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Communications to the Editor

Size Distribution of Complexes Formed between Poly(dimethyldiallylammonium chloride) and Bovine Serum Albumin

The formation of complexes between globular proteins and synthetic polyelectrolytes is generally evidenced by phase separation. The polymer-rich phase may be a liquid, i.e., a "complex coacervate",[1] or a solid precipitate. Examples of the latter have been observed for hemoglobin and dextran sulfate,[2] potassium poly(vinyl alcohol sulfate), and carboxyhemoglobin in the presence of poly(dimethyldiallylammonium chloride),[3] lysozyme and poly(acrylic acid),[4] and RNA polymerase and poly(ethylene imine).[5] Systems that exhibit coacervation include gelatin and polyphosphate[6] and serum albumin and poly(dimethyldiallylammonium chloride).[7]

There are several reasons for investigating such systems. Complexation of enzymes with polyelectrolytes offers a route to immobilized and, possibly, more stable forms of enzymatically active insulin,[8] catalase,[9] and urease and penicillin amidase.[10] Other changes in protein activity that accompany complex formation are also of interest, such as the reduction of oxygen affinity for hemoglobin in complexes with polyanions[11] which could lead to new blood substitutes.[12] The stoichiometry of colloid titrations of proteins with polyelectrolytes may help to shed light on the number and location of ionizable groups on the protein surface.[13,14] Complex formation between proteins and strong synthetic polyelectrolytes may provide useful models for theories about the nonspecific affinity of DNA-binding proteins[15] such as RNA polymerase, which appear to interact with nucleic acids largely through electrostatic effects.[16] Last, on a more practical level, selective coacervation or precipitation of proteins by polyelectrolytes could offer a novel approach to protein separation.[4,7,17]

We have previously studied the coacervation of several globular proteins with poly(dimethyldiallylammonium chloride) (PDMDAAC).[7] Phase separation occurs abruptly upon the addition of base to neutral or acidic mixtures of the two macroions at some well-defined pH. The value of pH_{crit} is nearly linear with ionic strength and also increases with protein isoelectric point. In order to apply this phenomenon to protein separation, a clearer understanding of the mechanism of phase separation is needed. The following are among the relevant questions that may be posed: Is phase separation preceded by molecular complex formation? Are such (hypothetical) complexes purely intrapolymer? How many proteins bind per polymer chain, and is the binding highly cooperative?

Most of the foregoing queries hinge on the identification of a soluble, equilibrium polyion–protein complex. Such soluble complexes have been identified for hemoglobin and anionic polyelectrolytes[11,18] and for serum albumin and poly(4-vinyl-*N*-ethylpyridinium bromide).[19] However, despite the great variety of techniques one could apply to these systems, no reports deal with the characterization of polyion–protein complexes in the region of incipient phase separation. We have therefore measured the apparent dimensions of complexes of PDMDAAC with bovine serum albumin (BSA) at pH's below and above pH_{crit}. Since PDMDAAC is a strong polycation, its dimensions are invariant with respect to pH.

PDMDAAC, a commercial sample "Merquat 100" from Calgon Corp. (Pittsburgh) with nominal molecular weight 2×10^5 and reported polydispersity of $\bar{M}_w/\bar{M}_n \simeq 10$ was dialyzed and freeze-dried before use. Bovine serum albumin was from Sigma Chemical Co. Solutions were prepared by combining one volume of 3.0 g L^{-1} PDMDAAC with two volumes of 10.0 g L^{-1} BSA in 0.010 M 9:1 NaCl/sodium acetate and then adjusting the pH by addition of 0.10 M NaOH. (Multivalent anion buffers, such as phosphate, lead to slight precipitation of PDMDAAC.) Turbidity was measured with a Brinkman PC600 fiber optics probe colorimeter, equipped with a 420-nm filter and a 2-cm path length probe tip. All values are reported as the corrected turbidity, i.e., [ln %T(blank) − ln %T(solution)], where the blank is a polymer-free sample. Solutions for QELS, 3.0 g L^{-1} in polymer and 10.0 g L^{-1} in protein, were filtered (0.20 μm Milipore), and, in some cases, centrifuged (2000 rpm, 15–20 min) in the 1-cm cylindrical sample cell, which was then placed in the toluene refractive index matching bath (25.0 °C) of a Malvern RR102 spectrometer. The light source was a 20-mW He–Ne laser (Jodon); scattered light was collected at 90° by using a PM frontal aperture of 0.5 mm. The PMT output was analyzed with a Nicomp TC-200 computing autocorrelator. Photon counts were acquired until computed distributions were stable, usually corresponding to a "fit error" of less than 5 and "residual"[20] of less than 5, normally requiring the acquisition of 50 000 counts above base

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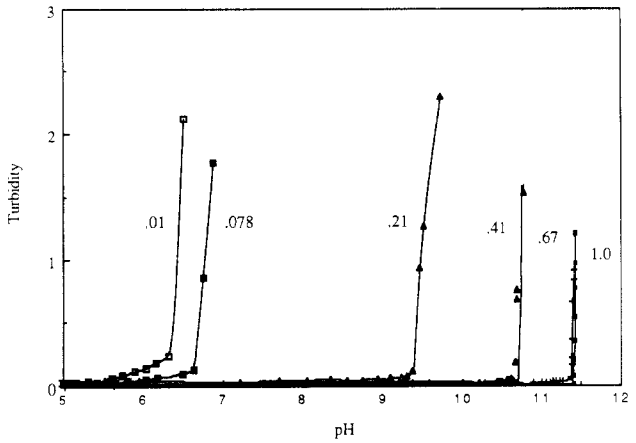

Figure 1. Turbidimetric titrations of 1.0 g L^{-1} PDMDAAC with 6.7 g L^{-1} BSA in 9:1 NaCl/sodium acetate at ionic strengths shown.

line and a typical run time of 15 h. The channel width was adjusted to encompass 2.0 decays. The autocorrelation function was analyzed by using both the method of cumulants[21] and a technique similar to that of Provencher's[22] in that a nonlinear least squares, nonnegatively constrained method is used to achieve the inverse Laplace transformation of the autocorrelation curve but differing from CONTIN in the degree of smoothing and the degree of coupling of the individual exponentials.[23] We have shown that this technique produces the same bimodal size distributions as the nonnegative-constrained least-squares method of Morrison and Grabowski[24] when applied to autocorrelation curves obtained for soluble complexes of PDMDAAC with mixed anionic/nonionic micelles.[25]

Turbidimetric titrations of PDMDAA + BSA are shown in Figure 1 for several ionic strengths (I). The rapid onset of turbidity at a critical pH corresponds in part to the formation of a coacervate which may be removed by low-speed centrifugation. The increase in pH_{crit} with I can be easily understood: with increasing ionic strength, attractive Coulombic interactions between the protein and polyion are screened and a larger net negative protein charge (z) is required for phase separation.[7]

Voorn and Overbeek[26] analyzed complex coacervation as a phase transition which takes place abruptly when the favorable electrical free energy compensates for the unfavorable loss of entropy. In contrast, Veis suggested that molecular association of oppositely charged polyions occurs first, followed by slow rearrangement of these aggregates to form the coacervate.[27] If such molecular complexes exist in the current system, the complexation process could be studied by a wide range of relevant solution techniques.

QELS results for mixtures of PDMDAAC and BSA at pH values near pH_{crit} are presented in Figure 2. The multiexponential curve-fitting procedure yields the set of decay constants and corresponding coefficients given by eq 1 and, hence, an approximate distribution of apparent

$$g^{(1)}(\tau) = \sum_i A_i \exp(-\Gamma_i \tau) \quad (1)$$

Stokes radii.[28] Here, $g^{(1)}(\tau)$ is the normalized, base line corrected, first-order autocorrelation function, τ is the delay time, and A_i is the probability of a scattering center with decay constant Γ_i.[28] This multiexponential algorithm broadens single-exponential peaks, so that QELS histograms in which only BSA contributes to the scattering look anomalously polydisperse. However, in the interest of consistency, all comparisons were made by using a multiexponential fit, even when only one decay constant was observed.

There are numerous hazards in the literal interpretation of such results, as follows. (1) The multiexponential curve-fitting process is a "delicate" operation and modest scatter in the experimental curve produces drastic artifacts in the computed distributions. (2) Some of the solutions display turbidity values large enough to suggest secondary scattering. (3) The measured apparent diffusion coefficient may be smaller or larger than the desired self-diffusion coefficient, depending on the presence of attractive or repulsive interparticle interactions, respectively. (4) The validity of the Stokes–Einstein equation for the diffusion of particles in polymer solutions has been called into question,[29] and, even if it were valid, the meaning of an equivalent Stokes radius for such complex particles may be debated.

The foregoing considerations notwithstanding, considerable circumstantial evidence supports a literal interpretation of these data: (1) The apparent Stokes radius from QELS of the polymer alone (ca. 35 nm) is in excellent agreement with the viscosity radius R_η, calculated as $R_\eta = 5.5 \times 10^{-9} ([\eta]M)^{1/3} = 33$ nm (the units of R_η and $[\eta]$ are cm and $cm^3\ g^{-1}$, respectively).[30] (2) The fastest diffusion mode in these distributions, with an apparent Stokes diameter of ca. 6 nm, is in good agreement with the result for BSA alone, or in the presence of polymer but below pH_{crit}. (3) The observed distributions are stable with respect to run time (after 2 h) and reproducible. (4) The coefficient of slowest diffusion mode is diminished upon centrifugation, so this mode presumably corresponds to a large species. It seems likely that many of the potential difficulties noted before may be alleviated by the dimin-

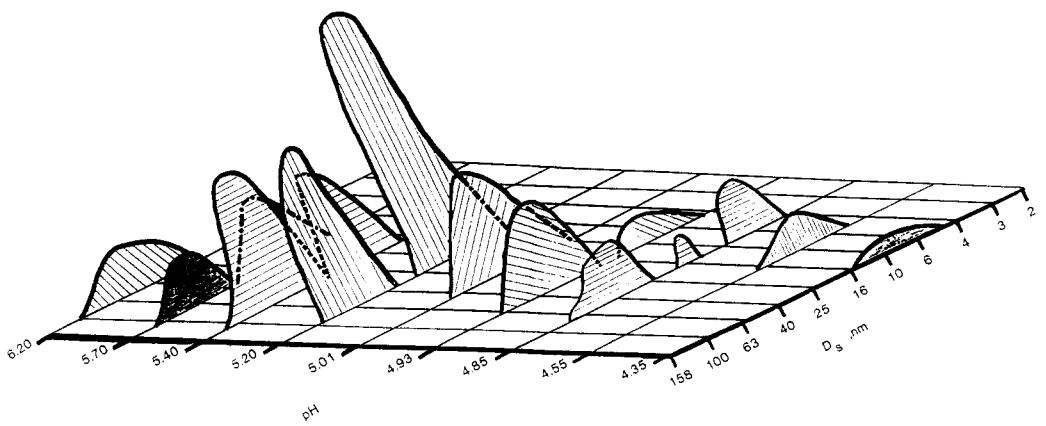

Figure 2. Apparent distributions of equivalent Stokes diameters, from quasi-elastic light scattering analysis of mixtures of 1.0 g L^{-1} PDMDAAC and 6.7 g L^{-1} BSA, in 0.01 M 9:1 NaCl/sodium acetate buffer, at pH values shown.

Table I
Apparent Stokes Diameters of Species Present in BSA/PDMDAAC Mixtures in 0.010 M 9:1 NaCl/Sodium Acetate

pH	turbidity (as %T)	variance[c]	rel % abundance[a] of species with Stokes diam shown[b] 7 ± 1 nm	25 ± 5 nm	50 ± 10 nm
4.35	95.3	0.32	100	0	0
4.55	95.3	0.32	100	0	0
4.85	95.3	0.63	46	54[d]	0
4.93	95.2	0.70	18	82	0
5.01	95.2	0.73	0	100	0
5.20	94.9	0.70	0 (0)	38 (56)	62 (44)
5.40	93.7	0.61	0 (0)	44 (100)	56 (0)
5.70	90.0	0.52	0 (0)	25 (39)	75 (61)
6.30	77.3	0.53	0 (0)	20 (36)	80 (64)

[a] From sum of coefficients in eq 1. [b] Values in parentheses obtained on centrifuged samples. [c] The square of the normalized standard deviation of the apparent diameter distribution, from cumulants analysis. [d] The decay observed corresponding to D_s = 16 nm (see Figure 2) is thought to be an artifact of the curve-fitting process and is included in this category.

ution of long-range interactions, since the Debye length at the ionic strength used here is on the order of 5 Å.

With these considerations in mind, we turn to the results of Figure 2 which are also displayed in Table I. These findings suggest the following conclusions. (1) There is no binding of proteins to polyion below pH ≅4.6 (note that free polymer scatters too little to contribute to the autocorrelation function). (2) Complexes (25 ± 5 nm) are in equilibrium with free protein in the range 4.6 < pH < 4.9; the size of these species are very nearly equal to that of the free polymer so they may well be highly solvated intrapolymer complexes, in which a number of protein molecules are bound to a single polyion chains without collapse of the latter. (3) No free protein is observed above pH 5, so that all protein is presumably bound. (4) At pH ≥5.2 larger aggregates are formed.

The somewhat erratic consequences of centrifugation on the QELS results are not fully understood. Centrifugation always reduces the relative abundance of the interpolymer aggregates ($D_s \cong 50 \pm 10$ nm) but not in a consistent fashion. It is not clear at present whether these aggregates are themselves removed by centrifugation or whether (more likely in our opinion) the coacervate is removed, with a concomitant perturbation of the aggregate concentration, through some equilibrium process.

The critical pH for bulk phase separation seems to be significantly larger than the pH at which intrapolymer complexes are first formed, and the aggregation of molecular complexes appears as an intermediate process. Such aggregates can only form when the net charge of intrapolymer complexes is close to zero. One may speculate that, near the isoelectric point of the protein (ca. 4.8 for BSA), each polyion chain binds some number of proteins which depends, inter alia, on polymer contour length, chain flexibility, and protein dimensions. The net charge of this complex, however, remains positive until the pH substantially exceeds the isoelectric point. Under such conditions intrapolymer complexes may begin to associate.

Future investigations will be designed to elucidate the structure and probable charge state of complexes and aggregates. Of particular interest will be the effect of polyion/protein stoichiometry on the progressive formation of associated species, as disclosed by both dynamic and static light scattering methods.

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