Association between Protein Particles and Long Amphiphilic Polymers: Effect of the Polymer Hydrophobicity on Binding Isotherms

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ABSTRACT: Association isotherms of bovine serum albumin (BSA) onto hydrophobically modified polyacrylates (HMPA) in aqueous solution were determined by frontal analysis continuous capillary electrophoresis experiments. A set of alkyl-grafted polyacrylates was studied by varying the degree of grafting and the length of the dangling alkyl groups, keeping constant the molar mass and the polydispersity. Binding isotherms indicate that both the concentration of free BSA and the hydrophobicity of the HMPA control the association, which is anti-cooperative. In the presence of a large excess of protein, the number of bound proteins per HMPA chain was found to increase linearly with an increase in the percentage of the dangling groups along the backbone. Although HMPA modified with dodecyl groups required higher protein concentrations to reach the saturation than those with octadecyl grafts, the results suggest that the saturation composition of the complexes correspond to three to four alkyl chains bound per protein. The quantitative analysis of the isotherms reveals that Hill's equation fits well the binding data, leading to an estimate of the anticooperativity index and of the association constant as a function of the polymer structure.

Introduction

Since mixtures of polymers and proteins find numerous applications in the industry as well as in biological sciences, interest in the characterization of protein−polymer complexes has increased tremendously.[1−13] Among the potential developments in this field, the use of polyelectrolytes was cited in drug delivery[1] and in protein purification[2] and separation[3] processes, as well as for enzyme immobilization.[4] From the biological point of view, aqueous solutions containing polyelectrolytes and proteins are also attracting attention for their parallel with complex systems such as nucleic acid−protein.[5]

It has been recognized that the interactions between proteins and polyelectrolytes may lead to the formation of soluble complexes, complex coacervation, or precipitation.[6] Phase separation and composition of the complexes were found to depend on several parameters such as the polymer molecular weight and charge density, the concentration of both partners, the choice of the ionic strength, and the pH.[6] Electrostatic interactions are generally believed to be the primary driving forces for the formation of complexes between globular proteins and polyelectrolytes.[7] This pattern was evidenced in systems where both partners bear opposite charge although it has been recently shown that complexation can also occur when the net protein charge has the same sign as that of the polyelectrolyte. This latter case occurs, however, close to the isoelectric point of the protein, and it has been attributed to the existence of opposite charge patches onto the protein and the low global charge of the protein.[8] On the other hand, when the polyelectrolyte contains hydrophobic groups anchored along the backbone, the association with proteins arises mainly from hydrophobic interactions.[9,10] Consequently, complexes between hydrophobically modified polyanions and proteins were obtained at pH far below or far above the isoelectric point of the protein, with highly positively or highly negatively charged proteins. To date, BSA has been one of the proteins that has been studied more due to its physiological properties: it is a fatty acid carrier in vivo that can strongly bind long alkyl chains. In a previous paper,[11] light scattering measurements gave evidence of complexation between bovine serum albumin (BSA, negatively charged) and hydrophobically modified polyacrylates (HMPA). The hydrophobic origin of the association was clearly revealed by the fact that the association did not take place with the unmodified polyacrylate. The apparent hydrodynamic radius of the complexes led us to speculate two different structures of the complexes depending on the polymer size: in the case of short polymers, BSA might be surrounded by several polymer chains whereas for large polymers we proposed several BSA molecules bound to one polymer chain. Estimate of the composition of the complexes, for short HMPA[11] and long HMPA,[12] respectively, were consistent with the proposed structures.

However, little attention has been paid to a quantitative characterization of the equilibrium between the bound and unbound partners. The magnitude of the binding constant, the cooperativity of the association, or the composition of the complex often remains unknown both in polyelectrolyte/protein and in HMPA/protein systems. This was probably due to difficulties in the separation of unbound from bound proteins. Very recently, Dubin et al. have applied the frontal analysis continuous capillary electrophoresis (FACCE) technique to study the binding of proteins to polyelectrolytes.[13,14] This method, which consists of the continuous injection of substrate−ligand mixtures into a separation capillary

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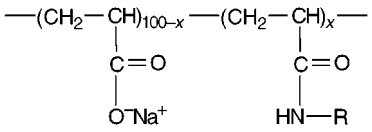
and as such sounds as a resurgence of electrophoretic moving boundary methods, enables a determination of the free ligand concentration without complete separation, avoiding perturbations of binding equilibrium. It has been revealed as a suitable technique to obtain isotherms quickly and easily.

The main goal of this report is to yield more detailed information about hydrophobic contributions to the formation of soluble HMPA−BSA complexes. A set of HMPA of adjusted hydrophobicity was synthesized from the same poly(acrylic acid) precursor. Average molecular weight and polydispersity were thus kept constant (derivatization from the same backbone), enabling the study of the sole influence of polymer hydrophobicity without variation of the chain length. In this work, FACCE was employed to obtain binding isotherms of different HMPA/BSA systems, varying both the length of the hydrophobic moiety along the polymer and the degree of modification. The stoichiometry of the complexes as well as the characteristic index of the association equilibrium were obtained. A quantitative approach of the equilibrium in terms of different equations is presented.

Material and Methods

Materials. The monomer of bovine serum albumin (BSA) was purchased from Sigma Chemical Co. (St. Louis, MO) as 98% pure crystallized and freeze-dried protein. Poly(acrylic acid) of average molecular weight 150 000 was supplied by Polysciences Inc. (Warrington, PA). Gel permeation chromatography measurements performed in $LiNO_3$ solution gave a molecular weight M_w of 130 000 and a polydispersity index of 4 for this sodium polyacrylate precursor. The hydrophobically modified poly(acrylic acid)s (HMPA) were synthesized according to a reaction described previously[15] by grafting dodecylamine or octadecylamine at random along the backbone of the precursor. The HMPAs have the same polymerization degree as their precursor and they were obtained in the sodium salt form with the following structure:



where x is the molar modification ratio and R is an alkyl chain with y carbon atoms ($y = 12$ or 18). Dispersity in composition was low, and x defined within 1% as revealed by a separation and analysis of these HMPA mixtures.[16] Another polymer was also synthesized by grafting the azobenzene group at 1.2 molar percentage at random along the backbone. The following code was adopted: 150-*x*Cy is a polymer sample containing x mol % of C_yH_{2y+1} dangling groups. 150-1.2azo is the polymer with the azobenzene dangling groups.

High-purity water obtained from a Millipore α-Q water system (Bedford, MA) was used to make all the solutions. Borate buffers of different ionic strengths (30 mM and 100 mM) at pH 9.2 were prepared with analytical reagent-grade disodium tetraborate decahydrated salt purchased from Prolabo (Paris, France). Mesityl oxide was obtained from Sigma Chemical Co. (St. Louis, MO)

Polymer stock solutions were prepared in water under magnetic stirring at least 18 h before use. The protein was dissolved in water to minimize the formation of aggregates and dialyzed in Slide-A-Lyzer dialysis cassettes (cutoff 10 000) (Pierce Chemical Co.) against water in order to remove the possible presence of small oligopeptides or additional salts. The dialyzed protein concentration was checked by absorbance measurements at 280 nm. BSA stock solutions were prepared daily to avoid bacterial growth.

Mixtures containing protein and polymer at different concentrations (0.15−3.00 wt % and 0.01−0.35 wt %, respectively) were made from the stock solutions and the final salt content of 30 mM was adjusted by adding the appropriate volume of 30 and 100 mM borate buffers. After vigorous shaking, samples were kept at room temperature for at least 1 h before study.

Apparatus. Experiments were carried out with an Applied Biosystems (ABI) Model 270A instrument equipped with a variable wavelength UV detector (Santa Clara, CA). Bare silica capillaries of 50 μm × 45 cm (effective length, 25 cm) from Supelco (Bellefonte, PA) were used. Data acquisition was performed using a Spectra-Physics model SP4400 Integrator (San Jose, CA). The experiments were conducted at a constant voltage of 10 kV and at 25 °C. The detection was performed at 279 nm, where only the proteins absorb. The running electrolyte was 30 mM sodium borate buffer, pH 9.2. It was filtered prior to use through a 0.45 μm membrane (Millipore). Mesityl oxide (0.1% v/v in borate buffer) was used as neutral marker.

Procedures. The capillary was flushed daily with 0.1 M NaOH for 10 min, followed by a water rinse for 5 min, and finally was allowed to equilibrate with the run buffer for 5 min Frontal analysis experiments were initiated by immersing the inlet of the capillary in the sample vial. A positive voltage was then applied at the inlet of the capillary to achieve continuous sample introduction and simultaneous separation process. According to their mobilities, the sample species were detected as two discrete and adjacent plateaus. The first one corresponded to the free protein whereas the second one was representative of the continuous sampling. The concentration of the free protein was determined from the height of the first plateau through a plateau height calibration that was acquired by injecting known concentrations of neat protein before the sample runs. The bound BSA concentrations were derived for each sample by subtracting the free from the total protein concentration.

After each electrophoretic run, the capillary was rinsed with the run buffer for 4 min to restore the surface state of the capillary that might have been altered by protein adsorption. Then, a short plug of the mesityl oxide solution was hydrodynamically injected into the capillary (negative pressure of 16.7 kPa for 1 s) to check the electroosmotic flow ($m_{eo} = 6.7 \times 10^{-4}$ cm^2 V^{-1} s^{-1} under these conditions), thus ensuring that the surface conditions stayed unchanged at the start of each run.

Results and Discussion

Adsorption of sample components on the wall of bare silica capillaries is one of the main limitations of capillary zone electrophoresis of proteins. It can be avoided or minimized (i) by working above the isoelectric point of the protein, pI, where the protein carries a net negative charge like the capillary surface, and (ii) by using shorter and/ore wider capillaries. The experiments were therefore carried out at pH 9.2 (pI of BSA = 4.9) in the shortest capillary allowed by the instrument. The good course of the method and the absence of significant adsorption was daily reassured by injecting the protein sample in borate buffer in free zone electrophoresis. The resulting electropherograms (Figure 1a) allowed us to check the migration time (4 min) as well as the apparent electrophoretic mobility of the protein (2.6×10^{-4} cm^2 V^{-1} s^{-1}) periodically. Sharp and symmetric peaks were obtained. In keeping with the zone pattern of the protein alone, Figure 1b exemplifies the migration of BSA in the frontal analysis mode. The protein was again detected 4 min after the voltage was applied, but now as an abrupt increase of the absorbance, generating a very well-defined plateau. Upon injection of BSA/polymer mixtures, the combined migration and electroosmosis resulted in the movement of both free BSA

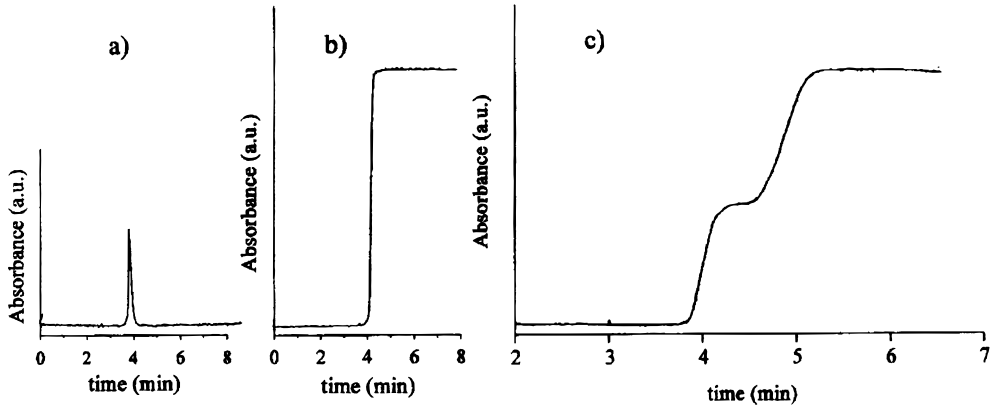


Figure 1. Electropherograms: (a) 0.5 wt % BSA in zone electrophoresis; hydrodynamic injection (1 s, 16.7 kPa); (b) 1 wt % BSA in frontal analysis continuous capillary electrophoresis (FACCE); (c) 1 wt % BSA + 0.12 wt % 150-3C12 in FACCE. Electrolyte: 30 mM borate buffer, pH 9.2. Detection: UV, 279 nm. Applied voltage: 10 kV (electric field: 220 V/cm).

and protein/polymer complexes toward the detection window. Owing to the permanent intrance of sample at the inlet of the capillary, the species formed two adjacent zones (Figure 1c) due to the different mobilities of the free protein and of the complexes. The first one, occurring at 4 min, was thus likely to correspond to a zone of pure unbound protein that was detected faster than the complex. The second zone was observed for at least 10 min without variation in height. Its absorbance value coincided with that of the initial sample, indicating the end of the analysis. This technique is finally equivalent to the extraction of a negligible fraction of free BSA (a few nanoliters) from the whole sample, which does not imply any significant effect on the equilibrium. Under these electrolyte conditions, the electroosmotic flow (m_{eo}) was high enough to sweep the polyanions of interest toward the cathode. Indeed, the apparent mobilities of the polyanions of interest are the sum of m_{eo} (positive and high) and of their negative effective mobilities. As a result, the polyanions were detected in the increasing order of their charge/mass ratio. Owing to the lower charge/mass ratio of the free protein as compared to the complexes (high charge density of HMPAs), free BSA was detected before bound BSA. The method should also discriminate different BSA/HMPA complexes provided that a variation in composition could induce a modification of their mobilities. These species would be detected between the two plateaus observed. Unfortunately, the absence of a significant drift and the rapid increase in absorbance between the plateaus did not enable any estimate of the dispersity in the composition of complexes. In addition, the possible presence of unbound polymer was not detected because the "naked" polymer does not absorb the light at 280 nm. The reproducibility of the plateau height, together with the good linearity of the calibration curves ($r^2 > 0.9994$, periodically checked), assured an accurate determination of the free protein concentration.

Figure 2 shows some of the electropherograms obtained for 0.5 wt % BSA + 150-3C18 at different concentrations. The presence of increasing amounts of polymer leads to a decrease of the height of the first plateau. Minor fluctuations in the height of the second plateau remained within experimental uncertainties, indicating that the total concentration of BSA was the same in all the samples. For each sample the free BSA concentration, $[BSA]_f$, was determined from the height of the first plateau and the bound BSA concentration

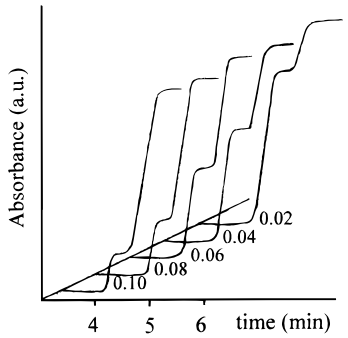


Figure 2. FACCE electropherograms obtained for the system BSA/150-3C18 at constant protein concentration (0.5 wt %) and different polymer concentrations (as indicated on each electropherogram, in wt %). Experimental conditions were as in Figure 1.

was then calculated by subtracting the free from the total BSA concentration. The average number of proteins per acrylate unit, $\bar{\nu}$, was calculated as the ratio of the mole number of bound BSA to the total mole number of monomer in the sample. One isotherm, $\bar{\nu}$ vs $[BSA]_f$, was obtained varying the polymer amount at constant protein concentration. Looking for the best operating conditions, additional series of experiments were performed at five different total BSA concentrations, varying the HMPA content. Figure 3a shows that the isotherm curves obtained for 0.2, 0.5, 1.0, 1.5, and 3 wt % BSA can be superimposed. Another set of experiments at fixed polymer concentration but varying the protein concentration was also carried out (Figure 3b), showing again a good superimposition. In this latter series, however, the discrepancy was slightly larger, owing to variations in the absorbance of the initial samples. To obtain the best precision the experiments were mainly done varying the polymer-to-protein ratio at constant BSA concentrations. Altogether, the measurements showed that $\bar{\nu}$ was mainly a function of $[BSA]_f$ irrespective of the total polymer concentration. In other words, the binding of BSA onto HMPA was found to be very similar to an adsorption onto a surface. The equilibrium must be described in terms of free protein concentration and density of binding onto the polymer.

Binding Isotherms. The influence of HMPA hydrophobicity on the HMPA−BSA association was considered by varying the modification percentage and the length of the alkyl chain, keeping constant both the average chain length and the polydispersity of the polymer. Thus, a series of FACCE experiments was

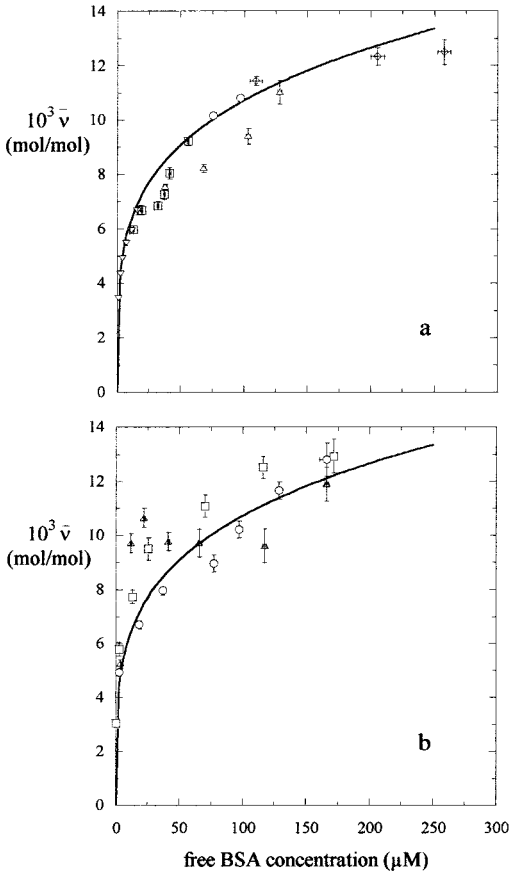


Figure 3. Binding isotherms determined from FACCE analysis of the system BSA/150-3C18. Each type of symbol corresponds to the experiments performed as follows: (a) keeping constant the protein concentration and varying the polymer amount (BSA concentrations, in wt %: (▽) 0.2, (□) 0.5, (△) 1.0, (○) 1.5, and (◇) 3.0 in 30 mM borate buffer pH 9.2); (b) keeping constant the polymer concentration and varying the BSA amount (150-3C18 concentrations, in wt %: (△) 0.05, (□) 0.1, and (○) 0.2, in 30 mM borate buffer pH 9.2). The common curve line is to guide the eye.

performed with HMPA of different hydrophobicities in dilute solution over a broad range of concentrations of both polymer and protein. The corresponding isotherms are plotted in Figure 4. For the sake of comparison, this graph also includes some results presented in our preceding paper.[12] From a qualitative point of view, all the binding isotherms appeared to be similar in shape, revealing the anti-cooperative character of the interaction. Namely, after an initial rapid increase of $\bar{\nu}$ at low $[BSA]_f$, the subsequent binding of BSA becomes more and more difficult, decreasing the slope of the isotherms progressively. At high $[BSA]_f$ ($\sim$ 1%), the number of BSA molecules bound per acrylate unit reached a maximum, $\bar{\nu}_{max}$. Only the BSA/150-1C18 and BSA/150-3C18 systems, however, exhibited a well-defined plateau. In the other cases, a slight drift in $\bar{\nu}$ was still observed at high BSA concentration. Langmuir equation cannot fit such a shape (see section Quantitative Analysis, below). It is also noteworthy that the feature of the isotherms is similar to that previously reported for BSA−polyelectrolyte interactions at pH 7.5, although in that case the plateau was not observed.[13]

Influence of the Hydrophobicity of HMPA. Comparison of the curves at a fixed free BSA concentration reveals that $\bar{\nu}$ increases with the modification degree of HMPA with a given alkyl dangling group (150-1C12 < 150-3C12 < 150-7C12 < 150-10C12). In addition,

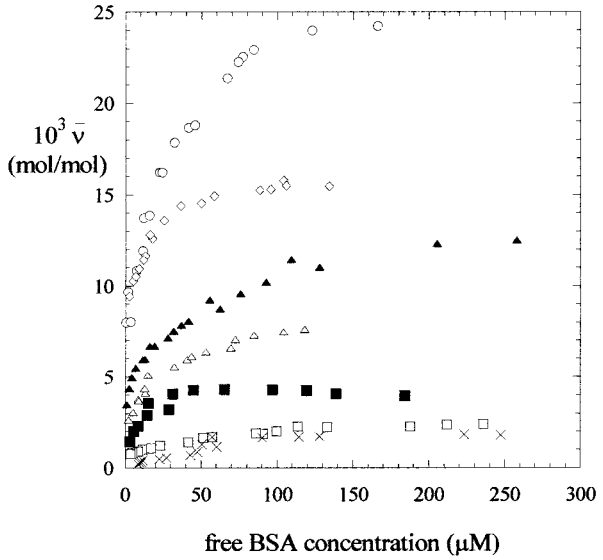


Figure 4. Isotherms of the association between BSA and different HMPAs in 30 mM borate buffer pH 9.2. Symbols stand for the polymers: (□) 150-1C12, (△) 150-3C12, (◇) 150-7C12, (○) 150-10C12, (■) 150-1C18, (▲) 150-3C18, and (×)-150-1.2azo. Data corresponding to the systems involving 150-3C12 and 150-7C12 have recently been published.[12]

HMPA with longer alkyl chains were found to associate more protein in similar conditions (150-1C12 < 150-1C18 and 150-3C12 < 150-3C18). These trends confirmed the hydrophobic character of the HMPA−BSA interaction reported previously.[10−12] They also showed that 1% of dodecylacrylamide groups was high enough to induce the complexation, although only to a limited extent. The maximum values of $\bar{\nu}$ appeared to be almost proportional to the density of C12 dangling groups along the HMPA chain. $\bar{\nu}_{max}$ values obtained are compiled in the second column of Table 1. Together with the constant value of the C12 group/BSA ratio at $\bar{\nu}_{max}$ for different HMPAs, it is therefore likely that systems were close to saturation at the highest concentration of BSA studied. In the case of HMPA bearing octadecyl acrylamide groups, this trend seems also fulfilled, although it was delicate to go deeply into this point with only two polymers. Figure 5 shows the $\bar{\nu}_{max}$ values of all the systems as a function of the number of alkyl chains per acrylate unit. The reciprocal of the slope of the fits yielded 4.3 C12 and 2.6 C18 alkyl chains per BSA molecule. These values are of the same order of magnitude than those reported for the binding of fatty acids of different chain length to serum albumin as well as with the potential number of specific sites of the protein (six). Indeed, it has been found that albumin typically binds up to two long-chain (C16−C20) fatty acids (LCFA) and this number can be allowed to rise to four or six under specific conditions, even in the cases of fatty acids with shorter chain or smaller molecules. From crystallographic data it was found that the serum albumin has three stronger LCFA sites, two of them being located in its less negatively charged half.[17]

We can also notice that, at low protein concentration, the isotherms of 7C12 and 10C12 hardly differ. As the structure of HMPA and absence of impurities were verified by ^{1}H NMR and capillary zone electrophoresis[16] analysis, this similitude reflects some sort of upper threshold in the strength of the association when highly hydrophobic polymers are used. Such similarity in the composition of the complexes formed by BSA/150-7C12

Table 1. Bound BSA per Acrylate Unit Obtained from the "Apparent" Plateau of the Isotherms ($\bar{\nu}_{max}$) and Fitting Parameters of the Isotherms Derived from (a) Hill's Equation and (b) Hill's Equation far from Saturation ($n \to \infty$)

		part a			part b	
HMPA	$10^3\,\bar{\nu}_{max}$	K	z	n	K	z
1C12	2.2	0.49 ± 0.04	0.37 ± 0.06	7.2 ± 3.9	0.52 ± 0.02	0.28 ± 0.01
3C12	7.5	2.00 ± 0.18	0.44 ± 0.08	14.2 ± 4.2	2.13 ± 0.10	0.27 ± 0.01
7C12	15.5	10.60 ± 0.74	0.41 ± 0.08	19.8 ± 1.9	7.80 ± 0.20	0.16 ± 0.01
10C12	24.2	4.64 ± 0.55	0.62 ± 0.06	32.5 ± 2.6	6.81 ± 0.38	0.27 ± 0.01
1C18	4.4	0.62 ± 0.24	1.07 ± 0.20	4.6 ± 0.3	1.03 ± 0.16	0.40 ± 0.05
3C18	11.3	3.36 ± 0.11	0.29 ± 0.04	48 ± 30	3.34 ± 0.07	0.24 ± 0.01

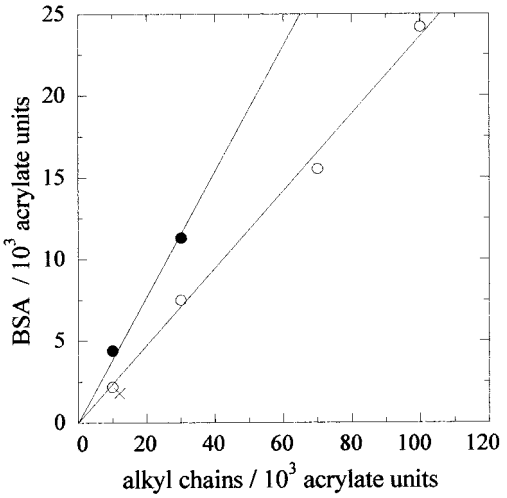


Figure 5. Number of proteins per acrylate unit under conditions close to saturation as a function of the average number of alkyl groups per acrylate unit for HMPA−C12 (○), HMPA−C18 (●), and the HMPA−1.2azo (×).

and BSA/150-10C12 at low free protein concentration (up to ~12 BSA particles per one thousand acrylate units) might be attributable to conformation transitions of the polymer chains and formation of primary complexes involving only accessible parts of HMPA. Indeed, it was shown that highly hydrophobic HMPA chain can collapse in intramolecular micelles sharing several alkyl dangling groups from the same backbone. In our experimental conditions, 150-1C12 and 150-3C12 are expected to exhibit essentially the same conformation as the precursor[16,18] (i.e., total accessibility of alkyl groups) whereas intramolecular collapse should occur slightly for 150-7C12 and markedly for 150-10C12. Correlation between intramolecular collapse and the leveling-off of the association strength will deserve further work. In the presence of excess BSA, however, the constant value of $\bar{\nu}_{max}$—corresponding to about 4 C12 groups per bound protein—indicates that this possible intrachain association would not survive close to saturation with proteins. In other terms, excess protein would be able to break the intrachain micelles.

Effect of the Type of the Dangling Groups. The comparison of the isotherms corresponding to C12 and C18 HMPAs (1% and 3% of modification, Figure 4) revealed that HMPAs modified with C18 bind the BSA molecules more strongly than the C12 modified ones do. On the other hand, polymers which were reported to exhibit similar self-associating behavior (such as 1C18~3C12 or 3C18~10C12),[19] significantly differ with respect to their association with BSA. The HMPA self-association is related to the formation of hydrophobic microdomains involving cooperative association of several tens of alkyl groups. In this case, a longer alkyl chain length can compensate for a lower grafting degree, resulting in similar formations of hydrophobic clusters in 150-3C12 and 150-1C18 solutions, for instance. On the contrary, in BSA/HMPA systems, each protein molecule is associated to a few alkyl chains whose number is of the order of the discrete binding sites on the protein surface. From this point of view, several small alkyl groups cannot balance one long group because the association is likely to involve only one dangling group per protein site. It is therefore expected that BSA could be more strongly bound in the presence of long dangling groups (effect on the equilibrium constant). Indeed, as it is shown in Figure 4, $\bar{\nu}$ was increased on replacing C12 by C18 subtituents at constant $[BSA]_f$.

To gain insight on the hydrophobic contributions to the HMPA−BSA interaction, additional experiments using a polyacrylate grafted with a group different from a linear alkyl chain were carried out. The polymer backbone was grafted at random with the azobenzene group, up to 1.2 mol % (150-1.2azo). As we can see in Figure 4 the association is weaker than for the two 1% modified HMPA ($\bar{\nu}_{max}$ = 1.8 BSA particles per one thousand acrylate units instead of 2.2 for 150-1C12 and 4.4 for 150-1C18). The isotherm of the BSA/150-1.2azo system was however similar to the 150-1C12 one. The azobenzene group presumably does not bind to the same protein sites as the alkyl groups but rather to those which are specific for aromatic groups that were located close to the two strongest LCFA sites.[17] In addition, the azobenzene is not expected to form similar micellar clusters as do the C12 groups. All these features are hints for differences between self-association of HMPA and association modes in HMPA/protein systems, both, however, involving the percentage of alkyl groups along the polymer and the hydrophobicity of the dangling group.

Quantitative Analysis of the Isotherms. The multiple binding affinity model corresponding to multiple classes of binding sites was chosen as a simple and generic approach of our systems. In general, binding of several ligands to one macromolecule can be described by Hill's equation.[20] Applied to the adsorption of BSA onto HMPA, it can be written

$$\bar{\nu} = \frac{K[BSA]_f^z}{1 + K'[BSA]_f^z} = \frac{nK[BSA]_f^z}{n + K[BSA]_f^z} \quad K = K'n \quad (1)$$

where K and K' are constants related to the intrinsic binding constant and to the number of sites, n, per HMPA. z is the Hill coefficient, an empirical exponent that measures the cooperativity of binding. If $z = 1$, no cooperativity is observed and only one constant governs the equivalent and independent binding sites along the polymer. On the contrary, if $z \neq 1$ the initial binding affects the subsequent binding of ligand: $z > 1$ means that a second ligand binds more easily than the first

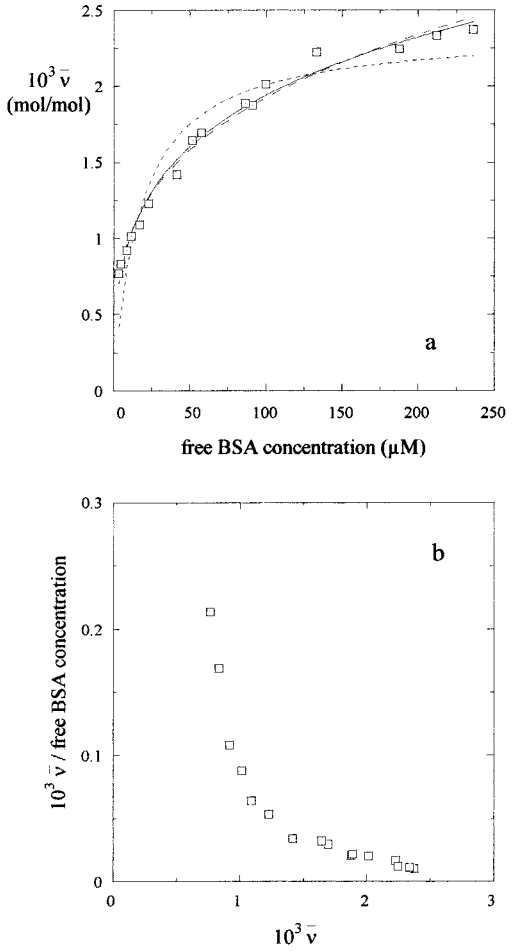


Figure 6. (a) Nonlinear curve-fitting of the BSA/150-1C12 isotherm. Data are fitted according to (—) Hill's equation, (– – –) Hill's equation taking $n = 60$, and (- - -) bi-Langmuir equation. (b) Scatchard plot.

one (cooperativity); $z < 1$ reveals that an additional ligand is weakly bound as compared to the previous one (anticooperative binding).

Equation 1 was found to hold satisfactorily to the studied isotherms, considering HMPA as the macromolecule and BSA as the ligand (see Figure 6a as an example). The parameters obtained are listed in Table 1. The results show that K as well as n increases with the percentage of the dangling groups along the polymeric backbone. This trend reflects the hydrophobic origin of the association on a quantitative basis as it is qualitatively discussed above. The values for Hill's coefficient are inferior to 1, denoting an anticooperative association between BSA and HMPA.

The sensitivity of the fit to small variations of the parameters was considered. Obviously, the value of n affects the curves at high $[BSA]_f$ whereas K and z determine the initial increase of $\bar{\nu}$. When n was fixed at the maximum value with physical significance (i.e., as the number of alkyl groups per 1000 acrylate units) and the other two parameters were floated, the curve fit scarcely differed from the "full" three-parameter fit. Differences between the values of K and z obtained by these two approaches felt inside the error interval. Even upon fixing n beyond the value of the number of alkyl chains per 1000 acrylate units, eq 1 held well to the isotherms (see Figure 6a as an example) and K and z were not significantly modified. Obviously, parameter n has not the main importance in the fit. In such a case

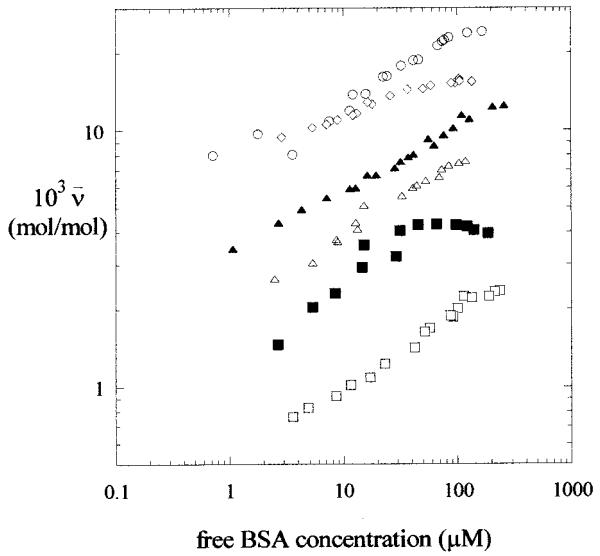


Figure 7. Double logarithmic plot of the isotherms of Figure 4.

we could consider n going to infinity, which would lead to Hill's equation being expressed in terms of only two parameters z and K:

$$\bar{\nu} = K[\mathrm{BSA}]_f^z \qquad (2)$$

The isotherms of Figure 4 were reanalyzed in terms of eq 2 paying special attention to the data points preceding the "apparent" saturation (ignoring the last 3–5 data-points corresponding to the highest BSA concentrations). The K and z values so obtained are also collected in Table 1. Values of z indicated a high anticooperativity, taking a value close to 0.3 in all the cases except for 150-7C12. Accordingly, the double logarithmic plot of the isotherms (Figure 7) reflected a linear increase of $\bar{\nu}$ over two decades of free BSA concentration. In addition, the slopes were roughly the same for all the polymers. The similitude between the isotherms of 150-7C12 and 150-10C12 at low BSA, enabled us nevertheless to use also $z = 0.3$ in a restricted concentration range in the case of 150-7C12. The absence of significant variation of z with HMPA structure is likely to reflect a common origin of anticooperativity, i.e., a decrease of the mean association strength upon binding, which does not depend directly on hydrophobicity. The anticooperative effect cannot be explained only by steric and Coulombic repulsions between bound proteins because it was clearly observed at very low $\bar{\nu}$, in the presence of a large excess of the charged acrylic units. Although a detailed interpretation of the constant value of z is premature, a few simple origins may however be regarded: (i) The presence of important fluctuations in the grafting density of alkyl groups along the HMPA backbones might induce a broad range of association constants with BSA, depending on this "local hydrophobicity". (ii) The wrapping of polymer around BSA should help propagating the influence of the binding along the backbone over hundreds of monomer units. Depending on the conformation of the bound polymer loops, a single BSA could possibly involve a long-range reorganization of the polymer.

Irrespective of the underlying mechanism of anticooperativity, the absence of variation in z enabled comparison of K values from different systems. Indeed,

the dimension of K, depending on z, (here $(L/mol)^{0.3}$) can be considered independent of the HMPA nature. K was thus a good index of association strength. For polymers carrying dodecyl chains (excepting 150-7C12, for reasons discusssed aboved), K increased linearly with the percentage of modification [$K = 0.68(\%\ C12)$; $r^2 = 0.998$]. The comparison of the values of K for HMPA carrying C12 and C18 alkyl groups at constant modification percentage shows that the length of the alkyl group strengthens the association.

Seeking after the most appropriate binding model to characterize the association, we also used the multiple equilibria theory[13,21] to analyze the isotherm data. The appearance of two slopes in the Scatchard plot (see Figure 6b) as well as the knowledge of two specific regions for hydrophobic ligands in BSA,[17] suggested the possibility of fitting the isotherms with a two-class binding site model expressed as

$$\bar{\nu} = \frac{n_1 K_1 [\mathrm{BSA}]_f}{1 + K_1 [\mathrm{BSA}]_f} + \frac{n_2 K_2 [\mathrm{BSA}]_f}{1 + K_2 [\mathrm{BSA}]_f} \qquad (3)$$

where subscripts 1 and 2 define the two classes of binding sites. This model assumes that binding at one site does not affect binding at any other site. As we can see in Figure 6a, eq 3 does not appear to suit the BSA/150-1C12 system better than the simplified Hill's equation (eq 2), despite the fact that four adjustable parameters were used. Moreover, this second model predicts a proportionality between $\bar{\nu}$ and $[\mathrm{BSA}]_f$ at low protein concentration. The slopes of the double logarithmic plot in Figure 7 show that it is not the case, indicating that the binding of less than one BSA per one thousand of acrylate units induces a non-Langmuir behavior.

Saturation Conditions. The fitting of the data to Hill's equation reveals that, despite the slow slope of the curves at high free protein concentration in Figure 4, the saturation of the polymer might not be completed in the range of concentrations assayed. Only the BSA/150-1C18 system exhibited a clearly defined plateau on this double logarithmic plot. The saturation of 150-1C18 corresponded to 4.4 bound BSA/1000 acrylate units, i.e., 2.3 alkyl grafts per protein. The length of the octadecylacrylamide groups could explain the difference between 150-1C12 and 150-1C18, despite their same modification percentage. The larger size of the octadecyl groups facilitates the accessibility to the protein binding sites enhancing the strength of the association. As a consequence, the saturation of 150-1C12 needs the presence of a higher concentration of free BSA, presumably beyond our experimental limits. In this sense, when the analysis were performed at higher protein concentrations, the plateaus were distorted and the first one shortened. It appeared difficult to obtain accurate determinations of $\bar{\nu}$ under these conditions. To keep a good accuracy of the determination of both free and bound BSA under close to saturation conditions, high concentrations of BSA and hence of polymer were needed, rendering these sample solutions viscous. Moreover, an intrinsic limit of the separation scheme was unfortunately attained when the concentration of counterions of the protein reached a value close to the concentration of the carrier electrolyte. The conductivity of the zones containing analytes was significantly higher than that of the zone of the pure buffer resulting in possible reverse stacking and Joule heating. To limit such problems, the electrolyte concentration might be increased. This point will deserve further studies, in connection with the effect of the ionic strength on the association equilibrium.

Measurements under saturation conditions would also allow us to verify if the 4.3 C12 and 2.6 C18 alkyl chains per BSA molecule, at higher free protein concentrations, still remain constant. Indeed, it would be interesting to know whether the number of alkyl groups per BSA at saturation corresponds to a number of sites in the BSA (typically two to three sites) irrespective of the length of the dangling groups or the saturation is modulated by the strength of the association, leading to a lesser number of alkyl chain per BSA molecule with longer dangling group.

Conclusions

Isotherms for the association between bovine serum albumin and hydrophobically modified polyacrylates have been obtained in a wide range of protein concentration varying the hydrophobicity of the polymer and keeping constant their molar mass and polydispersity. The binding of the two partners to form soluble complexes appears anticooperative, as the shape of the isotherms and the Hill's equation parameters have qualitatively and quantitatively showed, respectively. A modification rate as low as 1% of C12 dangling groups, along the backbone is large enough to induce association of several proteins per chain. Complexes are formed irrespective of the polymer concentration: only the free BSA concentration controls the association. However, the hydrophobicity of the polymer strongly influences the strength of the association and the composition of the complexes. Namely, at constant modification percentage, the increasing length of the alkyl group strengthens the association; at a constant length of the alkyl side groups, the higher density of modification increases the number of bound proteins, especially close to saturation of the HMPAs. Finally, binding does not depend simply on the global hydrophobicity, as for HMPA self-association, but more subtle rules have to be considered, taking into account both hydrophobicity and grafting density of pendant groups. As a consequence, HMPA-C12 is likely to require higher protein concentrations than that of HMPA-C18 in order to reach saturation. In both cases, a few alkyl chains per protein (2.6−4.4) corresponds closely to saturation conditions, in excellent agreement with the presence of three strong specific binding sites for long-chain fatty acids in BSA. Concerning the quantitative analysis, Hill's equation, using a minimum of two adjustable parameters, fits the isotherms better than the Scatchard plot.

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