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Influence of Polymer Structure on Protein Partitioning in Two-Phase Aqueous Systems

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Copolymers of acrylamide and styrene with two distinct structures were synthesized to study the effect of polymer structure on protein partitioning in two-phase aqueous systems. Micellar copolymerization was used to prepare a multiblock copolymer, while homogeneous copolymerization was used to prepare a random copolymer, both with the same composition and molecular weight. Phase behavior studies of the copolymers with poly(ethylene glycol) in water showed little difference in the phase boundaries. However, the partitioning of bovine serum albumin between the two aqueous phases was sensitive to the polymer structure. A molecular picture is proposed for the interactions between the protein and block copolymers. The effect of pH on the protein partition is analyzed in terms of the hydrophobic interactions, and the polymer and protein partitioning was correlated using a model based on the Flory−Huggins theory.

1.0. Introduction

Progress in technology based on biochemistry and cell biology depends to a great extent on the development of efficient separation methods. Economic analysis shows that purification is often the most important aspect of biomolecular production and processing. This is particularly true of protein processing which, because of the complexity of the starting material, often requires many steps to reach purity levels required for medical and food applications.

Liquid−liquid distribution methods (Albertson, 1971, 1986; Abbot and Hatton, 1988) are of special interest because they can be scaled up rather easily. The basis of separation by a two-phase system is the selective distribution of substances between the phases. For soluble substances, distribution takes place mainly between the two bulk phases, and the partition is characterized by the partition coefficient, K, defined as

$$K = \frac{C_t}{C_b} \tag{1}$$

where C_t and C_b are the concentrations of the partitioned substances of the top and bottom phase, respectively. The choice of a suitable phase system is the key step in all partition work. The factors that influence separation and purification are the partition coefficient, polymer cost, polymer recovery and recycling, density and viscosity of the polymer solutions, and the end use. The partition coefficient is influenced by molecular weights, concentrations, temperature, polydispersity, and functional groups.

In this work, we studied the influence of the polymer structure on protein partition coefficient. For this purpose we synthesized block and random copolymers of acrylamide and styrene with the same composition (around 1 mol % styrene) and molecular weight and studied their phase behavior with poly(ethylene glycol).

The water-soluble polymer of acrylamide/styrene was chosen as the second polymer because of the following reasons. First of all, a reasonably simple procedure is available for synthesizing it in multiblock and random forms. Second, the effect of the polymer structure will be highlighted due to the large difference in the hydrophobicity of the two monomers. Third, acrylamide contains the amide groups which interact strongly with proteins. And finally, the two monomers are common and relatively cheap.

2.0. Materials

Acrylamide was used as received from Fluka AG, Chemische Fabrik. Styrene from the same company was vacuum distilled prior to use. Sodium dodecyl sulfate (SDS) and potassium persulfate were purchased from BDH Limited Pool, England. Poly(acrylamide/styrene) (PAS), both random and block, having the same molecular weights were synthesized. All aqueous solutions were prepared using deionized water exclusively. Other materials used were methanol and serum albumin (BDH Limited, Pool, England), formamide (Fluka AG, Chemische), poly(ethylene glycol) (PEG) with molecular weights of 10 000 and 35 000 (Merck-Schuchardt), and sodium phosphate (J. T. Baker Chemical Co.).

3.0. Polymer Preparation

Copolymerization of a hydrophobic monomer with a hydrophilic monomer can result in an amphiphilic polymer, the specific nature of which can be controlled via polymerization parameters. The dual hydrophilic/hydrophobic nature provides these materials with unique interaction and solubilization characteristics. Water-soluble amphiphilic polymers are of particular interest due to the presence of microdomains that may impart unusual properties to a given system. In this work, acrylamide−styrene copolymers were synthesized that provide hydrophobic sites in aqueous solutions. Two different polymerization processes are used:

(i) *Micellar Copolymerization:* For the production of a multiblock copolymer of acrylamide−styrene.

(ii) *Homogeneous Copolymerization:* For the production of a random copolymer of acrylamide−styrene.

3.1. Micellar Copolymerization. There are major differences between the micellar copolymerization and the more conventional polymerization carried out in the

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Table 1. Composition and Molecular Weights of the Synthesized Polymers

copolymer (sample code)	[styrene][a]	[acrylamide][b]	[sodium dodecyl sulphate][c]	[potassium persulfate][d]	[formamide][e]	reaction time (h)	intrinsic viscosity	apparent mol wt
P(AM/Sty)9	2.25	5	2	1		4.8	2.0	1.798×10^5
P(AM/Sty)52	2.25	5	2	8		1.5		
P(AM/Sty)57	1.7	5	2	8		1.5		
P(AM/Sty)56	1.12	5	2	8		1.5	1.74	1.456×10^5
P(AM/Sty)30	2.25	5		1	10	48		
P(AM/Sty)77	1.12	5		1	10	1.5	1.78	1.507×10^5

[a] Mole percent in the feed = [styrene]/[styrene] + [AM]. [b] Monomer concentration in weight percent. [c] Weight percent based on volume of water. [d] Weight percent relative to monomer feed. [e] Percent by volume of water.

presence of a surfactant, i.e., emulsion or microemulsion polymerization. These are the amount of surfactant added, the solubilities of the monomers, and the phase where polymerization occurs.

In the micellar process, initially reported by Evani (1982, 1984), Bock et al. (1984), and Turner et al. (1985), the hydrophobic monomer (styrene in this work) is solubilized within the surfactant micelles, whereas acrylamide is dissolved together with the initiator (potassium persulfate in this work) in the aqueous continuous medium. The surfactant used in this study was sodium dodecyl sulfate (SDS) at concentrations between 4 and 20 times its critical micellar concentration. The reaction mixture was optically transparent.

The experimental procedure used for the micellar process is as follows. Aqueous solutions of acrylamide were placed in 500 mL Erlenmeyer flasks covered with septum caps and were degassed by gentle bubbling with nitrogen for 30 min while stirring. The sodium dodecyl sulfate was added. This was followed by injection of styrene with a syringe into the reaction mixture which was stirred continuously until a homogenous solution was obtained (within 0.5−1 h). Then the flask containing the reaction mixture was placed in the temperature-controlled oil bath at 50 °C under magnetic stirring. Polymerization was initiated by the injection of potassium persulfate solution with a syringe. The reaction mixtures and times are given in Table 1.

The polymers were precipitated by carefully pouring the solutions into methanol which was constantly stirred and six times in excess for the ease of precipitation. After filtration through filter paper, each polymer was washed repeatedly with methanol to remove all traces of water, surfactant, and residual monomer. After filtering again, it was dried under vacuum at 50 °C for 2 days and then the polymer (residue) was crushed and dried again for an additional few hours. Details about the experimental procedures and materials are reported elsewhere (Ijaz, 1995).

3.2. Homogenous Copolymerization. The procedure for this method of synthesis was analogous to that described in the previous section, except that for the homogeneous copolymerization there is no surfactant. In the homogenous process it was important to dissolve the styrene monomer first in formamide before adding the aqueous acrylamide solution.

3.3. Procedure for Protein Partitioning. The PAS/PEG/water system was used for the partitioning of the protein bovine serum albumin (BSA). The partitioning of the BSA was studied in block and random copolymers of acrylamide styrene. A solution of 0.01 M sodium phosphate was used to control pH at 7.0. The polymer solutions were prepared by weight in duplicate for both random and block PAS. Into one of the resulting two-phase systems, a known quantity of protein solution was added. The protein-free two-phase system served as the reference solution in the spectrophotometer for the measurement of protein concentration. The resulting polymer solutions were gently centrifuged for 15 min and then equilibrated for 48 h at 23 °C. In order to determine the concentrations of proteins in each of the coexisting phases, samples from each phase of the solution were separated using a syringe or small pipette. First, without disturbing the fragile liquid−liquid interface between the two phases, a sample of the top PEG-rich solution phase was carefully collected. Following this, the remainder of the top phase was sucked from the interfacial region using a Pasteur pipette. The interfacial sample, which typically contained a mixture of top and bottom phases, was then discarded. The remaining solution, namely the bottom PAS-rich phase, was withdrawn and then prepared for the measurement of protein concentration as follows.

3.4. Determination of Polymer Concentration. The samples were diluted prior to the measurement of the protein absorbance. If the samples were not diluted, streaks appeared in the polymer solutions as they were pipetted into spectrophotometric cuvettes, which in turn scattered light during the measurement of the protein absorbance. The absorbance of bovine serum albumin was measured at 282 nm using a Perkin-Elmer Lambda 5, UV/vis spectrophotometer, utilizing the corresponding protein-free two-phase system as a reference.

3.5. Polymer Molecular Weight and Composition. The average molecular weights of the synthesized polymers were obtained from the intrinsic viscosities which were determined in aqueous solutions at 30 °C. Viscosity measurements were carried out with an Ostwald's viscometer at 0.125, 0.25, 0.5, and 1.0 wt %. In these experiments, care must be taken to filter the solutions properly, which would otherwise result in erroneous flow times. Each reading is taken three times, and the average value is used for the calculation of the molecular weight of the copolymers. The molecular weights were determined from the Mark−Houwink−Sakurada equation for polyacrylamide (Kurata and Stockmayer, 1963):

$$\frac{[\eta]}{100}\left(\frac{\text{cm}^3}{\text{g}}\right) = (6.8 \times 10^{-4})M^{0.66} \qquad (2)$$

Although the constants in the above equation are for the polyacrylamide homopolymer, the small percentage of polystyrene, around 1.12 mol %, in the copolymer justifies the use of the same equation for comparing the molecular weights of the multiblock and random copolymers (Hill et al., 1993). The molecular weights are reported in Table 1. The block copolymer sample 56 and the random copolymer sample 77 were selected for phase behavior and partitioning studies because they have close molecular weights and gave very clear aqueous solutions. In addition, the composition of the final copolymer should be the same as the feed composition (1.12 mol %), as was shown by Hill et al. (1993).

4.0. Phase Behavior

The study of phase diagrams is an important step in this work. The binodal curve distinguishes between

single- and two-phase regions and provides information about the concentration of both the polymers required for protein separation. Phase diagrams may be constructed in many different ways. The turbidity method is used in this work.

The phase boundaries of random and block copolymers of acrylamide and styrene with poly(ethylene glycol) with average molecular weights of 10 000 and 35 000 in aqueous solution were determined. The samples of polymer solutions were prepared with different concentrations. The PEG solution and deionized water were taken into two different 10 mL burrettes. A predetermined quantity of PAS was taken to a flask, and PEG solution was added dropwise until the transparent system turned turbid. Then water was added dropwise until the system became transparent again. At this point, the final composition of the two polymers calculated corresponds to one point on the binodal curve. After obtaining the first point, a concentrated solution of PEG was added again to obtain a turbid dispersion, and dilution with water was repeated to obtain a second point on the binodal curve. This procedure was continued until a sufficient number of points for the construction of the binodal curve were obtained. The experiments were carried out at 23 °C.

The binodal curve for the PAS/PEG/water system shows a typical behavior of such systems in Figure 1. It was found that the changes in the molecular weight of PEG affect the binodal curve considerably. An increase in the molecular weight of PEG pushes it downward. With PEG-35000 the binodal curve shifts downward compared with PEG-10000 as shown in Figure 1a,b. This implied that smaller concentration of PEG-35000 is required for phase separation compared with that of a PEG-10000 in PAS/PEG/water system. Another interesting finding was that both block and random copolymers of acrylamide−styrene with same molecular weight exhibit almost identical phase diagrams with PEG-35000 as shown in Figure 1b,c.

In order to facilitate the use of aqueous two-phase systems, a sound theory is needed for the correlation of phase equilibrium behavior. Several theoretical models have been proposed for the thermodynamic behavior of aqueous two-phase systems and protein partitioning. Brooks et al. (1985) and Albertsson et al. (1986) have shown that the lattice model of Flory (1942) and Huggins (1942) could be used to qualitatively predict protein partitioning trends. Here we use the version suggested by Diamond and Hsu (1992):

$$\ln(K_1) = A_1(w_1'' - w_1') \quad (3)$$

$$\ln(K_2) = A_2(w_1'' - w_1') \quad (4)$$

where w'' and w' are the polymer weight fractions in the upper and lower phase and A_1 and A_2 are functions of the polymer molecular weights and the interactions between the polymers and water. Equations 3 and 4 provide a means for correlating phase equilibrium data for aqueous polymer systems. The correlation results are shown in Figure 2. Straight lines are obtained as expected. Figure 2a,b reveals that the slope of the PAS (block) line becomes more negative as PEG molecular weight increases from 10 000 to 35 000 because the slope is a function of polymer molecular weight in addition to the interaction between the polymers and water. The phase behavior of the PAS (random)/PEG (35 000)/water system is shown in Figure 2c.

5.0. Protein Partitioning

5.1. Effect of Polymer Structure and Concentration. It was found that, at pH = 7.0, the block copolymer showed much better selectivity for protein partitioning compared to that of the random copolymer, as shown in Figure 3. The polymer molecular weight affects the partitioning, but since the molecular weights of the block and random copolymers are similar, it was concluded that the polymer structure is the reason for the difference in the protein partitioning. The polyacrylamide repeat units, $CH_2CHCONH_2$, interact more favorably with the protein than the poly(ethylene glycol) repeat unit, CH_2-CH_2O, due to the presence of the amide group in the former. In the block copolymer, the highly hydrophobic monomer (styrene) is arranged in a block form, which may be providing concentrated repulsive interactions with the mostly hydrophilic surface of the protein (BSA) as compared with the random copolymers where the hydrophobic monomer is distributed randomly into the

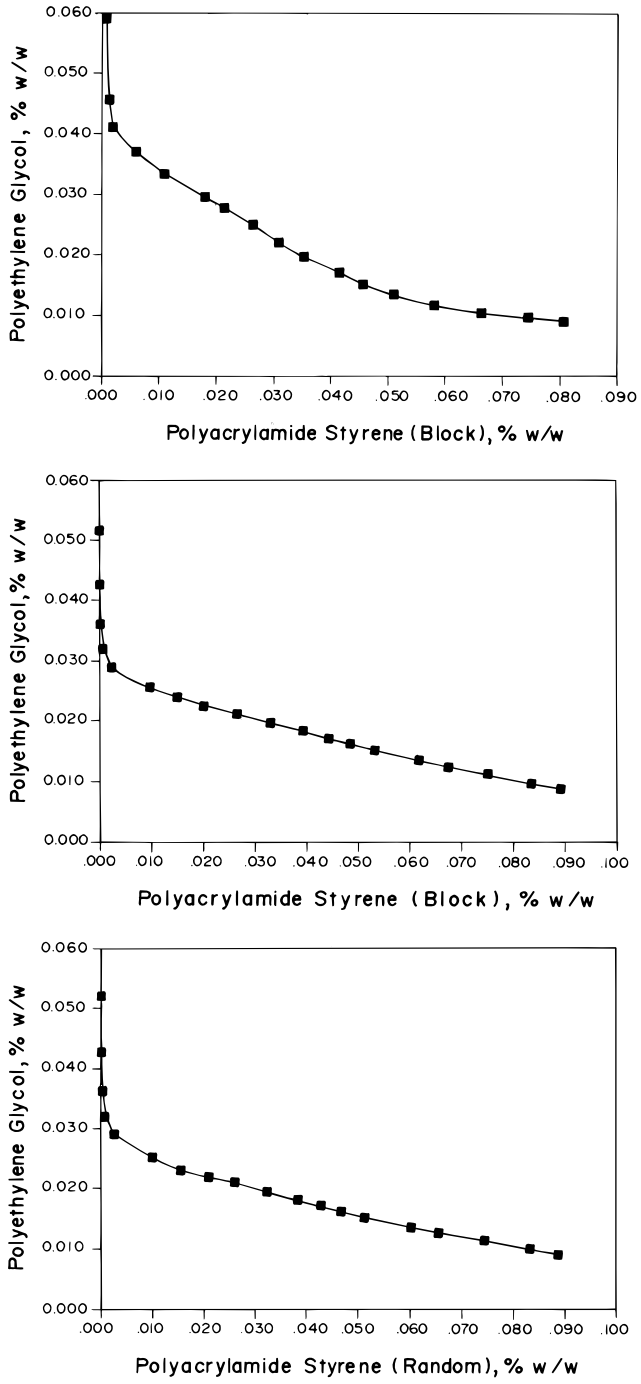

Figure 1. Phase diagrams of poly(ethylene glycol) (PEG) with poly(acrylamide-*co*-styrene) in water at 23 °C: (a) PEG-10000 with block PAS, (b) PEG-35000 with block PAS, (c) PEG-35000 with random PAS.

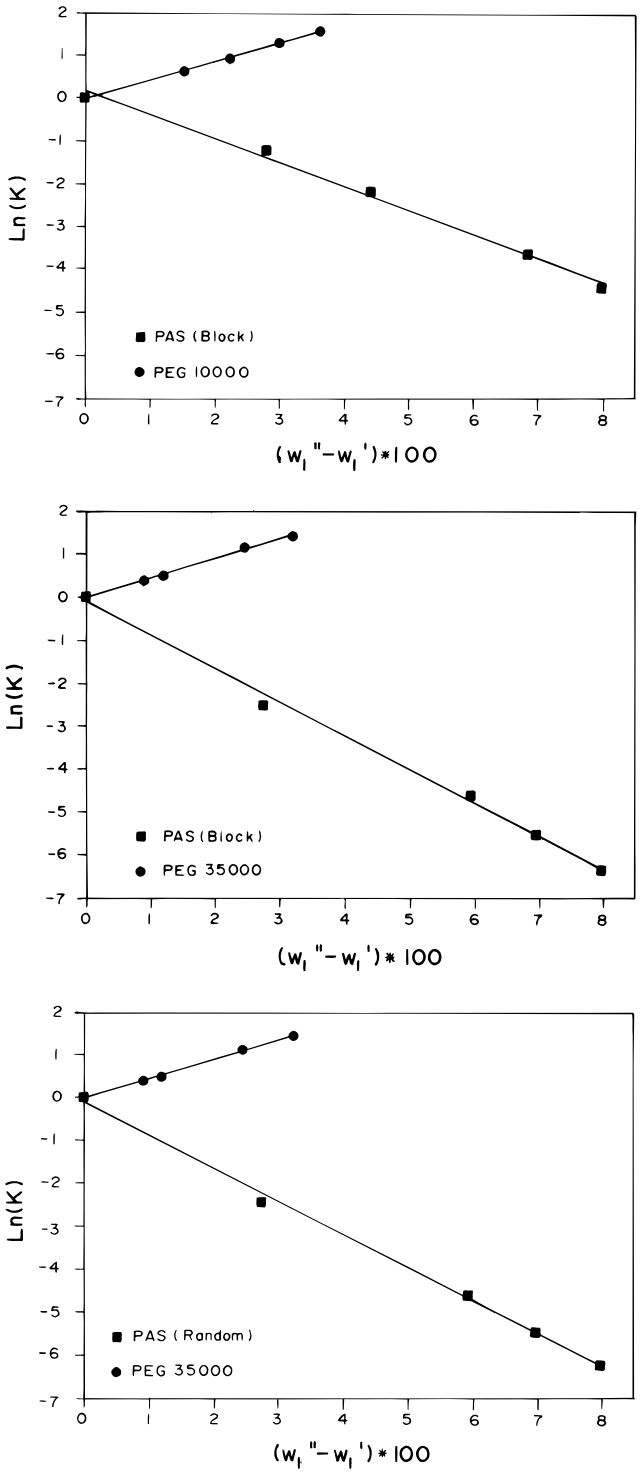

Figure 2. Correlation of the phase diagrams of poly(ethylene glycol) (PEG) with poly(acrylamide-*co*-styrene), PAS in water using the method of Diamond and Hsu (1992): (a) PEG-10000 with block PAS, (b) PEG-35000 with block PAS, (c) PEG-35000 with random PAS.

polymer chain, diluting the hydrophobic interactions. Therefore, in the block copolymer system, the protein molecules prefer the PEG-rich top phase to avoid the hydrophobic styrene blocks. In the random copolymer system, the styrene monomer occurs mostly in single units which have little effect on the overall interactions between the polyacrylamide and the protein. It is also possible that these lonely styrene units will interact favorably with one of the few hydrophobic sites on the protein surface.

It was observed that the partition coefficient of BSA increases by increasing the concentration of PAS as

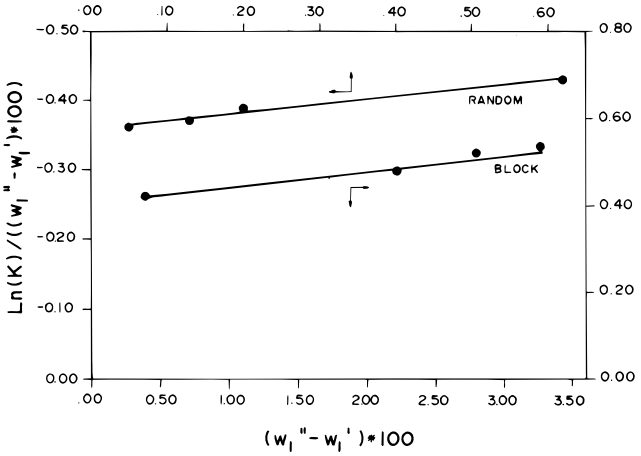

Figure 3. Bovine serum albumin (BSA) partitioning in PEG (35 000)/PAS random and block systems. Points represent experimental data, and lines represent the correlation of Diamond and Hsu (1990).

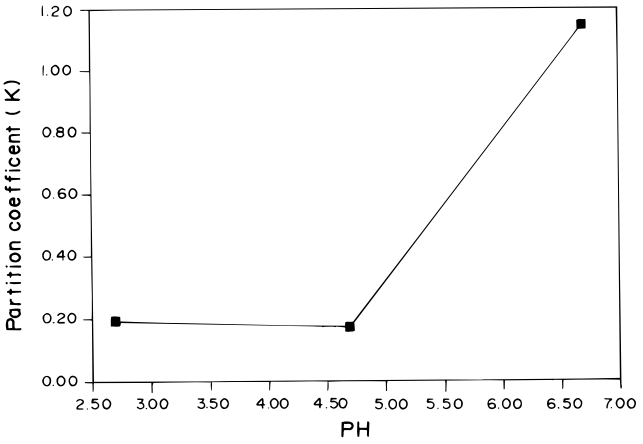

Figure 4. Effect of pH on bovine serum albumin (BSA) partitioning in PEG-35000 with block PAS.

shown in Figure 3. The shifting of protein molecules was from the higher molecular weight PAS phase toward the lower molecular weight PEG phase. All these studies were carried out at pH = 7.0. The effect of pH on the partitioning of BSA is presented next.

5.2. Effect of pH. The protein partition coefficient, *K*, defined as the ratio between the concentration of protein in the top and the bottom phases depends on , among other factors, the pH of the system. Hence an investigation was made to determine the effect of pH on protein partitioning. The system was prepared in exactly the same way as described in sections 3.3 and 3.4 except that the amount of buffer was varied in order to adjust the pH. The buffer used was 0.1 M HCl. The pH was measured with a microelectrode. The rest of the procedure for protein concentration measurement is the same as discussed in the above-mentioned sections.

The effect of pH on the partitioning of protein was studied at the isoelectric point of BSA (pI = 4.7). At this pH value the protein carries equal positive and negative charges. This implies that the hydrophobic repulsion becomes weaker as compared with the hydrophilic attraction between the acrylamide units and the protein. Therefore, at pH = 4.7, the net effect was shifting of BSA toward the PAS-rich bottom phase as shown in Figure 4 (the data points are connected to highlight the expected behavior). This result is in agreement with the fact that, in the absence of styrene, BSA is present in the polyacrylamide-rich phase. On the other hand, at a pH of 2.7, there was little change (slight increase) in the partition coefficient. It is due to the fact that BSA has

five isomeric forms, E, F, B, N, and A, depending on the pH value. Below a pH of 4.0, albumin becomes fully uncoiled (form E) as reported by Forcinit (1991). This resulted in the appearance of a net charge on the protein molecule and in the exposure of some hydrophobic parts of BSA. The charges repel the styrene blocks, and the hydrophobic parts on the protein attract them. The balance of these two factors caused little change in the partition coefficient compared to its value at the isoelectric point.

5.3. Correlation for Protein Partitioning. Several theoretical models have been proposed for the thermodynamic behavior of aqueous two-phase systems and protein partitioning. Brooks et al. (1985) and Albertsson et al. (1986) have shown that the lattice model of Flory (1941) and Huggins (1942) could be used to qualitatively predict protein partitioning trends. The following form proposed by Diamond and Hsu (1990) was used in this study:

$$\frac{\ln(K)}{(w_1'' - w_1')} = A^{**} + b^*(w_1'' - w_1') \qquad (5)$$

Equation 5 represents a simple second-order semilogrithmic relationship for correlating protein partitioning in aqueous two-phase systems, where the intercept A^{**} is a function of the molecular weights of the protein and the polymers, the interaction parameters for the protein−water, protein−polymer, and polymer−water pairs, pH, and concentration. Similarly, the slope b^* is a function of the molecular weight of the protein, the polymer−water interaction parameters, pH, and concentration. It is quite interesting that A^{**} and b^* contain χ_{01} and χ_{02}, which represent the interactions between water and PEG and water and PAS, respectively. The consistency of the protein partitioning data is indicated by the linear behavior in Figure 3.

6.0. Summary and Conclusions

A pausible physical picture is proposed for the interactions between protein and block copolymers that can be used to design polymer systems with enhanced partitioning coefficients. It is recommended to carry out more experiments with different copolymers to shed more light into the process. It is proposed that the copolymer blocks can be used to provide concentrated attractive or repulsive interactions with proteins. For the poly(ethylene glycol)/poly(acrylamide-*co*-styrene)/water system, the protein bovine serum albumin showed different levels of interaction with the block copolymer compared to the random copolymer, where the hydrophobic monomer is distributed randomly in the polymer chain. Observation showed that the block copolymer (PAS) forced most of the bovine serum albumin protein molecules out of the PAS phase and into the PEG phase. The presence of strong hydrophobic units (styrene) in the block form provides enough repulsive interactions to drive the BSA into the PEG-rich phase. In the random copolymer of the same composition and molecular weight, the hydrophobic interactions were diluted by the randomness of their distribution and, therefore, most of the BSA molecules remained in the PAS phase.

The study of phase behavior clearly demonstrated that, for the system under consideration, the polymer structure has little effect on the phase boundaries. This was probably due to the small number of the styrene units compared to the acrylamide units. However, protein partitioning was more sensitive to the copolymer structure.

The effect of pH on the partition behavior of protein (BSA) was also studied. At three different values of pH, it was found that the partition coefficient has a strong dependence on the pH of the system. The partition coefficient of BSA has a minimum value close to its isoelectric point (pI = 4.7), where the protein carries equal positive and negative charges. Therefore, at the isoelectric point, the hydrophobic repulsion is at its minimum. This causes the accumulation of most of the protein molecules in the PAS-rich bottom phase. It was found that the partition coefficient did not change much by decreasing the pH of the system. At pH = 2.7, a slight increase in the value of the partition coefficient is observed due to the particular form that the protein takes at this pH value.

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