / mL) at 25 °C. At a given interval, an aliquot (100 μL) was submitted to HPSEC. The complexation was monitored as a function of time by UV detection at 280 nm, and the initial velocity of the complexation (nM/s) was determined.

Isolation of the CHP−BSA Complex. The CHP−BSA complex was isolated by preparative gel chromatography. A typical procedure is the following. A 1.53-mL solution of BSA (6.52 mg/mL) was added to a 6.02-mL suspension of CHP-55-1.0 (11.39 mg/mL) in 10 mM PBS (pH 7.0). After incubation for 12 h at 25 °C, the resulting mixture was subjected to a preparative gel column (Sephacryl S300 HR column, ∅ 2.5 × 45 cm). For all the samples fractionated, the concentration of polysaccharide was determined by the phenol−sulfuric acid method,[18] while that of BSA was determined spectrophotometrically using the absorbance at 279 nm (ϵ_{279} = 44 500 $M^{-1}cm^{-1}$) or by the fluorescamine method.[19,20] A fractionated suspension of the CHP−BSA complex was concentrated by an ultrafiltration method (Amicon 8010, Diaflo ultrafiltration membranes YM10 (cutoff molecular weight, 10 × 10^3)). Finally, macroscopically transparent suspension of the CHP−BSA complex was obtained; with the complex, the concentration of CHP-55-1.0 was 9.85 mg/mL (17.9 μM), while that of BSA was 1.20 mg/mL (18.2 μM).

Circular Dichroism (CD) Measurement. CD spectra were obtained by using quartz cuvettes (1 or 10 mm) on a JASCO J-720 spectrometer equipped with a thermoregulated cell compartment. A relative α-helix content of BSA was computationally calculated using the Chang−Wu−Yang program from the spectrum over a range of 200−250 nm.[21] Unfolding of BSA induced by a denaturant, urea, was monitored by following the change of mean residual ellipticity at 222 nm as a function of the urea concentration. An appropriate amount of urea was added to a BSA solution or a CHP−BSA complex suspension, and the CD spectrum of the mixture was measured at 25 °C. For study of thermal stability of complexed BSA, the sample was heated from 25 to 95 °C at 0.75 °C/min, and the change in mean residual ellipticity was monitored at 222 nm as a function of the temperature.

Results

CHP Self-Aggregate. Table 1 shows radii of gyration (R_G) of various CHP self-aggregates. The monodispersive nanoparticle was formed by self-aggregation of CHP in water. Static light scattering experiments show that the aggregation number of CHP is approximately 10 and it is independent on the degree of substitution (DS) of cholesterol.[11,22] However, the size of the nanoparticles decreased with an increase in the DS of cholesterol. The nanoparticles were colloidally so stable that the size of the nanoparticle did not change at all even after a week at room temperature.

Complexation between BSA and CHP Self-Aggregate. The complexation between the CHP self-aggregate and BSA

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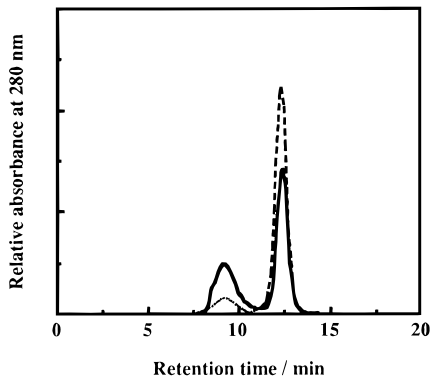

Figure 2. Chromatograms of the CHP-55-1.7 self-aggregate (0.91 μM) (dotted line), BSA (2.3 μM) (broken line), and the mixture of the two after 24 h (solid line) in 10 mM PBS (pH 7.0) at 25 °C.

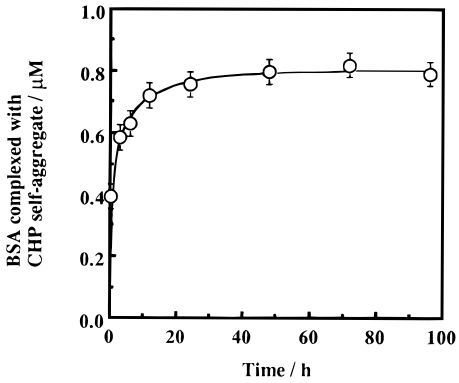

Figure 3. Complexation between BSA (9.09 μM) and CHP-55-1.7 self-aggregate (0.91 μM) as a function of time in 10 mM PBS at pH 7.0 and 25 °C.

was monitored by HPSEC (Figure 2). The chromatogram of free BSA indicates that BSA exists as monomer under the conditions employed. The peak intensity of free BSA (broken line at $R_t = 12.4$ min) decreased upon the co-incubation with CHP-55-1.7 self-aggregate, while that of the complex (solid line at $R_t = 9.4$ min) increased. The peak of the complex overlapped with that of the CHP self-aggregate (dotted line). The chromatogram indicates that BSA certainly bound to the CHP self-aggregates. Figure 3 shows the time course of the complexation between BSA (9.1 μM) and CHP-55-1.7 self-aggregate (0.91 μM). The complexation was rather slow and reached an equilibrium after 48 h. Other CHP self-aggregates also showed a similar trend. For all the cases, approximately one BSA molecule was complexed by one nanoparticle of the CHP self-aggregates (Table 1).

The CHP−BSA complex was isolated by preparative gel chromatography. The size of the nanoparticles was determined by HPSEC. For all the CHP−BSA complexes, R_G was almost the same, approximately 17 nm, even though the original size of the parent aggregate was different (Table 1). BSA was scarcely released from the complex even after being kept for a week at 22 ± 2 °C, and the isolated CHP−BSA complex was colloidally stable enough.

In order to clarify the mechanism of the complexation, initial velocity of the complexation between BSA and various CHP self-aggregates was determined by HPSEC. Figure 4 shows the initial velocity of the complexation as a function of the initial BSA concentration. The CHP self-aggregate with the lower DS of cholesterol complexed more quickly with BSA. The initial velocity increased with an increase in the initial BSA concentration and gradually leveled off to show saturation kinetics.

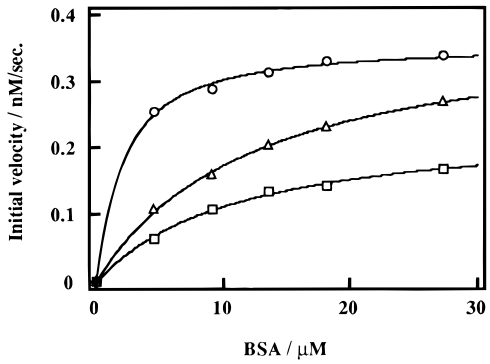

Figure 4. Initial velocity of the complexation between BSA and various CHP self-aggregates as a function of the initial concentration of BSA ((○) CHP-55-1.1, 0.91 μM, (△) CHP-55-1.7, 0.91 μM, and (□) CHP-55-3.4, 0.91 μM) in 10 mM PBS at pH 7.0 and 25 °C.

Table 2. Kinetic Parameters of the Complexation between BSA and CHP Self-Aggregate

CHP self-aggregate	K/M^{-1}	k_{+2}/s^{-1}
CHP-55-1.0	$(59.0 \pm 1.0) \times 10^4$	$(3.9 \pm 0.1) \times 10^{-4}$
CHP-55-1.7	$(8.5 \pm 0.7) \times 10^4$	$(4.3 \pm 0.1) \times 10^{-4}$
CHP-55-3.4	$(9.3 \pm 1.5) \times 10^4$	$(2.6 \pm 0.1) \times 10^{-4}$

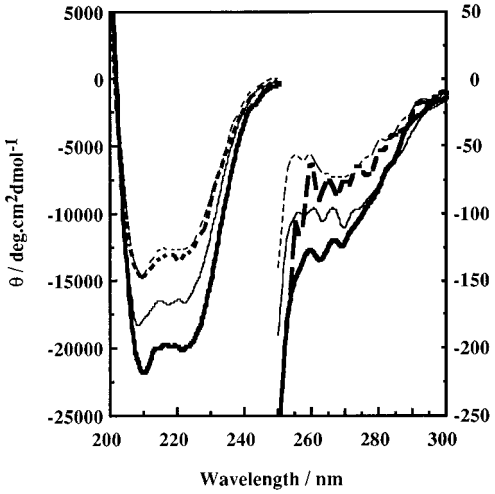

Figure 5. CD spectra of free BSA (thick solid line), CHP-55-1.0−BSA complex (thin solid line), CHP-55-1.7−BSA complex (thick dotted line), and CHP-55-3.4−BSA complex (thin dotted line) in 10 mM PBS at pH 7.0 and 25 °C.

Structure of Complexed BSA. In the CHP self-aggregate itself, no CD was observed under the condition employed. The CD spectra of BSA changed in far- and near-UV regions upon the complexation (Figure 5). The helical content of free BSA is 66%. After the complexation, however, it decreased to 57% for CHP-55-1.0, 44% for CHP-55-1.7, and 46% for CHP-55-3.4. The higher order structure of BSA changed upon complexation. The structural change of BSA upon complexation was not much when BSA was complexed by the CHP self-aggregate with the lower DS of cholesterol.

Stability of Complexed BSA against Denaturant. In the presence of 9 M urea, free BSA was unfolded to form a random coil structure.[23] However, the CD spectra of the complexed BSA scarcely changed even under the same conditions. The difference in the mean residual ellipticity ($\Delta\theta$) at 222 nm between the absence and presence of 9 M urea was 15000 for free BSA, 7200 for the CHP-55-1.0 complex, 2700 for the CHP-55-1.7 complex, and 1900 for the CHP-55-3.4 complex (Figure

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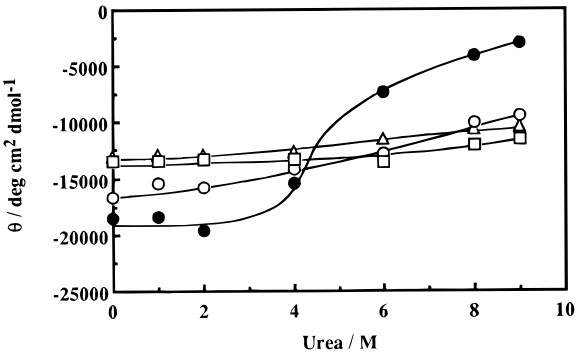

Figure 6. Mean residual ellipticity (θ) at 222 nm of (●) free BSA, (○) CHP-55-1.0−BSA complex, (△) CHP-55-1.7−BSA complex, and (□) CHP-55-3.4−BSA complex as a function of urea concentration.

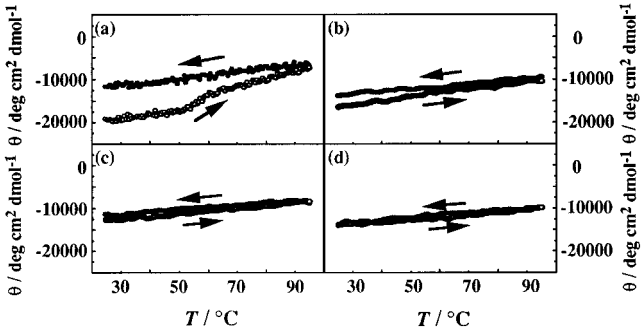

Figure 7. Mean residual ellipticity (θ) at 222 nm of (a) free BSA, (b) CHP-55-1.0−BSA complex, (c) CHP-55-1.7−BSA complex, and (d) CHP-55-3.4−BSA complex as a function of time in 10 mM PBS at pH 7.0.

6). The unfolding of BSA induced by urea was remarkably suppressed upon complexation with the CHP self-aggregates. The CHP self-aggregate with the higher DS of cholesterol more effectively suppressed the unfolding of BSA.

Thermal Stability of Complexed BSA. The thermal stability of the complexed BSA was investigated by CD (Figure 7). An aqueous solution of free or complexed BSA was heated up to 95 °C from 25 °C at 0.75 °C/min and then cooled down again to 25 °C with the same rate. The change of the mean residual ellipticity (θ) was monitored at 222 nm. The θ value of free BSA drastically increased at approximately 60 °C. This temperature is corresponding to the denaturation temperature of BSA.[24] Because the thermal unfolding of free BSA is irreversible,[24] denatured BSA gradually aggregates to give precipitates after cooling to 25 °C. Different from the case of free BSA, the θ value of complexed BSA increased little upon heating, and the change was rather reversible. BSA did not release from the complex, and no precipitation was observed in the CHP−BSA complex system even after heating. The shape of the CD spectra of complexed BSA did not change much even after heating. Once BSA was complexed with the CHP self-aggregate, its secondary structure was well maintained even with heating.

Discussion

Characteristics of Complexation. The CHP self-aggregate is regarded as a self-assembled hydrogel nanoparticle, in which the associated cholesterol groups provide cross-linking points (Figure 1). The average aggregation number of cholesterol molecules to form the cross-linking point is estimated to be 4−5 for all the CHP self-aggregates studied.[22,25] For example, one nanoparticle contains 41 cholesterol groups for CHP-55-1.0 self-aggregate, 59 for CHP-55-1.7, and 140 for CHP-55-3.4. This means that there exist approximately 9−40 hydrophobic domains, cross-linking points, in one hydrogel nanoparticle of the self-aggregate. Assuming a hydrodynamically equivalent sphere, the average polymer density and water content of the nanoparticle are calculated from R_H and M_W.[25] The water content of one nanoparticle is calculated to be 84 wt % for CHP-55-1.0, 79 wt % for CHP-55-1.7, and 50 wt % for CHP-55-3.4. CHP with the higher DS of cholesterol forms more densely packed particles due to an increase in the number of cross-linking points in one hydrogel nanoparticle.

Characteristics of the complexation between BSA and the CHP self-aggregate are summarized as follows. (1) One CHP nanoparticle complexed with one BSA monomer (MW 67000, stokes radius 7.68 nm[26]) irrespective of the structure of the CHP self-aggregate. As previously reported, one CHP nanoparticle complexes with one α-chymotrypsin dimer (MW 50000),[9] four molecules of cytochrome *c* (MW 12500), and ten molecules of insulin (MW 5735).[13] The maximum number of globular proteins complexed by one CHP self-aggregate would depend on the molecular weight (or size) of the protein. The binding site of protein within the hydrogel nanoparticle seems to have a limited capacity. (2) The particle size of the complex was almost the same irrespective of the difference in the size of the parent CHP self-aggregate. An originally small CHP self-aggregate showed a large increase in the particle size upon complexation. The CHP self-aggregate seems to be rather flexible to complex with BSA. (3) The higher order structure of BSA changed upon complexation. The more densely packed nanoparticle more strongly perturbs the structure of BSA upon complexation. (4) The complexed BSA was scarcely unfolded even in the presence of 9 M urea. Under the same conditions, free BSA was completely unfolded. (5) The thermal unfolding and the self-aggregation of BSA were suppressed upon complexation. The resistance of the protein against heating or urea-induced denaturation increases by addition of sorbitol or sucrose.[27,28] The thermal stability of enzymes also improves upon immobilization within a gel matrix.[29] The stabilization of BSA upon complexation in this work would be ascribed to the formation of multiple non-covalent interactions between BSA and the hydrogel of CHP self-aggregate. Taking all this into account, BSA must be incorporated into the inside of the hydrogel matrix of the CHP self-aggregate nanoparticle, and is not simply adsorbed onto the particle surface.

Kinetics of the Complexation. A plot of the initial velocity of the complexation versus the initial concentration of BSA (Figure 4) indicates that the complexation proceeds by at least two steps. The first stage seems to be a fast pre-equilibrium, which is followed by a slower process (eq 1).

$$\text{CHP agg} + \text{BSA} \overset{\text{fast } K}{\rightleftharpoons} \underset{\text{complex } \mathbf{I}}{(\text{CHP agg}\cdot\text{BSA})} \overset{\text{slow } k_{+2}}{\underset{k_{-2}}{\rightleftharpoons}} \underset{\text{complex } \mathbf{II}}{\text{CHP}-\text{BSA}} \quad (1)$$

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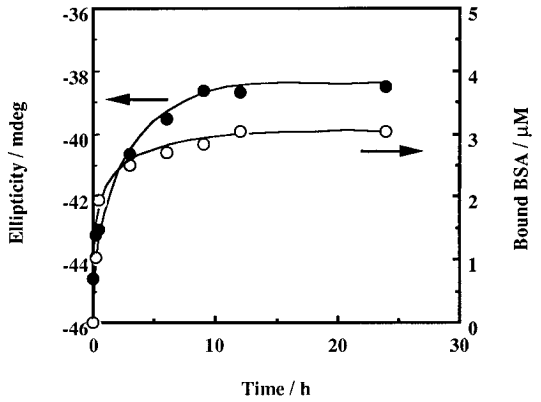

Figure 8. Time course of the ellipticity change of (●) BSA at 222 nm and the concentration of (○) complexed BSA upon complexation between CHP self-aggregate (8.2 μM) and BSA (3.0 μM) in 10 mM PBS at pH 7.0.

The rate constant of backward reaction at the second stage (k_{-2}) seems almost negligible from the fact that the dissociation of BSA from the isolated complex (complex II) is extremely slow. Hence, the initial velocity (v) of the complexation can be expressed by eq 2.

$$v = \frac{\mathrm{d}}{\mathrm{d}t}[\text{complex } \mathbf{II}] = k_{+2}x \tag{2}$$

where x is the concentration of complex **I** and expressed by eq 3 under the present conditions:

$$K = \frac{x}{(c_0 - x)(b_0 - x)} \tag{3}$$

where c_0 is the initial concentration of the CHP self-aggregate, while b_0 is the initial concentration of BSA. K represents the equilibrium constant of the first pre-equilibrium (eq 1). Consequently, eq 4 is derived as a function of the initial concentration of BSA (b_0):

$$v = \frac{k_{+2}}{2}[(b_0 + c_0 + 1/K) - \{(b_0 + c_0 + 1/K)^2 - 4b_0c_0\}^{1/2}] \tag{4}$$

Both the rate constant (k_{+2}) and the equilibrium constant (K) of complex **I** formation were obtained by computational fitting of the data of Figure 4 using the nonlinear least-squares minimization method. The result is shown by the solid line in Figure 4.

The fast pre-equilibrium constant (K) largely depends on the DS of cholesterol of CHP. In order to obtain further information about complex **I**, the time course of the CD spectral change upon complexation was investigated (Figure 8). From the equilibrium constant (K), the concentration of BSA bound to complex **I** can be estimated. For example, with CHP-55-1.7, approximately 40% of BSA initially added should bind to the CHP self-aggregate to give complex **I**. If the higher order structure of BSA changes during complex **I** formation, the CD spectrum of BSA should change significantly at the initial stage. However, the θ value at 222 nm monotonously increased with almost the same rate as that observed with complex **II** formation (Figure 8). The higher order structure of BSA changed slightly in the pre-equilibrium. The structure of BSA would change mostly at the stage of complex **II** formation due to its tight complexation into the hydrogel matrix of the self-aggregate.

The CHP self-aggregate provides both hydrophobic domains of associated cholesterol groups and water-filled hydrophilic regions of the entangled polysaccharide skeleton (Figure 1).[11] Such an amphiphilic microenvironment and a hydrogel network structure would play an important role for the complexation with a soluble protein. Soluble proteins have both hydrophilic and hydrophobic patches on their surface.[30] Especially, BSA has hydrophobic domains on its surface and strongly interacts with lipids or surfactants.[31] For complexation between the CHP self-aggregate and BSA, therefore, an association of cholesterol moiety to the hydrophobic patch of BSA must be partly responsible. The microenvironment of the CHP self-aggregate inside would become more hydrophobic as the DS of cholesterol increases. However, both the pre-equilibrium constant, K, and the rate constant, k_{+2}, tended to decrease with an increase in the DS value. The hydrophobicity would not be a major factor for both complex **I** and **II** formation.

CHP with the higher DS of cholesterol forms a more densely packed nanoparticle, and the distance between the cross-links within the hydrogel nanoparticle decreases with an increase in the DS of cholesterol. The CHP-55-1.0 self-aggregate, which is the least densely packed hydrogel with the larger network structure, showed the largest pre-equilibrium constant, K. CHP-55-3.4 showed the smallest rate constant, k_{+2} in the slow second stage (incorporation to the hydrogel matrix). The initial rate of complexation strongly depended on the size of the protein. For example, complexation with a smaller insulin proceeded very quickly and reached an equilibrium within 10 min.[13] The network structure of the CHP hydrogel nanoparticle would be one of the major factors in determining the complexation.

We reported the thermodynamic parameters of the overall complexation process by means of isothermal titration calorimetry;[32] for example, $\Delta G_b^\circ = -11.1 \pm 1.1$ kcal mol^{-1}, $\Delta H_b = -12.9 \pm 0.1$ kcal mol^{-1}, and $T\Delta S_b^\circ = -1.7 \pm 1.8$ kcal mol^{-1} for CHP-55-1.0, and $\Delta G_b^\circ = -10.8 \pm 0.3$ kcal mol^{-1}, $\Delta H_b = -22.1 \pm 0.1$ kcal mol^{-1}, and $T\Delta S_b^\circ = -11.3 \pm 0.7$ kcal mol^{-1} for CHP-55-1.7. The heat associated with complexation was exothermic. The complexation was enthalpically driven, not entropically. These negative changes in enthalpy and entropy terms for the complexation may not be explained only by contribution of hydrophobicity. The heat of complexation must be due to the formation of direct or water mediated hydrogen bonding between CHP and the protein. The ΔH_b value for CHP-55-1.7 was significantly smaller than that for CHP-55-1.0. However, there was no significant difference in the change of Gibbs free energy between the two. The favorable negative enthalpy change for the complexation was compensated for by the unfavorable decrease in the entropy. This would reflect strong hydration or mobility restriction of the protein upon tight complexation. The tighter complexation between BSA and CHP-55-1.7 self-aggregate would result in both the larger structural change of BSA and the higher stability against thermal or urea-induced unfolding of complexed BSA compared with the complexation with CHP-55-1.0 self-aggregate.

The hydrogel nanoparticle of the CHP self-aggregate is regarded as a host of a macromolecular guest such as protein. This is an example of well-controlled macromolecular association between two water-soluble macromolecules. This nanoparticle could be widely utilized in biotechnology and medicine because hydrophobized polysaccharides are able to conjugate

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additional functional units such as cell-specific saccharide determinants[10,33] or thermoresponsible moieties.[34]

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