Association between Hydrophobically Modified Polyanions and Negatively Charged Bovine Serum Albumin

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Attractive interactions between negatively charged bovine serum albumin (BSA) at pH 7 and 9 and poly(sodium acrylate) were obtained by substituting a small fraction of acrylic units with alkylacrylamide units. Using light scattering, equilibrium dialysis, and viscometry, we investigated, in dilute solution, the association between BSA and two sets of modified polyacrylates of mean molecular weight 5000 and 150 000, respectively. The formation of complexes was revealed by pronounced increases of the scattering depending on the hydrophobicity of the synthetic polymer. It was not observed with entirely hydrophilic polyacrylates under the same conditions. In the case of long polyacrylates, the apparent hydrodynamic radius of the complexes was slightly larger than that of the free polymer. The polydispersity in size of the complexes seemed low. In the case of short polyacrylates, the complexation can be depicted as the "adsorption" of several polymer chains per protein. In contrast, complexes with long polyacrylates contain a single chain that accommodates several proteins.

Introduction

Research on the interactions between polymers and proteins has received considerable attention with respect to both practical and fundamental interests of such systems at the boundary between polymer/polymer and polymer/colloid mixtures.[1−16] In the field of biochemistry, these systems can help to model vital phenomena such as protein-mediated polynucleotide behaviors (e.g., DNA/histone collapse). Owing to their interaction with proteins, poly(acrylic acid)s were also found to be antiviral agents.[2] On the other hand, several applications were suggested, such as enzyme stabilization[3] or protein isolation and purification by coprecipitation with synthetic polyelectrolytes or polyampholytes.[4,5] Since the pioneering work of Morawetz,[6] a huge majority of these investigations were focused on the formation of soluble or insoluble complexes between a polyelectrolyte and a soluble globular protein. In the latter systems, the Coulombic origin of the associations and the similarities to the usual polyelectrolyte complexes (polyanions/polycations) are now recognized.

Only a few papers report the possible influence of driving forces other than Coulombic. At low pH, hydrogen bonds were assumed to explain the observed attraction between pepsin and poly(ethylene glycol).[7] Akiyoshi and colleagues reported the formation of supramolecular assemblies between soluble proteins and self-aggregates of pullulan grafted with cholesterol dangling groups.[8,9] They suggested two possible driving forces of the association: a direct participation of the cholesterol groups (hydrophobic association) and hydrogen-bond formation with the backbone of the pullulan. Finally, little attention has been paid to the importance of hydrophobicity even though it was several times assumed to play a major role.[8−11]

Among water-soluble proteins studied in the presence of polyelectrolytes, bovine serum albumin (BSA) is one of the more hydrophobic. Owing to the prime importance of BSA in the control of osmotic pressure in vivo and the transport and storage of nutriments or drugs, the structure of the protein and its association with a tremendous number of molecules—such as fatty acids, aromatic compounds—were extensively investigated.[17] It can also form complexes with polyanions and polycations, the stability of which is strongly dependent on pH. As shown by several authors,[6,11,12−14] polycations are bound to BSA only above its isoelectric point (pI $\approx$ 4.3−4.9), when the total charge of the protein is negative. In contrast, complexes with polyanions not only can be formed below the pI but also can be formed a few pH units above it. Under these conditions, a total protein charge up to −25 does not hinder the association with poly(vinyl sulfonate) for instance.[12] On the basis of modification of the intrinsic fluorescence of BSA, Teramoto et al.[11] proposed that at high pH, polyanions interact specifically with binding site II of the protein. Dubin et al.'s measurements by light scattering led to a similar conclusion. The latter group claims the existence of a nonuniform charge distribution and positive charge patches just above the pI, despite a total charge on the order of −10 to −25. This assumption was proposed in the past to explain the adsorption of proteins on ion-exchange resins.[18] It is also supported by electrostatic-potential calculations as a function of pH on RNase, a protein of known structure.[15] Above pH 9, however, all the authors agree that complexes between BSA and polyanions disappear completely. At such a high pH value, the global Coulombic repulsion between the partners must oppose the local attraction with possible residual charge patches or binding sites.

In the present paper, we aim at a systematic approach of hydrophobically driven association between a set of modified polyacrylates of adjusted hydrophobicities and bovine serum

† R. Audebert died while the manuscript was being written. He initiated our research on proteins/polyamphiphile interactions. His faith and determination have greatly contributed to the launching and development of the present work.

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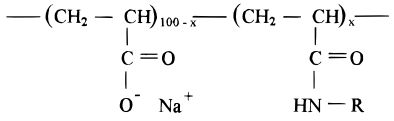
albumin (BSA) as a model protein. We report the formation of complexes between hydrophobically modified polyacrylates (HMPA) and BSA, at pH 7 and 9, in dilute solution over a broad range of concentrations of both protein and polymer. We were able to examine by light scattering, viscometry, and dialysis the formation of complexes as a function of the hydrophobicity or molecular weight of HMPA. The hydrophobic origin of the association was clearly revealed by a comparison with the case of hydrophilic precursors that, as expected, do not associate with BSA under the same conditions. This work confirms the proposed mechanism of thickening, upon addition of protein, observed previously in semidilute solutions of HMPA.[16]

Material and Methods

Materials. Bovine serum albumin (fraction V) was purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Poly(acrylic acid) samples of average molecular weight 5000 and 150 000 were obtained from Aldrich Chemical Co. (Milwaukee, WI) and Polysciences Inc. (Warrington, PA), respectively. The hydrophobically modified poly(acrylic acid)s (HMPA) were synthesized according to a reaction described previously[19] by grafting dodecylamine or octadecylamine at random along the backbone of the precursor. The HMPA have the same polymerization degree as their precursor polymer. They were obtained in the sodium salt form:



where x is the molar modification ratio and R is an alkyl chain with y carbon atoms ($y = 12$ or 18). They will be denoted by the codes 150-xCy and 5-xCy and were derived from a precursor of molecular weight (MW) 150 000 and 5000, respectively. Both contain x mol % of C_yH_{2y+1} dangling groups.

All the experiments were carried out in buffer solutions at pH 7.0 (phosphate buffer) or pH 9.0 (borate buffer). For the light scattering and some viscometric measurements, extra salt (0.3 M NaCl or $NaNO_3$) was added. Deionized water was obtained from a Millipore Milli-Q water system.

Polymer stock solutions were routinely prepared under magnetic stirring at least 18 h before use. The protein was previously dissolved in water to minimize the formation of aggregates, and the final protein samples were obtained by addition of buffer to the appropriate volume of aqueous protein solution. Mixtures were prepared from the stock solutions of polymer and protein, and after vigorous shaking, samples were kept at room temperature at least 2 h before the measurement.

Static and Quasi-Elastic Light Scattering. The measurements were made using an ionized argon laser (SP2020, Spectra Physics, CA, 3 W) at a wavelength $\lambda = 514.5$ nm. Detection optics and photomultiplier (PCS5, Malvern Instruments, England) were mounted in a rotating arm (PCS100, Malvern Instruments, England) that allows measurements at scattering angles θ from 10° to 150°. A digital correlator (K7025, Malvern Instruments, England) calculated the homodyne intensity−intensity correlation function $G(q, t)$ (where q is the amplitude of the scattering vector given by $q = (4\pi n/\lambda)\sin(\theta/2)$, n is the refractive index of the medium). G was related to the correlation function of the scattered field by

$$G(q, t) = A(1 + \beta g^2(q, t))$$

where A is the experimental baseline and β the fraction of correlated light (about 0.7). The normalized electric-field time-correlation function g was analyzed either by the method of cumulant (a) or as the sum of two exponential decays (b):

$$\log g = -\Gamma t + \frac{\mu_2}{2}t^2 + \frac{\mu_3}{6}t^3 \quad \text{(a)}$$

$$g = a_1 \, e^{-\Gamma_1 t} + a_2 \, e^{-\Gamma_2 t} \quad \text{(b)}$$

Γ_1 and Γ_2 are the inverse of the mean relaxation times and μ_i and a_i adjustable constants fitting the curve. The sensitivity of these fits to the choice of time range (about 5% of the values of Γ) was lower than the fluctuations obtained when several mixtures of the same composition were compared (relative error $\approx$ 10% on Γ). From the mean relaxation time we obtained the related diffusion coefficient $D = \Gamma/q^2$ and the apparent Stokes radius $R_h = kT/(6\pi\eta D)$ (where k is the Boltzman constant, T is the absolute temperature, and η is the viscosity of the solvent).

Benzene was used as an intensity reference. The temperature was regulated at 25 °C. Samples were filtered through 0.2-mm polysulfone filters (Whatman) prior to the measurement.

Viscometry. Initial HMPA solutions (at a concentration below 0.2%) in buffer at pH 9 (30 mM sodium borate and possibly 300 mM NaCl) in the presence of BSA (from 0 to 4 wt %) were diluted in the same buffer at constant BSA concentration. Linear extrapolations to zero polymer concentration of both specific and inherent viscosities gave intrinsic viscosity as a function of total BSA concentration in the solvent. The experiments were carried out at 25 °C in a Ubbelohde capillary viscometer of 0.46 mm in diameter (model TI1 from Sematech, France).

Dialysis. Dialysis measurements were performed at room temperature with Slide-A-Lyzer dialysis cassettes (cutoff of 10 kD) (Pierce Chemical Co.). A volume of 3 mL of a BSA solution at 0.5% (w/w) was loaded in the cassette and dialyzed against 230 mL of a polymer solution (0.005−0.12%). A quantity of 400 ppm of NaN_3 was added to the protein solutions in order to prevent the possible development of microorganisms and BSA degradation. All experiments were done in 20 mM borate buffer pH 9. In all the cases the equilibrium was reached after 20 h. It was checked that no change occurred beyond this time by going on the dialysis for a additional 52-h period. Aliquots from each side of the dialysis cells were periodically taken and their content of total organic carbon measured in a DC-80 total organic content system (Dorhmann, Xertex Co., CA). This provided the concentration of HMPA on both sides of the dialysis chamber (free polymer outside and free plus bound inside). Prior to BSA/HMPA experiments an additional dialysis of BSA against buffer solution was carried out in order to check that the protein does not diffuse across the dialysis membrane. This fact was also ascertained by measuring the absorbance at 280 nm of the different aliquots taken during the dialysis time. On the other hand, the preservation of BSA in its native form in the presence of HMPA for several weeks was checked by measurements of the specific rotation of the protein (not shown).

Results and Discussion

Modification of poly(acrylic acid) precursors provides the advantages of access to sets of HMPA having the same mean molecular weight and same polydispersity as the precursors. Such a feature has allowed us to characterize the complexes formed between BSA and hydrophobically modified polymers with two different mean MW (5000 and 150 000) as well as to

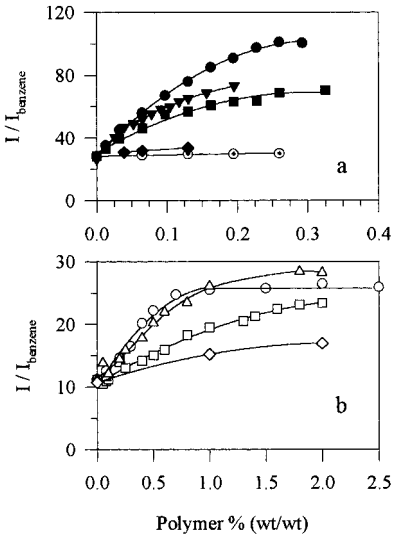


Figure 1. Intensity of light scattered at 90° by mixtures. (a) BSA (2%) and long HMPA in 0.3 M NaCl and 30 mM borate buffer, pH 9: (●) BSA + 150-4C18; (■) BSA + 150-3C12; (▼) BSA + 150-5C12; (⊕) BSA + precursor; (◆) intensity of BSA 2% + intensity of 150-3C12 alone. (b) BSA (0.5%) and short HMPA in 0.3 M $NaNO_3$, 30 mM borate buffer, pH 9: (○) BSA + 5-3C18; (□) BSA + 5-3C12; (△) BSA + 5-10C12; (◇) intensity of BSA 0.5% + intensity of 5-10C12.

analyze the effect of the hydrophobicity without contributions of molecular weight or polydispersity.

Evidence for Associations between BSA and HMPA. Static light scattering was carried out because it is especially sensitive to the formation of aggregates. In the past, it has been used to detect polyelectrolyte complexes and BSA/polysaccharide associations.[20,21] The intensity of light scattered by BSA/HMPA mixtures was found to be highly sensitive to the composition of the sample (Figure 1). Moreover, the scattering depended on the hydrophobicity of the polymer. The addition of a small amount of HMPA in a solution of BSA can increase the intensity scattered at 90° up to 3−4 times its initial value. In contrast, no variation of the intensity was observed when a hydrophilic precursor was added in BSA solutions at constant protein concentration (Figure 1a). As reference values of intensity, we added the intensity scattered by the solution of BSA alone plus that scattered by solutions of HMPA alone. The scattering from the HMPA being much lower than that of the protein, the reference values that correspond to the absence of interactions between the partners remain close to the intensity measured from pure BSA solutions. The observed increase of up to 4 times the reference intensity therefore indicates the presence of strong interactions between HMPA and BSA. Similar results were found for BSA concentrations over the range 0.5%−4%.

From the light scattering eq 1, we recognize several origins of the variations of the scattered intensity I in mixed systems, namely, variation of refractive index increment dn/dc, of the second virial coefficient A_2, of the size or shape of the present species (through the form factor $s(q)$), and of their MW:

$$Kc/(I - I_0) = (1/\mathrm{MW} + 2A_2c)1/s(q) \qquad (1)$$

where $K = [2\pi^2 n/(\lambda^4 N_a)](\mathrm{d}n/\mathrm{d}c)^2 I_0/R^2$, I_0 is the background intensity, c the polymer concentration, R the distance between the detector and the sample, and N_a Avogadro's number.

Under the conditions of our experiments, effects of both A_2 and $s(q)$ are negligibly small in the absence of association. In effect, the size of the partners (radii is less than 30 nm for HMPA and less than 5 nm for BSA; see next part below) leads to $s(q)$ of about unity. In addition, the angular dependency of the total intensity of the mixtures was modest (typically 20% increase from 120° to 40°), and it did not markedly depend on the composition of the mixture; large variations of the form factor can be dismissed. On other hand, in the concentration range of our experiments (BSA < 3% w/w, long HMPA < 0.1%, and short HMPA < 2%), the intensity scattered by a solution of protein alone (as well as of HMPA only) was proportional to the concentration of macromolecules (not shown); the presence of NaCl in the solvent (also buffered at pH 9) was enough to reduce the virial coefficient at a negligible rate. It turned out also that the refractive index increment of BSA is not modified by mixing with precursor as deduced from the identical variations of the scattered intensity with protein concentration in the presence or in the absence of precursor (not shown). Under these conditions, the formation of species having a higher MW than the initial partners is the only explanation of the increase of scattered light. It is a clear indication that HMPA form aggregates with BSA, whereas precursors do not. A few molar percentage of hydrophobic dangling groups along the backbone is large enough to induce the association with BSA whatever the MW of polyacrylates.

All the HMPAs induce similar behavior, namely, a sharp and linear increase of the intensity at low polymer concentration followed by a much slower variation above a threshold concentration. Beyond this similarity, some quantitative differences depended on either MW or hydrophobicity. At constant MW, we remark that the more hydrophobic the polymer, the higher the scattered intensity. Whatever the BSA concentration (between 0.5% and 4%), HMPA can be ranked with respect to their ability to enhance the scattering as follows: 150-3C12 < 150-5C12 < 150-4C18 and 5-3C12 < 5-10C12 ≈ 5-3C18. This classification falls in with previous studies of self-aggregation of HMPA in water[22] and with comparisons of HMPA association with surfactants.[23,24] At constant HMPA structure, polymers having different MW (e.g., 5-3C12 and 150-3C12) do not enhance the scattering at the same rate. Small amounts of long HMPA (final concentration less than 0.1% in Figure 1a) are sufficient to increase significantly the scattering intensity, whereas under the same conditions, the addition of short polymers has a negligible effect. It appeared necessary to add a weight fraction of short HMPA roughly 10 times higher than that of long ones to reach the same intensity enhancement. Undoubtedly, the type of aggregates formed (their mean constitution, size, molecular weight, ...) or the concentration of these aggregates is modulated by both hydrophobicity and MW of HMPA. However, the interpretation of these measurements can be consistent with several possible changes at the microscopic level. We tentatively propose three different origins.

(i) Equilibrium between free and bound macromolecules could be shifted toward free BSA and/or free HMPA when either the MW or the hydrophobicity is reduced. The association with short polyacrylates could indeed be weaker than with longer ones. Similarly, a polymer of high hydrophobicity should enhance the fraction of aggregated species. In this case, the scattered light depends primarily on the amount of associated species.

(ii) Formation of aggregates containing several HMPA molecules and several proteins might be promoted by both long chains and high hydrophobicity of HMPA. In other words, the mean size (consequently, the MW) of the aggregates might be controlled by the formation of cross-links between different macromolecules as shown previously in semidilute solutions.[16]

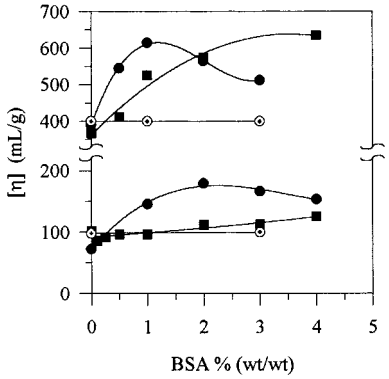


Figure 2. Variation of intrinsic viscosity of "long polymers" with concentration of BSA at pH 9.0 in 30 mM borate buffer (top) and in 0.3 M NaCl plus 30 mM borate solution (bottom): (●) 150-4C18; (■) 150-3C12; (⊕) precursor.

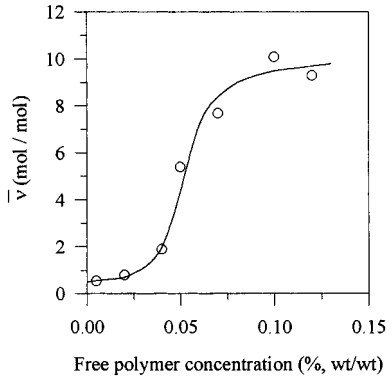


Figure 3. Isotherm of the association between 5-3C18 and BSA in 20 mM borate buffer, pH 9.0. ν is the ratio of the number of bound polymers to the total number of BSA.

(iii) Even in the absence of interpolymer association, longer HMPA could accommodate a larger quantity of bound protein, giving rise to species of higher MW than with short HMPA. In this case, the intensity is primarily modulated by the MW of complexes containing a single HMPA molecule.

From static light-scattering measurements only, it is extremely difficult to determine what mechanism is appropriate. At this step, for accurate determinations of the mean molecular weight of the associated species, the measurement of the concentration of possible free BSA and free HMPA is required. In some cases, the ambiguity was removed by the use of supplementary techniques.

Behavior of Long HMPA in Excess BSA. We have carried out previously viscosity measurements on similar systems[16] because the technique is sensitive to the formation of connections between macromolecules and to gelation threshold (i.e., what is expected in case ii above). This approach was extended to the dilute regime by measurements of specific and inherent viscosity of long HMPA as a function of BSA concentration. A similar viscometric approach was not accurate enough in the case of short HMPA. Because the values of the intrinsic viscosity of these short polymers are small, high polymer concentrations were required to measure the specific viscosity (beyond 2%). With long HMPA (MW of 150 000), it was possible to study the mixtures previously characterized by static light scattering. Variations of specific and inherent viscosities with polymer concentration were linear whatever the BSA concentration and below a polymer concentration threshold (0.1% w/w at "low ionic strength", i.e., 30 mM borate buffer; polymer up to 0.2% at "high ionic strength", i.e., 0.3 M NaCl and 30 mM borate buffer). The corresponding Huggins and Kramer constants (about 0.3 and −0.2, respectively) do not vary significantly with BSA concentration. They are close to the usual values for polyelectrolytes in aqueous media.[25] It points out therefore that under these conditions, the usual repulsive interactions between the HMPA molecules are involved. Reversible interpolymer associations that depend on dilution would have induced nonlinear variations of viscosities. Therefore, they do not take place in this concentration range: the composition and MW of the complexes do not vary significantly with dilution. Under these conditions, the extrapolated intrinsic viscosity $[\eta]$ is expected to follow the coil expansion of the complexes formed in a large excess of protein. We observed that the variation of $[\eta]$ with the concentration of protein (Figure 2) stayed within the experimental errors in the case of the precursor, whereas the intrinsic viscosity of HMPA was highly sensitive to the presence of protein. In the absence of BSA, similar values of $[\eta]$ are obtained for all the HMPA and their precursor (previously reported[25] and exemplified in Figure 2 at two ionic strengths). Because of the polyelectrolyte nature of HMPAs, their coil expansion is strongly modulated by the concentration of salt that could be changed by the addition of BSA. But the absence of the effect of protein on the precursor shows that BSA does not significantly change the ionic strength. Therefore, Coulombic effects cannot be involved in the differences observed in the presence of proteins. Differences between precursor and HMPA point out the existence of interactions with hydrophobic dangling groups, whereas the acrylate backbone is not affected by the presence of BSA. At "low ionic strength", the maximum of the expansion coefficient $\alpha = [\eta]/[\eta]_{\text{precursor}}$ is about 1.7 for 150-4C18 as well as for 150-3C12. At "high ionic strength", α reaches 1.8 for 150-4C18 but stays below 1.2 for 150-3C12. In the latter case, it is likely, however, that α continues to increase beyond the maximum concentration of the protein we studied (4% w/w, Figure 2). In terms of the apparent radius of polymer coils, this expansion remains modest, since it yields a ratio of the radii of complexes to that of the precursor on the order of $\alpha^{1/3}$. At the maximum, the radii of complexes is 1.2 times that of the free polymers. From these measurements, we can conclude that at concentrations of polymer below 0.1%, most of the long HMPA are not involved in large aggregates but probably form single-chain complexes in equilibrium with free proteins. The schematic picture of the complexation is the formation of a slightly expanded necklace, slightly larger than the free polymers, containing bound BSA along one HMPA.

Mean Composition of the Complexes with Short HMPA. To study the association with short HMPAs, we turned to the equilibrium dialysis technique. In this set of experiments we dialyzed the protein at a given concentration (0.5%) against a large volume of polymer solution (outer volume about 75 times the inner one). Under the experimental conditions, the protein remained inside whereas the polymer readily crossed the membrane. From the content of total organic carbon and the absorbance at 280 nm of the samples inside and outside the dialysis bag, we calculated the concentrations of protein and polymer of both sides as a function of dialysis time. At equilibrium, the polymer concentration outside the bag was assumed to be equal to the free polymer inside. The fraction of polymer bound to BSA was easily calculated by subtracting the total concentration of the inside polymer from the concentration of free HMPA. The mean number of polymer "adsorbed" per protein ν corresponds to the ratio of the concentration of bound polymer to the total BSA concentration. The isotherm obtained for the system BSA/5-3C18 in 20 mM borate buffer pH 9 is shown in Figure 3. Each data point corresponds to an

TABLE 1: Apparent Hydrodynamic Radius of HMPA or HMPA/BSA Complexes (at 0.1% Polymer, in 0.3 M NaCl, 30 mM Borate Buffer, pH 9) Deduced from the Slow Relaxation Mode in QELS[a]

BSA % (w/w)	R_h (nm)	
	150-3C12	150-4C18
0	29 ± 1	25 ± 4
1	30 ± 3	32 ± 3
2	30 ± 3	29 ± 3
3	32 ± 3	35 ± 3
4	34 ± 5	30 ± 4

[a] Each value is the mean of at least three measurements of different samples. Errors correspond to the largest difference between the mean and the measured values.

individual dialysis sample at equilibrium (i.e., 1-day to 3-day old). The shape of the isotherm is similar to those previously reported for protein−surfactant interactions.[26] From extremely low HMPA concentrations up to 0.04%, the ratio ν is about 1 (mol/mol) and remains practically constant. Further additions of polymer lead to a continuous adsorption of 5-3C18 molecules to BSA, and beyond 0.07% the binding approaches saturation at $\nu = 9-10$ (i.e., about 18 hydrophobic chains per molecule of protein). Like in the case of binding of surfactants onto proteins,[26] we observed a sharp increase of ν above a threshold amphiphile concentration, which reflects the cooperative character of the association. The constant polymer-to-protein ratio obtained at saturation ($\nu = 9-10$) is consistent with previous measurements of the binding of similar polymers onto membrane proteins.[27] And it is also a hint for the formation of complexes having a defined composition, possibly single BSA molecules surrounded by an HMPA layer. With the latter assumption, a 1/1 complex might be formed at low HMPA concentration. Above a threshold concentration, the large increase of the number of bound polymers up to saturation might reflect the formation of a micelle-like layer around each protein. This picture was ascertained by measurements of the size of the complexes.

Size of the Complexes. QELS is a well-suited technique for measurement of the hydrodynamic radius of species in dilute solution. In mixtures, however, all the macromolecules and their possible complexes contribute to a part of the scattering. Owing to the huge enhancement of the scattered light when complexes are formed, in our systems we expect that most of the scattered light can be attributed to complexes. More precisely, we have shown that the presence of free polymer was practically negligible (less than 10%) in terms of scattered intensity in both cases of short and long HMPA and that the fraction of scattered light due to free protein can be lower than 25% (when the total intensity was 4 times the reference ones (see Figure 1)). Moreover, conditions can be found that enable us to cancel most of the contribution of free protein.

Case of Short HMPA. Dialysis experiments showed that the presence of free polymer at a concentration above 0.1% (i.e., total HMPA > 0.6%) results in the formation of saturated complexes of constant composition. It is likely that all the BSA are embedded in complexes in this case. The correlation function was fitted by the method of cumulants.

Case of Long HMPA. The correlation function g cannot be fitted well by the method of cumulants. It was successfully analyzed as the sum of two exponential decays (see Material and Methods, eq b). For all mixtures the fit yielded one fast mode, similar to that obtained with a solution of free BSA, plus one slow mode, similar to that obtained with solutions of HMPA only. Provided that each mode can be related to a diffusion of

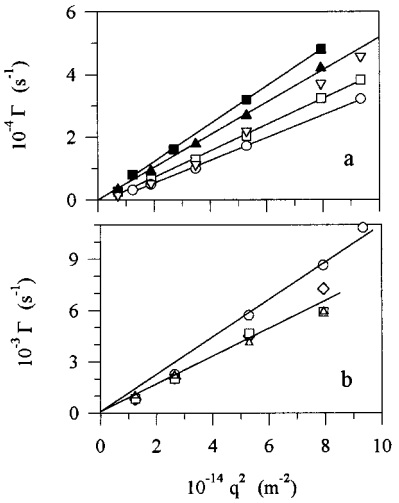


Figure 4. Inverse of the QELS relaxation time as a function of q^2 for BSA and BSA + HMPA mixtures. Part a is for the following: (▽) 0.5% BSA in 0.3 M $NaNO_3$, pH 9.0; (▲) 0.5% BSA in 0.3 M $NaNO_3$, pH 7.0; (■) fast mode from 2% BSA + 0.1% 150-3C12 in 0.3 M NaCl, pH 9.0; (○) 0.5% BSA + 0.6% 5-3C18 in 0.3 M $NaNO_3$, pH 9.0; (□) 0.5% BSA + 0.6% 5-3C12 in 0.3 M $NaNO_3$, pH 9.0. Part b shows the slow mode from (○) 0.1% 150-4C18, (◇) 0.1% 150-3C12, (△) 0.25% BSA + 0.1% 150-3C12, and (□) 0.75% BSA + 0.1% 150-3C12. Experimental conditions were 0.3 M NaCl, pH 9.0. The systems formed by 0.1% 150-4C18 in the presence of BSA (0.75% and 1%) were also measured, giving values very close to those obtained with 150-3C12 systems. For the sake of simplicity these data have not been pictured.

particles, this suggests that the fast mode and the slow mode can be attributed to free BSA and complexes, respectively (we calculated that even if most of the long HMPA remain free, they would induce only $^1/_8$ to $^1/_{10}$ of the intensity corresponding to the slow mode).

The fact that the inverse relaxation times are proportional to q^2 (Figure 4) confirms that in all the cases (long and short polymers) we observed diffusive modes. Under these conditions, some of the relaxation times can be interpreted as induced by concentration fluctuations of HMPA/BSA complexes. More precisely, a linear dependence of Γ on q^2, predicted by the relation $\Gamma = Dq^2$, is observed in Figure 4 in the case of mixtures of BSA and 150-3C12 (fast mode and slow mode), 5-3C12, or 5-3C18 at pH 9. Concerning the BSA alone, the proportionality is slightly lost at pH 9 whereas it is recovered at pH 7. This feature can be explained by the existence of a small amount of large particles (only apparent at small angles) due to the aggregation of BSA at pH 9 but not present at pH 7.[12,28] With regard to the BSA/HMPA mixtures at pH 9, the presence of polymer reduced the polydispersity and the linearity was recovered. A careful look at the points revealed nevertheless that proportionality is not perfect. An interpolation to $q^2 = 0$ gave a slightly negative value. It is a hint of the presence of remaining aggregates, although at an extremely small rate. The experiments carried out on HMPA/BSA systems at pH 7 gave values very close to those obtained at pH 9 (data not shown). Together with the absence of aggregates of BSA itself at pH 7, this implies that the slight deviation from proportionality is due to the presence of either aggregates of HMPA or of HMPA/BSA complexes.

For better insight of the complexation process, we preferred to discuss the results in terms of the apparent hydrodynamic radius of the various species in the mixtures. In agreement with that estimated from intrinsic viscosity, the apparent radius of the complexes involving the long HMPA was slightly larger than that of free polymers (Table 1). The values of the radius

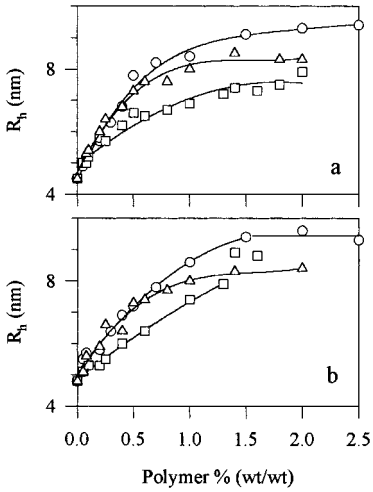


Figure 5. Apparent hydrodynamic radius R_h as measured by QELS in mixtures of 0.5% BSA and HMPA as a function of the polymer concentration in 0.3 M $NaNO_3$ at (a) pH 9.0 and (b) pH 7.0: (○) BSA + 5-3C18; (□) BSA + 5-3C12; (△) BSA + 5-10C12.

are essentially independent of the concentrations in the range we studied (BSA, 0.5%−4%; polymer, 0.02%−0.2%). An accurate value of the expansion cannot be obtained by this mean because the fluctuations (±10% in Γ, 3−4 nm in the radius) were about half the observed increase (<8 nm). On the basis of these measurements, we can claim nevertheless that the association between several long HMPA molecules was not observed in dilute media. To explain why both the intrinsic viscosity and the hydrodynamic radius remain so close to those of free polymers over a large concentration range, we consider that most of the complexes contain only one single chain of HMPA. With respect to the short HMPA, the apparent hydrodynamic radius was computed as a function of the polymer concentration at constant BSA concentration and at both pH 7.0 and 9.0 (Figure 5). In practice, a mean radius was calculated from the analysis of g by the method of cumulants. It contains a contribution from free BSA (4.5 nm), free polymer (3.5−4 nm), and complexes (about 8 nm; see below) too close to be separated by a mathematical analysis. It is probably one reason for the initial linear increase of the mean size of the species with the polymer concentration (below 0.5−0.6%). At polymer concentrations above 0.6%, a less drastic increase was observed in the case of 5-3C12 whereas for the more hydrophobic HMPA (5-3C18 and 5-10C12) the radius of the complexes reached a constant value. Such a threshold concentration is in total agreement with the dialysis results. As described above, the saturation of BSA corresponded to concentrations of free polymer beyond 0.1% and ν of about 9. The total polymer concentration at saturation for a BSA concentration of 0.5% is therefore 0.6%. Beyond this concentration, provided that the scattering from free polymer can be neglected as seen above, saturated complexes are only responsible for the scattered light. The value between 8 and 9 nm reached at high HMPA concentrations was therefore attributed to saturated complexes. 5-3C18 and 5-10C12 form complexes of similar dimensions as expected from their similar hydrophobicity.[22−24] On other hand, 5-3C12/BSA complexes appeared smaller, but in the polymer concentration range studied the saturation was not reached. The study at both pH 7 and 9 revealed that there is no effect of the pH on the apparent hydrodynamic radius. The size of the complexes with short HMPA could correspond to that of a single BSA surrounded by several polymer molecules assuming that the thickness of the polymer layer is close to the radius of polymer in solution. Such an assumption is supported by both theoretical[29] and experimental approaches[30] of polymer adsorption onto surfaces.

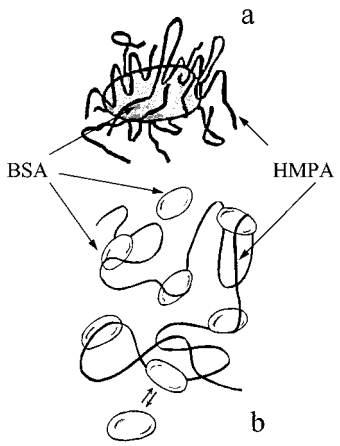


Figure 6. Schematic illustration of the association between BSA protein and HMPA: (a) short HMPA; (b) long HMPA.

Conclusion

In alcaline media, HMPA interact strongly with BSA in dilute solution whereas hydrophilic precursors do not. Associations between the two partners were found more pronounced when polymers with the same chain length are more hydrophobic. Complexation correlate well with the hydrophobicity of the polymers at both high and low ionic strengths. In these mixtures, Coulombic repulsion plays a minor role compared with hydrophobically driven attractions. This suggests that HMPAs can be useful tools for handling globular proteins in solutions. Obvious applications include specific complexation for separation and purification methods, stabilization of enzymes in dispersed form because of the "shield" of polymer, etc. Despite the possible formation of large aggregates, or networks found previously in semidilute media, the size of the complexes is small in dilute solutions. Over large concentration ranges the apparent hydrodynamic radius of the complexes was found to be essentially independent of the composition of the mixtures. Polydispersity seemed low as suggested by the QELS analysis. Measurements are consistent with two extreme structures depending on the MW of HMPA; with short polymers (MW ≈ 5000, apparent radius about that of the protein), up to nine macromolecules can form an external shell around the central BSA core (Figure 6a). In the case of long HMPA (MW ≈ 150 000, apparent radius about 6 times that of BSA), we propose a sort of necklace formed by the binding of several BSA along a single HMPA macromolecule (Figure 6b). Assessment of these possible structures deserve further investigation.

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