Formation of an Intrapolymer Complex from Human Serum Albumin and Poly(ethylene glycol)

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Complexation between human serum albumin (HSA) and poly(ethylene glycol) (PEG) was studied using different experimental techniques: quasi-elastic light scattering (QELS), static light scattering (SLS), electrophoretic light scattering (ELS), dialysis, and fluorescence spectroscopy. The QELS study for aqueous HSA−PEG mixtures at different levels of pH and ionic strength (NaCl) showed the formation of a water-soluble complex, the size of which varied depending on both the ionic strength and the molecular weight of PEG but remained unaltered when the mixing ratio of PEG to HSA was varied. The study of the complexation in the presence and absence of 1 M urea as a function of pH by QELS and fluorescence spectroscopy strongly suggested that hydrogen bonding plays an important role in the complex formation. A combination of SLS and dialysis at pH 2 and at the ionic strength 0.1 demonstrated that the complexation yielded an "intrapolymer" complex in which several HSA molecules bound to a PEG chain. In addition, ELS indicated that the resulting intrapolymer complex behaves like a free draining coil during electrophoresis.

Introduction

The complex formation of proteins with water-soluble synthetic polymers in an aqueous system is interesting from two points of view. The first concerns the way in which globular proteins interact with flexible chain macromolecules through electrostatic, hydrogen bonding, and hydrophobic interactions, an understanding of which could provide a better explanation of the mechanisms of macromolecular interaction available in nature.[1,2] The second concerns the extent to which biochemical activity is maintained in the resulting complexes, the answer to which is central to the molecular design of composite protein−polymer systems, such as immobilized enzymes,[3] as well as to the process design for protein separation using water-soluble polymers.[4]

A number of studies have dealt with the formation of protein−polyelectrolyte complexes in aqueous salt-free and salt-containing systems under different pH conditions (refs 1−5 are reviews; refs 6−26 are original papers). Turbidimetric titration,[5,10−12,17−22,26] quasi-elastic light scattering (QELS),[5,17,19−21,26] static light scattering (SLS),[5,6,9,20,26] electrophoretic light scattering (ELS),[5,19,20,26] and fluorescence spectroscopy[23−25] have been employed. Biochemical methods such as the measurement of enzymatic activity have also been employed in the appropriate cases.[2,3,18,22] By combinations of QELS, SLS, and ELS techniques, it has been demonstrated that poly(diallyldimethylammonium chloride) and bovine serum albumin give forth an "intrapolymer" complex under special pH and salt concentration conditions.[5,20] Although the formation of intrapolymer complexes is believed in other

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Table 1. Properties of PEG Samples Used in the Present Study

sample	$[\eta]^a$ (100 mL/g)	molecular weight[b]	$(d\tilde{n}/dc)^c$ (mL/g)
PEG(1)	0.232	1.3×10^4	0.1372
PEG(2)	0.382	2.9×10^4	
PEG(3)	0.683	6.2×10^4	
PEG(4)	3.084	42.8×10^4	0.1371

[a] Denotes the intrinsic viscosity determined in pure water at 25 °C using an Ubbelohde viscometer with a fall time of 305 s for water at 25 °C. [b] Determined in pure water at 25 °C by SLS. [c] Measured at 25 °C with an electrophotometric differential refractometer (for details, see text).

systems,[26] especially at low polyelectrolyte concentrations, such complexes readily aggregate upon change in pH or polyion concentration; therefore, many difficulties arise in the characterization of an intrapolymer complex by means of QELS, SLS, and ELS. Complexes of proteins with neutral polymers are less prone to exhibit such aggregation effects.

Kokufuta et al. have recently demonstrated that pepsin forms a water-soluble complex with poly(ethylene glycol) (PEG) at pH 3.0 but not at pH 4.5, presumably through the hydrogen bonding of the protein COOH groups with the ether groups in PEG.[27,28] Neither precipitation nor turbidity appeared in this system after the mixing of the protein and polymer at different ratios. This is clearly different from protein-polyelectrolyte systems which can display turbidity to a greater or lesser degree at conditions corresponding to strong interaction. To establish the generality of the observations in refs 27 and 28, we have studied another protein−PEG system. The present paper reports that an intrapolymer complex with a well-defined size is prepared by mixing PEG with human serum albumin (HSA) in aqueous NaCl-containing systems at different pH levels, in particular at pH 2−3.

There are several studies in which attention should be called to the formation of a PEG−protein complex, for instance, basic and technological studies on protein partitioning in two-phase aqueous polymer systems[29] and a biochemical study on PEG-induced changes in lactate dehydrogenase activity.[30] In addition, with the intention of developing novel drug delivery systems,[31] block and graft polymers with PEG segments have also been studied in terms of the formation of polymer−protein complexes, although the authors did not pay much attention to the role of the PEG moieties in the complex formation. The information from the present study would help to develop or establish these PEG-based biochemical and biomedical technologies.

Experimental Section

Materials. HSA was commercially obtained from Sigma Chem. Co., product # A9511. PEG samples were obtained from two different commercial sources: PEG(1) from Nishio Industrial Co. and the others from Wako Pure Chemical Co. The molecular weight of PEG, $\bar{M}_{PEG}$, was determined by viscometric measurements (see Table 1). All water was deionized, distilled, and filtered through a Gelman 0.2 μm filter.

Preparation of Complex. Since the amino acid sequence of HSA is known,[32] we may count the number of the ionizable groups: 116 total acidic groups (98 carboxyl and 18 phenolic OH) and 100 total basic groups (60 amino, 16 imidazolyl, and 24 guanidyl). The absolute molecular weight of HSA was 66 436, as determined from the numbers and molar masses of the constituent amino acid residues. The complexation of HSA with PEG was studied at different molar ratios (R_m) of the ether groups in PEG to all of the HSA acidic groups. Aqueous NaCl solutions (containing 1 M urea in a special case) with different levels of pH and ionic strength were used as a solvent. R_m can be related to the weight ratio (R_w) of PEG to HSA by $R_w = 0.0768R_m$, because the content of the acidic groups is 1.75×10^{-3} mol/g and the molecular weight of the repeating units for PEG is 44. The HSA concentration (C_{HSA}) was kept constant throughout all the experiments, while the PEG concentration (C_{PEG}) was varied.

Aqueous HSA−PEG mixtures at desired R_m ratios were used in QELS and fluorescence spectroscopy without separation of the complex formed. For SLS and ELS, a HSA−PEG mixture (10 mL) at $R_m = 1$ and $C_{HSA} = 0.1$ mg/L was dialyzed against a large amount of the same solvent used for the preparation of the protein−polymer mixture. The dialysis was allowed to continue until a peak assigned to free HSA was no longer observed in the QELS pattern.[33] The dialyzed sample solution was then subject to the measurements without determination of the complex concentration (C_x). The procedures of the dialysis will be described later.

QELS. The measurements were carried out at a scattering angle of 90° with a Brookhaven system (Holtsville, NY) equipped with a 256-channel digital autocorrelator (BI-2030 AT) and a 2 W Ar laser (Stabilite 2017, Spectra-Physics Lasers). A 400 μm pinhole aperture was employed for the EMI photomultiplier tube, and Decalin (decahydronaphthalene) was used as the refractive index matching fluid to reduce stray light. We analyzed the autocorrelation functions with the CONTIN program; the principle of this analytical method was reported in detail in our previous papers.[5,20,21,26]

SLS. The same Brookhaven system described above was employed for SLS measurements. The calibration was made by using pure (>99.5%) toluene, and the optical alignment was ensured by less than 3% deviation from linearity in the $I \sin \theta$ versus θ plot over the range of $40° \le \theta \le 140°$. Each measurement was carried out for 1 s. We determined the Rayleigh ratio (R_θ) on the basis of the average of five such measurements.

Changes in the refractive index with concentrations for PEG and HSA, that is, $(d\tilde{n}/dc)_{PEG}$ and $(d\tilde{n}/dc)_{HSA}$, were measured at 25 °C with an Otsuka electrophotometric differential refractometer (model DRM-1021). The measurements were carried out using unpolarized light from an iodine arc with a spectrum filter whose wavelength was 488 nm. The instrument constant was obtained by using a standard KCl solution (0.01481 g/mL) with a known $d\tilde{n}/dc$ value of 0.1344.

Electrophoretic Light Scattering. The measurements were performed at the fixed scattering angle 8.7° with a Coulter DELSA 400 apparatus (Hialeah, Florida). The electric field was applied at the constant current 0.3 mA. The temperature of the thermostated chamber was maintained at 25 °C. The principle applied in determining the electrophoretic mobility has been described in detail in our previous papers.[5,20,21] The mobilities obtained in this work were repeatable to within less than 5%.

Fluorescence Spectroscopy. Most sample solutions were bubbled with nitrogen gas before spectroscopic measurements in order to minimize the effects of oxygen on the fluorescence spectra. The spectra were measured with a Hitachi model F-4500 spectrophotometer. A high-quality quartz cuvette with a 2 or 10 mm path length was employed for both spectroscopic measurements.

Dialysis Experiments. Dialysis measurements were carried out in order to determine the mass ratio of bound HSA to PEG. We employed two Spectra/Pro CE (cellulose ester) dialysis membranes with the molecular weight cut offs (MWCOs) 50 000

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(33) Prior to detailed descriptions of our QELS results, we should note that there was little change in the size of complex particles during the course of dialysis.

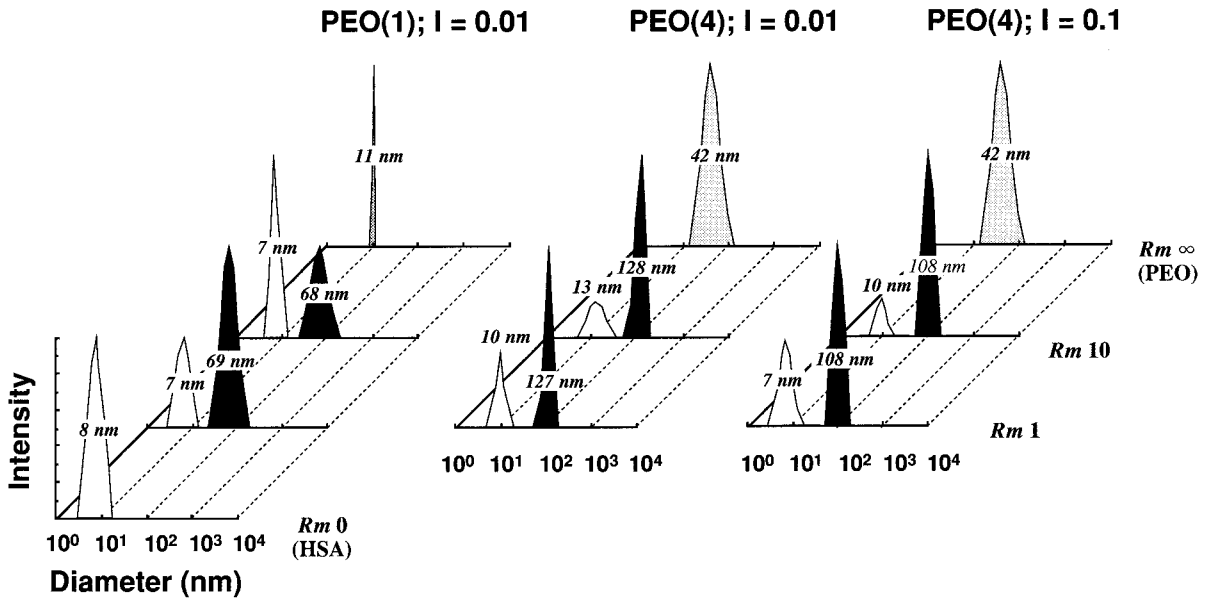


Figure 1. Results of QELS for free HSA, free PEG, and HSA−PEG mixtures at $I = 0.01$ and 0.1. $C_{HSA} = 0.1$ mg/mL for both free HSA and the HSA−PEG mixture. R_m denotes the mixing ratio based on the functional groups of the protein and polymer (see text).

and 100 000; the former was used for HSA-PEG(1) and the latter for HSA-PEG(4) systems.[34] An aqueous HSA-PEG mixture at $R_m = 1$ or 10 was prepared with 0.1 M NaCl as the solvent, and then 10 mL of the mixture was dialyzed against the same solvent (90 mL) as used for the sample preparation until equilibrium was established (usually within 100 h). The amount of HSA plus PEG in the solution against which the dialysis had been performed was determined as a chemical oxygen demand (COD) by the oxidation of HSA and PEG with potassium permanganate. At the same time, the protein concentration was determined by spectrophotometric measurements in which a calibration curve obtained by plotting the absorbance at 280 nm against the HSA concentration was employed. Therefore, the amount of PEG was calculated as the difference between the results of both measurements. The error inherent in the present method was within ±4%.

Results and Discussion

Complex Formation between HSA and PEG. We studied the complexation at pH 2 and at the ionic strengths (I) 0.01 and 0.1 (NaCl) at different R_m ratios. Our previous study has demonstrated that at pH 2, at which all the protein basic groups are completely protonated, the diameter of HSA did not differ from those at neutral pH ranges.[26] Figure 1 shows typical examples of QELS results. Two varieties of particles with different sizes were observed in the HSA-PEG mixtures at $R_m = 1$ and 10. The size of the small particles was very close to that of free HSA and independent of the following factors: R_m, $\bar{M}_{PEG}$ and I. Therefore, we may assign this particle to uncomplexed free HSA.

Since the size of large particles varied depending on $\bar{M}_{PEG}$ and I, the effects of both were studied in detail (Table 2). An increase in $\bar{M}_{PEG}$ increased the size of the large particles, whereas the particle size at $I = 0.1$ was always smaller than that at $I = 0.01$. A polymer chain with bound HSA would carry positive charges at pH 2 due to the protonated guanidyl, amino, and imidazolyl groups. Repulsions among bound proteins are reduced by an increase in I due to the electrostatic screening effect. This leads to a contraction in the polymer chain. Therefore, these results indicate that the large particle is the HSA-PEG complex.

(34) Preliminary experiments revealed that both uncomplexed polymer and protein completely passed through the membrane used for each system.

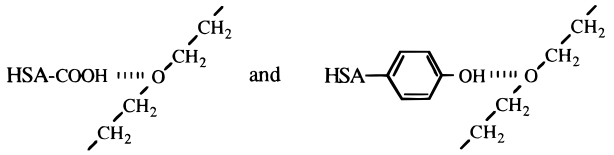
Table 2. Apparent Stokes Diameters ($\bar{d}_s$) for HSA−PEG Complex Particles as a Function of R_m and Ionic Strength (I)[a]

	$\bar{d}_s$ (nm)			
	$I = 0.01$		$I = 0.1$	
sample	$R_m = 1$	$R_m = 10$	$R_m = 1$	$R_m = 10$
PEG(1)	69	68	55	52
PEG(2)	82	83	70	69
PEG(3)	89	91	71	73
PEG(4)	127	128	108	108

[a] Determined under conditions of $C_{HSA} = 0.1$ mg/mL, pH 2, and 25 °C.

Our samples were assumed to be multicomponent systems of PEG, HSA, and the complex of these two. An excess of uncomplexed PEG would remain in the systems at high R_m (e.g., $R_m = 10$), although it did not appear in the QELS pattern. However, our QELS studies dealt with protein−polyelectrolyte complexes,[26] and pepsin−PEG complexes[28] have demonstrated that the contribution of free polymers (and also of free proteins) to the scattering signal assigned to the complex was essentially negligible. The same conclusion can be obtained here, because there was little change in $\bar{d}_s$ with R_m (see Table 2).

Effect of pH on the Complexation. Our previous studies for the pepsin−PEG system[27,28] have suggested that hydrogen bonding of the ether groups in PEG with the carboxyl and phenolic OH groups in the protein plays a major role in the formation of a water-soluble complex;



thus, the complex was formed at pH 3 but not at pH 4.5. The complexation between HSA and PEG was therefore studied over a wide pH range including the isoelectric point (pI; 4.9 ± 0.2) of HSA. A typical result is shown in Figure 2. The observed QELS pattern at pH 3 was the same as that at pH 2 in Figure 1. However, at pH 5 (close

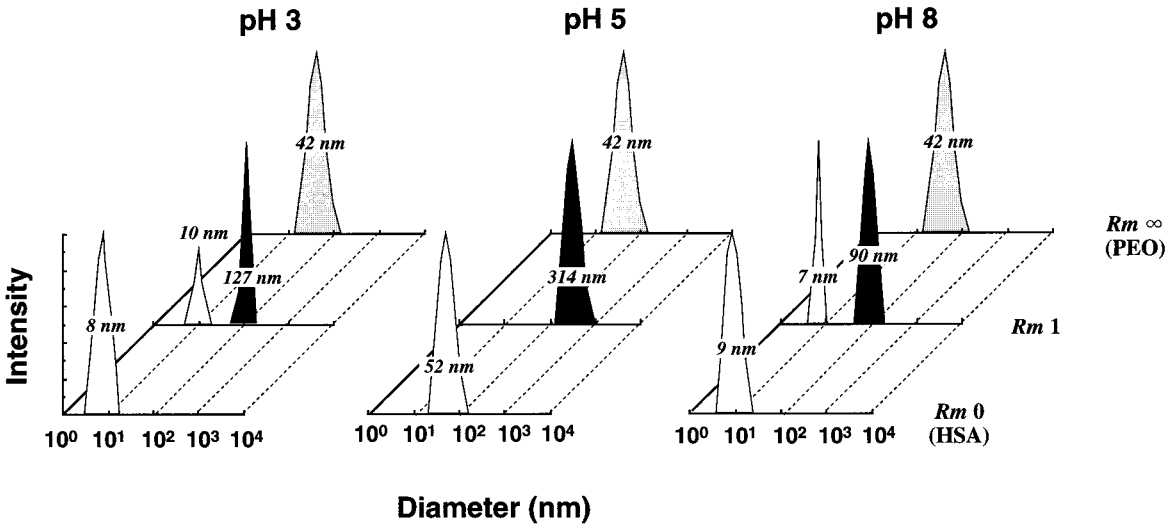
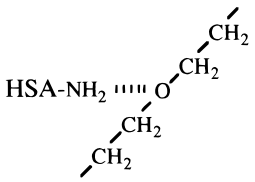


Figure 2. Results of QELS for free HSA, free PEG, and HSA−PEG mixtures at $I = 0.01$ and at different levels of pH. PEG(4) was used in all the measurements. $C_{HSA} = 0.1$ mg/mL for both free HSA and the HSA−PEG mixture.

to pI), aggregation of free HSA was observed, and hence a HSA−PEG mixture ($R_m = 1$) included only very large particles ($\bar{d}_s \sim 300$ nm). Although there was no aggregation at pH 8, at which a large part of the carboxyl groups would be deprotonated, the particles with a 90 nm size still remained in the system.

The formation of a complex between PEG and HSA at pH 8 was unexpected from the results for the pepsin−PEG system. However, taking into account the fact that pepsin as a typical acid gastric protease has a lot of acidic groups (43 carboxyl and 16 phenolic OH) but a few basic groups (2 amino, 1 imidazolyl, and 2 guanidyl) (e.g., see ref 35), we become aware of the difference between pepsin and HSA with respect to the ratio of the acidic to the basic groups. In contrast to pepsin, HSA has 100 total basic groups, the number of which is about the same as that (116) of acidic groups. Thus, it is likely that the basic groups, most of which are deprotonated at pH 8, could form hydrogen bonds with PEG; for example,



Effect of Urea on the Complexation. The above results are indicative, yet not conclusive, of the formation of HSA−PEG complexes via hydrogen bonding. Thus, we attempted to study the effect of urea on the complexation using QELS and fluorescence spectroscopy. Urea has been frequently employed in the biochemical field as a tool to identify hydrogen bonds, since it is generally believed that urea can break up intra- or intermolecular hydrogen bonding of proteins in aqueous systems. Figure 3 shows typical examples of QELS results in the presence and absence of urea at pH 2 and $I = 0.01$. The urea concentration was adjusted to 1 M (mol/L), which is one-eighth lower than the most commonly employed concentration (8 M) in the denaturation of proteins. Therefore, we did not observe a change in the size of free HSA either in the presence or the absence of urea. For the HSA−PEG mixture, however, 1 M urea inhibited the complex formation, even under the conditions at which the complex was formed (pH 2 and $I = 0.01$). This could suggest that hydrogen bonding plays a role in the complexation between HSA and PEG, at least at pH 2.

It has long been believed that urea disrupts the cluster structure of water molecules,[36] that is, a "structure breaking effect." Consequently, one may argue that urea weakens the hydrophobic hydration of a solute, leading to a reduction in the hydrophobic interaction (but not in hydrogen bonding) between PEG and HSA. We thus employed fluorescence spectroscopy as a second tool for studying the complexation. The UV spectrum of an aqueous HSA solution showed an absorption band around 280 nm due to tryptophan and tyrosine residues; thus, the wavelength for the excitation in our fluorescence spectroscopic studies was fixed at 280 nm. An emission maximum (λ_{em}^{max}) was then observed around 340 nm in the spectrum of free HSA at pH 8, the value of which was in fair agreement with that in the literature.[24] The fluorescence around this λ_{em}^{max} is mainly due to the tryptophan residues in the HSA molecule and on its surface; therefore, λ_{em}^{max} is shifted upon changes in the "microenvironment" around the tryptophan residues (e.g., see ref 24). The best known example of this is a blue shift of λ_{em}^{max} upon a decrease in pH, the phenomena of which can be observed at pH < pI for HSA. However, Teramoto et al. have observed a strong blue shift, other than this pH-induced blue shift, when HSA formed a complex with polyions having opposite charges.[24] We also observed a blue shift, not only in the complex formation of papain with polyanion but also in the aggregation of papain molecules due to an increase in the protein concentration.[23] These results clearly indicate that a blue shift of λ_{em}^{max} can be observed when many proteins are bound together by one or more polyions to enhance an "interprotein" interaction, that is, a protein−protein interaction within the complex.

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(36) For previous studies showing that urea acts as a water structure breaker, see the introduction of the following articles: (a) Kuharski, R. A.; Rossky, P. J. *J. Am. Chem. Soc.* **1984**, *106*, 5786. (b) Tanaka, H.; Touhara, H.; Nakanishi, K.; Watanabe, N. *J. Chem. Phys.* **1984**, *80*, 5170. However, we should note that both articles deal with a molecular dynamics simulation of a dilute aqueous urea solution and report that urea has little effect on water structure under an infinitely low concentration.

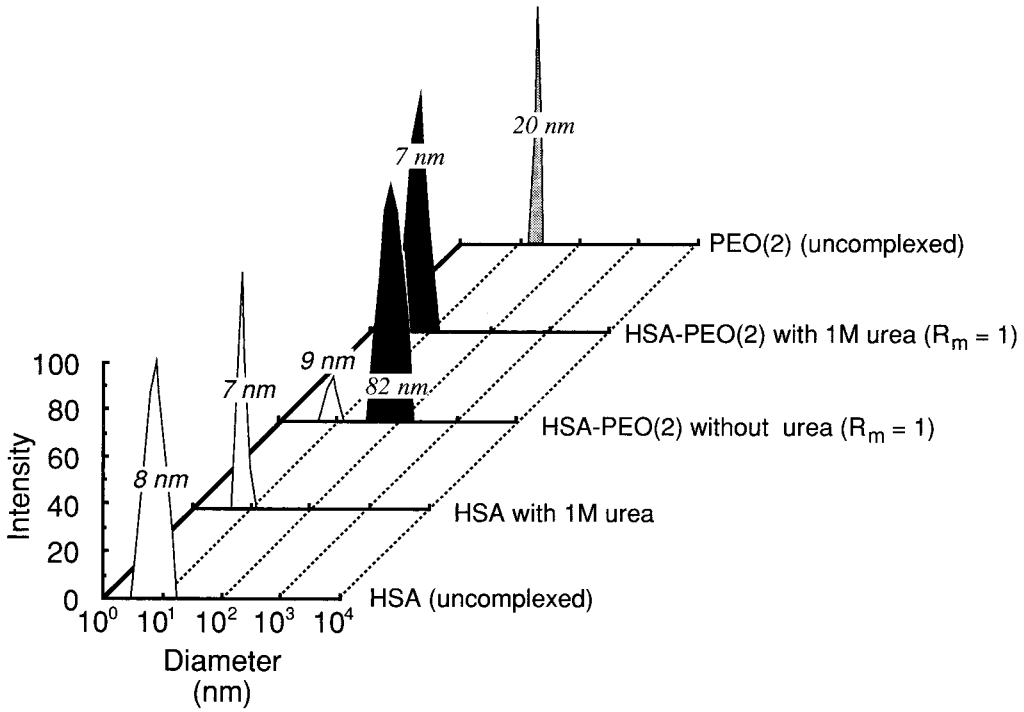

Figure 3. Effect of 1 M urea on the size of free HSA, free PEG, and the HSA−PEG(2) complex (C_{HSA} = 0.1 mg/mL; R_m = 1) at I = 0.01 and pH 2.

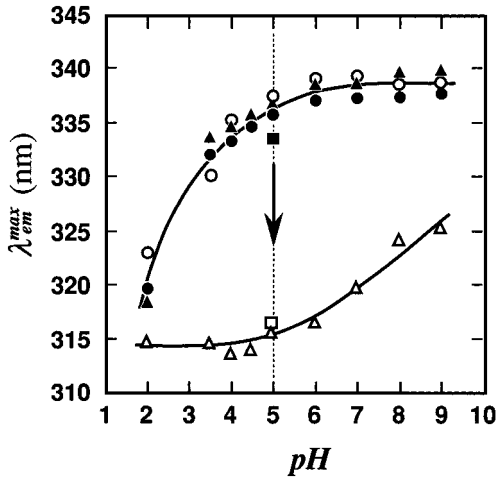

Figure 4. pH Dependence of the emission maximum (λ_{em}^{max}) for free HSA and the HSA−PEG(3) mixture (C_{HSA} = 0.01 mg/mL; R_m = 1) in the presence and absence of 1 M urea: open circles, free HSA (C_{HSA} = 0.01 mg/mL) in the absence of urea; closed circles, free HSA (C_{HSA} = 0.01 mg/mL) in the presence of urea; open triangles, HSA−PEG mixture in the absence of urea; closed triangles, HSA-PEG mixture in the presence of urea. The change in λ_{em}^{max} with free HSA concentration in the absence of urea at pH 5 (close to pI) was also shown: open square, C_{HSA} = 0.04 mg/mL; closed square, C_{HSA} = 0.1 mg/mL. The arrow shows an increase in C_{HSA} from 0.01 to 0.1 mg/mL.

Taking the above into account, we studied a shift of λ_{em}^{max} as a function of pH in the presence and absence of 1 M urea (Figure 4). Also shown in Figure 4 is the dependence of λ_{em}^{max} on the free HSA concentration at pH 5 (close to pI). The results in Figure 4 are outlined as follows: (i) A decrease in the pH of the free HSA solution brings about a decrease in λ_{em}^{max} at pH < 5 (i.e., pH-induced blue shift). (ii) A blue shift of λ_{em}^{max} is also observed when increasing the concentration of free HSA at pH 5 (aggregation effect). (iii) Urea (1 M) little affects the pH curve of λ_{em}^{max} for free HSA. (iv) In the presence of urea, there is little difference in the pH versus λ_{em}^{max} curves for free HSA and the HSA−PEG mixture. (v) In the absence of urea, however, PEG causes a marked blue shift over all the pH ranges studied, particularly at pH < 5, at which the λ_{em}^{max} values are close to that (315 nm) observed under conditions where the free HSA molecules associate with one another to form aggregates with a 314 nm size (see Figure 2). From results iv and v, it is clear that urea inhibits the complex formation. In addition, result iii means that 1 M urea has no influence on the microenvironment around the tryptophan residues. Consequently, we may say that hydrogen bonding, but not hydrophobic interaction, plays an important part in the complexation between HSA and PEG.

Structure of the Complex. We performed SLS experiments to study the structure of the HSA−PEG complex. The weight-average molecular weight ($\bar{M}_x$) and root-mean-square of gyration (R_g) for the complex may be estimated by the following equation:

$$\frac{KC_x}{R_\theta} = \frac{1}{\bar{M}_x}\left(1 + \frac{16\pi^2}{3\lambda^2} R_g{}^2 \sin^2\frac{\theta}{2}\right) + 2A_2C_x \qquad (1)$$

Here, C_x denotes the weight concentration of the complex, R_θ is the Rayleigh ratio, θ is the scattering angle, λ is the wavelength of the light in the medium, A_2 is the second virial coefficient, and K is given as

$$K = (2\pi^2/\lambda_0{}^4 N_A)\tilde{n}_0{}^2(\mathrm{d}\tilde{n}/\mathrm{d}c)_x{}^2 \qquad (2)$$

where λ_0 is the wavelength of the light in a vacuum, N_A is Avogadro's number, $\tilde{n}_0$ is the refractive index of the medium, and $(\mathrm{d}\tilde{n}/\mathrm{d}c)_x$ (in mL/g) is the change in refractive index with complex concentration. Therefore, we can obtain both $\bar{M}_x$ and R_g from eq 2 if C_x and $(\mathrm{d}\tilde{n}/\mathrm{d}c)_x$ are known. However, it is no easy matter to isolate an "intact" complex from a HSA-PEG mixture. We thus employed the previously developed analytical approaches[5,20] for SLS data. According to this, C_x and $\bar{M}_x$ are related to the

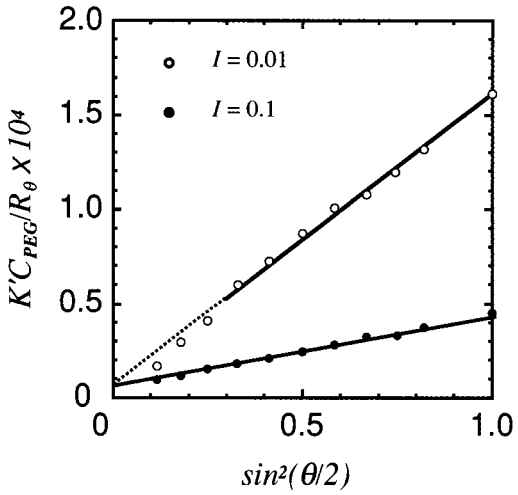

Figure 5. Results of SLS for the HSA−PEG(4) complex at $I = 0.01$ and 0.1. The complex was obtained by dialyzing a HSA−PEG mixture ($C_{HSA} = 0.1$ mg/mL; $R_m = 1$; pH 2; and $I = 0.01$ or 0.1) against a large volume of the same solvent (HCl−NaCl mixture; pH 2; $I = 0.01$ or 0.1) used for the preparation of the protein-polymer mixture. The dialysis was completed when QELS showed that there was no detectable peak of the free protein.

concentration (C_{PEG}) and molecular weight ($\bar{M}_{PEG}$) of PEG by

$$C_x = C_{PEG}(1 + \beta) \tag{3}$$

$$\bar{M}_x = \alpha \bar{M}_{PEG}(1 + \beta) \tag{4}$$

where β represents the mass ratio of bound protein to the polymer and α is the number of polymer chains within one complex particle, that is, the number of aggregated intrapolymer complexes. Moreover, $(d\tilde{n}/dc)_x$ is related to the measurable $(d\tilde{n}/dc)_{PEG}$ and $(d\tilde{n}/dc)_{HSA}$ values by

$$\left(\frac{d\tilde{n}}{dc}\right)_x = \frac{1}{(1+\beta)}\left(\frac{d\tilde{n}}{dc}\right)_{PEG} + \frac{\beta}{(1+\beta)}\left(\frac{d\tilde{n}}{dc}\right)_{HSA} \tag{5}$$

Substitution of eqs 3−5 into eq 1 yields

$$\frac{K'C_{PEG}}{R_\theta} = \frac{1}{\alpha \bar{M}_{PEG} B^2}\left(1 + \frac{16\pi^2}{3\lambda^2} R_g^2 \sin^2\frac{\theta}{2}\right) + 2A_2 C_{PEG}\left(\frac{(1+\beta)}{B}\right)^2 \tag{6}$$

where

$$K' = (2\pi^2/\lambda_0^4/N_A)\tilde{n}_0^2 \tag{7}$$

$$B = \left\{\left(\frac{d\tilde{n}}{dc}\right)_{PEG} + \beta\left(\frac{d\tilde{n}}{dc}\right)_{HSA}\right\} \tag{8}$$

As a result, we can analyze SLS data without using the concentration and refractive index increment of the complex because all the terms in eq 6, except β, are measurable quantities. In the actual analyses of SLS data, we ignore the particle interaction term, that is, the second virial coefficient.

Figure 5 shows a typical example of $K'C_{PEG}/R_\theta$ vs $\sin^2(\theta/2)$ plots for the HSA−PEG(4) system at pH 2. The plots at $I = 0.1$ provided a good straight line over all the ranges of θ (40−140°), whereas at $I = 0.01$ deviations were observed at $\theta < 60°$, perhaps due to polyelectrolyte effects at low ionic strengths. Such deviations were also observed for the HSA-PEG(1) system; thus, we adopted only the data at $I = 0.1$ for the estimations of $\bar{M}_x$ and R_g. Assuming the formation of an intrapolymer HSA−PEG complex (i.e., $\alpha = 1$), the intercept and slope of the straight line yield β and R_g, respectively. In addition, we can obtain $\bar{M}_x$ by the introduction of β into eq 4. These results are listed in Table 3.

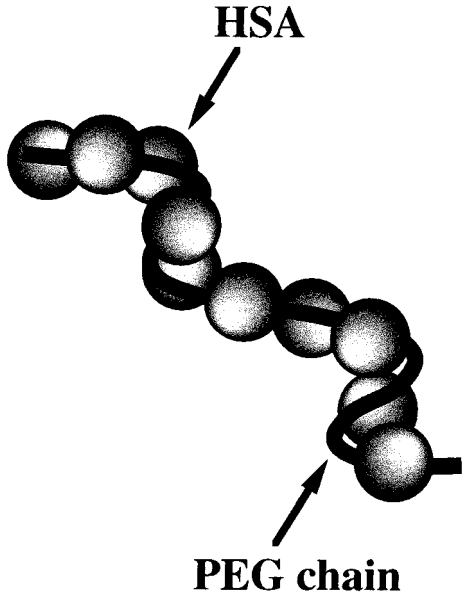

Figure 6. Schematic illustration of an intrapolymer HSA-PEG complex in which several proteins bound to a polymer chain.

Table 3. Results of SLS and Dialysis Experiments for Complexes of HSA with PEO(1) and PEO(4) at $I = 0.1$ and at pH 2

sample	β SLS	β dialysis	$\bar{M}_x$	R_g (nm)	R_h (nm)	$\rho = R_g/R_h$
PEG(1)	33.4	33.2	4.92×10^5	77	28	2.8
PEG(4)	3.03	3.20	18.6×10^5	148	54	2.7

We made two assumptions to analyze SLS data by the above approaches; that is, $A_2 \sim 0$ and $\alpha = 1$. Therefore, the validity of such assumptions should be judged by another experimental method. For this purpose, we carried out the dialysis experiments through which β can be determined. As can be seen from Table 3, there is a good agreement between the values of β from the SLS and the dialysis experiments. This evidently indicates that the assumption of $\alpha = 1$ is correct; in other words, $\bar{M}_x$ corresponds to the molecular weight of an "intrapolymer" complex.

From R_g and the corresponding hydrodynamic radius (R_h, half of $\bar{d}_s$ in Table 2), we obtain $\rho = R_g/R_h$ for the intrapolymer HSA−PEG complex. Hydrodynamic theory[37] shows that ρ changes from infinity to 0.775 when the polymer structure changes from a long rod to a sphere, with values from 1.3 to 1.5 for random coils. As can be seen from Table 3, ρ ranges from 2.8 to 2.7. This result suggests an extended structure of the HSA−PEG complex (see Figure 6). Such an extended structure appeared to be due to "steric" rather than "electrostatic" repulsion among the positively charged and polymer-bound proteins, since the ionic strength used here was considerably high. Moreover, a marked blue shift of λ_{em}^{max} due to the complexation, suggestive of an interprotein interaction within the complex, has been observed in Figure 4.

(37) Konishi, T.; Yoshizaki, T.; Yamakawa, H. *Macromolecules* **1991**, *24*, 5614.

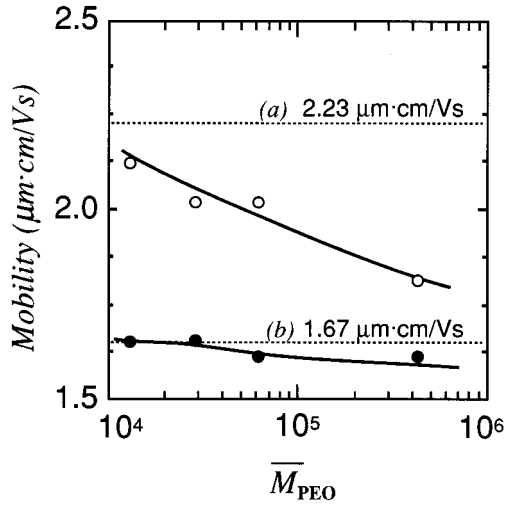

Figure 7. Electrophoretic mobilites of HSA complexes with PEG samples [i.e., PEG(1) to PEG(4)] with different molecular weights at pH 2 and at $I = 0.01$ (open circles) and 0.1 (closed circles). Also shown in this figure for the purpose of comparison are the mobilities (dashed line) of free HSA at pH 2 and at $I = 0.01$ (a) and 0.1 (b). The same dialysis procedure as described in Figure 5 was employed to prepare each complex, except for the use of the polymers with different molecular weights.

In addition to the above, we should discuss the big difference in β between PEG(1) and PEG(4) observed in Table 3. This result means that a $\bar{M}_{PEG}$-dependent increase in the number (n) of HSA molecules bound per chain of PEG is not constant over a wide $\bar{M}_{PEG}$ range, but the increment of n with $\bar{M}_{PEG}$ decreases with increasing $\bar{M}_{PEG}$ (see the following section). Such an $\bar{M}_{PEG}$ effect is then explicable in terms of the common knowledge that an intramolecular interaction among the segments assigned to a polymer chain in a good solvent becomes stronger as its molecular weight is increased. Thus, an increase in $\bar{M}_{PEG}$ depresses the protein binding which should accompany an extension of the PEG chain due to the interprotein interaction. Taking this into account, it is predictable that the binding of HSA to PEG with an enormous $\bar{M}_{PEG}$ takes place only on the surface of the polymer coil. Under such a situation, we would not observe an extended structure of the complex as shown in Figure 6. To confirm this, it is necessary to employ a PEG with $\bar{M}_{PEG}$, the value of which is one or more orders of magnitude greater than that of PEG(4), but many difficulties would arise in handling such a high-molecular-weight sample.

Electrophoretic Behavior of the Complex. We employed the ELS technique for the examination of the complex charge. The dependence of the electrophoteric mobility (U) on $\bar{M}_{PEG}$ is shown in Figure 7. It was found that U decreases more or less with increasing $\bar{M}_{PEG}$, in particular at $I = 0.01$, due to the effect of $\bar{M}_{PEG}$ on n as stated above. However, a large difference in U between the complexed and free proteins was not observed, even when PEG(4) was used in the complexation. There was no influence of R_m on U at $I = 0.01$ and 0.1 (data not shown). Seemingly, these results imply that during electrophoresis the complex consisting of PEG-bound proteins with positive charges behaves like a "free draining coil", which is a generally accepted model in many theoretical and experimental studies on the electrophoretic behavior of polyelectrolyte ions.[38] Nevertheless, one may argue that since an increase in $\bar{M}_{PEG}$ results in increases both in the size of the complex and in the number of bound proteins at the same time, it is possible to observe a constant U without the influence of $\bar{M}_{PEG}$ (i.e., a "nonfree draining" behavior). Thus, we attempted to analyze the mobility data in more detail.

Table 4. Comparison of Calculated and Observed Electrophoretic Mobilities for Complexes of HSA with PEO(1) and PEO(2) at pH 2 and at $I = 0.1$

sample	R (nm)	kR	$f(kR)$	n	Z	U_{calcd} (μm·cm/(V s))	U_{obs} (μm·cm/(V s))
PEG(1)	28	28.6	0.91	7	315	0.37	1.61
PEG(4)	54	56.2	0.95	21	945	0.31	1.62

Both SLS and dialysis experiments have demonstrated that the complexation between HSA and PEG yielded the intrapolymer complex. When such a particle behaves as a nonfree draining coil during electrophoresis, its mobility may be given by Henry's equation:[39]

$$U = \frac{f(\kappa R)(1 + \kappa r)}{6\pi\eta R(1 + \kappa r + \kappa R)} Ze \qquad (9)$$

where $f(\kappa R)$ is Henry's function, κ is the Debye−Hückel parameter, R is the radius of a complex particle ($R \sim R_h$), r is the radius of a small ion, e is the electric charge, and η is the viscosity of the medium. Moreover, Z is the charge of a complex particle and may be given by $Z = nZ_{HSA}$, where Z_{HSA} is the charge of a bound HSA molecule. Then, we may obtain n from β in Table 3 using the relation

$$n = \beta(\bar{M}_{PEG}/M_{HSA}) \qquad (10)$$

The charge of free HSA (Z_{HSA}) was then estimated to be +45 from the mobility (1.67 μm·cm/(V s)) at $I = 0.1$ and pH 2 using eq 9. As a result, we can estimate the mobility for a nonfree draining complex particle. The results are listed in Table 4, together with all the parameters used for the calculation. The calculated values were about one-fifth the observed ones. Even though some errors exist in our estimation, these results strongly suggest that the intrapolymer complex consisting of HSA and PEG did not behave as a nonfree draining particle during electrophoresis.

It is significant to compare the n values for PEG(1) and PEG(4) in Table 4. For this purpose, we attempted to estimate n for PEG(4) from β for PEG(1). Since $M_{PEG} = 428\,000$ for PEG(4) and $M_{HSA} = 66\,436$, we obtain $n \sim 215$. This is almost 4 times that determined using the β values from the SLS and the dialysis experiments for PEG-(4). The reason for this deviation has been discussed in the previous section.

Conclusions

The complex formation between HSA and PEG has been studied using different experimental techniques. The results obtained are summarized as follows. (i) A water-soluble complex was formed by mixing HAS with PEG in aqueous acidic solvents containing different amounts of NaCl. (ii) The particle size of the resulting complex increased with increasing PEG molecular weight but decreased with increasing NaCl concentration. (iii) The complexation did not take place in the presence of 1 M urea. (iv) A combination of SLS and dialysis experiments demonstrated that the complex formed is an intrapolymer complex in which several HSA molecules bound to a PEG chain. (v) Such an intrapolymer complex behaves like a

(38) (a) Hermans, J. J. *J. Polym. Sci.* **1955**, *18*, 529. (b) Overbeek, J. T. G.; Stigter, D. *Recl. Trav. Chim.* **1956**, *75*, 543. (c) Nagasawa, M.; Noda, I.; Takahashi, T.; Shimamoto, N. *J. Phys. Chem.* **1972**, *76*, 2286. (d) Kokufuta, E. *Polymer* **1980**, *21*, 177 and other references therein.

(39) Henry, D. C. *Proc. R. Soc.* **1931**, *A133*, 106.

free draining coil during electrophoresis. (vi) Detailed analyses of QELS and fluorescence data strongly suggested that hydrogen bonding plays an important part in the complexation.

The picture of intrapolymer complex formation reported here could provide a key for allowing us to explore the principles underlying the molecular interactions between globular colloidal particles and flexible chain macromolecules. Moreover, the information from the present study would help to develop and establish PEG-based biochemical and biomedical technologies; for example, the process design for protein separation by aqueous polymer two-phase partitioning as well as the molecular designs of protein−polymer composites, such as immobilized enzymes and drug delivery systems.

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