Protein Separation via Polyelectrolyte Coacervation: Selectivity and Efficiency

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Selective phase separation with polyelectrolytes can be used to separate a mixture of proteins. The efficiency of separation was examined using the cationic polyelectrolyte poly(diallyldimethylammonium chloride) and the model proteins bovine serum albumin, β-lactoglobulin, γ-globulin, and ribonuclease A. The coacervation yield for individual proteins and the degree of separation, for selected protein pairs, were studied as a function of polymer molecular weight, ionic strength, and pH.

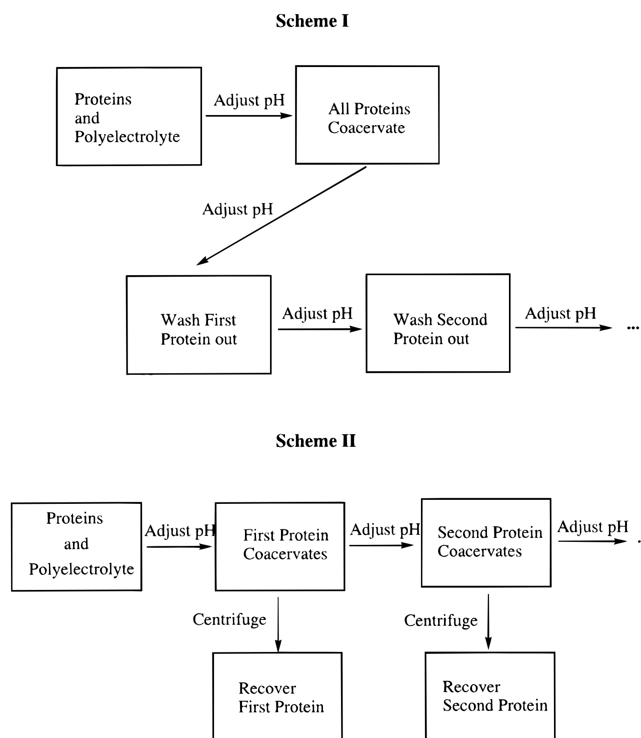
Introduction

Protein–polyelectrolyte complex coacervation provides a novel alternative to conventional protein separation methods (Dubin et al., 1994). In polyelectrolyte complex coacervation, oppositely charged macromolecules form complexes and then a separate dense liquid phase or coacervate (Burgess, 1994), as opposed to the separate solid phase formed in precipitation. Protein separation arises during polyelectrolyte coacervation as a consequence of selective protein–polyelectrolyte complexation. This phenomenon may afford the possibility of large-scale protein purification in a manner that is fast, efficient, and inexpensive compared to traditional protein separation methods such as large-scale chromatography, filtration, or centrifugation (Ladisch et al., 1990).

The phenomenon of protein–polyelectrolyte coacervation has been studied for several decades (Bungenburg de Jong, 1949; Tsuchida and Abe, 1982; Kokufuta et al., 1982; Burgess and Carless, 1984; Clark and Glatz, 1987; Niederauer and Glatz, 1994), but application in protein separation is still developmental. Industrial scale separation could employ one of the two schemes shown in Chart 1. After the proteins are separated and concentrated into the coacervate, polyelectrolyte contained within can be removed via ultrafiltration or selective precipitation (Dubin et al., 1994). The efficiency of recovering proteins via a well-designed polyelectrolyte coacervation is relatively high; this requires careful optimization of variables such as pH, ionic strength, and solute concentrations for appropriate protein–polyelectrolyte pairs. Thus, Morawetz and Hughes (1952), Clark and Glatz (1987), Sternberg and Hershberger (1974), and Strege et al. (1990) all reported removal of over 90% of the proteins in solution upon careful selection of experimental conditions for appropriate protein–polyelectrolyte combinations.

Two key factors that affect the applicability of polyelectrolyte coacervation to protein separation are the *efficiency* and *selectivity* of the process. However, these factors have not been addressed and studied adequately in previous research. Since the current literature does not express agreement on well-defined parameters to evaluate the separation process quantitatively, it is necessary that factors describing selectivity and efficiency in this research be established. The efficiency of protein coacervation or precipitation with polyelectrolyte may be equated to the percent of the protein in the condensed phase (yield). Selectivity may be defined as (Strege et al., 1990)

Chart 1. Industrial Scale Protein Separation



$$S = \frac{[A]_c/[B]_c}{[A]_s/[B]_s} \quad (1)$$

where $[A]_c$ and $[B]_c$ are the respective concentrations of two proteins in the coacervate phase and $[A]_s$ and $[B]_s$ are their concentrations in the supernatant. By definition, S is always greater than or equal to unity, the latter corresponding to completely indiscriminate coacervation.

Optimization of this purification method in terms of efficiency and selectivity depends on a knowledge of the factors that govern the binding affinity between proteins and polyelectrolytes. Generally speaking, protein binding and subsequent coacervation depend on pH, ionic strength (I), protein/polyelectrolyte ratio (r), and polyelectrolyte molecular weight (MW). Therefore, the conditions for inducing coacervation in the separation process and for redissolving coacervates in the recovery process may be

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Table 1. pI and MW of Proteins

protein	pI	MW
ribonuclease	9.45	14 700
β-lactoglobulin	5.2	36 600
γ-globulin	7.0	150 000
BSA	4.9	66 000

determined through turbidimetric titration of protein/polyelectrolyte solutions made at different I and r. Such experiments need to be carried out for each polyelectrolyte–protein pair and may depend also on polymer molecular weight.

In prior studies, we have considered in some detail the interaction between the polycation poly(diallyldimethylammonium chloride) (PDADMAC) and bovine serum albumin (Mattison et al, 1995; Xia et al., 1993a), or for several protein–polyelectrolyte pairs (Xia et al., 1993b), but did not consider the separation of protein mixtures. In this paper, we focus on the effect of four major parameters, pH, I, r, and MW, on the selectivity and efficiency of polyelectrolyte–protein coacervation for various protein pairs. Bovine serum albumin (BSA), β-lactoglobulin, γ-globulins, and ribonuclease A were selected as model proteins on the basis of their isoelectric points (pI's), stability, molecular weights, availability of pH titration data, and expense. Poly(diallyldimethylammonium chloride) (PDADMAC) was chosen as the model polyelectrolyte because its charge is independent of pH, because narrow MWD fractions were available, and because it is a common commercial product. Given the large number of experimental variables (protein type, polymer MW, pH, I, and r), a vast number of possible experiments can be visualized, with the current results representing only a selected subset.

Experimental Section

Materials. Poly(diallyldimethylammonium chloride) (PDADMAC) (Merquat 100), from Calgon Corp. (Pittsburgh, PA) with a nominal MW of 2×10^5 and Mw/Mn > 10 was dialyzed (molecular weight cutoff = 12 000–14 000) and freeze-dried before use. Fractions of PDADMAC were prepared and characterized by Xia et al. (1995). Bovine serum albumin was purchased from Boehringer Mannheim (Indianapolis, IN) (Lot 100062). All the other proteins, β-lactoglobulin (Lot T1126), γ-globulins (Lot G5009), and ribonuclease A (Lot R5125), were obtained from Sigma. The MW and isoelectric point (pI) of the proteins are listed in Table 1. All the proteins were used without further purification.

Methods. ***1. Turbidimetry.*** Turbidity was measured at 24 ± 1 °C with a Brinkmann PC800 colorimeter equipped with a 2 cm path length probe, at 420 nm wavelength. In "Type I" titrations, NaOH was used to change the pH of a macromolecular solution maintained at constant ionic strength. Protein/polyelectrolyte solutions were prepared by mixing predetermined amounts of polymer and protein solutions in a selected ionic strength medium at pH = 4.00. NaOH (0.1 M) solution was added stepwise from a microburet under constant stirring. The pH and the transmittance (%T) of the solution were recorded after each titrant increment, allowing 1–2 min for the solution to reach equilibrium.

The experiments were usually conducted in 0.1 M NaCl, although to study the effects of ionic strength, some experiments were carried out in 0.02, 0.04, or 0.05 M NaCl. The ionic strength contributions of polymer, protein, or added NaOH titrant were always less than 1 mM and could therefore be neglected relative to the concentration of NaCl.

2. Separation and Analysis. After the formation of coacervate, solutions were centrifuged at 4000 rpm (Model CL, International Equipment Co.) for about 15 min to separate coacervate from supernatant. The separated coacervate was then redissolved for further analysis in 5 mL of 0.02 N HCl adjusted to the same ionic strength as the supernatant.

3. SEC Measurement. Size exclusion chromatography (SEC) was carried out on a Superose 12 column (Pharmacia, 30 × 1 cm). The system incorporated a minipump (Milton Roy) and a 100 μL injection loop. UV (Gilson 112 UV/vis, Gilson λ = 254 nm) and refractive index (Waters Associates R401) detectors were linked in series to simultaneously monitor both protein and polymer concentrations. The column was calibrated with Pullulan standards (P-82, Shodex Standard, Showa Denko). The flow rate of the mobile phase (HAc–NaAc buffer, pH = 4.0, I = 0.2 M) was 0.52 mL/min. In this mobile phase no protein/polymer complexation occurs. Conversion of UV peak heights to protein concentrations was accomplished by using calibration curves constructed with known concentrations of proteins. Concentrations of PDADMAC were determined from RI peak heights in the same way. The SEC analysis, which was the major source of error in the measurement of selectivity, contributed an uncertainty of ±5%.

4. UV Measurement. UV measurements were made at 280 nm with a Hewlett Packard 8450A diode array spectrophotometer. Extinction coefficients of the proteins were measured by recording absorbance of protein standard solutions. Unknown protein concentrations were determined based on the measured absorbance and calculated extinction coefficients.

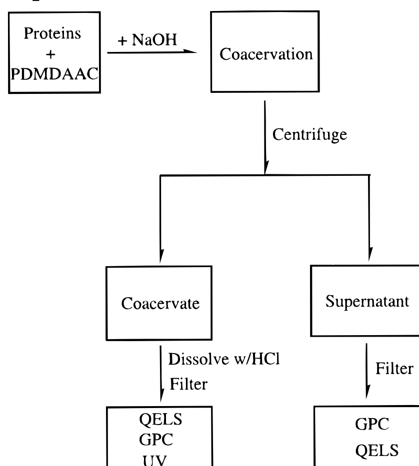
5. Dynamic Light Scattering (DLS). DLS was carried out with a Brookhaven (Holtsville, NY) 72 channel BI–2030 AT digital correlator, using a 100 mW Ar-ion laser. The theoretical details of this method can be found in several references (Pecora, 1985; Schmitz, 1990). UV measurements were made before and after dust filtration of DLS samples with a 0.2 μm filter to make sure that no macromolecules were lost due to the filtration.

Experimental Scheme. To measure the efficiency and the selectivity of a particular protein–polyelectrolyte system, the optically clear mixture of protein and polymer at moderately low pH was first titrated with NaOH to the desired pH, and the solution was then centrifuged to separate coacervate from supernatant. The former was dissolved by adding HCl and was filtered prior to analysis by QELS, SEC, and UV to determine protein and polymer concentrations. The supernatant was filtered and then analyzed by SEC and DLS. The experimental scheme is shown in Chart 2.

Results and Discussion

1. Turbidimetric Titrations. Figure 1 shows the results of "Type I" titrations for different proteins in 0.1 M NaCl, 0.1 g/L polymer, and 0.5 g/L protein (r = 5). For each protein, the turbidity of the solution increases sharply at pH_ϕ, corresponding to phase separation. Proteins of similar pI values tend to have similar pH_ϕ, e.g. BSA (pI = 4.8, pH_ϕ = 7.6) and β-lactoglobulin (pI = 5.2, pH_ϕ = 7.2). Such "Type I" titrations may be used to identify the significant pH regions for the separation of multiprotein systems. As an example, for the case of BSA/RNAse, the insert in Figure 1 indicates region I, which corresponds to the coacervations of BSA alone, and region II, which corresponds to the coacervation of both proteins. Such information guides in the selection of

Chart 2. Experimental Procedure



conditions for optimum selectivity, for example, in the choice of ionic strength. The effect of ionic strength on pH_ϕ for the same protein pair is shown in Figure 2. The best protein separation should be obtained by maximizing $\Delta pH_\phi = (pH_{\phi,RNAse} - pH_{\phi,BSA})$, which is observed at $I = 0.05$ M. Such predictions are valid to the extent that the polyelectrolyte-binding properties of the two proteins are independent of each other.

2. Selectivity. Measurement of selectivity requires analysis of binary protein mixtures, which we chose to do by SEC. However, this method is best applied to protein pairs that differ sufficiently in MW so that their respective SEC peaks were resolvable. Solutions containing the two proteins and PDADMAC were brought to the point of selective coacervation (e.g., region I in Figure 4) by addition of NaOH. The coacervate was separated by centrifugation, redissolved by acidification, and analyzed by SEC to obtain the concentrations of proteins in the coacervate phase. From these concentrations and the initial values, it is possible to calculate S. Qualitative comparison of the SEC chromatograms for a mixture of RNAse and BSA, before and after coacervation (Figure 3), indicates that coacervate formation in pH region I is totally selective ($S = \infty$). Therefore, some proteins can be efficiently separated with "Type I" titration in pH region I. The case of BSA/γ-globulin provides another example of good separation in region I (Figure 4), although the SEC resolution is not adequate to calculate S. However, when the two proteins have similar MWs and isoelectric points, region I may be very narrow (see Figure 1) and the solution pH would have be carefully controlled to maintain good coacervation selectivity.

Selectivity is reduced in the pH region in which both proteins coacervate with PDADMAC (region II), but some separation may still be achieved. Region II is effectively inaccessible for BSA/RNAse since it involves such high pH that BSA forms a precipitate with PDADMAC, presumably due to protein unfolding. Therefore we consider a mixture of β-lactoglobulin ($pH_\phi = 7.1$), and γ-globulin ($pH_\phi = 8.7$) with PDADMAC (concentrations 0.2, 0.4, and 0.04 g/L, respectively) at $I = 0.04$ M, which also enables us to compare the selectivity in regions I and II. In region I (pH = 8.2), selectivity is infinite, with the coacervate containing only β-lactoglobulin, but is only about 2 in region II (pH = 9.6), with the coacervate enriched in γ-globulin. In addition, the coacervation yield for each protein also varies from region I to region II in a way that is different from the behavior of the single proteins. The yields for unmixed β-lactoglobulin and γ-globulin at pH = 9.6 are 64% and 31%, respectively; however, under the same pH and ionic strength, in the mixture of the two proteins, the yields are 36% and 52%, respectively. Obviously, the presence of the second protein affects the coacervation of the first, and the interaction among β-lactoglobulin, γ-globulin, and PDADMAC facilitates the selective coacervation of γ-globulin with PDADMAC, while reducing the coacervation of β-lactoglobulin. (Experiments are in progress to determine whether γ-globulin can actually displace β-lactoglobulin from its coacervate with PDADMAC.) Thus, selectivity is possible even in pH region II. Another example is provided by the mixture of PDADMAC, β-lactoglobulin, and BSA, far above region I at pH 9.0 (see Figure 1). S is found to be over 20, with β-lactoglo-

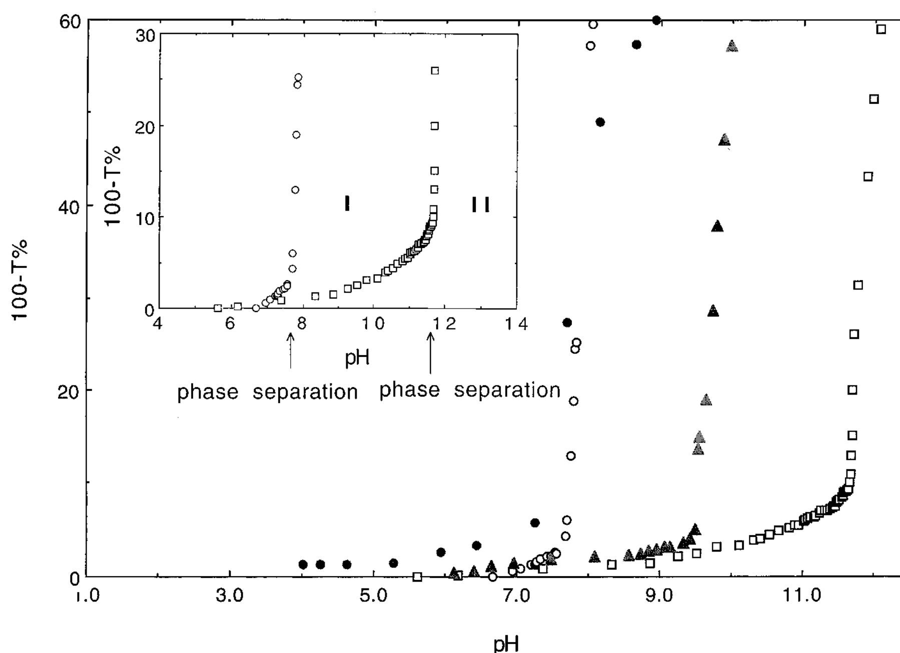


Figure 1. Type I turbidimetric titrations for different proteins (1 mg/mL) in 0.1 M NaCl, protein/polymer ratio = 5, ionic strength = 0.1. Protein (from left to right): β-lactoglobulin (●), BSA (○), γ-globulin (▲), RNAse (□). Insert: definitions of regions I and II, for BSA/RNAse.

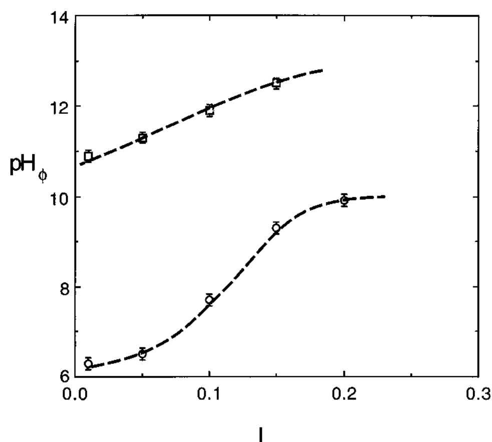


Figure 2. "Phase boundary" for BSA–PDADMAC (○) and RNAse–PDADMAC (□) (protein/polymer ratio = 5).

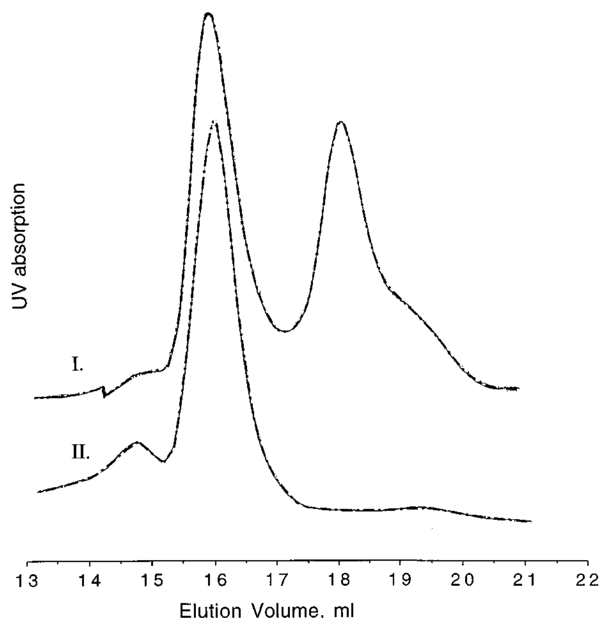


Figure 3. (I) SEC chromatograph of a BSA and RNAse mixture (both 1 mg/mL). (II) SEC chromatograph of coacervate from BSA (1 mg/mL)/RNAse (1 mg/mL)/PDADMAC coacervation. Small peak: BSA dimers. Coacervation conditions: protein/polymer ratio = 10, ionic strength = 0.1, pH = 9.0.

bulin the target protein, as shown in Figure 5. While pH_c is independent of polymer and protein concentrations (Dubin et al., 1995), pH_ϕ varies with r. The dependence of S on r is the subject of continuing studies.

3. Efficiency. ***a. Effect of Molecular Weight.*** The effect of polymer molecular weight on protein recovery was evaluated by comparing the coacervation yield for different PDADMAC fractions in 0.10 M NaCl. After pH adjustment to pH > pH_ϕ to induce coacervation, the mixture was centrifuged and the supernatant was analyzed by SEC. The amount of protein coacervated was obtained by subtracting the protein in supernatant from the total protein. Figure 6 shows that efficiency of protein coacervation in general increases with MW. The MW effect is much more evident for MW < 10^5 and is diminished at MW > 10^5. Similar MW effects have also been reported by Shieh and Glatz (1991) and Sternberg and Hershberger (1974).

One explanation of the above observation is based on the assumption that intrapolymer complexes (one polymer chain binding many proteins) are precursors of coacervation. This assumption appears to be justified by a wide range of studies, at least at low polymer concen-

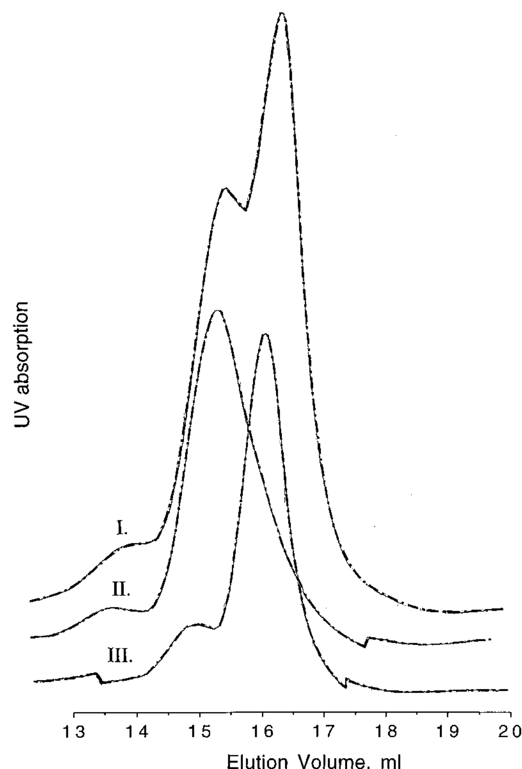


Figure 4. (I) SEC chromatograph of a mixture of BSA (1 mg/mL) and γ-globulin (1 mg/mL). First peak: γ-globulin. Second peak: BSA. (II) SEC chromatograph of supernatant of BSA (1 mg/mL)/γ-globulin (1 mg/mL)/PDADMAC coacervation. (III) SEC chromatograph of dissolved coacervate. Small: peak: BSA dimers. Coacervation conditions are the same as in Figure 3.

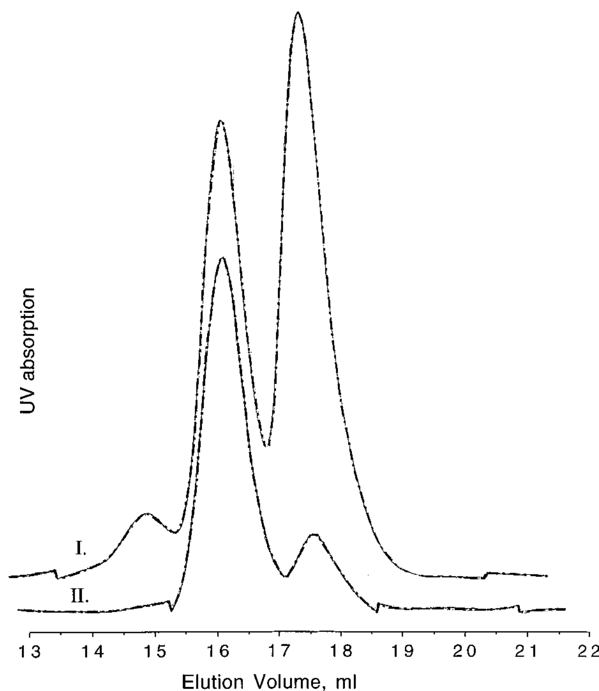


Figure 5. (I) SEC chromatograph of mixture of BSA (1 mg/mL) and β-lactoglobulin (1 mg/mL). First peak: BSA. Second peak: β-lactoglobulin. (II) SEC chromatograph of supernatant from BSA (1 mg/mL)/β-lactoglobulin (1 mg/mL)/PDADMAC coacervation. First peak: BSA. Second peak: β-lactoglobulin. Coacervation conditions are the same as in Figure 3.

tration (Xia and Dubin, 1994). In this case, we may speculate that polymers of larger MW form larger protein–polymer intrapolymer complexes of lower solubility. Alternatively we can suggest that phase separa-

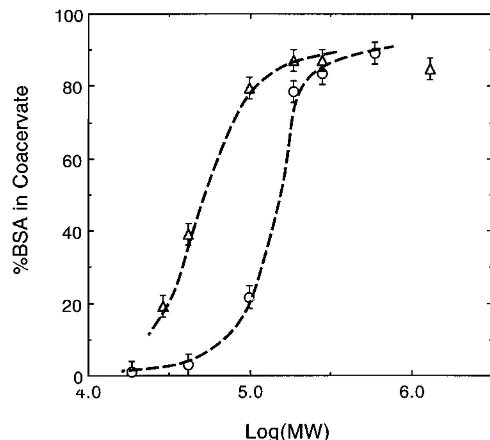


Figure 6. Effect of polymer molecular weight on BSA removal at pH 8.0 (○) and pH 9.0 (△); ionic strength = 0.1, protein concentration = 0.5 mg/mL, protein/polymer ratio = 5.

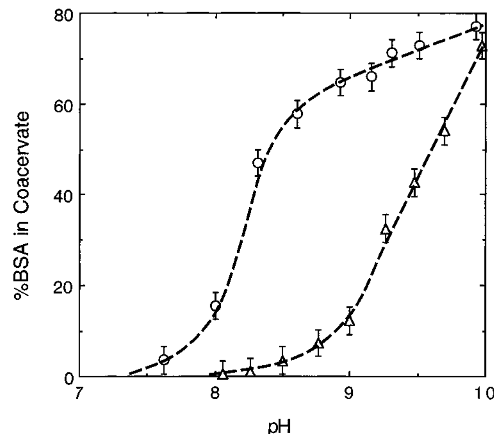


Figure 7. Effect of pH on BSA removal by PDADMAC fraction with MW 1.0×10^6 (○) and MW 9.8×10^4 (△); ionic strength = 0.1, protein concentration = 0.5 mg/mL, protein/polymer ratio = 5.

tion only occurs when the number of proteins bound per polyion produces a species with zero net charge. Since only an integral number of protein molecules can be bound, low-MW polymers may not be able to form uncharged complexes because these polymers lack the necessary sites to bind an integral number of proteins. Once the MW of polymer is sufficiently large, the solubility of the protein−polymer complexes becomes less and less dependent on the polymer chain length. On the other hand, at high pH and low ionic strength, the electrostatic interactions are more intense, leading to greater release of small ions and a concomitant drop in complex solubility. The requirements for overall charge neutrality are less restrictive, and relatively high efficiency of protein recovery is observed regardless of the molecular weight of PDADMAC, as shown in Figures 7 and 8. The effect of molecular weight thus differs in low ionic strength solution from that in high ionic strength solution (Figure 8).

b. Effect of the Protein/Polyelectrolyte Ratio on Protein Separation. The effect of the [protein]/[polyelectrolyte] ratio (r) was studied at $C_p = 0.1$ g/L and $I = 0.1$ M. BSA and PDADMAC were mixed at different ratios at pH = 8.0, 8.5, 9.0, or 9.5. As shown in Figure 9, the yield displays a maximum with increasing r value. The position of this coacervation maximum occurs at lower r values for solutions of higher pH. If complexation is treated via the formalism of ligand binding, in which unbound BSA is free ligand, and BSA in the coacervate corresponds to "bound" ligand, Figure 9 can then be viewed as a type of "binding curve" which shows a stronger binding at a higher pH, as expected. It is also worth noting that, when pH > 9.0, the r values for maximum coacervation are all close to 5.0. If it were assumed that the binding constant were very large, then these findings could be treated in stoichiometric terms, i.e. based on the net charge of the two species (only the protein charge is pH-dependent). However, at moderate ionic strength, this is not the case, and there is even evidence that protein-free polymer and unbound protein can be in equilibrium with the complex at certain conditions (Li et al., 1996).

c. Quantitative Determination of BSA Bound to PDADMAC. The average number of protein molecules bound per polymer chain, n', was determined at the point of maximum coacervation by measuring the concentrations of PDADMAC and BSA in the supernatant. The presence of polyelectrolyte in the supernatant phase was monitored before and after coacervation by QELS and SEC with RI detection. It was found that, after the point

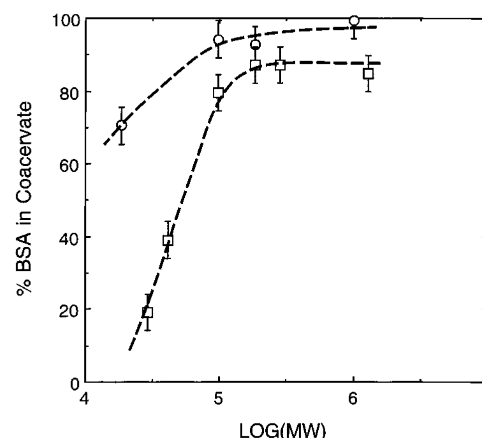


Figure 8. Effect of polymer molecular weight and ionic strength on BSA removal: ionic strength = 0.04 (○), ionic strength = 0.1 (□); protein concentration = 0.5 mg/mL, protein/polymer ratio = 5, pH = 9.0.

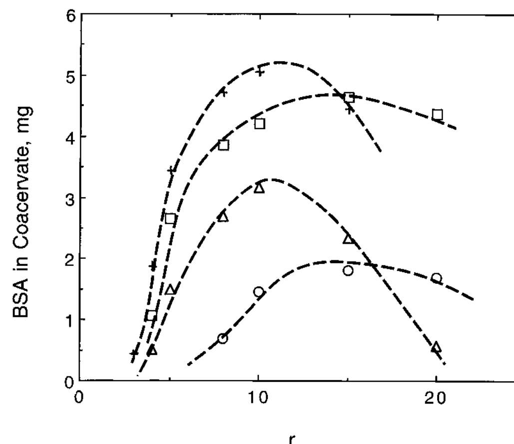


Figure 9. Effect of protein/polymer ratio on BSA removal at pH 8.0 (○), pH 8.5 (△), pH 9.0 (□), pH 9.5 (+), ionic strength = 0.1.

of maximum coacervation, no polyelectrolyte exists in the supernatant, i.e. all of the polyelectrolyte is in the coacervate phase. This result simplifies the task of calculating n'. Figure 10 shows that n' at the point of maximum coacervation increases sharply with increasing pH, then remains relatively constant when pH > 9.0. If maximum coacervation simply corresponded to stoichiometric neutralization of charges, then the number of proteins in the coacervate should decrease when their

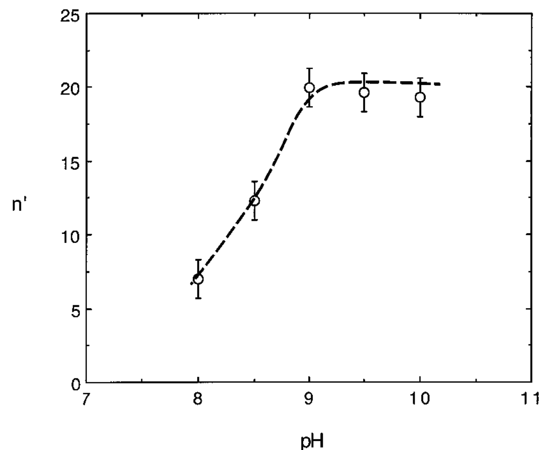


Figure 10. Effect of pH on number of BSA bound per polymer chain at the maximum coacervation, ionic strength = 0.1. n' is the number of BSA bound per polymer chain. Polymer MW = 2.6×10^5.

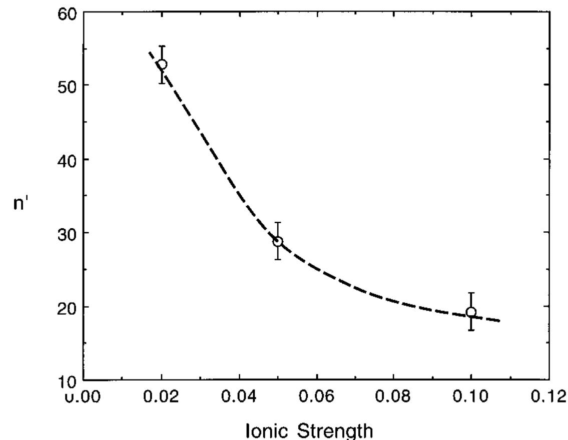


Figure 11. Effect of ionic strength on number of BSA bound per polymer chain at the maximum coacervation, pH = 9.0. n' is the number of BSA bound per polymer chain. Polymer MW = 2.6×10^5.

average charge is more negative, i.e. n' should decrease with increasing pH. Resolution of this question may require consideration of the fate of the counterions: the number of proteins bound could increase, with preservation on charge neutralization, if there is a increase in the expulsion of Cl− from the polymer, i.e. "tighter binding". "Stoichiometry" in solutions of moderate ionic strength is thus a more complicated matter than in salt-free polyelectrolyte−protein systems (Ahmed et al., 1994). The break point at pH = 9.0 is also not well understood. One possible explanation is that the polymer chains are saturated at the point of maximum coacervation when pH > 9.0. Figure 11 shows that n' decreases with increasing ionic strength. This clearly indicates that "binding affinity" is lower at high ionic strength.

Strege et al. (1990) determined n' in a different way. Assuming that neutral protein−polyelectrolyte complexes would form at the critical pH of coacervation, Strege et al. (1990) calculated n' by converting the critical pH of the solution into the protein charge density based on published protein pH−titration curves. The average binding number thus calculated is ca. 60 in the solution of $I = 0.1$ at critical pH (pH = 7.4). The difference between the results obtained in this research and that reported by Strege et al. is due in part to differences in solution conditions and in part to the point along the Type I titration at which the measurement is made.

Quantitative studies of protein−polymer phase separation may help to elucidate the mechanism of binding of proteins to polyelectrolyte. The determination of binding number would in particular provide insight. However, since the complexation is biphasic, simple binding models such as those proposed by Scatchard or Hill are not very suitable. Appropriate modeling of the system remains a challenge for future studies.

Summary

The selectivity of protein separation via polyelectrolyte coacervation depends mainly on pH. In the pH where only one protein coacervates with the polyion, selectivity is good. In the pH region where both proteins form coacervate with the polyelectrolyte, good selectivity can only be achieved for certain protein mixtures, in which the mutual effect of the two proteins enhances the binding to polymer of one protein while depressing the other. The efficiency of protein coacervation was found to increase with polymer molecular weight, with increasing pH, and with diminishing ionic strength.

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