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Stoichiometry and the Mechanism of Complex Formationin Protein-Polyelectrolyte Coacervation

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STOICHIOMETRY AND THE MECHANISM OF COMPLEX FORMATION IN PROTEIN-POLYELECTROLYTE COACERVATION

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ABSTRACT

The complexation and coacervation of bovine serum albumin (BSA) with poly(dimethyldiallylammonium chloride) (PDMDAAC) in pH 7.86 buffer solutions, at various ionic strengths (I), was investigated by turbidimetric titration, quasi-elastic light scattering, and electrophoretic light scattering. The results obtained support the following mechanism. Upon addition of PDMDAAC to BSA at $I = 0.01$ M, a stoichiometric complex is initially formed. Subsequent addition of polymer causes this complex to form a coacervate with concomitant charge neutralization. At higher ionic strengths (0.05 and 0.10 M), the initial complex is nonstoichiometric, and coacervates are formed by the aggregation of the complex. These coacervates are observed to be ~700 nm by optical microscopy.

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INTRODUCTION

Proteins used in the food and pharmaceutical industry have to be produced with a concern for manufacturing cost. In most instances, industrial proteins are crudely extracted in the most economical way from plants or animal tissues or the products of microbial fermentation. For each particular protein, a unique series of purification and recovery steps from the crude protein extracts is required. This process may be difficult and expensive because of the similarity between the desired protein and the other biomolecules present. Furthermore, harsh conditions must be avoided since the activity of the protein must be retained. In view of such considerations, protein phase separation by polyelectrolytes is an attractive process for protein purification and recovery. In this process the target proteins are obtained first through selective protein–polyelectrolyte phase separation, then recovered by adjusting pH or ionic strength [1, 2]. The efficiency of final protein purification and recovery depends largely on the phase separation step.

The phase separation of proteins by polyelectrolytes, commonly called precipitation, may in fact be more frequently coacervation. In coacervation, the polymer-rich phase is a second liquid phase, while precipitation corresponds to the formation of a solid phase [3]. The distinction between coacervation and precipitation becomes apparent upon centrifugation. It appears likely that precipitation is characteristic of oppositely charged polyelectrolytes with symmetric charge spacing at low ionic strength [4], while coacervation is typical of weakly charged and/or asymmetric polyelectrolyte systems.

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The phenomenon of macromolecular complex coacervation was first studied by Bungenberg de Jong [3]. Complex coacervates appear as microscopic droplets floating in the liquid; these may fuse with each other but do not mix with the solvent. The coacervate in aqueous solution represents a kind of aggregate colloidal particle. Complex coacervation is a property of mixtures of oppositely charged polyelectrolytes, of which protein–polyelectrolyte coacervation appears to be a special case. An important question in both situations is the relationship between complex formation and phase separation.

Even though protein-polyelectrolyte complexes have been studied for more than two decades, few studies have dealt with the mechanism of complex formation. In the purification of lysozyme from egg white by precipitation with polyelectrolytes, Glatz et al. [5] first suggested that the protein–polyelectrolyte flocs or coacervates are formed by aggregation of so-called "primary particles." Recently, Berg et al. [6] reported a mechanism for floc formation of lysozyme–polyacrylic acid. It was concluded that coacervation is due to polymer bridging at high polymer MW (over one million) and to charge neutralization at low polymer MW (several thousand). In both Glatz' and Berg's studies, the initial formation of the "primary particle" was not fully discussed; in particular, they did not elucidate the structure and mechanism of formation of the primary particle.

Other studies [7–9] dealing with protein–polyelectrolyte complex composition and properties have provided more focus on the molecular level. For the complexation of bovine serum albumin (BSA), ribonuclease, and lysozyme with both polycations and polyanions, Dubin et al. [10] proposed the formation of soluble complexes prior to phase separation. Kokufuta [11] employed colloidal titration to study complexation between human serum albumin (HSA), poly(dimethyldiallylammon-

ium chloride) (PDMDAAC), and potassium poly(vinyl alcohol sulfate) (KPVS) in pure water. Titrating the protein with the polyelectrolytes, he found turbidity maxima (referred to as end points) corresponding to conditions under which the mole numbers of quaternary ammonium groups in PDMDAAC and sulfate groups in KPVS were approximately identical to the contents of the acidic and basic groups in HSA. Therefore, it was concluded that the complexation between HSA, PDMDAAC, and KPVS involves "stoichiometric" binding.

A reaction is usually called "stoichiometric" when 1) the structure of the reaction product is well-defined, 2) the reaction has a large equilibrium constant, and 3) the process is rapid. Stoichiometric polyelectrolyte complexes are formed as a result of charge neutralization, i.e., the ratio between the oppositely charged groups in the complexes is 1:1 [4, 12]. These compounds are insoluble in water and organic solvents [4, 12]. This phenomenon forms the basis of "colloid titration" in which the concentration of a polyelectrolyte can be quantitatively determined by titration with an oppositely charged polyelectrolyte to a turbidimetric, colorimetric, or conductimetric end-point.

There are also reports of nonstoichiometric polyelectrolyte complexes. Kabanov [4] first reported that nonstoichiometric water-soluble polyelectrolyte complexes are formed by poly-*N,N*-dimethylaminoethyl methacrylate hydrochloride and sodium polyphosphate. It was found that the complexes in aqueous salt media undergo considerable conformational changes prior to phase separation. Conformation change was also found for some other nonstoichiometric protein–polyelectrolyte complexes in salt solution [13–15].

Consideration of the results of studies on the stoichiometry of the protein–polyelectrolyte complex suggests that stoichiometry may be related to the salt concentration, but further understanding is needed. In particular, it is not clear how stoichiometric and nonstoichiometric binding are controlled, and how the two different binding processes lead to phase separation. In the present paper we investigate complexation between BSA and PDMDAAC, focusing in particular on the ionic strength effect on the binding stoichiometry and coacervate formation.

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EXPERIMENTAL

Materials

Poly(dimethyldiallylammonium chloride), a commercial sample of Merquat 100 from Calgon Corporation (Pittsburgh, Pennsylvania) possessing a nominal molecular weight of 2×10^5, was dialyzed and freeze-dried before use. Bovine serum albumin was obtained from Sigma as 95–99% pure with a p*I* value of 4.9. Monobasic and dibasic sodium phosphate salts and sodium chloride of AR grade were obtained from Mallinckrodt Inc. Distilled and deionized water was used in all experiments.

Methods

Turbidimetric Titration

Turbidimetric titrations were carried out at 22°C in solutions of the desired ionic strength. A 2-mL microburet was used to deliver titrant, and the turbidity was

followed with a Brinkmann PC600 probe colorimeter (420 nm, 2 cm pathlength). Solutions were always stirred, and turbidity values were obtained after several minutes of stabilization in all cases. Two types of titration were involved in the study. In "Type I" titrations, NaOH was added to an initial solution of PDMDAAC, BSA, and NaCl at a pH around 4. A pH electrode connected to a Beckmann Φ 34 pH meter was used to monitor any pH change during the titration. The turbidity was monitored as a function of pH. In the other type of titration, referred as "Type III," BSA in phosphate buffer solution was titrated by PDMDAAC. The turbidity was recorded as a function of the concentration ratio of [PDMDAAC]/[BSA] = W (or $1/r$).

Quasi-Elastic Light Scattering (QELS)

QELS measurements were made at scattering angles from 30 to 150° with a Brookhaven (Holtsville, New York) 72 channel BI-2030 AT digital Correlator, using a Jodon 15 mW He-Ne laser (Ann Arbor, Michigan). Samples were made dust-free by filtering through 0.20 μm Acrodisc filters (Gelman) prior to analysis. We obtained the homodyne intensity-intensity correlation function $G(q,t)$, with q, the amplitude of the scattering vector, given by $q = (4\pi n/\lambda) \sin(\theta/2)$, where n is the refractive index of the medium, λ is the wavelength of the excitation light in vacuum, and θ is the scattering angle. $G(q,t)$ is related to the time correlation function of concentration fluctuations $g(q,t)$ by

$$Q(q,t) = A[1 + bg(q,t)^2] \tag{1}$$

where A is the experimental baseline and b is a constant related to the fraction of the scattered intensity.

The diffusion coefficients were calculated by using

$$D = \frac{\lambda^2}{16\pi^2 \sin^2(\theta/2)\langle\tau\rangle} \tag{2}$$

where $\langle\tau\rangle$ is the diffusion time constant, obtained from the CONTIN [16] calculation of $g(q,t)$ [17]. The diffusion coefficient, D, is directly related to the Stokes radius, R_S, by Stokes' equation

$$R_S = \frac{kT}{6\pi\eta D} \tag{3}$$

where k is the Boltzmann constant, T is the absolute temperature, and η is the viscosity of the solvent.

Electrophoretic Light Scattering (ELS)

ELS measurements were made at four scattering angles (8.6, 17.1, 25.6, and 34.2°), using a Coulter (Hialeah, Florida) DELSA 440 apparatus. The electric field was applied at a constant current of 5 mA. The temperature of the thermostated chamber was maintained at 25°C. In ELS, electrophoretic velocities are determined by measurement of the Doppler shifts of light scattered from molecules or particles drifting in an electric field. The measured Doppler shift frequency, $\Delta\omega$, is given by [18]

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$$\Delta\omega = \frac{2\pi n}{\lambda} Eu \sin\theta \tag{4}$$

where E(V/cm) and $u[(\mu m \cdot s^{-1})/(V \cdot cm^{-1})]$ are the applied electric field strength and electrophoretic mobility, respectively.

Optical Microscopy

Microscopic observation of BSA-PDMDAAC coacervates was carried out using a WILD MPS 51S compound microscope. Coacervates were prepared by mixing BSA and PDMDAAC in 0.10 M NaCl solution with NaOH to adjust pH to 7.9. The coacervate solution was placed on a slide and then covered by a cover slip for microscopic observation. The sizes of coacervates were estimated from a calibration scale.

RESULTS AND DISCUSSIONS

Figure 1 shows the Type I turbidimetric titrations curve of 0.10 g/L PDMDAAC in 0.60 g/L BSA solution at an ionic strength of 0.01 M NaCl. The curve displays an abrupt increase in turbidity at pH 5.1, about 0.2 pH unit above the isoelectric point of BSA, corresponding to coacervate formation. Prior to coacervate formation, we observe a ~2% turbidity increase at pH 4.6. This small turbidity increase is due to initial formation of a soluble complex; particles with a size larger than either BSA or PDMDAAC are detected at this pH by QELS [19].

Figures 2–4 show turbidimetric Type III titrations of various concentrations of BSA with PDMDAAC in pH 7.86 sodium phosphate buffer at ionic strengths of

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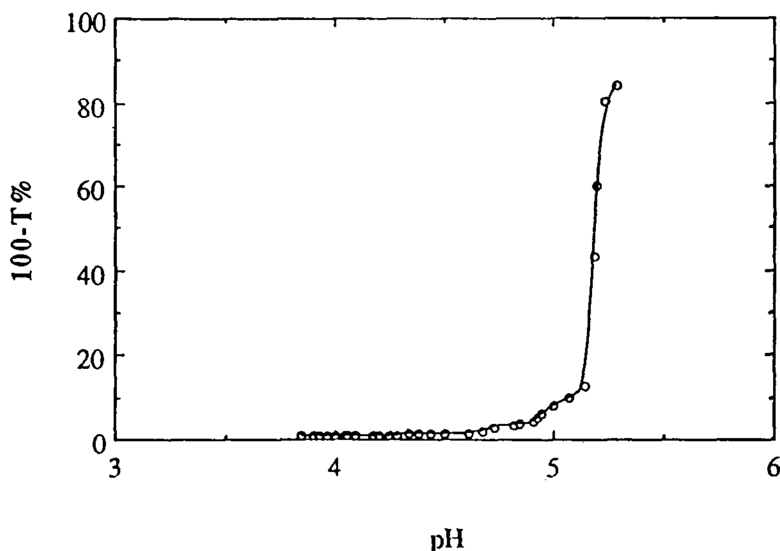

FIG. 1. Fluorescence emission spectra of native papain (a) and papain/KPVS complex (b) excited at 280 nm. Papain concentration, 10^{-6} mol/dm^3; KPVS concentration, 0.0025 mol/dm^3 (based on sulfate groups); papain:KPVS = 1.5:0.8 (volume ratio).

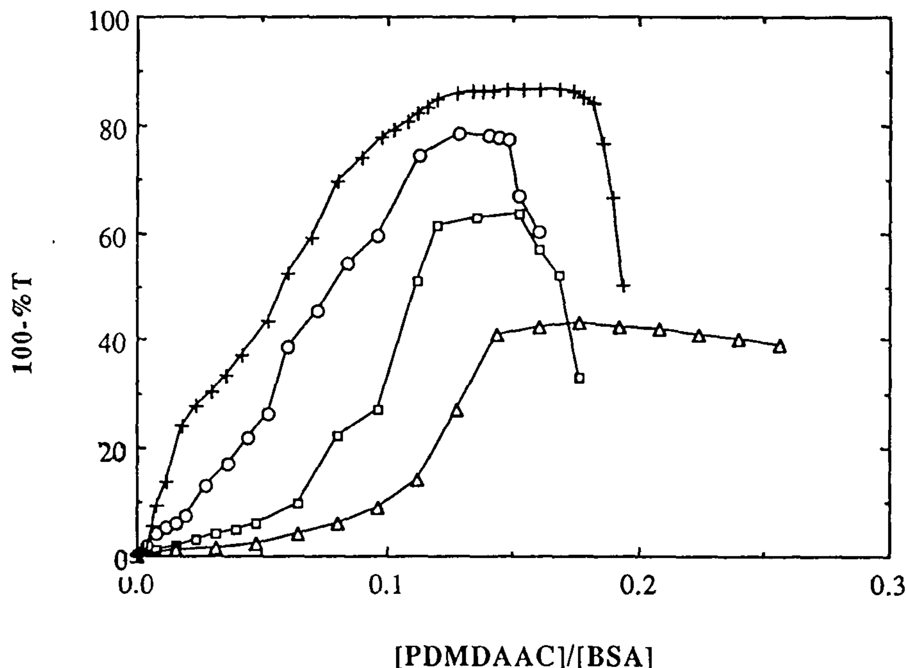

FIG. 2. Type III turbidimetric titrations of various concentrations of BSA with PDMDAAC at an ionic strength of 0.01 M and pH 7.86 phosphate buffer solutions. BSA concentrations (g/L): 0.36 (+), 0.12 (○), 0.06 (□), and 0.03 (△).

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0.01, 0.05, and 0.10 M, respectively. The value of pH 7.86 was chosen on the basis of the Type I titration in Fig. 1 to ensure coacervate formation. All solutions probably exhibit turbidity maxima. In the case of the high BSA concentration (0.36 g/L), the plateau is not meaningful because the sensitivity of the photodetector is not sufficient to record accurate transmittances for such turbid solutions. For the lowest ionic strength, the position of this turbidity maximum is relatively independent of protein concentrations, i.e., the turbidity maximum reveals a certain stoichiometric character. For higher ionic strengths, the position of the maximum shifts to larger relative polymer concentrations with decreasing protein concentration.

In order to understand the distinction between the behavior at high and low ionic strengths, measurements of the apparent size of the scattering species were conducted by QELS at a protein concentration of 0.60 g/L and at both low (I = 0.01 M) and high (I = 0.10 M) salt concentrations. Figure 5(a) and Fig. 6 are the QELS results obtained at low and high ionic strengths, respectively. A dramatic difference between the two ionic strength is obvious. Under the former condition, a remarkably stable species with $R_S \approx 100$ nm is observed to form at very low added polymer concentration, and it persists as the sole scattering species until a polymer concentration close to the turbidity maximum is attained. In contrast, QELS measurements at I = 0.10 show that the size of the scattering particle increases rapidly with added polymer, even at very low polymer concentration.

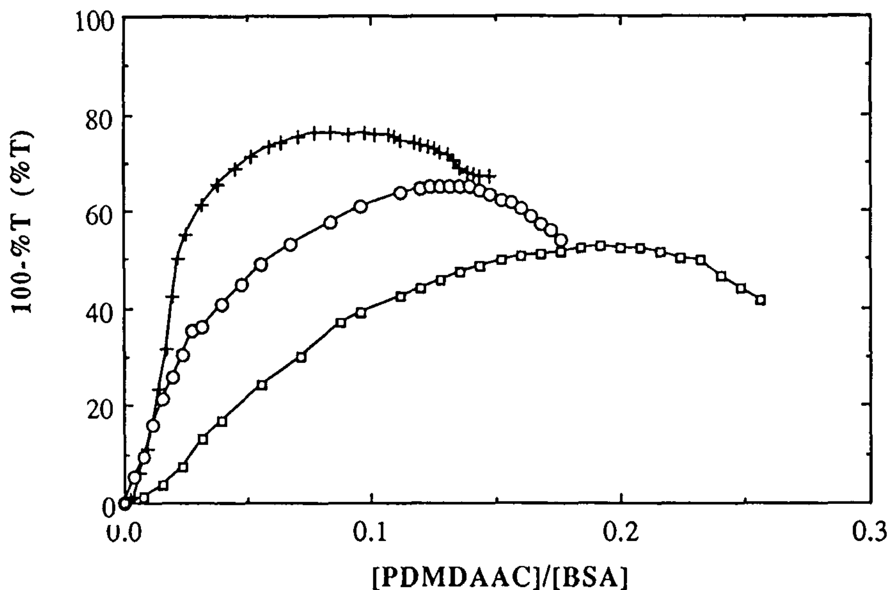

FIG. 3. Type III turbidimetric titrations of various concentrations of BSA with PDMDAAC at an ionic strength of 0.05 M and pH 7.86 phosphate buffer solutions. BSA concentrations (g/L): 0.36 (+), 0.12 (○), and 0.06 (□).

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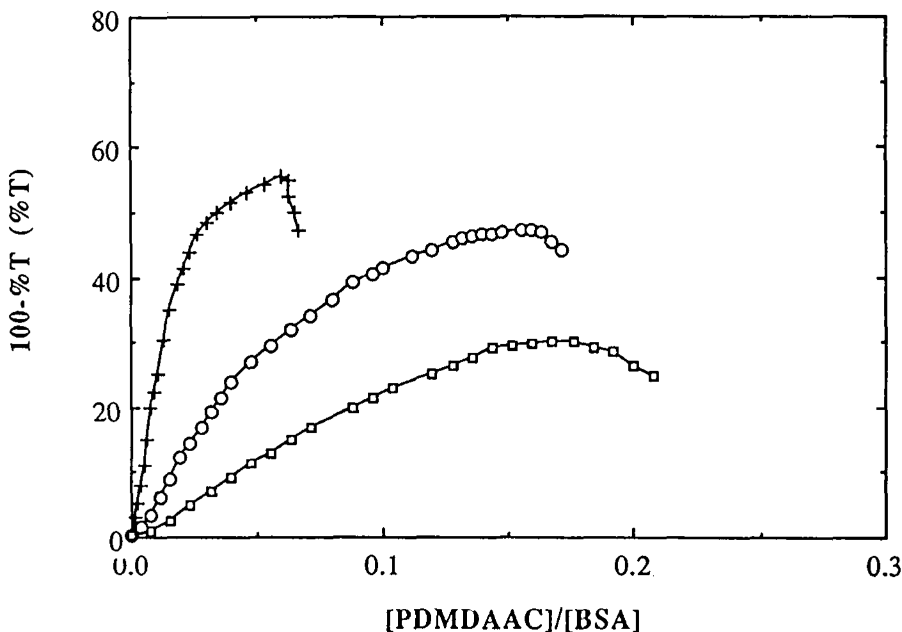

FIG. 4. Type III turbidimetric titrations of various concentrations of BSA with PDMDAAC at an ionic strength of 0.10 M and pH 7.86 phosphate buffer solutions. BSA concentrations (g/L): 0.36 (+), 0.12 (○), and 0.06 (□).

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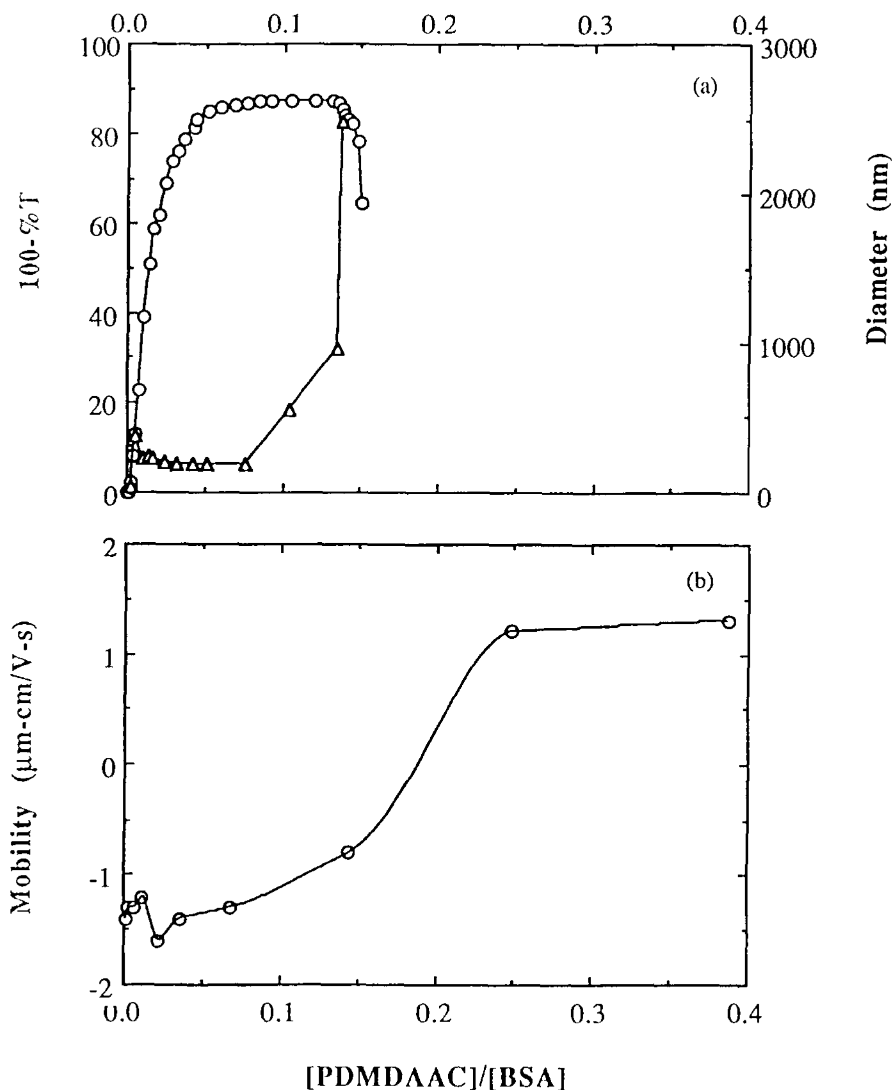

FIG. 5. (a) Diameter of the BSA-PDMDAAC complex as a function of polymer concentration in 0.60 g/L BSA and 0.01 M, pH 7.86 phosphate buffer: (△) QELS results, (○) the corresponding turbidity. (b). Mobility of the BSA-PDMDAAC complex at the same conditions as those in (a).

The preceding results are consistent with the following model for complexation and phase separation. At low ionic strength and at pH 7.86, the binding of BSA to PDMDAAC is quite strong, and in the limit of $r \rightarrow \infty$ each polymer chain binds a full complement of protein molecules, i.e., on the order of 120 proteins per polymer chain [20]. At pH 7.86, BSA carries a large negative net charge, $Z = -21$ [21], and therefore this primary complex also has a net negative charge. From electrophoretic light-scattering measurements, we find for this complex a mobility

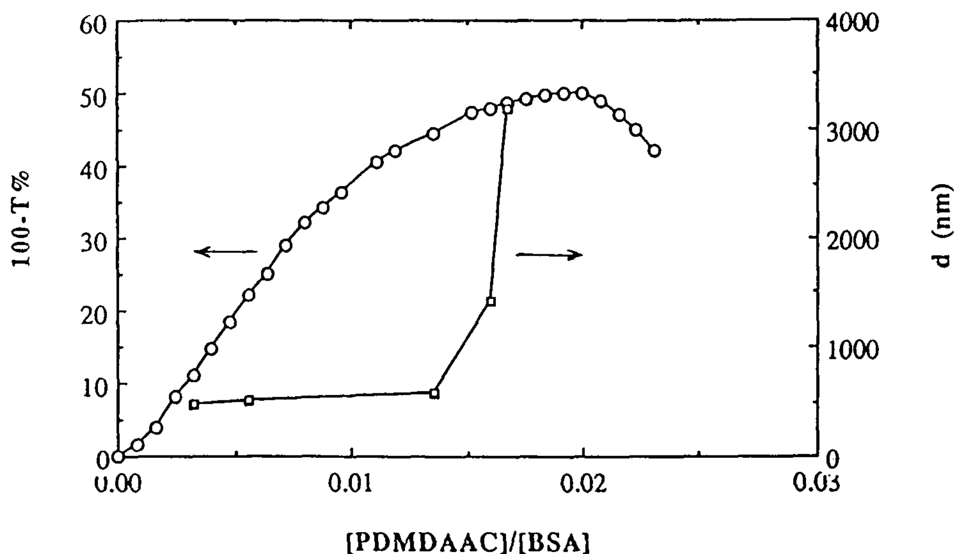

FIG. 6. Diameter of the BSA-PDMDAAC complex as a function of polymer concentration in 0.60 g/L BSA and 0.1 M, pH 7.86 phosphate buffer: (□) QELS results, (○) the corresponding turbidity.

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of -1.4 μm·cm/V·s, corresponding to a zeta potential of -18 mV, as shown in Fig. 5(b). Because the proteins are highly charged at this pH, they exert a repulsion on neighboring bound proteins, causing the chain to adopt an extended configuration with a hydrodynamic radius approximately four times larger than the value of the protein-free polymer. The net negative charge of this complex precludes its further association. Therefore, the turbidity increases only because of an increase in the number of such species. Because of the large binding constant, there is essentially no free polymer in these systems, only the primary complex and free protein. When a sufficient amount of polymer has been added to bind all the free protein, further addition of polycation leads to the coacervation of the primary complexes. The instability of the coacervate and the very large related particle sizes produce the ultimate decrease in turbidity. The maxima observed at low ionic strength therefore display a stoichiometric character, in the sense that a doubling of the protein concentration produces an approximate doubling of the amount of polymer added at the point where the turbidity no longer increases. In contrast to stoichiometric protein-polyelectrolyte complexes formed in pure water [8, 11] and stoichiometric polycation-polyanion complexes [4] which are neural particles, the BSA-PDMDAAC stoichiometric complex has a negative charge. This negative charge prevents the complex from forming coacervates through simple aggregation. Addition of polymer neutralizes this charge and overcomes the charge resistance to coacervation. This is consistent with the experimental results in Fig. 5(b), which show a constant mobility at low polymer concentration and then an increase from negative to positive values. The initial constant mobility corresponds to the stable stoichiometric complex, and the mobility change with polymer concentration, as

well as the observation of zero mobility, are consistent with the coacervation of the complex upon addition polymer.

As seen in Fig. 6, the size of the complex formed at higher ionic strength always increases with addition of polymer. Thus, the complexation is not stoichiometric because there is no well-defined product. Furthermore, the lowest value for R_S seen, ~250 nm, is very much larger than the dimensions of the protein-free polymer at this ionic strength. This result indicates that "primary" complexes are not particularly stable, but instead strongly tend to associate to form higher-order aggregates. Phase separation occurs when these aggregates become very large. We suggest that the size of the aggregates is not a function of the bulk solution stoichiometry alone, but instead increases strongly with total solute concentration. Thus, in two solutions that have the same polymer:protein stoichiometry, the one with the higher protein concentration will form larger aggregates. The turbidity is thus observed to increase more rapidly with the concentration ratio W of the polymer to the protein when the protein concentration is higher, as seen in Figs. 3 and 4. Also the size required for bulk phase separation is attained at lower W in this case.

There are two reasons why complexes may tend to aggregate more readily at higher ionic strength. First, the intrinsic polymer-protein binding constant may be expected to be smaller at larger I. Therefore, the number of proteins bound per polymer chain may be less and the charge of the "primary complex" closer to electrical neutrality. Second, the Debye length decreases from 30 to 10 Å as the

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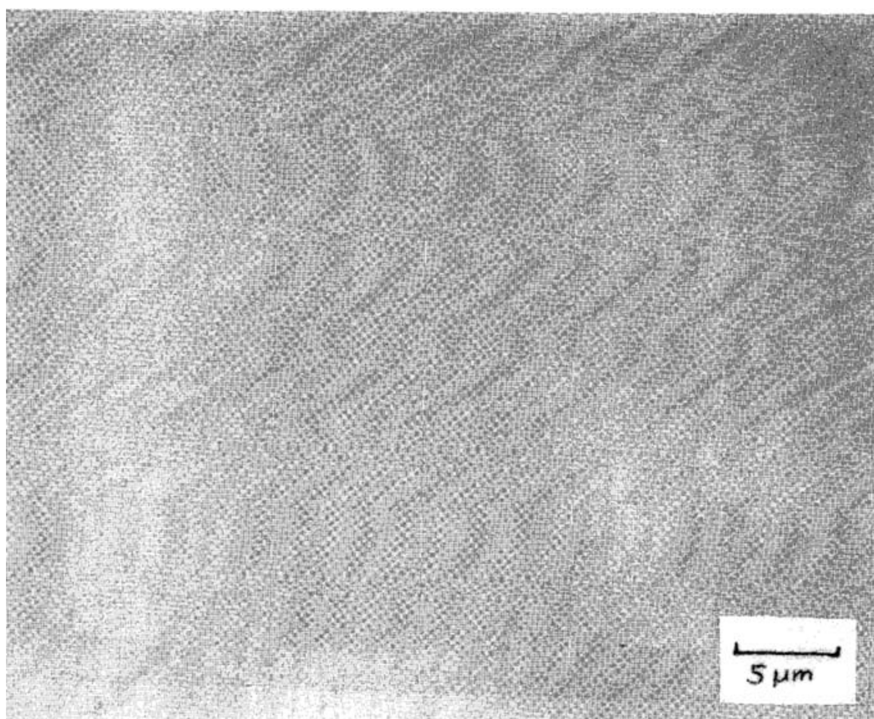

FIG. 7. Optical photomicrograph of BSA-PDMDAAC coacervate.

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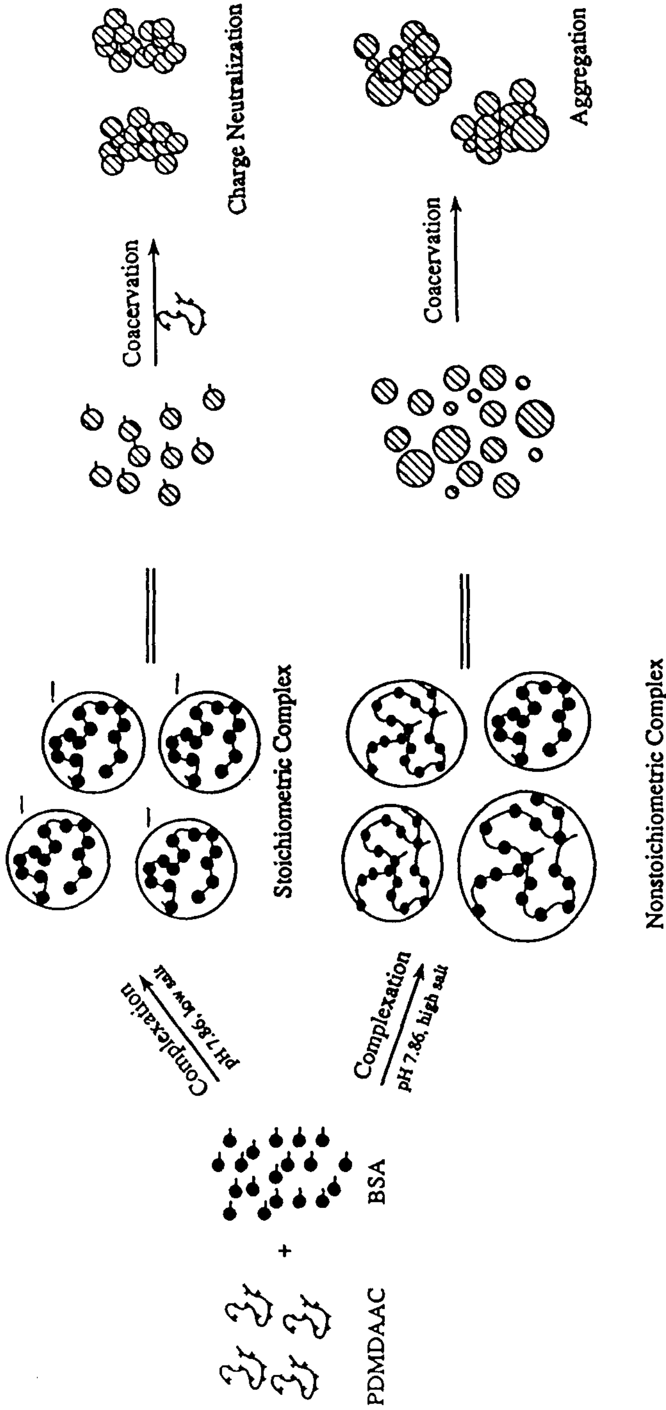

FIG. 8. Schematic diagram of complex formation in protein separation by polyelectrolytes.

ionic strength increases from 0.01 to 0.10 M. In the low I condition, two primary complexes can interact with each other in their entireties, and the net charge of the entire complex determines association or coacervation. In the second case, two primary complexes may interact with specific regions within each other's domain. Thus, a protein-rich portion of one complex (negatively charged) could interact with a protein-poor region (positively charged) in another complex. The "polarizability" of the primary complex may therefore promote higher-order aggregation. In all cases the higher aggregates or coacervates have a size of about 700 nm (Fig. 7) as observed by microscope.

In summary, we propose a mechanism for BSA–PDMDAAC complex formation and complex coacervation as shown schematically in Fig. 8. At low ionic strength, e.g., $I = 0.01$ M, BSA and PDMDAAC first form a negatively charged stoichiometric complex with a diameter of ~ 200 nm. The 200-nm particles then coacervate through charge neutralization upon further addition of the polycation. In the case of higher ionic strength, the initially formed complexes are nonstoichiometric with a size of ~ 500 nm in diameter. The nonstoichiometric complexes bear less charge and may exhibit charge polarization. The polarized particles then aggregate to form coacervates.

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