PROTEIN PARTITIONING IN pH-RESPONSIVE POLYMERIC AMINO ACID AND ASSOCIATING POLYMERS

BY

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A Thesis Presented to the

DEANSHIP OF GRADUATE STUDIES

KING FAHD UNIVERSITY OF PETROLEUM & MINERALS

DHAHRAN, SAUDI ARABIA

In Partial Fulfillment of the
Requirements for the Degree of

MASTER OF SCIENCE

In

CHEMICAL ENGINEERING

MAY 2002

KING FAHD UNIVERSITY OF PETROLEUM & MINERALS

DHAHRAN 31261, SAUDI ARABIA

DEANSHIP OF GRADUATE STUDIES

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DEDICATION

To my uncle
Dr. Shehu N. A. Saidu
and his family

ACKNOWLEDGMENT

In the name of Allah, the Beneficent, the Most Merciful. Praises and thanks be to Allah, the Lord of the worlds, and peace be upon the last messenger, Muhammad, his Family and Companions.

Acknowledgement is due to the King Fahd University of Petroleum & Minerals for providing facilities and KACST for financial support, towards this research. I also wish to acknowledge Ahmadu Bello University, Zaria for granting me Study Fellowship.

My gratitude and acknowledgment are due to Dr. Basel F. Abu-Sharkh, my thesis advisor, for his constant support, encouragement and guidance. Also, I am very grateful to my committee members: Dr. S.A. Ali, for his help with the polymer synthesis and providing me with training on phase behavior studies; Dr. I. Abu-Reesh, Dr. I. Hussein and Dr. R. Alnaizy for their invaluable comments and suggestions. I appreciate the help I received from the following: Mr. I. W. Kazi on the use of spectrophotometer; Mr. Mohammed Arab for providing NMR analysis of my samples; and Dr. M. M. Khaleed for his assistance on the use of electrochemical apparatus. I also want to express my gratitude to Mr. Waleed for typing the Arabic abstract of this thesis.

My thanks also go to the Chairman, Dr. A. A. Shaikh, and the entire faculty and staff of the chemical engineering department for various helps rendered to me during the course of my MS program. I want to particularly mention and acknowledge the tremendous

assistance as well as advice and encouragement I received from Mr. N. M. Tukur and Dr. U. A. El-Nafaty.

I am pleased to thank Mr. Jibril Baba El-Yakubu for introducing me to this great university and I remain highly indebted to Mr. Hassan Hammawa for the great role he played in my coming to KFUPM. I extend my regards to the African community in KFUPM and all my friends, most especially Yau, Yunus, Ahmad, Tijani, and Abdul Aziz (Abu Yazid), for providing me with wonderful company.

My sincere appreciation goes to my parents, brothers, sisters and the entire members of Waziri family for their love, sacrifice and prayers. A special thanks to Umar Hassan Waziri for keeping me constantly in touch with home. I greatly value your indefatigable efforts. I wish to thank my beloved one, Aisha, for her patience, encouragement, and prayers.

Finally, I pray to Almighty Allah to reward all those who contributed, either directly or indirectly, to the success of this work.

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THESIS ABSTRACT

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TITLE: PROTEIN PARTITIONING IN pH-RESPONSIVE POLYMERIC AMINO ACID AND ASSOCIATING POLYMERS

MAJOR: CHEMICAL ENGINEERING

DATE: MAY 2002

Partitioning of proteins in aqueous two phase systems has emerged as one of the important downstream processing techniques in biotechnology. The phase behavior of aqueous two-phase polymer system containing a new pH-responsive polymeric amino acid (PAA) and polyethylene glycol (PEG) was investigated as a function of pH, salt concentration, and polymer concentration. The effect of pH and salt concentration on the partitioning behavior of bovine serum albumin (BSA) and cytochrome c in the PAA-PEG system was also studied. Unlike the BSA, the cytochrome c was found to preferentially partition into the PAA-rich (bottom) phase under all conditions of pH and salt concentrations considered in the study. Electrochemical potential difference was found to play a major role in the partitioning behavior of the two model proteins. Correlation studies of both the phase behavior experimental data and the protein partitioning results were conducted using various theoretical models.

MASTER OF SCIENCE DEGREE

KING FAHD UNIVERSITY OF PETROLEUM & MINERALS

Dhahran, Saudi Arabia

May, 2002

ملخص الرسالة

الاسم: سعيدو محمد وزيري

عنوان الرسالة: فصل البروتينات في المحاليل المائية الثنائية لبوليمرات مستجيبة لدرجه الحموضة وبوليمرات مترابطة

التخصص: هندسة كيميائية

تاريخ التخرج: مايو ٢٠٠٢

يعتبر فصل البروتينات في المحاليل المائيه الثنائيه أحد ابرز التطورات في عمليات الفصل في التقنيه الحيويه. تمت دراسه حالة الطور في المحاليل المائيه الثنائيه لمحاليل تحتوى على بوليمر يستجيب للتغيير في درجه الحموضه ومكون من أحماض امينيه مع البولي اثيلين جلايكول. وقد تمت هذه الدراسة عند درجات مختلفه من الحموضه وتركيز الأملاح وتركيز البوليمرات. ثم تمت دراسه فصل البروتينات بوفين سيرم البومين وسيتو كروم سى في هذه المحاليل الثنائيه واعتماد هذا الفصل على درجه الحموضه والملوحه. وقد وجد إن بروتين سيتو كروم سى يفضل دائما الطور الغنى بالبولى امنولايت بعكس بروتين البوفين سيرم البومين. وقد وجد إن فرق الجهد بين الطورين يلعب دورا مهما في عمليه الفصل. كما تم تطوير نماذج رياضية لنمذجه عمليه الفصل وتصرف الطور باستخدام نظريات مختلفه.

درجه الماجستير في العلوم

جامعة الملك فهد للبترول والمعادن

الظهران، المملكة العربية السعودية

CHAPTER 1

INTRODUCTION

Advances made in molecular biology and recombinant DNA technology have made possible the production of unprecedented number of new proteins important to the food, pharmaceutical, medical and chemical industries. In most cases the proteins of interest are produced in a highly complex mixture of contaminating proteins, cell wall material, and nucleic acids. Separation from these complex mixtures is frequently complicated by the labile nature of the desired products and by the stringent final product purity specifications. This explains why downstream processing usually accounts for the highest part of the production costs of proteins. Thus, separation and purification of proteins is a critical element of modern process biotechnology because it provides a vital link between laboratory discoveries and large-scale productions.

In recent years there has been an on-going interest for the development of innovative and efficient separation and purification methods in the biotechnology industry. Liquid-liquid extraction, using aqueous two-phase systems (ATPS), shows promise as a cost effective, large-scale process that can achieve high selectivity and purity requirements. This novel separation methodology exploits the remarkable fact that many aqueous polymer-polymer systems, under appropriate solution conditions, can spontaneously separate into two water-based, yet immiscible, phases. The phases offer different physical and chemical environments, which allow for the selective partitioning of proteins and other

biomolecules while maintaining their native conformations and biological activities. The distribution of protein between the two phases is characterized by the partition coefficient, K, defined as

$$K = \frac{C_t}{C_b} \tag{1.1}$$

where C_t and C_b are the concentrations of the partitioned substances of the top and bottom phases, respectively. While the degree of separation G is determined by:

$$G = K\frac{V_t}{V_b} \tag{1.2}$$

where V_t and V_b denote the volume of the top and bottom phase respectively. The partitioning of proteins in aqueous two-phase systems depends mainly on the physicochemical properties e.g. protein hydrophobicity, charge and size. The partitioning can be influenced by changing polymers, polymer molecular mass, temperature, the pH or by addition of salts to the system. The partition coefficient of the protein of interest sets the specifications for the extraction equipment required. Moreover, knowledge of the partitioning characteristics of each protein is essential in other applications of aqueous two-phase systems.

Aqueous two-phase systems offer numerous advantages over conventional protein purification methods such as membrane systems, chromatographic methods and centrifugation methods. The advantages include:

1. High biocompatibility owing to;
   (a) Very low interfacial tensions (0.1 to 100 μN/m),
   (b) The fact that each phase contains principally water (85-99 wt %), and

(c) The fact that the polymers are also thought to contribute to protein stability, thereby decreasing the possibility of denaturation of labile biomolecules observed in non-aqueous systems;

2. Good resolution and yields, which can be enhanced dramatically, if needed, by the addition of affinity ligands to the phase systems;
3. Volume reduction and high capacity;
4. Non-flammable and non-toxic to personnel;
5. Easy to scale-up;
6. Direct use of available chemical engineering technology (liquid-liquid extraction equipment) for industrial-scale separation.

Despite the more than 40-year history of biotechnological application, the ATPS technique has not found extensive industrial application. This is due to the following limitations:

1. The high cost of the phase-forming polymers;
2. The need, in some cases, for additional centrifugation equipment to facilitate phase separation;
3. Viscosity problems;
4. The lack of engineering design correlation; and
5. The reluctance of the biotechnology industry to incorporate novel techniques in protein purification.

1.1 Statement of the Problem

The exploitation of aqueous two-phase systems for the separation and recovery of target proteins is gaining importance in biotechnology. However, the main problem of the method - how to separate the target protein from the phase-forming polymers - has not yet been completely solved. Stimuli-responsive polymers provide an elegant solution of this problem. In this research, we studied the influence of pH, polymer concentration and salt concentration on partitioning of proteins in two-phase aqueous solutions of pH-responsive polymeric amino acid and associating polymers.

1.2 Objectives

The main objective of this research is to develop a cost-efficient two-phase system for protein partitioning. Our aim is to:

- Facilitate polymer recovery by use of a new pH-sensitive polymeric amino acid as one of the phase-polymers.
- Enhance protein partition coefficient by hydrophobically modifying the second phase polymer.
- Mathematically model the phase behavior and the partition coefficient of proteins in the new polymer systems.

1.3 Scope of the Study

We studied the influence of pH, polymer concentration and salt concentration on partitioning of proteins in two-phase aqueous solutions of a new polymeric amino acid and associating polymers. The study is limited to the following:

- Phase systems studied are:
  1. Polymeric amino acid- PEG 35 000
  2. Polymeric amino acid-PVA 72 000
  3. Polymeric amino acid-urethanized PVA 72 000
  4. Polymeric amino acid-hydrophobically modified PVA 72 000
- Model proteins: Two model proteins were employed in this study. These are:
  - Bovine serum albumin
  - Cytochrome c

  The two proteins are very different in terms of isoelectric point and size.

1.4 Methodology

The research comprise of both experimental work and modeling. The following aspects are covered:

1. Study of phase behavior: The equilibrium phase behavior of the following systems was investigated.
   i. Polymeric amino acid-PEG 35 000
   ii. Polymeric amino acid-PVA 72 000

iii. Polymeric amino acid-urethanized PVA 72 000

iv. Polymeric amino acid-hydrophobically modified PVA 72 000

2. Study of protein partitioning behavior: The system that shows good phase separation is chosen for partitioning studies of the model proteins. An effect of pH, salt concentration and polymer concentration on the partitioning behavior is investigated.

3. Model the phase behavior and partition coefficient of proteins in the new polymer systems.

CHAPTER 2

LITERATURE REVIEW

2.1 Aqueous Two-Phase Polymer Systems (ATPS)

Aqueous two-phase systems are formed when two polymers are mixed at appropriate concentrations in the presence of water. The solvated polymers cause a phase separation. In general, one polymer will collect in the lighter, top phase, while the other will predominate in the bottom, denser phase. This phenomenon was noted as early as 1896 by a Dutch microbiologist named Beinjerinck [1, 4, 7]. Upon mixing gelatine and agar in water, he observed the formation of two liquid phases. Since then, many immiscible biphasic aqueous systems have been found utilising hydrophilic polymers in aqueous solution. Dobry and Boyer-Kawenoki [23] published studies on 78 different polymer-polymer-solvent systems. Their conclusion was that phase separation is the rule and total miscibility the exception. However, it was in the 1950's that Albertsson [7] realized the potential value of these systems for the extraction of biomolecules after discovering that different biological particles, when added to an ATPS, would partition uniquely between the phases. His pioneering work, which he describes in the first book on aqueous two-phase systems [4], mainly focuses on systems containing two polymers as phase polymers, but also gives some attention to aqueous systems with polymer and a salt as phase formers. Since then, the attention for these systems has grown rapidly and

especially in the last two decades a lot of work has been performed in order to acquire more fundamental knowledge about ATPS and also to develop feasible separation processes for various bioproducts.

The time of phase separation in ATPS is a function of the density and viscosity difference between the two phases, and the interfacial tension between the two phases. Since both phases contain predominantly water (usually 75 to 90 weight percent) the density of each phase is close to 1.0 (generally between 0.95 and 1.1). Similarly, the interfacial tension between the phases is also very low; typically between 0.1 and 100 μN/m. The viscosity of a polymer solution is a function of its concentration. Although higher molecular weight polymer solutions are more viscous, the viscosity of the phase system may still be reduced by increasing the molecular weight of the polymer used, since less polymer is required to effect phase separation. The time required for phase separation may vary from 5 min to 6 h [5]. The greater the difference between the phases is, the less the time required for phase separation to occur. In general, intermediate polymer concentrations (removed from the critical point, but also from very high concentrations) provide optimal conditions for phase separation kinetics. The time required for phase separation can be reduced by the use of centrifugation at low speed. Flygare *et al* [30] reported a dramatic enhancement in the phase separation process by inclusion of micron-sized magnetic particles in the ATPS.

2.2 Phase Separation in ATPS

The fact that two or more phases form in water with the addition of incompatible polymers or polymers and salts has been well known for several decades. But the molecular mechanisms that determine the phase separation are still not clearly understood. Normally, when solutions of small molecules are mixed together, the entropic contributions to the Gibbs free energy change on mixing outweigh the enthalpic contributions, that is,

$$\Delta G_m = \Delta H_m - T\Delta S_m \tag{2.1}$$

Thus, ΔG_m is a negative, and a homogeneous solution is the result. When two different polymer solutions are mixed in concentrations above a critical total concentration of solute, the enthalpic (like polymer attraction and unlike polymer repulsion) contributions to the Gibbs free energy change on mixing outweigh the entropic contributions. Thus phase separation ensues [64].

For a ternary system with one solvent component denoted by '0' and two solute components denoted by '1' and '2' respectively, the conditions necessary for phase equilibria between a top (") and bottom (') phase are:

$$\mu_i'' = \mu_i' \quad i = 0, 1, 2 \tag{2.2}$$

To apply Equation 2.2 appropriate analytical functions of chemical potential of each of the species are needed and various approaches have been used for aqueous two-phase systems.

Cabezas [17] presented a comprehensive review of the principal mathematical models and the relevant theories of phase formation in aqueous two-phase systems. He pointed out that there are roughly three schools of thought in the area of modeling of phase separation:

1. Models based on extensions of lattice theories such as the Flory-Huggins theory;
2. Models based on osmotic virial expansions descended from the original work of Edmond and Ogston;
3. Models incorporating integral equation theory as a major element; and
4. Models that do not fall into the above categories, such as group contribution schemes and excluded volume approximations.

2.2.1 Lattice Theory

This class of theories is formulated assuming concentrated polymer solutions, and the expressions are not expansions in solute concentration. Therefore, the question of applicability as the solution becomes more concentrated that arose with osmotic virial expansions is avoided completely. There is, however serious doubt that the same lattice expressions are applicable in very dilute solutions where there exists large regions of pure solvent between polymer coils.

2.2.1.1 Flory-Huggins Theory and its Extensions

The classical polymer solution theory of Flory [28, 29] and Huggins and its several modifications has been the basis of several efforts to model phase behavior of two-polymer aqueous two-phase systems. The attractiveness of the Flory-Huggins and related

theories include their relative simplicity, the fact that they give qualitatively good predictions or at least correlations for phase behavior and their ability to give mechanistic insight into the phase separation process. Flory and Huggins used the liquid lattice model in conjunction with statistical thermodynamics approach to calculate the total Gibbs free energy of mixing for a polydisperse polymer. The expression for the total Gibbs free energy of mixing at constant temperature and pressure for a multicomponent system is [27]:

$$\Delta G_m = \Delta H_m - T\Delta S_m = kT\left(\sum n_i \ln \phi_i + \sum n_i \phi_i \chi_{ij}\right) \tag{2.3}$$

The chemical potential is related to the Gibbs free energy of mixing:

$$\mu_i - \mu_i^{\circ} = N_A\left(\frac{\partial G_{mix}}{\partial n_i}\right)_{T,P,n_j} + z_b F\psi \tag{2.4}$$

where μ_i is the chemical potential of species i, μ_i° is the chemical potential at standard state, z_b represents the net charge of species and ψ is electrostatic potential.

Benge [13] gives an excellent critical review of the application of the Flory-Huggins theory to polymer solutions and two-phase systems covering work up to about 1986. Benge noted that the Flory-Huggins interaction parameters Π_{ij} show composition dependence in two-polymer ATPSs. To address some of the problems pointed out by Benge, a research group at Lund [75] has modified Evers *et al.*'s [26] extension of the Flory-Huggins theory by allowing the monomers to have internal degrees of freedom. This modification gives the interaction parameter Π_{ij} which is temperature dependent.

This approach gives reasonably correct phase behavior with temperature and composition for two-polymer ATPSs.

Since narrowly fractionated polymers are expensive, polymers with wide dispersion in molecular weight are commonly used for ATPS applications. However, this dispersion affects the liquid-liquid equilibrium. Koningsveld and Staverman [66] have extensively investigated phase separations in solutions containing polydisperse macromolecules by using Flory-Huggins model and concluded that polymolecularity of the macromolecules affects the phase diagrams to such an extent that it cannot be ignored in treatments that are intended to be quantitative.

Gustafsson and coworkers [39] studied the possibility of applying the Flory-Huggins model to predict ternary polymer-polymer-water phase diagrams. They found that the traditional Flory-Huggins model could be used to approximate the ternary phase diagram by fitting the binary interaction parameters required. Kang and Sandler [60] experimentally determined the binary interaction parameters from intrinsic viscosity and critical point data. They found a good agreement between the predicted phase diagrams using calculated binary interaction parameters and phase diagrams on polymer molecular weight were also shown to be in good qualitative agreement. The model required existing binodal data from which interaction parameters were calculated. Thus it may not be practical for two-phase systems that have not been studied previously.

Diamond and Hsu [20] applied Flory-Huggins theory to predict phase diagram behavior in ATPS. Their empirically extended relationship was expressed in the form

$$\ln K = A(w_1'' - w_1') \tag{2.5}$$

where w' and w'' represent the polymer weight fractions in the upper and lower phase and A is function of the polymer molecular weight and the interactions between the polymers and water. Recently, Hino and Praustnitz [49] applied Flory-Huggins theory to calculate phase separation in polymer-salt aqueous solutions.

2.2.1.2 The UNIQUAC Model

Abrams and Prausnitz [3] obtained a semitheoretical equation for the excess Gibbs energy of a liquid mixture using a generalized quasi-chemical analysis. The resulting UNIQUAC equation requires only two adjustable parameters per binary pair. Kang and Sandler [60] have applied the UNIQUAC equation to the prediction of the ternary polymer-polymer-water binodals. They developed a numerical procedure to predict the six binary interaction parameters required.

The simplest and possibly the most effective technique to include the effect of polydispersity and phase fractionation into phase diagram calculations is the pseudo-component method. Essentially, the method amounts to replacing the many individual components of different molecular masses contained in a polymer with a continuous statistical distribution of molecular masses. The distribution is then used in a

mathematically consistent quadrature procedure to choose a limited number of components of a specified molecular mass which can be used to represents the polydisperse polymer. In a later study, Kang and Sandler [61] incorporated the effect of polymer polydispersity in two-phase systems by using the pseudo-component method in conjunction with the UNIQUAC model. The calculated results exhibited good agreement with the observations of Albertsson that the transition between a homogeneous and a two-phase solution for polydisperse polymers is much broader than for phase polymers of uniform molecular weight distribution.

2.2.2 Osmotic Virial Expansions

Cabezas [17] noted that there are two different osmotic virial expansions, one based on the work of McMillan and Mayer [76] and the other on the work of Hill [48]. Being expansions in composition these theories are strictly applicable only at very low solute concentrations. But since the concentrations of polymers are rather low even at the crossover concentration, it is then fair to say that the theories are at least approximately applicable to the solutions commonly encountered in ATPS.

2.2.2.1 The McMillan-Mayer Theory

The McMillan theory has been used to calculate phase diagrams in aqueous two-phase systems by a number of different researchers over the years. King *et al.* [65] and Haynes *et al* [46] predicted phase separation in polymer-polymer systems in the absence and presence of salts on the basis McMillan-Mayer solution theory. Similar studies were

reported by Wu *et al.* [99] for phase separation in aqueous polymer-salt systems. Gaube *et al* [33] developed a thermodynamically-consistent osmotic virial expansion for predicting phase diagrams of PEG-dextran and PEG-sodium sulfate.

The McMillan–Mayer osmotic virial coefficients have been experimentally measured with a variety of techniques. Thus, Edmond and Ogston [24] experimentally determined values for the coefficients from osmotic pressure, sedimentation, equilibria and phase diagram critical point. King *et al.* [65] experimentally measured the coefficients using low angle laser light scattering and vapor pressure measurements. Gaube *et al.* [33] measured the coefficients by vapor pressure osmometry and membrane osmometry. The calculations of Edmond and Ogston and those of King *et al.* assume that the polymers are monodisperse while those of the Gaube *et al* do include the polydispersity and fractionation of the polymers.

2.2.2.2 The Hill Theory

The theory derived by Hill seems superficially similar to that of McMillan and Mayer, and indeed both give component chemical potentials as a power series in solute composition. However, the theory of Hill gives the component chemical potentials at constant temperature and pressure rather than constant solvent chemical potential μ_s, and it, therefore requires no osmotic pressure correction.

Cabezas and co-workers [18] developed predictive equations for binary interaction parameters as a function of molecular weight and polydispersity based on the solution theory of Hill. The second virial cross-coefficient is determined as a geometric mean of the individual second virial coefficients. The resulting equations for the second virial coefficients include a total of five parameters, the binary interaction coefficients and two scaling exponents. The parameters were determined by using a combination of experimentally determined binary interaction parameters and ternary phase diagrams. Once obtained the parameters were used to predict ternary phase diagrams.

Haynes and co-workers [47] combined the derivations of Hill and Ogston with an extended Debye-Huckel theory for chemical potentials of ions and solvent. In this derivation, the binary interaction parameters for ion-polymer and ion-ion are required in addition to polymer-polymer interaction parameters that must be determined. This results in a total of eight interaction parameters that must be determined. However all parameters could be experimentally determined or estimated without the need for binodal information to fit the parameters.

2.2.3 Integral Equation Theory

A recent and unique approach is the work of Haynes and others [45]. This approach combines integral equation theory, a hard sphere equation of state, perturbation theory, the McMillan-Mayer osmotic virial expansion and other elements. All these are brought together to derive a very general expression for a modified excess Helmholtz free energy

for an aqueous mixture of polymers, salts, and proteins. The resulting model is perhaps, the most complete and sophisticated available in the literature. Overall, the model represents the phase behavior of polymer-polymer and salt-polymer aqueous two-phase systems, and partitioning of proteins quite adequately, which is a very significant accomplishment. It is, however, a fairly complex model. For further progress to be made toward a practical design-oriented correlation, it will be necessary to characterize, quantify and tabulate polymer-polymer and polymer-ion interaction coefficients in aqueous systems. Compilation of a database of such fundamental thermodynamic parameters for aqueous polymer solutions will be an important tool in the successful application of any model in process design [17].

2.3 Kinetics of Phase Separation

The similarity in the properties of the phases in ATPS is a key factor in the mildness of aqueous two-phase operation, minimizing the damage and loss of activity of proteins being recuperated. Conversely, a small difference in physical properties leads to difficulties in the separation of the phases. This is easy to overcome in the laboratory using centrifugation. In a large scale plant, on the other hand, the possibility of using gravitational separation should always be considered, because of the advantage of lower initial inversion in equipment, mechanical simplicity, maintenance and operation costs [77]. Kaul *et al.* [62] studied the kinetics of phase separation in ATPS in terms of the physico-chemical properties of the phases (density, viscosity, interfacial tension) by measuring dispersion height as a function of separation time. They found that the kinetic

behavior depends greatly on which of the phases is continuous and that the properties of the continuous phase strongly influence the movement of the drops of the dispersed phase and hence phase separation.

Mistry *et al.* [78] developed a mathematical model to describe the continuous, steady state operation of an aqueous two-phase system for protein extraction. The model is based on steady state mass balance of the main system components and phase equilibrium data. Salamanca [92] investigated the effect of the tie-line location (phase volume ratio) on the kinetics of phase separation in batch ATPSs. They also studied the behavior of batch and continuous systems in the presence and absence of *Bacillus subtilis* extract in systems with continuous bottom phase. They demonstrated that the settling velocity was lower in the continuous than in the batch systems, and in both cases the initial rate was lower in the presence of *Bacillus* extract.

2.4 Factors Determining Protein Partitioning

Aqueous two-phase systems were discovered more than 100 years ago. However, it was Albertsson who first described their general application for purification of biomolecules in the 1950s. He discovered that different biological particles, when added to an aqueous two phase system, would partition uniquely between the phases. Thus, aqueous two-phase systems provide a powerful method for separating proteins by extraction. The distribution of proteins between the two aqueous polymer solution phases is characterized by a partition coefficient, K_p, defined as

$$K_p = \frac{C_{p,t}}{C_{p,b}} \tag{2.6}$$

where $C_{p,t}$ and $C_{p,b}$ are the protein concentrations in the top and bottom polymer solution phases, respectively.

The partitioning behavior of protein in an aqueous two-phase system is governed by several factors. Albertsson *et al.* [6] presented a detailed overview of the factors that determine how protein partition between phases, including size-dependent, electrochemical, hydrophobic, biospecific, and conformation-dependent contributions. The model represents the logarithm of the overall partition coefficient as a linear combination of each of these contributing factors:

$$\log K = \log K^o + \log K_{el} + \log K_{hfob} + \log K_{biosp} + \log K_{size} + \log K_{conf} \tag{2.7}$$

where the subscripts el, hfob, biosp, size, and conf denote the electrical, hydrophobic, biospecific, size, and conformational contributions to the partition coefficient. K^o contains all other factors not specifically accounted for in the other coefficients. Each one of these factors may be made to play a dominating role in a partition experiment.

2.4.1 Effect of Phase-Forming Polymers

The type, molecular weight and composition of the phase-polymers in an ATPS determine its main characteristics and therefore play an important role in determining the partition behavior of proteins. The type of phase polymers used determines the

hydrophobicity of the phases, which is important for Van der Waals interaction between chain segments of a given protein as well as between solvent and protein, and also influences protein solubility. Hydrophobicity increases in the aqueous solutions of polymers in the order dextransulfate<carboxymethyldextran<dextran< hydroxypropyldextran<methoxycellulose<polyvinyl alcohol<polyethylene glycol <polypropylene glycol. For the same polymer the hydrophobicity increases with increasing molecular weight, since the number of end-groups diminishes [70]. Hamad and coworkers [40] have shown that although polymer structure has little effect on phase behavior, it has a significant influence on protein partitioning. The specific structure of the polymer is important because it determine the ability to make derivatives or to attach ligands. The type of phase-polymers also affects the time of phase separation, viscosity, and interfacial tension; all of which must be considered in process equipment design.

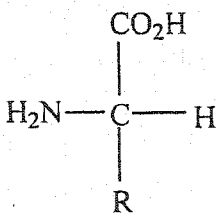
The effect of the molecular weight of phase-forming polymers is such that when the molecular weight of one polymer is decreased, the protein tends to favor the phase rich in this polymer. This influence of the polymer molecular weight on protein partitioning also depends on the size of the partitioned protein. High molecular weight proteins are more affected by changes in the molecular weight of polymers than small proteins [6]. Thus, polymers with different molecular weights can be used to improve separation of protein molecules differing in size. The partition coefficient correlates directly with the difference in molecular weight between the two-phase polymers: the greater the difference in molecular weight, the larger the deviation of the partition coefficient from unity. Therefore, phase compositions that are far removed from the critical point lead to

improved separation [64]. Besides the average molecular weight, the molecular weight distribution of the phase polymer also contributes to the relative hydrophobicity of the phases. In two-phase systems incorporating a polymer with a large molecular weight distribution some fractionation will occur leading to a higher average molecular weight in the polymer rich phase. In such cases the partition coefficient will be changed with the concentration of the polymer even if the two systems are on the same tie line.

Very close to the critical point, the compositions of the phases are similar, so that protein partitioning is relatively even. Increasing the polymer concentration removes the phase system more from the critical point and the two phases become more different, providing different environments for the proteins to be distributed. Hence, the higher the total concentration of the polymers, the further the phase compositions are from the critical point and the more extreme the protein partitioning between the phases becomes. However, increasing the polymer concentration also lead to increase in viscosity and this must be considered in process equipment design.

2.4.2 Effect of Proteins Properties

Proteins are complex biopolymers composed of a series of amino acids attached by peptide bonds. There are 20 different naturally occurring amino acids present in proteins each with a different residual group, R:



The properties of the residual groups, in conjunction with their structural positions, define the solution properties of the protein. Amino acids fall into five categories: aliphatic, nonpolar, aromatic, polar and charged.

Proteins have four structural categories. The most basic level of protein structure, called the primary structure, is the linear sequence of amino acids. Different sequences of the acids along a chain, however, affect the structure of a protein molecule in different ways. Forces such as hydrogen bonds, disulfide bridges, attractions between positive and negative charges, and hydrophobic and hydrophilic linkages cause a protein molecule to coil or fold into a secondary structure (α-helix or β-pleated sheet). The tertiary structure is the three dimensional conformation, representing how the secondary structure folds to obtain the most favorable thermodynamic state, with hydrophobic residues on the interior and hydrophilic residues on the exterior. The quaternary structure is the arrangement of the aggregation of several polypeptide chains.

The characteristics imparted to the protein by the amino acid side chains include solubility, charge, hydrophobicity, and intermolecular bonding [98]. Slight changes in size, charge, and surface chemistry of a protein can lead to noticeable changes in partitioning behavior [53]. The interaction of the protein with the phase polymers

depends strongly on the nature of the protein surface, and not average protein properties. The surface of the protein generally contains polar amino groups and charged side groups. However, the surfaces of some proteins contain significant hydrophobic regions. The variation of surface properties of the proteins or the hydrophobicity of the polymer can be used to enhance the partition of proteins to a certain phase. The proteins favor the phase that contains the more compatible polymer in terms of surface charge and hydrophobicity. Modification of proteins by addition of charged or hydrophobic groups was found to enhance the partition coefficient in certain polyelectrolyte systems [72]. The structure or conformation of a protein has much influence on its partitioning in two-phase systems. A molecular-thermodynamic analysis of protein partitioning in an aqueous two-phase systems shows that the partition coefficient for a native (globular) protein is very much different from that for a denatured (linear) protein; while the former is weakly dependent on protein molecular weight, the latter depends strongly on molecular weight. Native and denatured DNA and supercoiled and un-coiled DNA also display very different behavior, as do linear and cyclic oligosaccharides [53].

2.4.3 Effect of pH Value

Proteins are composed of amino acids having anionic, cationic, and hydrophobic side chains. The ionization state of these amino acids depends upon the pH of the solution. The pH influences dissociation of ionizable groups of the protein which in turn will alter surface charges and therefore partition. At low pH the basic side chains will be ionized and bear a positive charge. At high pH the acidic side chains will be ionized and bear a

negative charge. The point at which the net charge is zero is termed the isoelectric point (pI). This variable is unique to the individual protein.

The solubility of proteins is greatly dependent upon pH. The solubility varies inversely with how close the protein is to its pI [98]. As a general rule, proteins are least soluble at their pI. At pH values below its pI, a protein will be positively charged, and at pH values above its pI it is negatively charged. In either case, the net electrostatic effect is repulsion between adjacent protein molecules thus preventing coalescence. At the pI, there is no net charge on the molecule; individual molecules now have a greater tendency to approach one another due to electrostatic attraction between the oppositely charged groups. They tend to clump together (coagulate) and precipitate out of solution. Properties of some representative proteins are shown in Table 2.1.

2.4.4 Effect of Electrolytes

Partitioning of proteins and other charged biomolecules can be enhanced, in some cases dramatically, by the addition of certain electrolytes to a phase system. Albertsson [5] have shown that the addition of a single electrolyte to an initially uncharged aqueous two-phase system (e.g. PEG-dextran) will lead to the formation of a Galvani-type interfacial electrostatic potential difference $\Delta\square$ if the anion(s) and cation(s) of the dissociated salt have different affinities for the two phases. This interfacial potential difference will contribute to the overall partition coefficient of a protein macro-ion in direct proportion to the net charge z_p of the protein at the system pH, bearing in mind that a partitioned

TABLE 2.1 Protein Properties

Protein	Molecular mass(Da)	pI	Net charge at pH 7.1
BSA	69 000	4.8	-18
β-Lactoglobulin	35 000	5.1	-5
Myoglobin	17 500	7.1	0
Cytochrome c	13 000	9.4	6
Lysozyme	13 900	11.0	7

Source: Sivars *et al* [94]

protein macro-ion must carry with it sufficient number of counter-ions and co-ions to maintain phase electroneutrality. Following Guggeinheim [37], Albertsson has shown that the partition coefficient of either ion i is given by

$$\ln K_i = \ln K_i^o + \frac{z_i F}{RT}\Delta\psi \tag{2.8}$$

where K^o is the partition coefficient in the absence and K the partition coefficient in the presence of an electrical potential difference.

2.4.5 Effect of Temperature

The sensitivity of partition coefficients to changes in temperature is not very high. Large-scale stage extraction can be performed without extensive temperature regulation. Rises in temperature of 1-2°C in the liquid during processing have only negligible consequences for recovery of the desired protein and performance of separation with the exception that the system must be far from the binodal to ensure phase formation over this temperature interval [67, 71]. Large-scale operations are usually carried out at room temperature to avoid expenditure for cooling devices and energy. Two facts contribute to such desirable operating conditions. The polymers introduced stabilize proteins and in general high yields are obtained at ambient temperatures. In addition the viscosity of the dispersion and phases will be lower at 20°C compared to 4°C improving the performance of the separation unit [70].

2.5 Prediction of Protein Partitioning

Industrial equipment design for separation and purification of proteins in ATPSs is based on trial and error experiments [64]. To aid design and to optimize ATPSs for separation and purification of proteins and other biomolecules, several theories have been proposed for predicting thermodynamic properties and phase behavior of phase-forming systems and partitioning behavior of proteins and other biomolecules.

Early attempts were reported by Brooks *et al.* [15] and by Gustafsson et al [39] using Flory-Huggins lattice theory to correlate qualitatively phase diagrams and protein partitioning coefficient data in an aqueous mixture containing two polymer solutes. Diamond and Hsu [21] used a linearized form of Flory-Huggins theory to obtain a semi-empirical expression for protein partitioning in polymer-polymer ATPSs with salt buffer.

$$\ln K_p = A\left(w_1'' - w_1'\right) + b\left(w_1'' - w_1'\right)^2 + \frac{z_p F \Delta\psi}{RT} \tag{2.9}$$

In the absence of buffer salt the equation reduces to the following form:

$$\frac{\ln(K)}{\left(w_1'' - w_1'\right)} = A + b\left(w_1'' - w_1'\right) \tag{2.10}$$

On the basis of self-consistent mean-field Scheutjens-Fleer lattice theory for polymer adsorption Baskir and co-workers [12] developed a modified lattice theory to predict protein partitioning without, however, accounting for salt effects. This description, coupled with a Pitzer-Li long-range electrostatic term, was extended by Peng *et al.* [85] for partitioning of amino acids and proteins in polymer-salt systems. In the same spirit,

the polymer-scaling concept of de Gennes was applied by Abbott *et al.* [2] to describe interactions between proteins with flexible non-ionic phase-forming polymers toward investigating protein partitioning. On the basis of simple geometric arguments, the appropriate length scales are identified to describe interactions between protein and polymer; scaling relations are then proposed for the free energy accounting for the interactions of protein and polymer.

The osmotic virial expansion, first proposed by Edmond and Ogston [24], provided a simple theoretical framework that is commonly used for aqueous two-phase systems. On this basis, King *et al.* [65] and Haynes *et al.* [46] predicted phase separation and protein partitioning in polymer-polymer systems in the presence and absence of salts.

$$\ln K_p = a_{2p}\left(m_2'' - m_2'\right) + a_{3p}\left(m_3'' - m_3'\right) + \frac{z_p F\left(\Psi'' - \Psi'\right)}{RT} \tag{2.11}$$

Similar studies were reported by Zhou *et al.* [100] for amino acids partitioning in aqueous polymer-polymer systems. Recently, Jiang and Prausnitz [53] used a molecular-thermodynamic model to study the partitioning behavior of native and denatured proteins in ATPSs. These studies using the osmotic virial expansion were based on McMillan-Mayer solution theory. In contrast, on the basis of the constant-pressure-solution theory of Hill, Cabezas *et al.* [18] and Fortiniti and Hall [31] developed an isothermal-isobaric virial expansion to study phase behavior of aqueous two-phase systems.

Kang and Sandler [61] used the UNIQUAC model to predict binodals of polydisperse PEG-dextran ATPSs. Hartounian *et al.* [42, 43] studied phase behavior and protein partitioning in PEG-dextran system with a salt buffer by combining Guggenheim's extension [37] of the Debye-Huckel theory for long-range electrostatic interaction. They obtained the following simplified expression for the protein partitioning coefficient:

$$\ln K_p = A[TLL] + B\left(m'_{cat} - m''_{cat}\right) + C\Delta\Psi \qquad (2.12)$$

where A is a function of the composition of the phase-forming polymers, and B depends on the types of the salt and protein. The parameter $C = z_p F/RT$ is a function of the solution pH and the protein used.

Similarly, the UNIFAC model was used by Peng *et al.* [86] to predict phase behavior of polymer-salt systems; a semi-empirical group-contribution model was used to study amino acids and short-peptide partitioning in polymer-polymer and polymer-salt systems. Integral-equation theory has been applied by Haynes *et al.* [45] and by Kenkare and Hall [63] to study phase behavior and partitioning behavior of proteins in both aqueous polymer-salt and polymer-polymer two-phase systems.

2.6 Selectivity of Protein Extraction

There are various efforts to increase the selectivity of protein partitioning to make extraction predictable as well as to achieve sufficient purification during this step so that subsequent downstream processing steps are avoided, or at least reduced in number [44].

An approach that has been studied for a long time has been the incorporation of affinity ligands, which are able to bind to specifically and reversibly with the target protein, in one of the phases of the system. Normally this incorporation involves covalent coupling of the ligands to the phase-forming polymer. Thus, for applications on a large scale, an efficient recycling of ligands will be necessary. Different means for modification of ligands have been suggested to facilitate recycling [91, 58, 59]. The size and/or properties of the polymer to which the ligand is coupled may be varied, thereby changing the partition behavior. Employing phase-forming components, which would allow for desirable partitioning of the ligand, hence obviating the need for coupling, is also possible. The use of inexpensive phase components and ligands should make affinity extraction economically feasible and will yield a substantially enriched product. A recent approach being studied for enhancing the selectivity of extraction is genetic modification of the target protein by adding certain amino acids to target its partitioning into a desired phase of a predetermined two-phase system [25].

2.7 Selection of Phase-Forming Polymers

The design of an aqueous two-phase process requires consideration of a large number of variables. Initially there are choices regarding the type of phase forming polymers employed and the composition of the system. Choice of polymer in an ATPS can be a critical factor in the successful execution of a desired separation. This is true not only because of the effect of the polymer on the distribution of the protein between the phases,

but also because of the effect the polymer has on the physical characteristics of the ATPS.

The most frequently encountered two-phase systems in lab-scale separations of biomolecules are composed of low concentrations of PEG, fractionated dextran and water, mainly for legal but also for technical reasons. Both polymers are extensively studied, the toxicology is known and they are included into the pharmacopoeias of most countries. However, the high cost of fractionated dextran ($500/kg) allied to its biodegradability is a serious drawback for its application in large-scale processes [64]. In addition, the limited solubility of many proteins in the presence of PEG and the necessity for a second two-phase system to extricate the polymer from the partitioned protein are further disincentives for the widespread use of such systems. Removal of phase-forming polymers is generally accomplished by mixing the protein-containing phase with concentrated salt solution, which promotes the transfer of the protein from the polymer phase to the salt phase. An additional step to desalt the protein is then required before further processing. These problems have resulted in considerable efforts toward finding alternative phase-polymers.

2.7.1 Replacement of Dextran

Kroner [68] attempted to solve the problem of the high cost of fractionated dextran by replacing it with a crude dextran. While the crude fractions seem to have little effect on protein-partitioning behavior, their rheological properties are less favorable.

Nyugen and others [80] studied the use of food grade (crude) pullulan as a low-cost, low viscosity phase-polymer to replace dextran in the PEG-dextran system. In showing the applicability of pullulan similar to that exhibited in PEG-dextran systems, they demonstrated the separation of *cellulase* from *β-galactosidase* and the extractive hydrolysis of *lactose*.

Tjerneld [96] summarized studies on cellulose, polyvinyl alcohol, and derivatives of starch. Starch tends to form gels quite easily. However, hydroxypropyl derivatives of the starch were found to have better stability. This polymer is commercially available as Aquaphase PPT [64] and Reppal PES [88]. The use of this polymer with PEG has been demonstrated in the affinity purification of *lactate dehydrogenase* from swine muscle using PEG bound triazine dye. Tjerneld [96] studied the ethyl hydroxyethyl cellulose derivative in order to obtain a polymer that was cheap and required minimal quantities to form two-phase system. The polymer was found to work well with dextran. Less of both phase polymers were required, but the use of the most expensive phase polymer, dextran, is still required and the settling times are quite long. The use of PVA as a replacement for dextran has begun to be evaluated. PVA-PEG exhibits different partitioning properties from that of PEG-dextran systems. Enzymes were shown to partition strongly to the top, PEG, phase of these systems, and the partition coefficient was found to increase with increasing polymer concentration.

Szlag and Guiliano [95] studied the use of low-cost maltodextins to form two-phase systems with PEG. The advantages of these systems include low cost, low viscosity, high density differences to allow faster settling times, and high biocompatibility. However, the low cost is offset by the fact that higher concentrations of polymer are required.

Rastogi and Chand [90] evaluated gum acacia, a naturally occurring polysaccharide, for use as a phase-forming polymer. They determined the characteristics of gum acacia-PEG aqueous two-phase systems and showed feasibility in the separation of mixtures of proteins. The advantages of this polymer include low cost and low viscosity, good protein stabilizing characteristics, and high solubility in water.

2.7.2 Thermoseparating Polymers

Luong and Nguyen [74] developed a new affinity polymer from the observation that glycidyl acrylate residues react with amino groups of proteins. By combining this with N-isopropyl acrylamide residues, which are sensitive to temperature and salinity, they formed a block copolymer from the two, which they named GA-NIPAM. Recycling of the polymer is effected by adjustment of the salinity since the N-isopropyl acrylamide residues cause the polymer to precipitate at low salt or temperature conditions. However, the cost and commercial availability of the polymer will most likely be high.

Pietrusska *et al.* [89] developed a new starch modified polymer for application in ATPS for protein separation. Partial hydrolysis and acrylamide modification of starch to

different degrees make it suitable for forming ATPS with PEG in a moderate concentration range. They evaluated the potential of the polymer to form ATPS with the thermoprecipitating copolymer of 1-vinylimidazole with N–vinylcaprolactam.

Persson *et al.* [88] recently introduced an aqueous two-phase system composed of two thermoseparating polymers. The system contains random copolymers of ethylene oxide and propylene oxide (EOPO copolymers) and a hydrophobically modified EOPO polymer (HM-EOPO). In this system either phase can be used for the extraction of the target protein since both phases can be thermo-separated. After thermoseparation the protein can be recovered in a water phase and both of the polymers can be recycled.

In a later work, Persson *et al.* [87] developed a novel one-polymer aqueous two phase system containing only thermoseparating EOPO copolymers and water for separation of smaller biomolecules e.g. peptides. Proteins can be extracted in a one-polymer phase system containing a HM-EOPO. The target protein is partitioned to the HM-EOPO phase. The copolymer can be recycled after back-extraction of the protein to a new water phase. They also developed a system for protein purification based on EOPO copolymer-hydroxypropyl starch ATPS.

2.7.3 pH-Responsive Polymers

Hughes and Lowe [52] reported the use of polyampholytic, acrylic copolymers and polyvinyl alcohol as alternative two-phase systems for protein partitioning. In contrast to

conventional PEG-dextran two-phase systems, quantitative protein recovery from liquid phases containing polyampholytes could be achieved simply by isoelectric precipitation of the polymer. In addition, the acrylic copolymers are inexpensive, non-toxic and form two-phase systems at low concentrations.

Patrickios *et al.* [82] investigated the potential utility of random acrylic polyampholytes as novel phase-forming polymers in two-phase aqueous polymer systems for protein extraction. The polyampholyte-containing two-phase systems exhibit a number of properties that may be of advantage in developing novel protein separation strategies. They exhibit phase inversions, viscous and non-viscous phases and isoelectric precipitation. In particular, this latter property can be exploited for the recovery of proteins and the polyampholyte.

In another study, Patrickios and coworkers [84] investigated phase behavior of random and ABC triblock methacrylic polyampholytes in aqueous mixtures with PVA as a function of pH and salt concentration. They discussed the potential utility of the system for protein extraction.

Johansson [54] investigated new aqueous liquid-liquid two-phase systems based on bovine serum albumin in combination with either PVA or PEG for the partitioning of enzymes. He explained that by using a protein phase, the natural environment of the

enzymes can be mimicked and the function of enzymes and enzyme systems in concentrated form surrounded by another protein could be studied.

2.8 Protein Determination

In order to experimentally obtain protein partitioning coefficient it is important to have an accurate method for determining the protein concentration in both phases of the ATPS. There are several tests available for protein determination. But, the most widely used methods are based on those originally developed by Lowry *et al.* [73] and Bradford [14]. Protein determinations in ATPSs are usually assayed according to Bradford using Coomassie brilliant blue G staining in solution [9, 32, 55, 87, 89]. In contrast to the Lowry method, the presence of polymers does not have strong negative influence on the assay [55, 70]. Another possibility is to determine the absorbance at 280 nm. Although this method is rapid and non-destructive, it is not strictly quantitative. In both cases the phases are diluted 5 to 100 times before the assay and phases from a protein-free system are used as blanks.

2.9 Analysis of Literature

Recently, there has been much interest in the use of aqueous two-phase systems for the commercial purification and concentration of biotechnological products. In some cases, inexpensive polyethylene glycol/salt systems are limited by the high salt concentration necessary for the formation of the phases. Polymer/polymer phase systems are more

generally useful, but commercial exploitation of these products has been restricted by the high cost of the fractionated dextran used in the dextran/PEG phase systems on which most of the literature is based. The above literature review has pointed out that there is multitude of studies going on in search of cost-efficient polymers to provide aqueous two-phase systems with economic and environmental advantages that will allow their widespread use in protein separation.

Initial efforts were directed at developing other polymer pairs that combine to form phase systems having comparable properties to, but lower costs than dextran/PEG systems. But, recent works in finding alternative polymers show growing emphasis on phase-polymers that are recoverable because of economical and environmental reasons. The introduction of stimuli-responsive polymers in aqueous two-phase systems is a new development in this regard. In spite of the promising features of polyampholytes as shown by the pioneering work of Hughes and Lowe [52], there seems to be little effort in further investigations to assess the potential of these very interesting polymers for separation of proteins. Instead, most of the current researches are in the area of thermoseparating polymers.

CHAPTER 3

PHASE-FORMING POLYMERS

3.1 Introduction

The selection of the polymers to generate the phases is the starting point for any ATPS. The choice of polymer systems involves a complex evaluation of the properties of these polymers and the implications of their use for other aspects of the partitioning process. Carlson [19] has reviewed the factors influencing the choice of polymers for ATPS. Phase-forming characteristics, structure, density and viscosity, cost, recovery and end-use are recognized to be the major factors to be considered when choosing polymers for an ATPS. In this chapter we are going to discuss about the polymers used in this work and the reasons for their selection.

3.2 Polyampholytes

Macromolecules capable of possessing both positively and negatively charged moieties are commonly known as polyampholytes. The presence of ionic units along the polymer chain results in a complex solution behavior which is essentially controlled by electrostatic interactions. Coulombic attractions between oppositely charged sites afford

formation of inter- and intramolecular ionic interactions that are stronger than van der Waals forces, yet weaker than covalent bonds [93].

In contrast to polyelectrolytes, structure property relationships of polyampholytes are governed by coulombic attractions between anionic and cationic mer units. When the anionic or cationic species are present in sufficient excess (>=10-15 mol%), charge repulsions induce an extended conformation of the chain resulting in rheological behavior typical of polyelectrolytes. As the molar ratio of anionic to cationic species approaches one, coulombic interactions lead to globule-like conformations and, in many cases, insolubility in deionized water. These attractive interactions may be screened by the addition of electrolytes or change in pH, which induces a transition to a random coil conformation, often facilitating solubility.

Polybetaines are prepared from zwitterionic monomers in which the anionic and cationic groups are incorporated in a single mer unit. The anionic group is typically a sulfonate, carboxylate, or phosphate moiety, while the cationic moiety is usually an ammonium species. Polyzwitterions containing sulfonate moiety (sulphobetaines) have been most thoroughly investigated. In comparison with copolymers containing sulfonate and ammonium groups on different mer units, the majority of the poly-(sulfobetaines) systems are insoluble in deionized water with a few exceptions. The addition of a critical concentration of salt is again required to achieve solubility to enhance viscosity.

The isoelectric point of a polyampholyte can be determined mathematically from knowledge of the acid to base molar ratio, R, and the dissociation constants of the base and acid monomers, (pKb and pKac). Starting from the requirement that the total net charge is zero and assuming that the activity coefficients of the acid and base residues are equal to one, the following closed form expression for pI at low ionic strength can be derived:

$$pI = pK_b + \log\left\{\frac{1}{2}\left[\frac{1-R}{R} + \sqrt{\left(\frac{1-R}{R}\right)^2 + \frac{4}{R}10^{pK_{ac}-pK_b}}\right]\right\} \tag{3.1}$$

For extreme values of R the pI approaches asymptotically the limiting forms:

$$pI = pK_{ac} - \log R \qquad \text{for } R>3 \tag{3.2}$$

and

$$pI = pK_b - \log R \qquad \text{for } R<1/3 \tag{3.3}$$

The PI of a polyampholyte is a function of ionic strength. Anions, especially at high ionic strengths are known to bind to proteins resulting in an ionic strength-dependent shift of the pI to a lower pH. For some proteins this shift can be dramatically large, reaching four pH units [81].

3.2.1 Solubility of Polyampholytes

The solubility of polyampholytes has strong dependence on pH and salts [93]. The solution behavior is essentially controlled by the competition between repulsive (polyelectrolyte effect) and attractive (polyampholyte effect) electrostatic interactions.

These are directly related to the strength of the acid or base groups and the value of the pH (annealed polyampholytes) or to the copolymer composition (quenched polyampholyte).

The addition of salt screens the electrostatic interactions and weakens the attractions. The amount of salt required to dissolve the polymer depends on the nature of the salt with data following the Hofmeister series, the net charge, and charge density (the lower the charged density the less the added salts). Experimental studies as well as recent simulation have revealed the influence of the distribution of the charges along the chains on the properties of polyampholytes. In particular it was found that polyampholytes showing a trend for alternation are usually soluble over the entire range of pH even at the isoelectric point in contrast with other types of polyampholytes [81].

3.2.2 Application of Polyampholytes in Protein Partitioning

Traditionally, the removal of polymer from protein in an aqueous two-phase separation requires either back extraction into a salt rich phase, utrafiltration, diafiltration, or a chromatographic step, e.g., ion exchange. pH responsive polymers can be used in place of the commonly used PEG in polymer-polymer two-phase systems and, therefore, offer an obvious advantage for the cost-effective purification of target protein into a "clean" water phase and for the recycling of polymer.

Introduction of stimuli-responsive polymers, such as polyampholytes, in aqueous two-phase systems is a new development in the search for alternative phase-forming

polymers. What makes polyampholytes interesting is that they can be easily recovered by isoelectric precipitation and recycled. Recently, Al-Muallem *et al* [10] reported the synthesis of new ionic polymers using cheap and commonly available chemicals. The polymers, just like proteins, contain two pH-triggerable functionalities (N and CO_2^-) that make them exhibit pH responsive behavior. One of the polymers was found to be almost water-insoluble in acidic pH range. This behavior makes it a potential candidate for industrial applications since it can be effectively removed from solution by pH-controlled precipitation. Furthermore, in applications such as protein partitioning, the protein-like structure of the polymer is expected to enhance protein-polymer interactions.

3.2.3 Polymeric Amino Acid

The primary phase polymer we employed in this work is a novel amino acid-sulfur dioxide copolymer. The polymer was synthesized by cyclocopolymerization of diallyl salt having pendant ester functionality with sulfur dioxide. Butler's (1951) cyclopolymerization technique was used for the synthesis. The detail of the synthesis is available elsewhere [10]. The polymeric amino acid is an anionic polyelectrolyte (APE) having two pH-triggerable functionalities (N and CO_2^-). This makes it pH responsive. Addition of 1 equivalent HCl to the APE gave a water insoluble polybetaine (PB). However, if the APE is treated with less than one equivalent of HCl then a polyampholyte having a mixture of APE and PB will result. The APE starts to precipitate in salt-free water after addition of 0.95 equivalents HCl (pH ~7.0). Figure 3.1 shows the structural formulae of the APE and PB. By pH adjustment, through the addition of HCl, different mole ratios of PB:APE can be obtained. At pH values of 7.89, 7.41 and 7.37 the

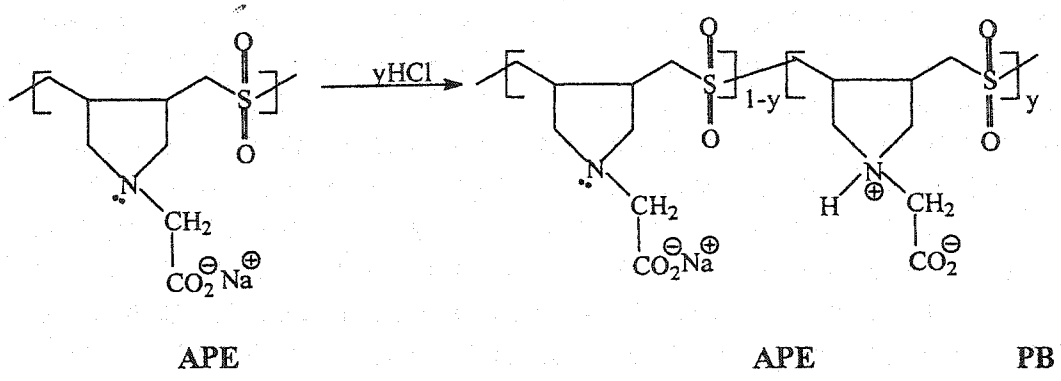


Figure 3.1 Structural Formula of the APE and PB

APE:PB molar ratios are 1:1.2, 1:4, and 1:9, respectively. Elemental analysis to determine the carbon, hydrogen, nitrogen, and sulfur content of the APE was carried out. The analytical result shows that the APE has the following composition: C, 34.4; H, 5.75; N, 5.15; S, 11.2. The intrinsic viscosity of the APE was determined to be 0.3 dl/g.

3.3 Preparation of Urethanized Polyvinyl Alcohol

Urethanized polyvinyl alcohol was prepared by treating polyvinyl alcohol (PVA) with urea in distilled dimethylformamide (DMF) at 150°C. The PVA has a degree of polymerization of 1,600, molecular weight of 72,000 and degree of hydrolyzation of 97.5-99.5 mol %. In a three-necked, 100 ml flask, equipped with a stirrer, a condenser, a thermometer and a magnetic stirrer were placed 20 g of the PVA, 100 g of distilled DMF and 27.3 g (1 mole urea to 1 base PVA monomer) of urea. The flask was maintained at a temperature of 148-152°C, under nitrogen gas to stop oxygen free radicals degradation in the reaction mixture. The reaction mixture became homogenous within a few minutes and proceeded, accompanied by evolution of gas. The reaction was left for 5 hours. The resulting polymer was precipitated into methanol, purified twice in excess methanol, and then dried under vacuum at 70°C until a constant weight was obtained. The final weight was found to be 21.1 g. NMR spectra of the product was then carried out to determine the degree of incorporation of the urea. The percentage incorporation was found to be 15%.

3.4 Polyethylene Glycol

PEG is one of the most commonly used and studied water-soluble polymers in aqueous two-phase systems. It was selected as a phase polymer in this study because of its low cost and availability in different molecular weights. In addition, the toxicology of PEG is well known and it is included in the pharmacopoeias of many countries.

CHAPTER 4

PHASE BEHAVIOR OF AQUEOUS TWO-PHASE SYSTEMS

4.1 Introduction

An aqueous two-phase system is produced when appropriate small amounts of two-chemically different water soluble polymers, or a water soluble polymer and inorganic salts, are added to water, causing the system to separate into two immiscible water-rich phases [5]. The high water content in aqueous two-phase system, typically greater than 80 % w/w, coupled with its low interfacial tension provide a benign environment for biomolecules not attained in solvent extraction. The system offers a technically simple, energy efficient, easily scalable and mild separation technique for product recovery in biotechnology [97]. Its major use has been in the concentration and purification of proteins and in the extractive bioconversion of enzymes. The separation technique is also becoming important in non-biotechnology areas such as industrial waste remediation. Notable examples include utilization of aqueous phase systems in removal of color from textile plant wastes, metal ions and organic pollutants from the environment, and aromatics from crude oil [44, 50, 51].

The most commonly used and investigated aqueous two-phase systems are composed of either polyethylene glycol (PEG)-Dextran (Dex) or PEG-inorganic salts. Large scale

application of PEG-Dex systems has been limited by the high cost of dextran and the need for enhanced selectivity during protein extraction. And for the PEG-salt systems the economical and environmental problem associated with large consumption of phase forming chemicals, which are difficult to regenerate, has been a major drawback. Due to these limitations there has been continuous interest for developing novel polymers that can be readily recovered from solution and also having properties that can enhance partitioning of substances between the phases. Unlike the PEG-Dex system, where the phase forming components are neutral, some of the new types of aqueous two-phase systems being developed consist of charged components. The use of charged polymers such as trimethylamino-PEG [56] and polyethylenimine [38] as phase forming polymers have been reported recently. And more recently, Al-Muallem *et al* [11] reported the synthesis of new ionic polymers using cheap and commonly available chemicals. The polymers, just like proteins, contain two pH-triggerable functionalities (N and CO_2^-) that make them exhibit pH responsive behavior. One of the polymers, polydiallyaminoethanoate-dimethyl sulfoxide copolymer, is an anionic polyelectrolyte (APE) and it was found to be almost water-insoluble in acidic pH range. This behavior makes it a potential candidate for industrial applications since it can be effectively removed from solution by pH-controlled precipitation. Furthermore, in applications such as protein partitioning, the protein-like structure of the polymer is expected to enhance protein-polymer interactions.

Since most of the studies of aqueous two-phase systems, including theoretical and empirical modeling studies, have focused on PEG-Dex and PEG-salt systems there is

very little understanding of the phase behavior of systems consisting of charged phase-forming components. Cabezas [18], in a comprehensive review of the theory of phase formation of aqueous two-phase systems, highlighted the seeming dearth of theoretical models for the phase behavior of aqueous two-phase systems formed with polyelectrolytes and also recommended the need for such studies.

This chapter reports the result of an experimental study and correlation on the phase behavior of a new anionic polyelectrolyte-nonionic aqueous two-phase system based on polydiallyaminoethanoate-dimethyl sulfoxide copolymer and PEG. The effect of potassium chloride concentration and pH on the position of binodal curve for the system was investigated. The correlation was carried out using a modified version of "the binodal model" developed by Guan *et al* [36], which is based on statistical geometric concepts.

4.2 Thermodynamic Basis of Two-Phase Formation

Mixing aqueous solution of two different polymers may lead to phase separation regardless of whether the polymers are ionic or non-ionic. As a matter of fact, for polymer mixtures miscibility of different aqueous phases is an exception rather than the rule. Two kinds of phase separations can occur depending on the nature of the polymers. Polymer incompatibility will lead to phase separation in a segregative way where two polymers are collected mainly in different phases, i.e. each phase is rich in one of the polymers. Association of unlike polymer species into a polymer-rich phase coexisting

with a polymer-poor phase can also occur. This type of phase separation is referred to as complex coecervation. Polymer mixtures with both polymers either ionic with similar charges or nonionic lead to segregative type of phase separation, whereas mixtures with one polymer having cationic backbone and the other with anionic backbone results in complex coecervation. Segregative phase separation occurs if the effective interaction between polymers is repulsive or if the two differ in their interaction towards the solvent.

Phase separation occurs because of the high molecular weight of the polymers combined with interaction between the segments of the polymers. The high molecular weight leads to an enthalpic domination of the system's free energy of mixing. The driving force for the demixing process in polymer-polymer-solvent systems is the enthalpy associated with the segregation of the components during phase separation. Water as the solvent is able to engage in a number of noncovalent interactions with the polymer, which makes the quantitative description of the phenomenon quite complex. Because the interactions increase with the size of the molecules, the phase separation in aqueous two-phase systems occurs at very low polymer concentrations because of their large size and corresponding small loss in entropy upon demixing.

4.3 Phase Diagram

A *phase diagram* graphically depicts the phase behavior, thereby delineating the potential working area, of an aqueous two-phase system. It provides information about the polymer compositions necessary to form a system with two phases that are in equilibrium, the

resulting concentration of phase components in the top and bottom phases, and the ratio of phase volumes. The phase diagrams are useful tools for finding systems with the desired properties, thus providing vital information required for protein separation work. Figure 4.1 is a phase diagram of polymer Y-polymer X-solvent system. The Y-rich phase is the top phase and the X-rich phase is the bottom phase. By convention, the component predominantly in the bottom phase is plotted as the abscissa and the component predominantly in the top phase as the ordinate. The concentrations are expressed as weight percents. Present on the diagram is a *binodal curve*, which divides a region of component concentrations that will form two immiscible aqueous phases from those that will form one phase. At points below the binodal curve (e.g. point D), the system is a homogenous liquid. Point Cp is the *critical point* at which the compositions of the two liquid phases are identical. The composition represented by point Cp is termed the *critical composition*. Above the critical point, the system splits into two separate phases. Coordinates for all potential systems will lie on a *tie-line*. The tie-line connects two *nodes* on the top and bottom phases. For instance, if the solution is mixed with compositions at point A, two phases will result at equilibrium. The equilibrium compositions of the top and bottom phases will be represented by the nodal points T and B respectively. Moving along the tie-line coordinates denote systems with differing total compositions and volume ratios, but with the same final concentration of phase components in the top and bottom phases. The ratio of segments AB (top phase) and AT (bottom phase) can be estimated graphically by using the weight ratio $V_t\rho_t/V_b\rho_b$= AB/AT where V and ρ are the volume and density of the top (t) and bottom (b) phase.

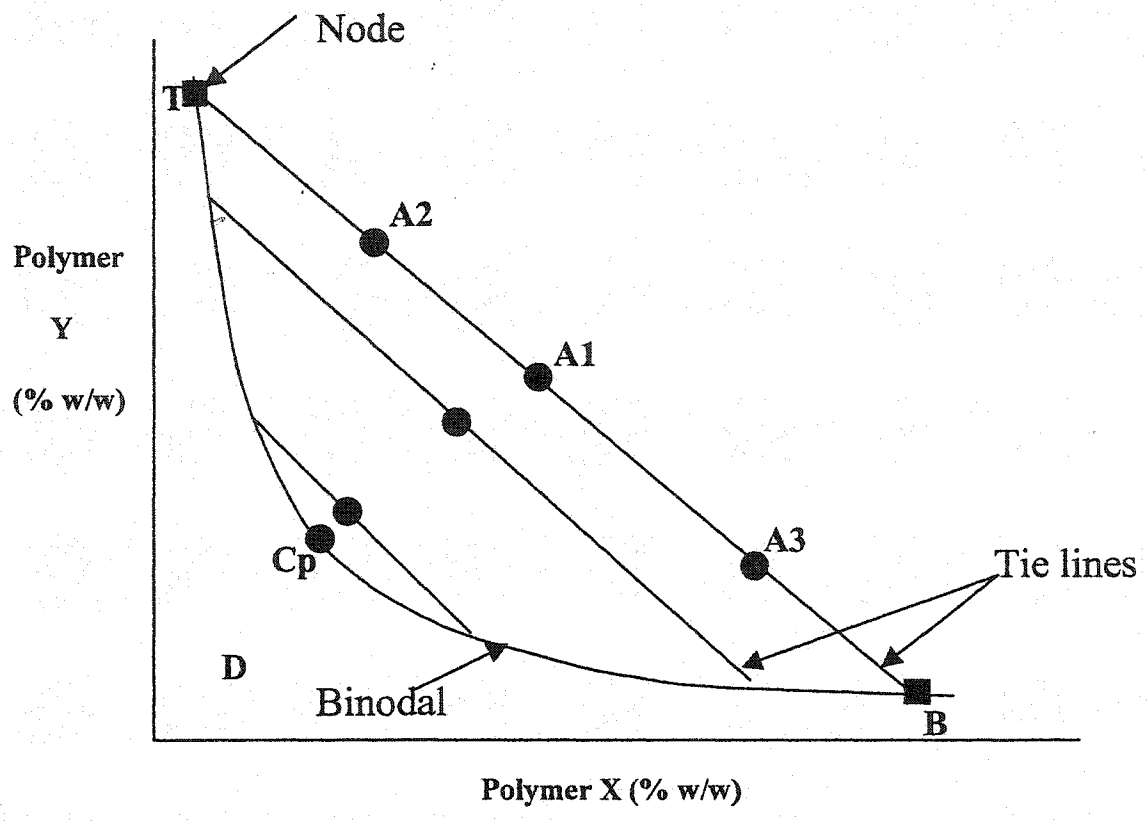


Figure 4.1. Illustration of the phase diagram. Bottom phase polymer (% w/w) is plotted on the abscissa and top phase polymer Y (% w/w) is plotted on the ordinate. A1, A2, and A3 represent the total compositions (●) of three systems lying on the same tie line with different volume ratios. The final composition of the top and bottom phase is represented by nodes T and B (■).

The tie line length (TLL) is a common parameter used to correlate experimental data for aqueous two-phase systems. It is a measure of the composition difference between the two phases at equilibrium and is defined as;

$$TLL = \sqrt{(w_1'' - w_1')^2 + (w_2'' - w_2')^2} \qquad (4.1)$$

where w_1 and w_2 represent the weight percent of polymer Y and X, respectively in the top (") and bottom (') phase. Tie lines are commonly parallel and hence the slope of the tie line can be calculated and used for the construction of further tie lines. As tie lines decrease in length they ultimately approach a critical point on the binodal where TLL=0. At this point the composition and volume of the two phases theoretically become equal.

4.4 Determination of the Binodal Curve

The binodal curve may be constructed using one of the following methods: turbidometric titration, cloud point method or node determination.

4.4.1 Turbidometric Titration

Turbidometric titration is a relatively quick and commonly used method for the determination of the binodal. When phase components are mixed, the mixture becomes turbid only if immiscibility occurs. This permits a visual measurement of the binodal when the polymers are monodisperse. In this method, a turbid two-phase mixture with a known concentration of the two-phases (w/w %) is quantitatively diluted with water (or

salt solution) until a homogenous solution is obtained. The final compositions of the two polymers calculated correspond to one point on the binodal curve. After obtaining the first point, a concentrated solution of one of the polymers is quantitatively added to the solution to again obtain a turbid dispersion. Dilution with water is repeated to obtain a homogeneous solution and composition of which represents a second point in the binodal curve. This procedure is continued until a sufficient number of points for the construction of the binodal curve are obtained.

4.4.2 Cloud Point Method

The cloud point method follows a similar principle to the turbidometric titration method. A concentrated stock of one of the polymers is added drop-wise to a known amount of concentrated stock of the second polymer. At a critical point (the cloud point) the mixture will become turbid and is indicative of two-phase formation. The composition, just prior to two-phase formation, is calculated and provides a point on the binodal. The mixture is then diluted to below the cloud point and the procedure is repeated.

4.4.3 Node Determination

The former methods are relatively inaccurate when using polymers that are polydisperse. Such polymers produce a gradual transition in turbidity rather than a sharp change, making the point of transition imprecise and difficult to determine. An alternative method, used in order to circumvent the problem of polydispersity, is by determination of the nodes of a series of systems, thus, providing points on the binodal. This method,

although more tasking, has an additional advantage of providing information for the evaluation of tie-line length. There are several techniques of determining the phase composition of the polymers. These include optical rotation (for phase components containing an asymmetric center that can rotate a plane of polarized light), refractive index, dry weight, conductivity measurements, or ^{1}H-NMR spectroscopy. The method employed in this study is ^{1}H-NMR spectroscopy. Hence, a detailed description of the method will be in the following section.

4.4.3.1 Node Determination Using ^{1}H-NMR Spectroscopy

In using this method, series of aqueous mixtures of polymer P and Q are prepared in 4 ml vials. The mixtures are then gently centrifuged for about 5 minutes to complete the separation process. After keeping the sample for 24 hours, the volume and density of the top and bottom layers are determined. Phase density can be determined using pycnometer or by weighing a known volume of phase in a volumetric flask using an analytical balance. In this study, phase densities were obtained by weighing a known volume of phase in a Pasteur pipette. The weights of the top and bottom phases are calculated from the volume and density of the phases. The mole ratio of the polymers in each phase is determined by running ^{1}H-NMR spectra on samples taken from the phases. A JOEL LA 500 MHz spectrometer was used to obtain the ^{1}H-NMR spectra of the polymer solutions in D_2O using dioxane as internal standard. These data are then used to evaluate the phase compositions of polymer P and Q in the system by employing the equations presented below.

Consider an aqueous two-phase system containing polymers P and Q. The polymer mole balance equations can be written as follows:

P component: $[P]_t V_t + [P]_b V_b = P_o/M_P$ (4.2)

Q component: $[Q]_t V_t + [Q]_b V_b = Q_o/M_Q$ (4.3)

Where

$[P]_t$, $[P]_b$ = molar concentration of polymer P in the top and bottom phase, respectively.

$[Q]_t$, $[Q]_b$ = molar concentration of polymer Q in the top and bottom phase, respectively.

V_t, V_b = the volume of the top and bottom phase, respectively.

P_o, Q_o = total mass of the polymer P and Q, respectively, in the system.

M_P and M_Q = molar mass of repeated units for polymer P and Q, respectively.

Equations (1) and (2) can be re-written as:

$$[Q/P]_t[P]_t V_t + [Q/P]_t[P]_b V_b = [Q/P]_t\{P_o/M_P\} \quad (4.4)$$

$$[P]_t[Q/P]_t V_t + [P]_b[Q/P]_b V_b = Q_o/M_Q \quad (4.5)$$

Subtracting equation (3) from (4) followed by simple rearrangement gives

$$[P]_b = \frac{(Q_o/M_Q) - (P_o/M_P)([Q]/[P])_t}{V_b\{([Q]/[P])_b - ([Q]/[P])_t\}} \quad (4.6)$$

The mass of the polymer P in the bottom phase is then calculated using

$$P_b = [P]_b V_b M_b \quad (4.7)$$

Once one of the polymer concentrations is known in a phase, then the rest of the concentrations in the two phases are easily calculated from the known volume, density and mass of the two phases.

4.5 Prediction of Aqueous Two-Phase Diagram

A set of equations for predicting phase separation, useful in process design, would greatly aid in the incorporation of ATPS on a more widespread basis in industrial applications. King [64] has provided a criterion for developing a correlation that is useful for engineering calculations. According to him, the equations must 1) be simple to use; 2) have minimal number of parameters that can be easily evaluated (preferably from independent measurements); and 3) provide accurate results. Moreover, a model that is predictive, in the sense that no adjustable parameters are required, is desirable. An added benefit would be the capability of providing an accurate physical picture of the molecular mechanisms occurring in the systems.

4.5.1 The Binodal Model

Based on statistical geometric arguments Guan et al [36, 37] developed a simple one parameter model for the binodal curve in polymer-polymer aqueous two-phase systems. They made the following fundamental assumptions in the development of the model; (i) molecules of the same species are distributed at random according to an exponential law and (ii) solute molecules of one species, characterized by effective excluded volume (EEV), almost fill the corresponding effective available volume i.e. the structure of the solution is geometrically saturated. Here only the essential features of the model will be highlighted since its extensive description can be found in the literature [36, 37].

In a pseudo-binary system i-*j*-*0* with molecular number densities v_i and v_j, the probability of finding no species *j* in an arbitrary located volume V_{ij0} is given by applying the Poisson distribution:

$$P(\underline{V} \geq V_{ji0}) = e^{-V_{ji0}v_i} \tag{4.8}$$

The volume V_{ji0} is termed the "effective excluded volume" of molecule *j* in the *i-j-0* pseudo-binary system. It represents the minimum volume in the molecular center network of species *i* which holds an individual *j* molecule.

Assumption (ii) in conjunction with Eq. 4.8 leads to the following equation:

$$e^{-V_{ji0}v_i} = V_{ji0}v_j + f_{ji0} \tag{4.9}$$

where f_{ji0} is the volume fraction of unfilled effective available volume after tight packing of *j* molecules into the network of species *i* aqueous solution.

For systems with *i* and *j* molecules having highly dissimilar sizes f_{ji0} will become insignificant thus Equation 4.9 can be simply written as:

$$e^{-V_{ji0}v_i} = V_{ji0}v_j \tag{4.10}$$

By treating the solution density, ρ, as a constant and using the root mean-square molar mass $\left(\langle M_{rms}\rangle = \sqrt{\langle M_w\rangle/\langle M_n\rangle}\right)$ for a polydisperse species it can be shown that Equation 4.10 assumes the following form:

$$\ln\left(\langle V^*\rangle_{ji0}\frac{w_j}{\langle M_{rms}\rangle_j}\right) + \langle V^*\rangle_{ji0}\frac{w_i}{\langle M_{rms}\rangle_i} = 0 \tag{4.11}$$

where $\langle V^*\rangle_{ji0} = N_A\langle \rho V_{ji0}\rangle$ is a new parameter called the average scaled EEV and w_i and w_j are the weight percent of *i*-mer and *j*-mer, respectively.

Using PEG-Dex aqueous two-phase system Guan et al [37] have shown that the above model equation only gives satisfactory agreement with experiment when the molar mass ratio of Dex to PEG $\geq ca.$ 4.

4.5.2 Modified Binodal Model

In this section we intend to develop a modified form of the binodal model which can be applied to systems where molar mass ratio of the phase-forming polymers is close to unity. From the derivation in the previous section it is clear that for such systems the term f_{ji0}, which appears in Equation 4.9, can not be simply ignored. The contribution of this term to the right hand side of Equation 4.9 can be taken care of, as a first approximation, by incorporating it into the first term, i.e.,

$$V_{ji0}v_j + f_{ji0} \approx V_{ij0}v_j \tag{4.12}$$

where $V_{ij0} > V_{ji0}$. With this approximation Equation 4.10 takes the following new form

$$e^{-V_{ji0}v_j} = V_{ij0}v_j \tag{4.13}$$

From Equation 6 it can be readily established that

$$\ln\left(\left\langle V^*\right\rangle_{ij0}\frac{w_j}{\left\langle M_{rms}\right\rangle_j}\right)+\left\langle V^*\right\rangle_{ji0}\frac{w_i}{\left\langle M_{rms}\right\rangle_i}=0 \tag{4.14}$$

It is important to note the main difference between the binodal model developed by Guan *et al* (Equation 4.11) and the modified form (Equation 4.14) presented here. The modified form is a two parameter model while Equation 4.11 contains only one parameter.

4.6 EXPERIMENTAL

4.6.1 Materials

Polymers used in this study are; poly(ethylene glycol) (MW 35,000), poly(vinyl alcohol)(MW 72,000), urethanized polyvinyl alcohol (UPVA), hydrophobically modified polyvinyl alcohol (HMPVA), polyacrylamide-methoxy styrene (PAA-MS) and anionic polyelectrolyte (APE). The PAA-MS has 1.7 % incorporation of styrene and the UPVA has a 15 % incorporation of urea. The poly(ethylene glycol) was purchased from Mark-Schuchardt. The Polyvinyl alcohol was obtained from Fluka. The UPVA was prepared using the method explained in chapter 3. The APE, HMPVA and PAA-MS were generous gift from Dr Ashrof Ali. Potassium chloride and other chemicals used are of analytical grade. All glassware are cleaned using deionized water.

4.6.2 Phase Behavior Studies

In this work, four different aqueous two-phase systems were studied. These are:

1. Three stock solutions of APE (~19%) were prepared, each treated with a different amount of HCl; 0.55, 0.8 and 0.9 equiv. HCl. The corresponding pH values of the APE stock solutions, measured using a Corning pH meter, were found to be 7.89, 7.41 and 7.37, respectively. PEG stock solution of concentration 25 % w/w was also prepared. All the stock solutions were prepared in 0.1 N KCl. Known weights of stock solutions of APE (treated with 0.55 equiv. HCl) and PEG were added to a 10 mL magnetically stirred conical flask until the clear system became turbid. Then the system was titrated, dropwise, with 0.1 N KCl until it became clear, i.e., one phase is formed. The weight of KCl solution added just prior to one phase formation was

noted. At this point, the final composition of the polymers calculated corresponds to a point on the binodal curve. After obtaining the first point, a concentrated solution of PEG was added again to obtain a turbid suspension, and dilution with 0.1 KCl solution was repeated to obtain a second point on the binodal. This procedure was continued until a sufficient number of points for the construction of the binodal curve were obtained. Similar experiments were conducted using the other two APE stock solutions (i.e. solutions treated with 0.8 and 0.9 equiv. HCl). Precise compositions of the phases were determined by analyzing the compositions of the top and bottom phases using ^{1}H NMR technique. To study the effect of salt concentration on the phase behavior of the systems, the experiments were repeated in 0.3 N KCl.

2. Stock solutions of PVA (10 % w/w) and APE (19 % w/w) were used to study the phase behavior of PAA-MS/APE/water systems.

3. Stock solutions of UPVA (15 % w/w) and APE (19 % w/w) were used to study the phase behavior of UPVA/APE/water systems.

4. Stock solutions of HMPVA (10 % w/w) and APE (19 % w/w) were used to study the phase behavior of HMPVA/APE/water systems.

4.7 RESULTS AND DISCUSSIONS

4.7.1 Phase Behavior

The APE does not form a two-phase system with any of the polymers considered in this study except PEG. On mixing the APE with PVA in the presence of large amount of water a one phase system was initially observed. Upon addition of more APE a cloudy suspension was noticed. After centrifuging the mixture a pseudo two-phase system was formed. Close examination reveals that what resulted was not actually a two-phase system because the bottom layer was completely immobile. Several other compositions were tried but the same result was observed in all the cases. Similar results were obtained when the APE was mixed with UPVA or HMPVA.

The APE forms two-phase system with PEG (molecular weight 35 000). While the PEG showed greater preference to remain in the top phase, the APE displayed predominant presence in the bottom phase. The experimental binodal data obtained for the aqueous two-phase system composed of the PEG-APE-water is presented in Table 4.1 and 4.2 and graphically depicted in Figure 4 .2. The binodal curves were constructed by turbidometric titration as explained in the experimental section. Figure 4.2 suggests that both pH and salt concentration have significant influence on the phase composition of the system.

TABLE 4.1 Binodal data for PEG-APE-water (0.1 N KCl) system at 296.15 K

pH 7.89		pH 7.41		pH 7.37	
APE (%w/w)	PEG (%w/w)	APE (%w/w)	PEG (%w/w)	APE (%w/w)	PEG (%w/w)
14.2	0.3	11.4	0.3	12.7	0.2
12.7	0.5	10.1	0.6	12.1	0.3
12.0	0.9	8.6	1.1	10.1	0.4
11.2	1.4	7.9	1.5	8.9	0.6
10.4	2.0	6.7	2.0	8.0	0.8
9.4	2.9	5.9	2.5	7.3	0.9
8.6	3.6	5.3	3.0	6.3	1.1
7.8	4.4	4.7	3.4	5.4	1.4
6.8	5.4	4.2	3.8	4.7	1.5
6.3	6.0	3.8	4.2	3.2	2.3
5.8	6.7	3.5	4.5	2.5	2.5
5.3	7.5	3.1	4.9	1.4	3.1
4.8	8.3	2.9	5.1	1.1	3.3
4.2	9.1	2.7	5.4	0.9	3.6
3.9	9.9	2.5	5.6	0.7	3.7
3.3	11.3	2.3	5.8	0.2	4.3
		2.2	6.1	0.1	4.4
		2.0	6.3		
		1.9	6.2		
		1.7	6.5		
		1.6	6.8		
		1.4	7.1		
		1.2	7.4		

TABLE 4.2 Binodal data for PEG-APE-water (0.3 N KCl) system at 296.15 K

pH7.89		pH7.41		pH7.37	
APE (%w/ w)	PEG (%w/ w)	APE (%w/ w)	PEG (%w/ w)	APE (%w/ w)	PEG (%w/ w)
11.4	0.5	10.0	0.4	9.7	0.4
8.2	1.1	8.8	0.8	8.3	0.8
7.5	1.7	8.1	1.1	7.3	1.1
6.7	2.3	7.3	1.5	6.3	1.5
6.1	2.8	6.6	1.7	4.9	2.0
5.7	3.2	5.7	2.0	4.3	2.4
5.2	3.6	5.1	2.5	3.8	2.7
4.7	4.0	4.9	2.8	3.1	3.1
4.5	4.2	4.2	3.2	2.8	3.3
4.1	4.5	3.9	3.4	2.5	3.5
3.9	4.8	3.5	3.7	2.3	3.7
3.6	5.1	3.1	3.9	2.0	3.8
3.4	5.3	3.0	4.0	1.8	3.9
3.1	5.6	2.7	4.2	1.7	4.1
2.9	5.9	2.5	4.4	1.5	4.2
2.6	6.3	2.3	4.6	1.3	4.4
2.4	6.7	2.1	4.7	1.2	4.5
2.2	7.0	2.0	4.8	1.1	4.6
2.0	7.2	1.9	4.9	1.0	4.7
1.9	7.4	1.7	5.0	0.9	4.8
1.8	7.6	1.6	5.2	0.8	4.9
1.3	8.2	1.5	5.4	0.6	5.4
1.0	9.0	1.4	5.5	0.3	5.9
0.6	10.1	1.3	5.7	0.2	6.4
0.4	11.7	0.8	6.4	0.1	8.2
0.2	14.2	0.5	7.2		
		0.3	8.3		
		0.1	10.2		

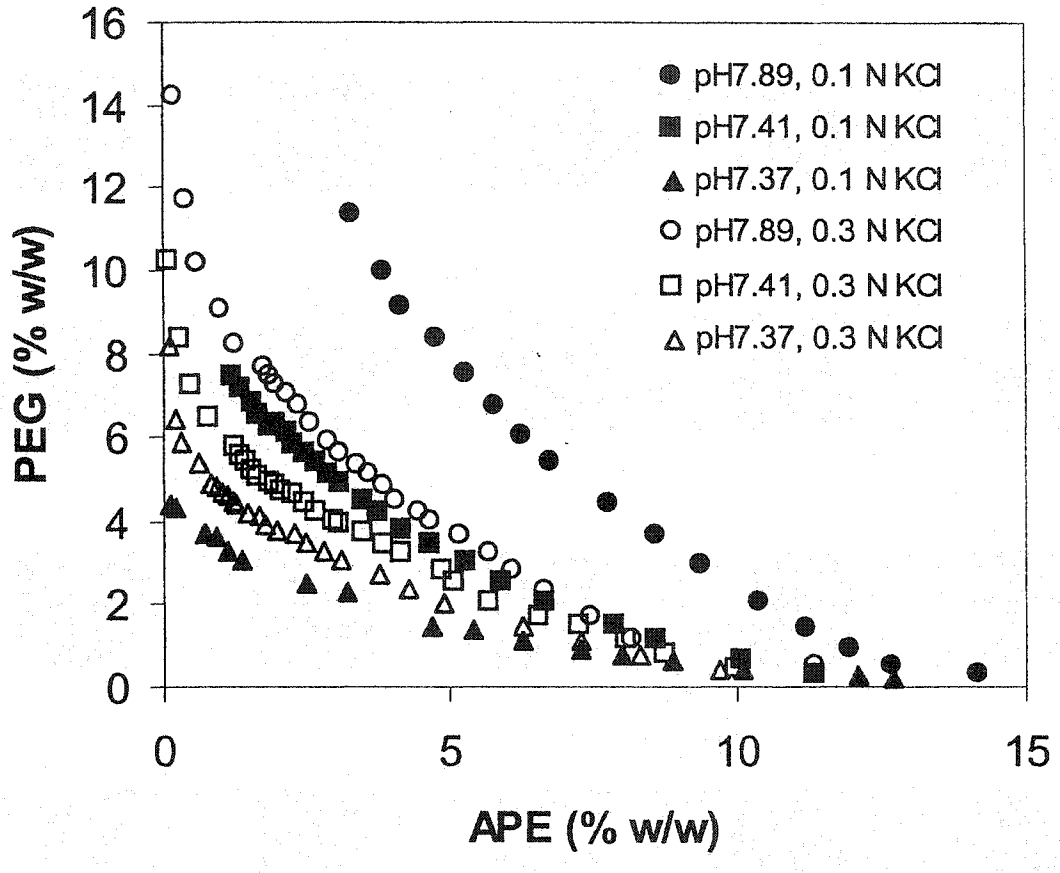


Figure 4.2. Effect of pH and salt concentration on the binodal of the PEG-APE-water system at 296.15 K.

lower, indicating that smaller concentration of the phase polymers is required to form It can be seen that the binodal curve shifts downward as the pH of the medium becomes aqueous two-phase system at low pH values. The observation is true for both concentrations of KCl investigated in this study. This behavior is probably due to decrease in hydrodynamic volume of the APE as the concentration of HCl in the solution increases. In a previous study [10] on the effect of added HCl on the viscosity behavior of the APE it was shown that increase in HCl concentration leads to reduction in intrinsic viscosity of the APE solutions. Since it is well-known that hydrodynamic volume of polymers in solution is directly proportional to their intrinsic viscosity it can be deduced that increasing the HCl concentration reduces the net charge of the polymer chain. The decrease in net charges leads to a consequent reduction of intramolecular electrostatic repulsions that cause the chain to assume a more coiled structure of smaller hydrodynamic size (see Figure 4.3). The coiling of the polymer results in less interaction between the APE and PEG and as a result lower concentration of polymers are required for phase separation.

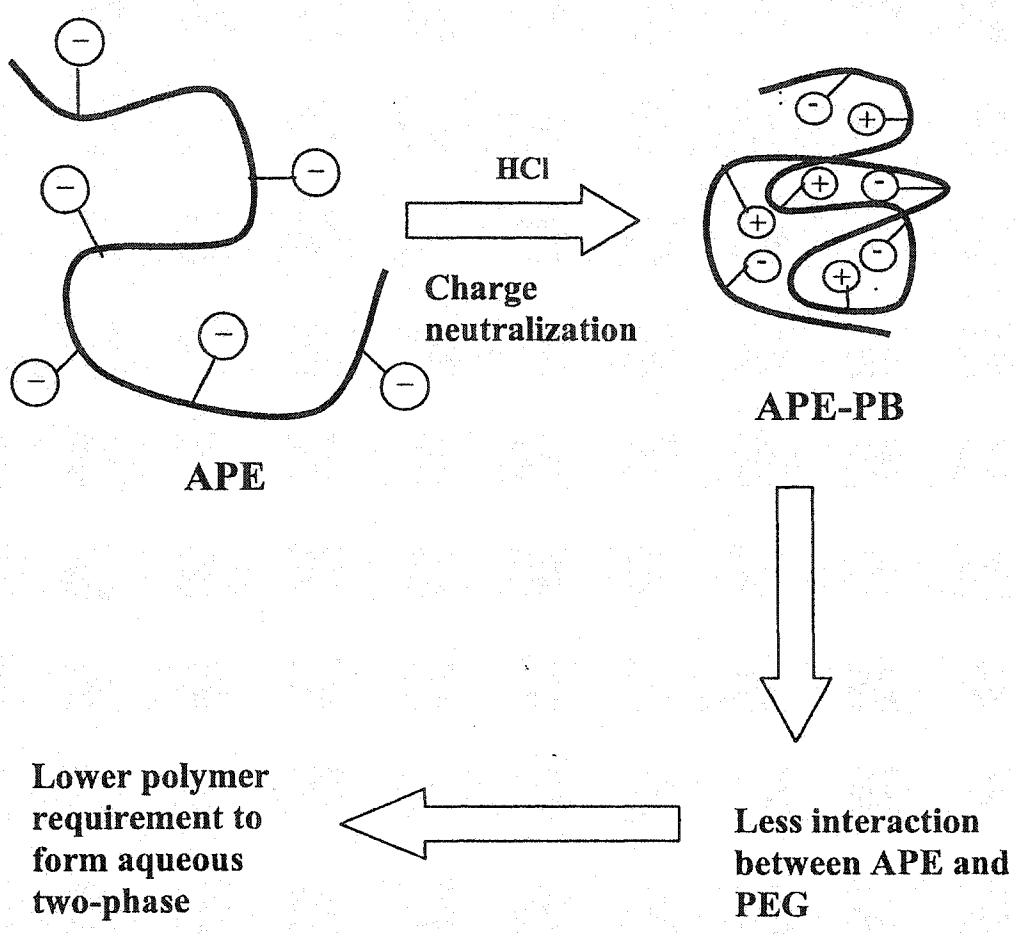


Figure 4.3 Effect of pH on the behavior of APE in solution.

The effect of added electrolyte on the phase behavior of the APE-PEG system can also be seen in Figure 4.2. It is interesting to note that at pH7.89 much lower polymer concentrations are required for phase separation at 0.1N KCl compared to 0.3 N KCl. The same trend was observed at pH7.41. But at pH7.37 the opposite trend was noticed, i.e. the binodal moves upward upon addition of more salt. According to Munk [79], the addition of small amounts of electrolytes to solution of simple linear polyelectrolytes lead to Debye-Huckel shielding effect making the polymer undergo a conformational transition, adopting a smaller, more entropically favored conformation. This behavior is depicted in Figure 4.4 They further explained that the opposite behavior is typical of aqueous solutions of polyampholytes. In polyampholytes, the addition of salt will result in reducing electrostatic attraction rather than reducing electrostatic repulsion (anti-polyelectrolyte effect). The behavior we observed at pH7.89 and 7.41 was probably due to the more polyelectrolytic nature of the polymer at these pH values compared to pH7.37, where the polymer has a APE:PB mole ratio of 1:9 and hence become more polyampholytic in nature. The polyampholytic nature of the polymer at pH7.37 makes it assume an extended rod-like conformation upon addition of more salt. This chain expansion can lead to more interactions between the phase polymer and hence higher total polymer requirement for phase separation at 0.3 N KCl compared to 0.1N KCl.

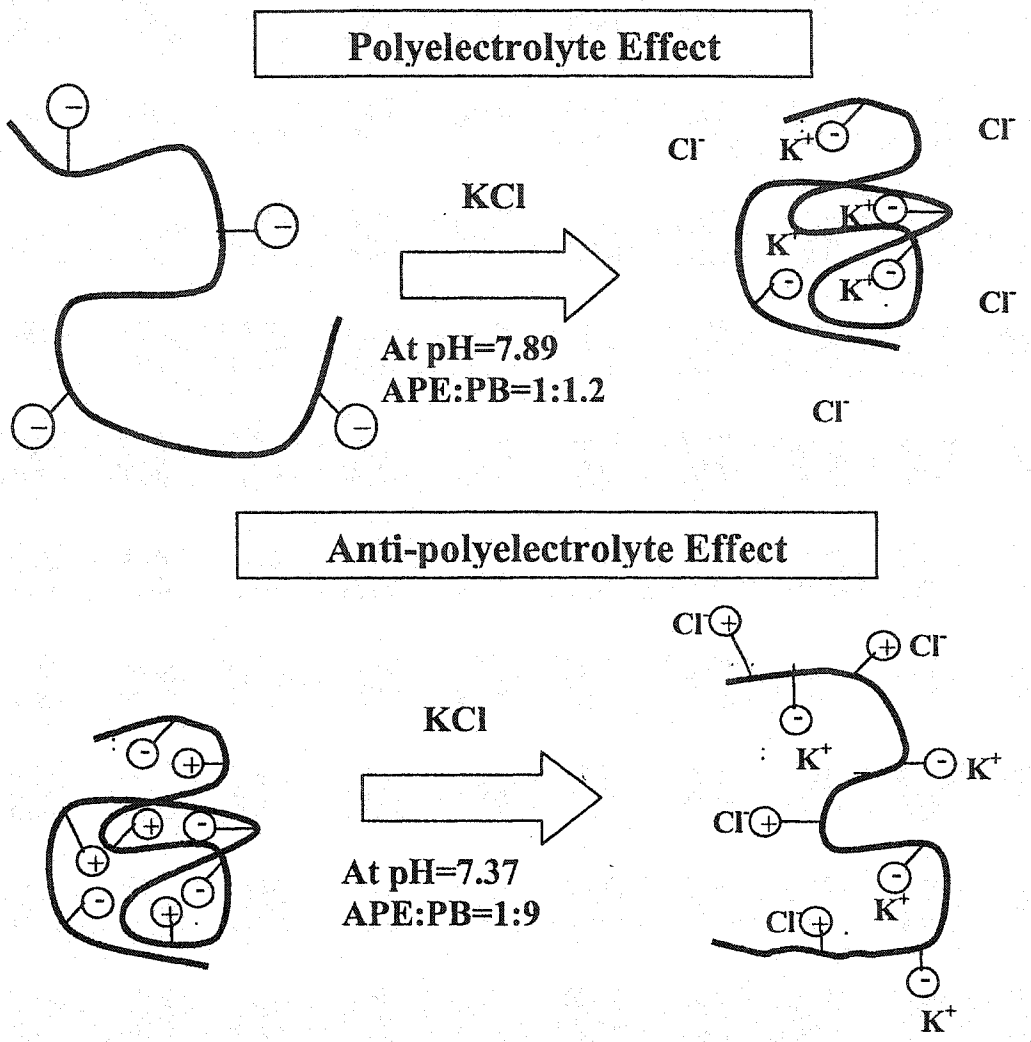


Figure 4.4 Behavior of charged macromolecules in solution upon addition of salt.

Tie lines were constructed for systems at different pH values and salt concentrations by ^{1}H NMR technique. The phase compositions of the systems used for the construction of the tie lines are given in Tables 4.3 and 4.4. The tie lines are helpful in constructing two-phase systems with similar volume ratio of the top and bottom phases. Figures 4.5-4.8 show the phase diagrams constructed from the binodal data given in Tables 4.1 and 4.2 and the nodal points data presented in Tables 4.3 and 4.4.

Phase separation in the system under study takes place at a relatively low total polymer concentration. This can be seen from a comparison of our system with a commonly studied two-phase system formed by PEG and dextran as presented in figure 4.9. The data for the PEG3400-DexT70 system was reported by Diamond and Hsu [21].

TABLE 4.3 Phase compositions of the PEG-APE-Water (0.1 N KCl) system at 296.15 K

System	Total system (% w/ w)			Top phase (% w/ w)		Bottom phase (% w/ w)		
No	APE	PEG	pH	APE	PEG	APE	PEG	Vol ratio
1	7.18	3.46	7.41	2.08	5.94	13.67	0.31	1.32
2	13.43	6.49	7.41	0.00	12.50	27.96	0.00	1.18
3	9.30	6.10	7.41	0.90	9.90	23.20	0.20	1.50
4	8.45	4.00	7.41	1.20	7.40	16.00	0.30	1.12
5	6.87	2.17	7.37	5.44	4.05	8.39	0.16	1.11
6	8.10	1.71	7.37	4.00	2.93	12.54	0.39	1.12
7	5.81	7.94	7.37	2.13	11.25	14.60	0.04	2.60

$$* \ Volume\ ratio = \frac{volume\ of\ top\ phase}{volume\ of\ bottom\ phase}$$

TABLE 4.4 Phase compositions of the PEG-APE-Water (0.3 N KCl) system at 296.15 K

	Total system (% w / w)			Top phase (% w / w)		Bottom phase (% w / w)		
System	APE	PEG	pH	APE	PEG	APE	PEG	Vol ratio
1	9.19	3.23	7.37	0.20	6.60	17.16	0.21	1.00
2	6.37	2.92	7.37	0.25	6.20	11.40	0.30	1.00
3	5.78	2.61	7.37	0.30	5.90	8.96	0.64	1.00
4	5.7	2.49	7.37	0.50	5.60	8.30	0.80	1.00
5	9.39	4.96	7.41	0.10	10.00	17.54	0.41	1.00
6	6.93	2.96	7.41	1.74	5.30	12.80	0.45	1.00
7	6.53	2.66	7.41	2.70	4.20	11.91	0.42	1.00
8	6.41	2.33	7.41	3.50	3.70	11.00	0.40	1.00
9	10.64	8.58	7.89	0.20	13.90	20.30	3.57	1.00
10	7.54	5.08	7.89	0.50	10.70	13.60	0.44	1.00
11	7.41	4.62	7.89	0.80	9.50	13.00	0.40	1.00
12	6.68	4.04	7.89	1.30	8.20	11.40	0.50	1.00

$$*Volume\ ratio = \frac{volume\ of\ top\ phase}{volume\ of\ bottom\ phase}$$

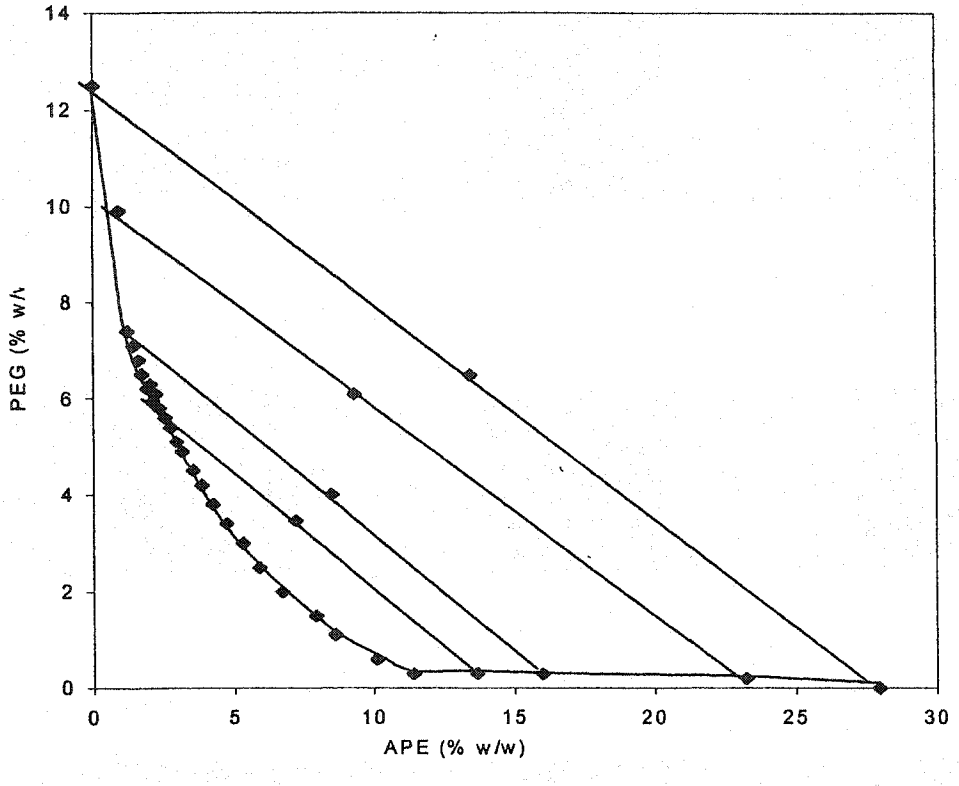


Figure 4.5 Phase diagram for the system APE-PEG-Water (0.1N KCl) at 296.15 K and pH7.41.

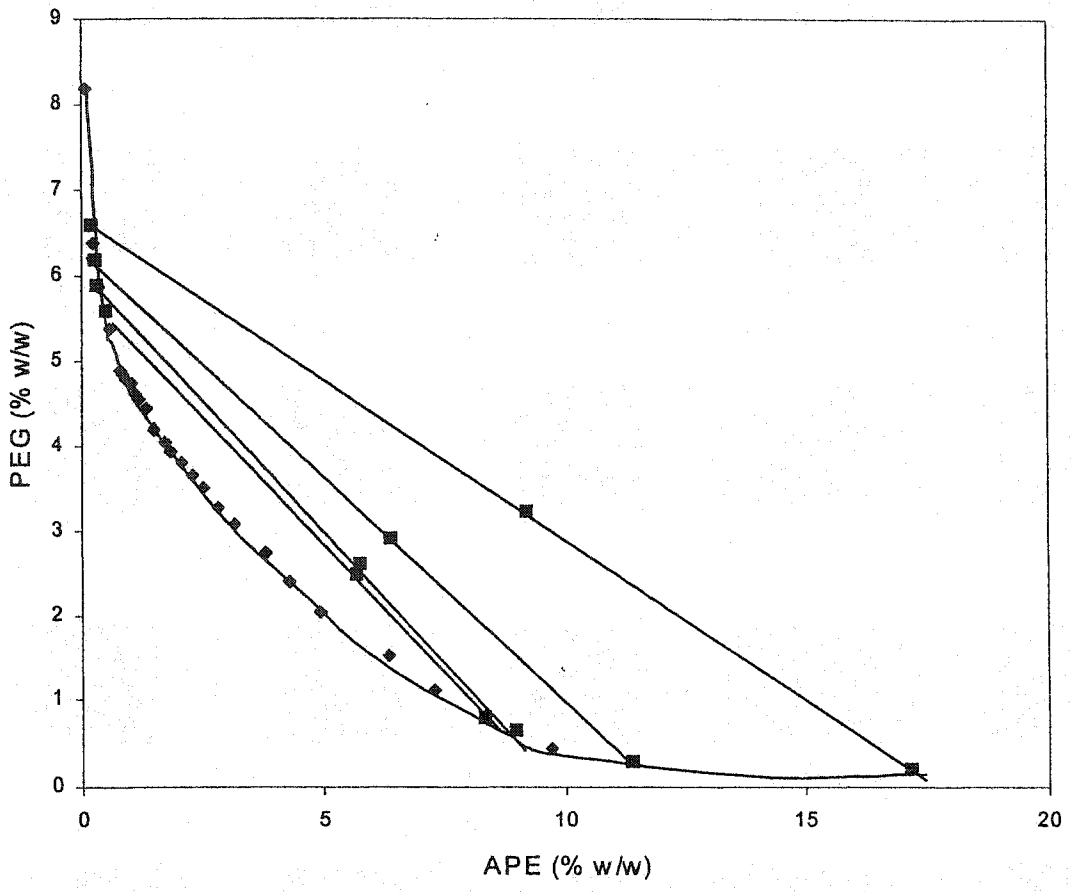


Figure 4.6 Phase diagram for the system APE/PEG/Water (0.3N KCl) at 296.15 K and pH7.37.

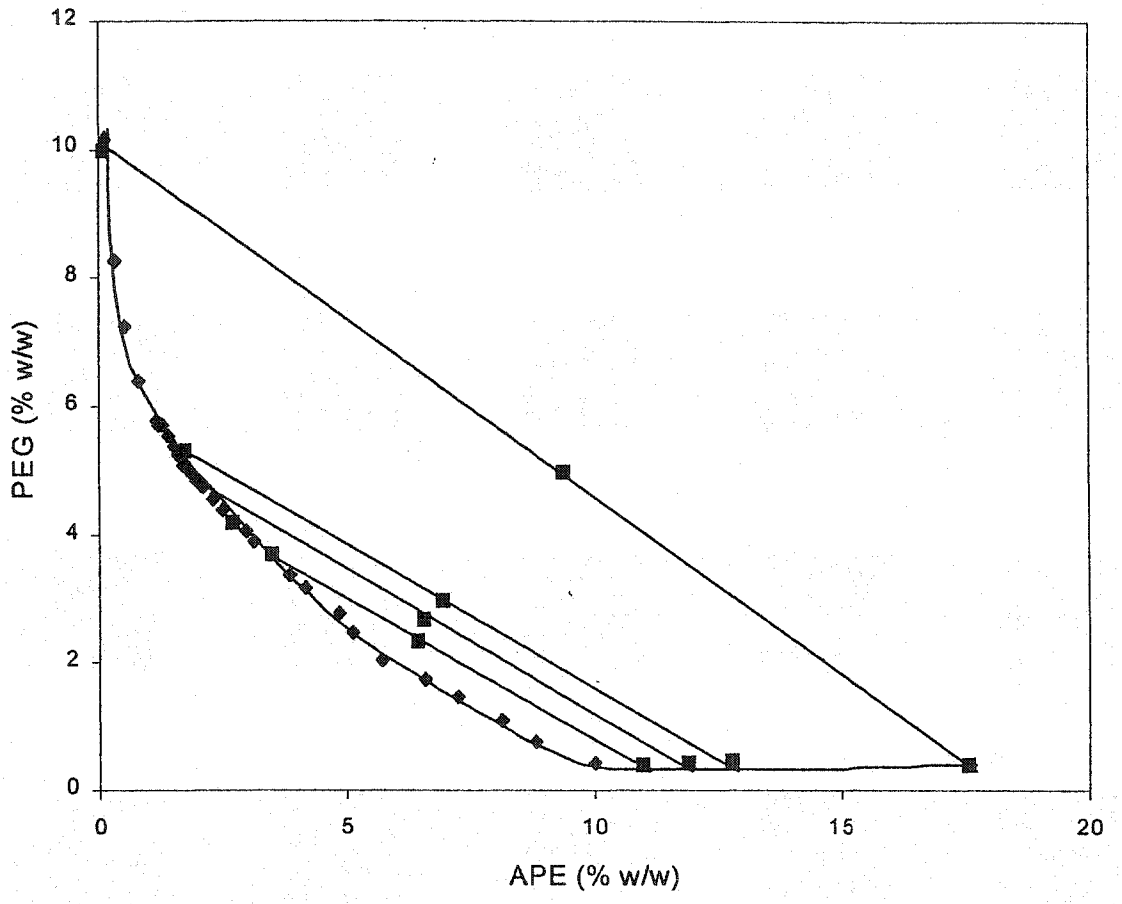


Figure 4.7 Phase diagram for the system APE/PEG/Water (0.3N KCl) at 296.15 K and pH7.41.

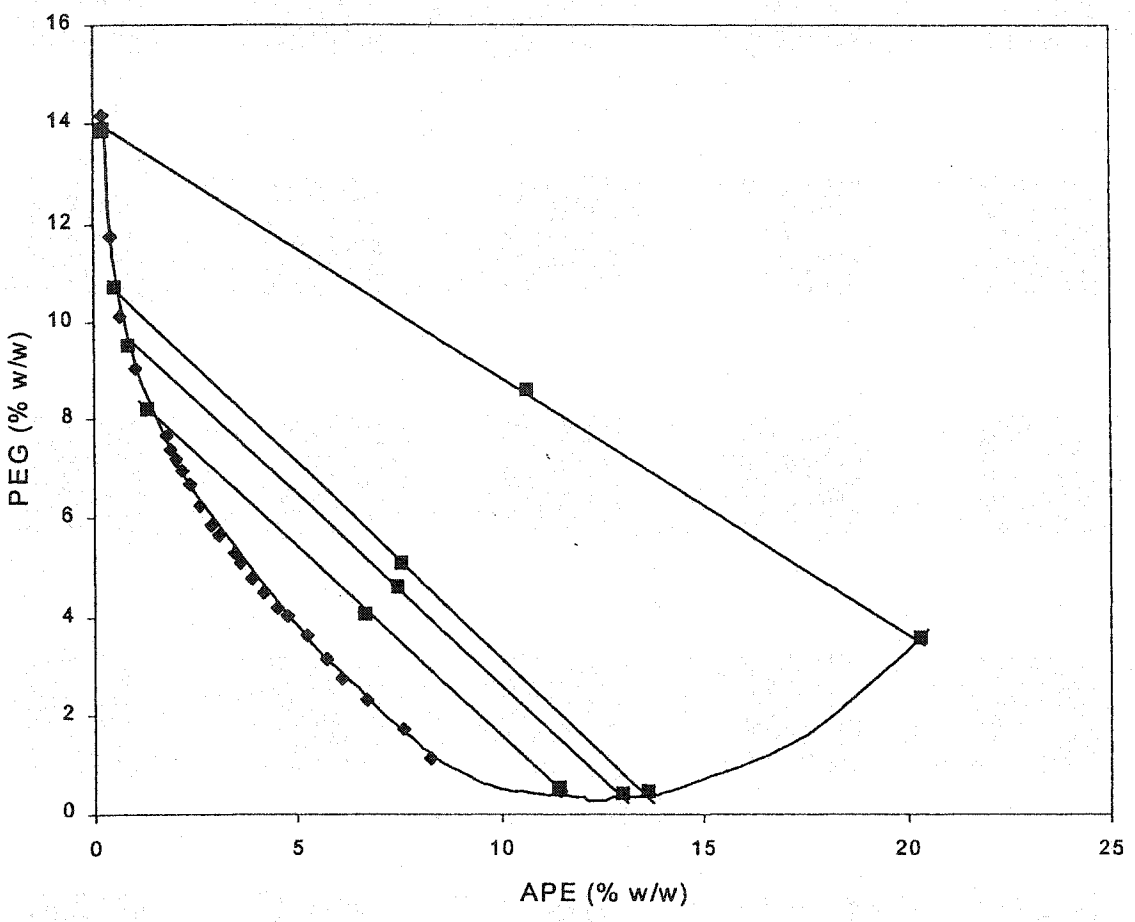


Figure 4.8 Phase diagram for the system APE-PEG-Water (0.3N KCl) at 296.15 K and pH7.89.

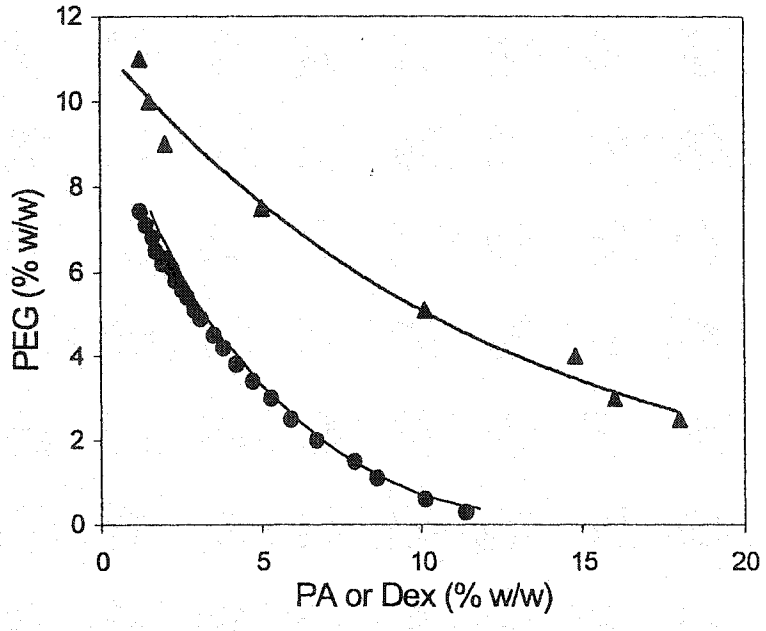


Figure 4.9. Comparison of phase diagrams of PEG-Dex-Water and PEG-APE-Water (0.1 KCl, pH7.41) systems at 296.15 K. (▲) PEG-Dex-Water; (●) PEG-APE-Water. Data for the PEG-Dex-water system was from Reference [21].

4.7.2 Correlation of Phase Behavior

The applicability of the modified binodal model developed in Section 4.5.2 was tested using the experimental data presented in Tables 4.1 and 4.2. The modified binodal model is reproduced below.

$$\ln\left(\langle V^* \rangle_{ij0} \frac{w_j}{\langle M_{rms} \rangle_j}\right) + \langle V^* \rangle_{ji0} \frac{w_i}{\langle M_{rms} \rangle_i} = 0 \qquad (4.14)$$

The PEG-APE-water system used in this study has a molar mass ratio of APE to PEG equal to 0.94. In using the modified binodal model (Equation 4.14) the subscripts *i, j*, and *0* refer to APE, PEG and water, respectively. Comparison of the experimental and predicted binodals of the PEG-APE aqueous two phase systems are shown in Figures 4.10-4.15. It can be seen from the figures that the model can satisfactorily reproduce the experimental results. The values of the model parameters and the correlation coefficients are given in Table 4.5. The parameters were evaluated using a least square algorithm. The parameter V^*_{ji0} stands for the EEV of PEG in an APE solution, while V^*_{ij0} is the EEV of APE in a PEG solution. The latter represents the smallest spacing of PEG which will accept an individual APE molecule. The numerical value of this parameter would be expected to decrease as the pH of the aqueous phase system increases. This is because an increase in pH makes the polymer (APE) more polyelectrolyte in nature thereby increasing electrostatic repulsion between similar charged groups. The repulsion makes the polymer assumes a more extended conformation which causes it to occupy a smaller volume. The values of V^*_{ij0} presented in Table 4.5 conform to this expectation. The trend is that the higher the pH the smaller the value of V^*_{ij0}.

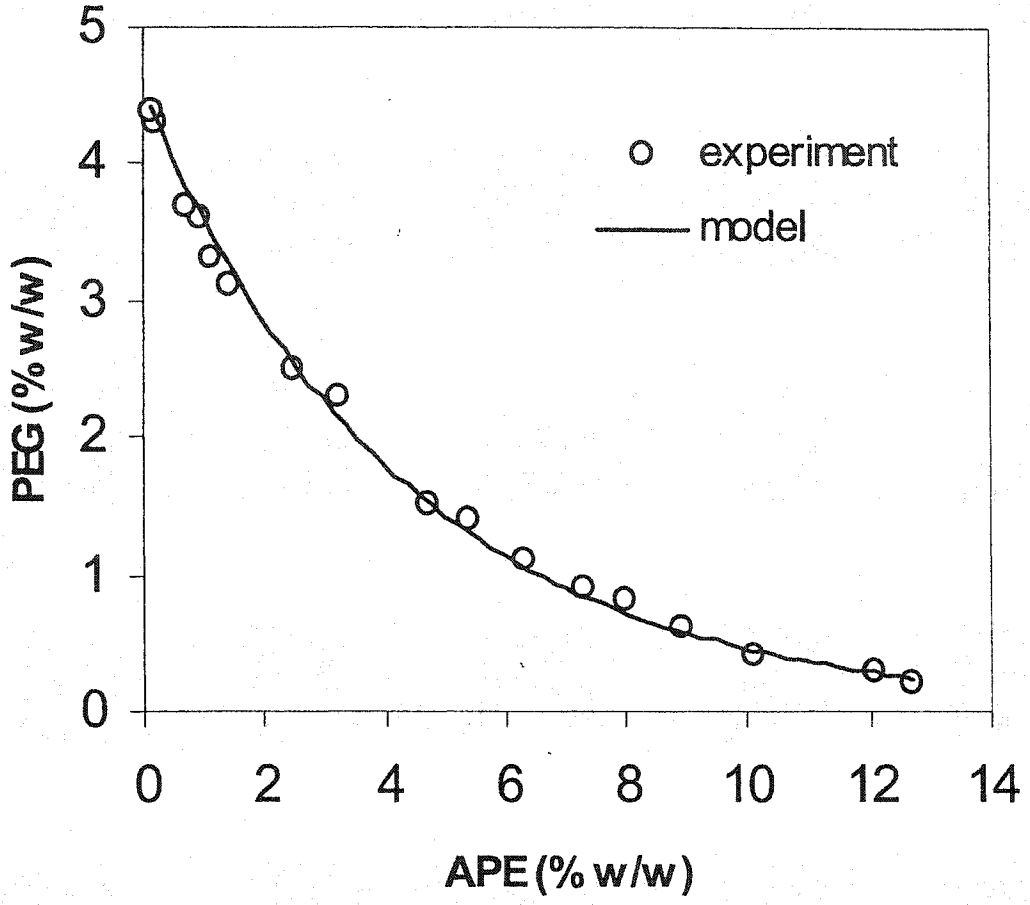


Figure 4.10 Comparison of experimental and predicted PEG-APE-water (0.1N KCl) binodal at pH 7.37 and 296.15 K.

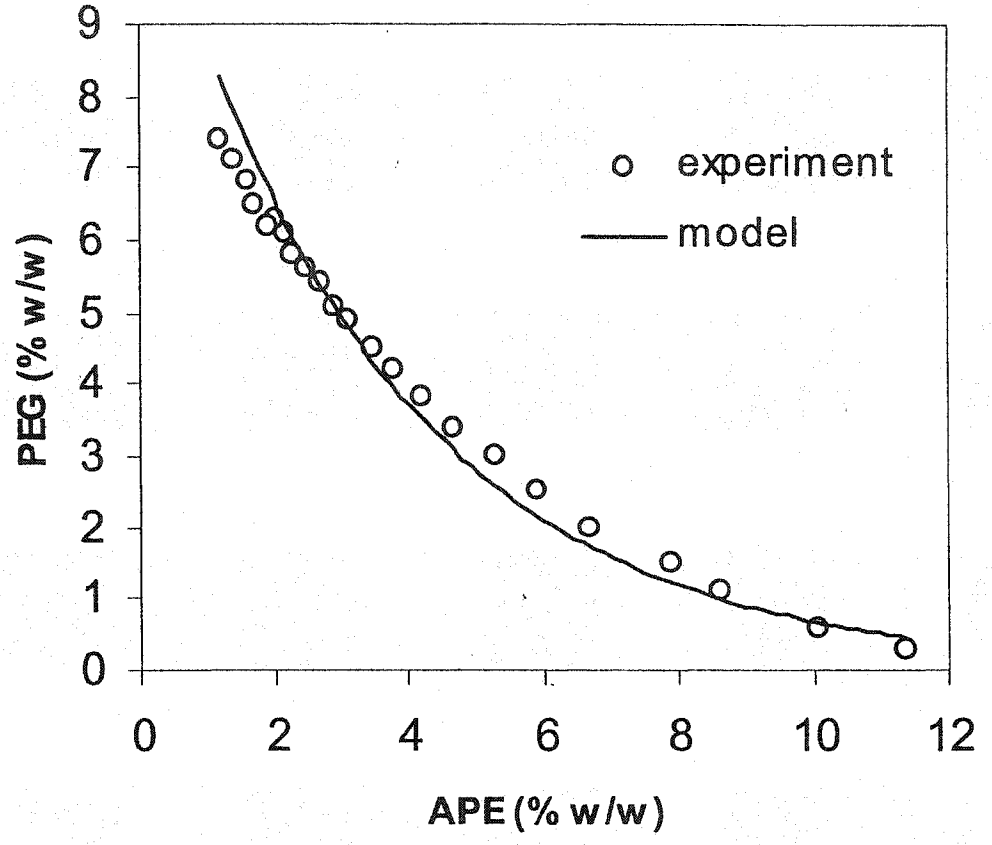


Figure 4.11 Comparison of experimental and predicted PEG-APE-water (0.1N KCl) binodal at pH 7.41 and 296.15 K.

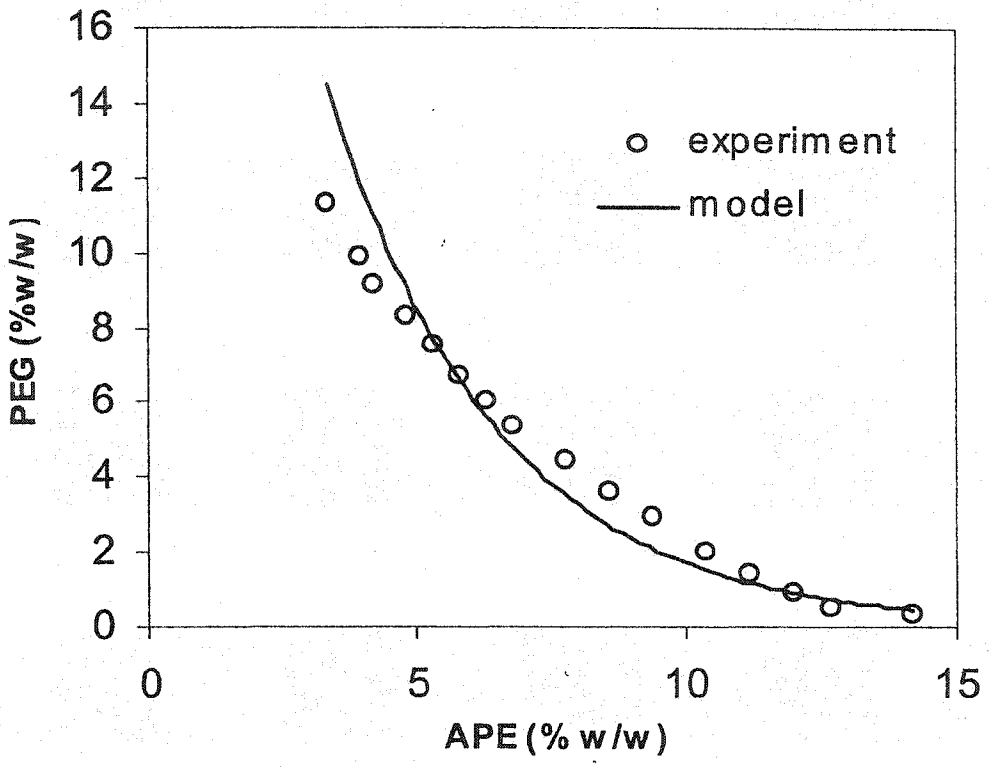


Figure 4.12 Comparison of experimental and predicted PEG-APE-water (0.1N KCl) binodal at pH 7.89 and 296.15 K.

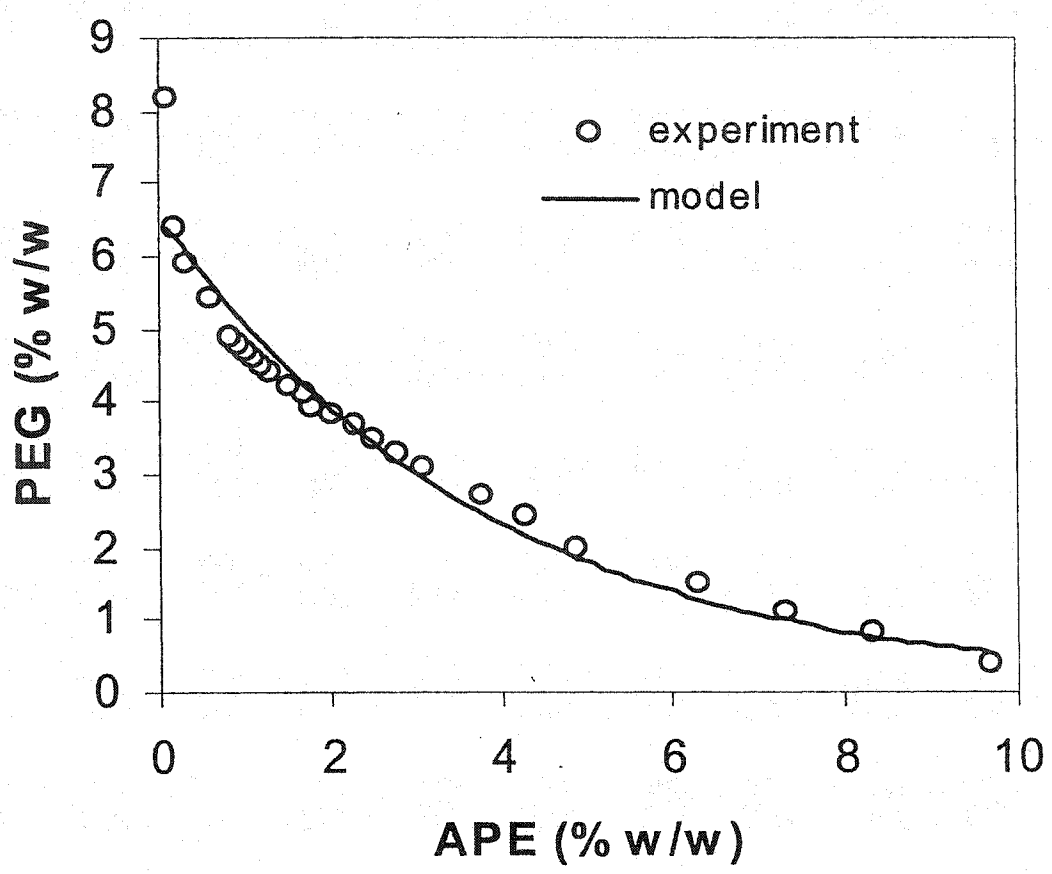


Figure 4.13 Comparison of experimental and predicted PEG-APE-water (0.3N KCl) binodal at pH 7.37 and 296.15 K.

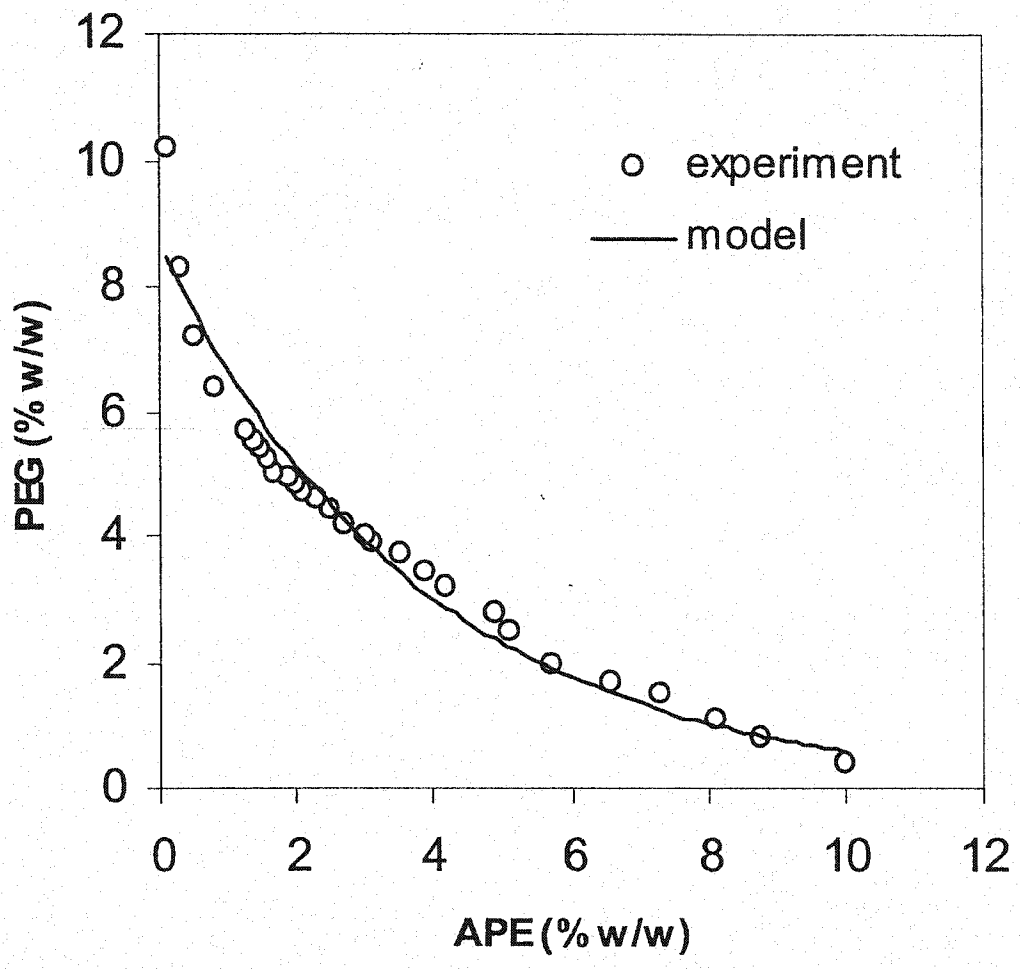


Figure 4.14 Comparison of experimental and predicted PEG-APE-water (0.3N KCl) binodal at pH 7.41 and 296.15 K.

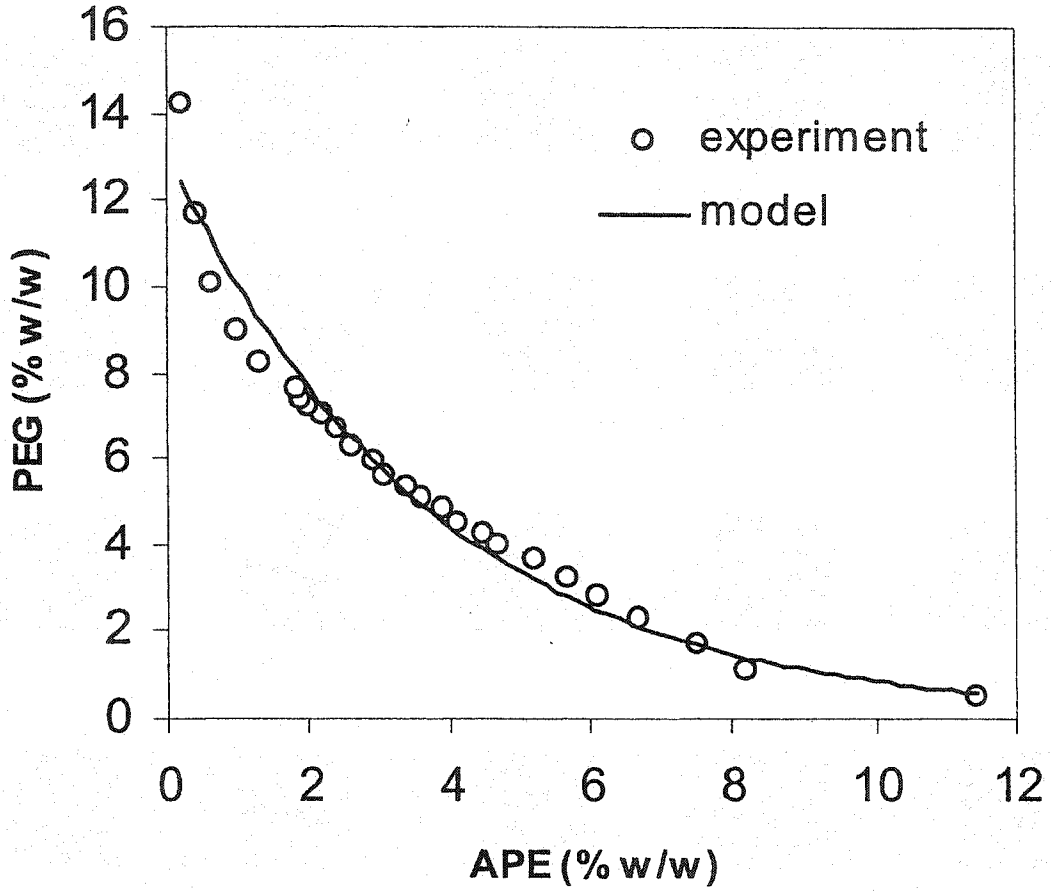


Figure 4.15 Comparison of experimental and predicted PEG-APE-water (0.3N KCl) binodal at pH 7.89 and 296.15 K.

TABLE 4.5 Model parameters obtained by fitting the experimental data in Tables 4.1 and 4.2 to Equation 4.14.

PEG-APE aqueous sysems		Model parameters		
		$10^3 \times \langle V_{ij0}^* \rangle$	$10^3 \times \langle V_{ji0}^* \rangle$	r
pH	KCl concentration (N)	(kg kmol^{-1})	(kg kmol^{-1})	
7.37	0.1	7.754	7.558	0.997
7.41	0.1	3.009	9.344	0.988
7.89	0.1	0.847	10.405	0.977
7.37	0.3	5.357	8.444	0.992
7.41	0.3	4.078	8.649	0.986
7.89	0.3	2.653	8.987	0.989

In order to correlate the tie lines, the empirically extended relationship of Diamond and Hsu [20] that is based on Flory-Huggins theory was employed:

$$\ln(K_1) = A_1(w_1'' - w_1') \tag{4.15}$$

$$\ln(K_2) = A_2(w_1'' - w_1') \tag{4.16}$$

where w' and w'' represent the polymer weight fractions in the upper and lower phase and A is function of the polymer molecular weight and the interactions between the polymers and water. The correlation result for the system at pH 7.41 and 0.1 N KCl is shown in figure 4.16. It can be observed that the correlation of the type described above can be used to qualitatively describe the phase behavior of the two polymers system even though it has been developed for nonelectrolyte systems. It is important to note that at pH 7.41 the APE is close to the isoelectric point and its behavior and chain conformation are fairly similar to that of the uncharged polymer. This is probably what leads to the observed correlation. Development of a more accurate model for phase behavior of systems containing a polyelectrolyte and an uncharged polymer is therefore needed.

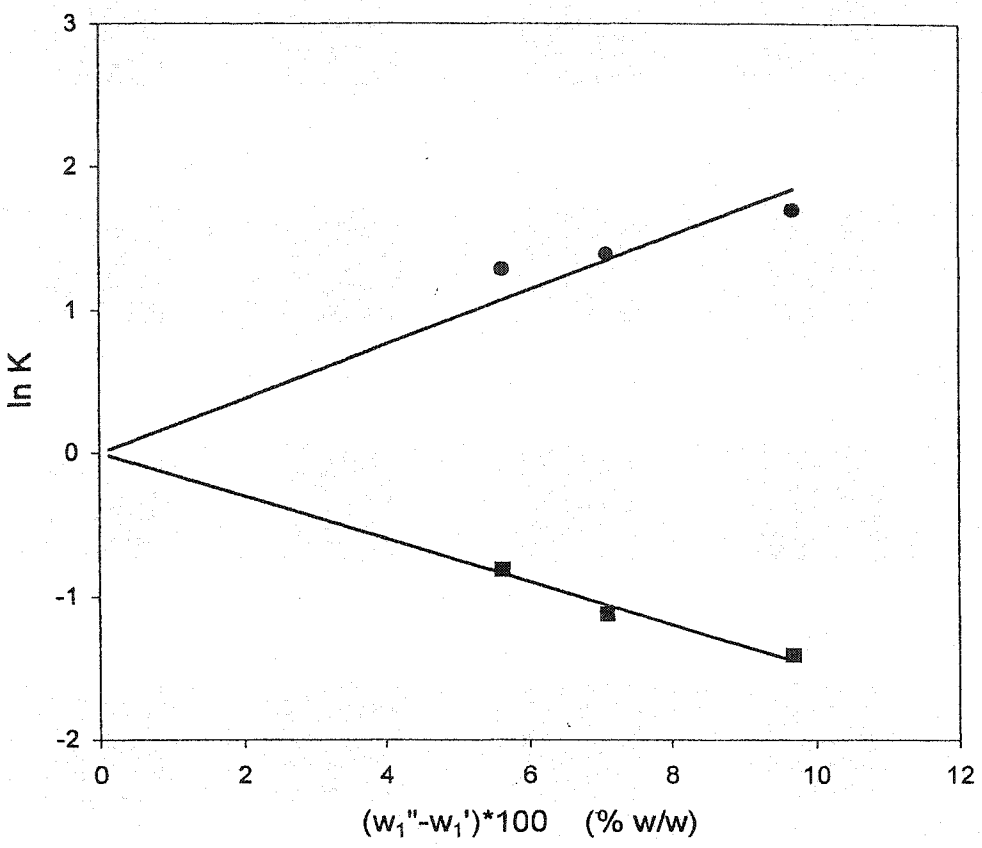


Figure 4.16 Correlation of the phase diagram of PEG with APE (pH 7.41) in 0.1N KCl using the method of Diamond and Hsu [20]. (■) APE; (●) PEG.

CHAPTER 5

PROTEIN PARTITIONING

5.1 Introduction

It is over 40 years since the potential of aqueous two-phase systems was first realized by a Swedish biochemist, P.A. Albertsson, for separations of cells, cell particles, and proteins. Separation could be achieved by a batch procedure or by counter-current distribution. Since then, ATPS has become a powerful tool for separation of a range of biomaterials, including plant and animal cells, microorganisms, fungi and other spores, virus, chloroplasts, mitochondria, membrane vesicles, proteins, and nucleic acids. Without a doubt, the application of ATPS that has attracted the most interest in biotechnology is its use as a primary operation for isolation of proteins from crude feedstocks. ATPS are being employed today at process scale by a few industries for protein recovery [44]. Even polyphase systems containing three or four polymer phases have been constructed and used for bioseparations. For example, proteins have been partitioned between three phases of PEG-Ficoll-Dextran systems where PEG is concentrated in the top phase, Ficoll in the middle phase, and dextran in the bottom phase [41].

The basis of separation in a two-phase system is the selective distribution of substances between the two phases. Generally, small molecules are more evenly distributed between the phases; the partitioning of macromolecules is extremely variable, whereas that of particles is relatively one-sided. With increase in the size, molecules become more sensitive to the phase composition. The partitioning of soluble molecules occurs between the two bulk phases, top and bottom. This is normally characterized by the term, partition coefficient, K, which is the ratio of the concentration of the protein (e.g. moles/liter, mg/mL or units/mL) in the top phase (C_t) to the bottom phase (C_b):

$$K = C_t / C_b \tag{5.1}$$

The partition coefficients for proteins in the most commonly employed phase systems generally fall within the range 0.1 to 10. For large molecules (such as high molecular weight DNA and RNA) and particles (such as cells and virial particles), partition coefficients > 100 to < 0.01 are observed [5, 69]. Small ions tend to partition equally between the two phases.

The partition coefficient is governed by a number of parameters relating to the properties of the phase system and the protein, the interactions between the two, and also temperature. This makes the prediction of partitioning, particularly of large molecules, a difficult task. The partitioning can, however, be made selective. For instance, selectivity can be introduced by manipulating the system properties to make a particular kind of interaction predominant. The partitioning coefficient is independent of protein concentration and volume ratio of the phases. Kula [69] has shown that the protein

partitioning coefficient is independent of its own concentration up to approximately 30 % w/w.

Proteins targeted for separation are commonly individual members of a large family of closely similar molecules. Bulk properties of proteins differ by virtue of only minor changes of the macromolecule surface exposed to solution. Conventional purification technologies attempt to exploit limited groups of such properties (e.g., charge, size, hydrophobicity, etc.) in a controlled manner, but difficult to achieve in a single step. Thus, downstream operations require sequential exploitation of one type of interaction after another in multiple steps. The application of traditional separation media demonstrates why some 50-80% of the total cost of purification of a therapeutic protein is incurred at the purification stage and why the replacement of a multi-step process by a single step can have a revolutionary economic effect [57]. In contrast, partitioning behavior in ATPS exploits the entire molecular surface of the protein with the phase forming polymers. Alteration of the solution properties of the phase system influences those molecular forces and directs partitioning of the proteins. Hence, separation by partition in ATPS may often be used to substitute the conventional separation forms.

Single-step partitioning is invariably the first stage in determining the separation possible in a two-phase system. To start with, the phase system(s) is often arbitrarily chosen, and partitioning of the protein of interest between the phases is studied. This is followed by further systematic changes in the phase composition either to achieve a desired separation or to study the influence of the changes on partitioning. The changes may be performed

by altering the different parameters of the system one by one. A significant difference between the partitioning of the species of interest from the others present in a sample can be used for its separation in a single step; for smaller differences, multi-step extraction is required. Two proteins can be resolved from a mixture by partition in a system in which their K values differ enough. Usually, several partition steps are necessary to get an acceptable resolution unless the K values are extreme (e.g., $K_1<0.01$ and $K_2>100$) [55].

Aqueous two-phase systems are easy to use, involving two-unit operations: equilibration and phase separation. Equilibration is rapid, involving mixing of the components that constitute the phase system with the material subjected to partitioning, and dispersing the phases to obtain equilibrium of phase compositions and partition. This is followed by separation of the liquid phases. The phase separation under gravity is not rapid as in water-organic solvent systems, varying between a few minutes and a few hours because of a rather low difference in the densities of the phases (about 0.05-0.15 g/cm^3), their viscosities, and the time required by the small droplets, formed during mixing, into larger droplets [97].

However, despite the definite advantages of ATPS, applications of the technique are limited because of poor understanding of the mechanisms of partitioning which has made the method development wholly empirical, the need for selectivity during protein extraction, and the cost of the phase-forming components and the associated waste water treatment. This limitations form a major focus of study in aqueous two-phase technology.

5.2 Prediction of Protein Partitioning

If protein partitioning could be reliably predicted, extraction in ATPS could be optimized by calculation only. However, the quantitative modeling of protein partitioning poses an extremely complex problem because of its dependence on a broad array of factors as well as the lack of understanding of the molecular mechanisms involved in the partitioning process.

Complementary modeling attempts using lattice model techniques, virial expansions, UNIQUAC, the scaling-thermodynamic approach, and others have been successful in highlighting several molecular-level mechanisms influencing protein partitioning. The models developed provide good start for understanding and predicting protein partitioning. However, a model allowing a priori calculation of protein partitioning for a wide range of phase polymer molecular weights and polymer and salt concentrations without the measurement of a large number of parameters is not yet available because many of the physical phenomena associated with these complex systems are not well understood [1, 11].

5.3 Experimental

5.3.1 Materials

The description of the polymers used in this study has already been given in chapter 4. BSA (prd 44155) was purchased from Biochemical, BDH. Cytochrome c (from horse heart), lot 49#7022, was obtained from Sigma. They were used without further purification. The physicochemical properties of BSA and cytochrome c have been given in Table 2.1. It should be noted that the two proteins are very different in terms of isoelectric point and size.

5.3.2 Protein Assays

Three stock solutions of APE, each treated with different equivalents of HCl, were prepared in 0.1 and 0.3 N KCl. Stock solution of PEG (25 % w/w) was also prepared in 0.1 and 0.3 N KCl. Series of aqueous two-phase systems were made from the stock solutions in duplicate. Known weight of protein (BSA or cytochrome c) was added to one of the identical two-phase systems, while the other serves as a blank for protein determination. The samples were gently mixed by shaking, centrifuged for 10 minutes and then equilibrated for 48 hours at room temperature. Samples from both phases of each of the two-phase systems were withdrawn with a Pasteur pipette and diluted with deionized water in 25 mL volumetric flasks. The dilution serves two purposes. First, it helps in reducing interference from phase polymers on the absorbance reading. If the samples were not diluted, streaks appeared in the polymer solution as they were pipetted

into spectrometric cuvettes, which in turn scattered light during the measurement of the protein absorbance. Secondly, the dilution is done in order to ensure that the absorbance of the unknown samples fall within the linear range of the calibration curve. The diluted samples were analyzed for their protein content (BSA and cytochrome c) by measuring their absorbance at 280 nm using a Shimadzu UV-1601PC spectrophotometer. The instrument has dual beam configuration with accuracy of ± 0.004 Abs. Standard 1 cm path-length quartz cells were used for the study. Diluted phases from the protein-free systems were used as blanks. The protein concentrations were determined from calibration curves for the respective proteins. The calibration curves were constructed using standard solutions of the proteins in deionized water. The results are shown in Figures 5.1 and 5.2. The concentrations are within the limit of Beer's-Lambert law linearity in both cases. The extinction coefficients for BSA and Cytochrome c under the current experimental conditions are 0.648 mL cm^{-1} mg^{-1} and 1.560 mL cm^{-1} mg^{-1} respectively.

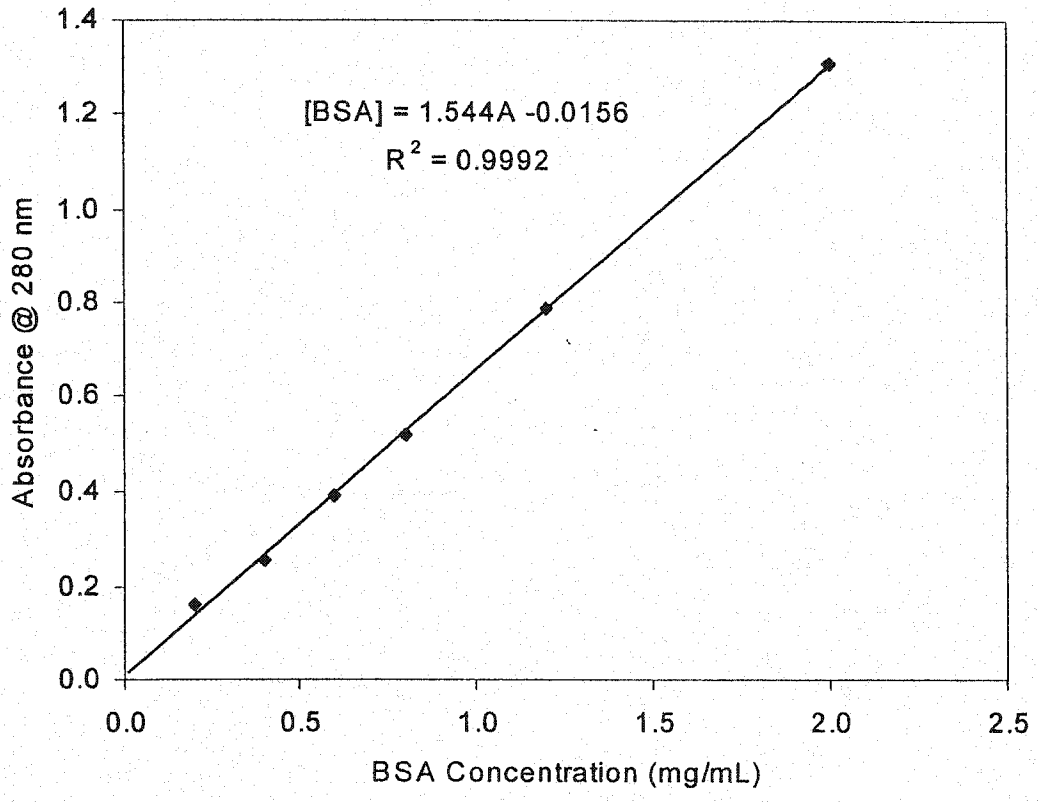


Figure 5.1. Standard curve for BSA in deionized water at 280 nm.

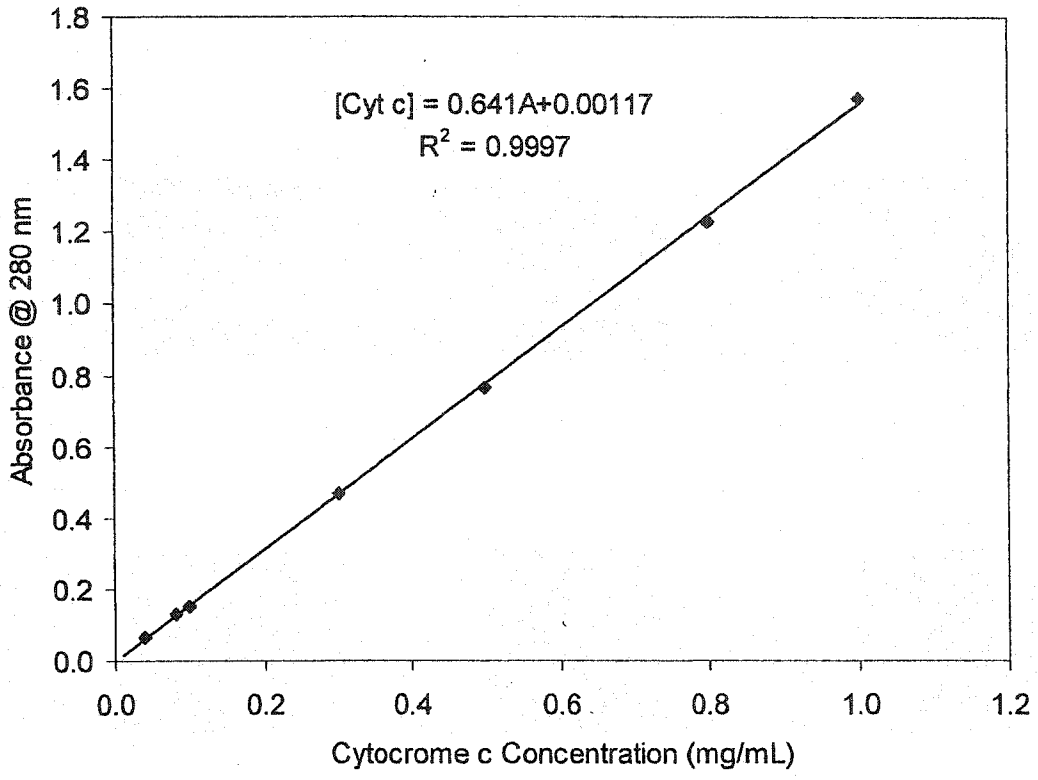


Figure 5.2. Standard curve for Cytochrome c in deionized water at 280 nm.

5.3.3 Potential Difference

Electric potential difference between the two aqueous phases was measured according to the protocol of Brooks and Norris-Jones [15]. An outline of the procedure is as follows. Glass microcapillaries are drawn to an internal tip diameter of about 30 μm and filled with 1.0 M KCl. Reversible Ag/AgCl electrodes are inserted in the KCl-filled microcapillaries (salt-bridges). The electrodes are connected to a millivoltmeter, while the tips of the two salt bridges are immersed in the top phase of the ATPS. Once the voltage has reached a steady level, one of the capillaries is smoothly lowered through the interface. The capillary is moved in and out of the bottom phase and the voltage averaged over 5 excursions to provide the electrostatic potential difference estimate. A diagram of the set-up is given in Figure 5.3.

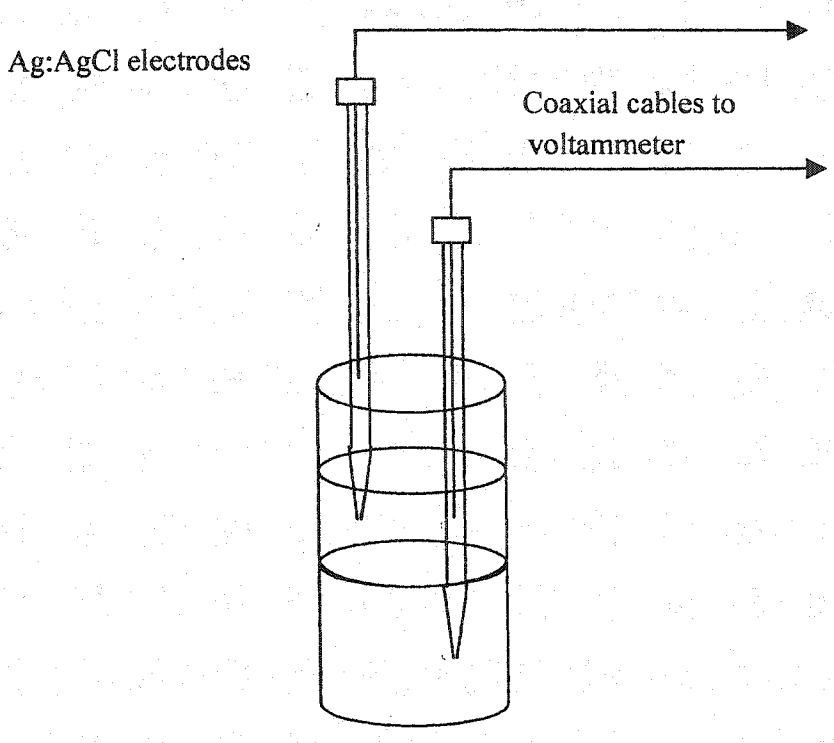


Figure 5.3 Schematic of electrostatic potential difference measurement using a microelectrode.

5.4 Results and Discussions

Partitioning behavior of two model proteins (BSA and cytochrome c) was carried out in an aqueous two-phase system comprising of the polymers PEG and APE. The partitioning was investigated as function of polymer concentrations, salt concentrations and pH.

Table 5.1 gives a summary of the results of the partitioning study. The partition coefficients for proteins in the most commonly employed phase systems generally fall within the range 0.1 to 10 [55]. Therefore, it is remarkable to note that partitioning coefficient values lower than 0.01 were recorded in the aqueous two-phase system under investigation. For instance, in one of the phase systems (observation 9 in Table 5.1) both BSA and cytochrome c partitioned almost exclusively into the APE-rich phase, with K_{BSA}=0.004 and $K_{cytochrome\ c}$= 0.006. The partitioning behavior of cytochrome c under this condition can be easily explained based on electrostatic interactions. At pH 7.98, cytochrome c (pI=9.4) has a net positive charge. Hence, there is likelihood of favorable electrostatic interaction between it and the net negatively charged APE. In the case of BSA (pI=4.8), since it is negatively charged at pH 7.89, it would be expected to partition more into the top phase assuming that electrostatic interaction is the predominant mechanism governing its partitioning behavior. The fact that a contrary behavior was observed clearly suggests that there are opposing factors competing with the electrostatic mechanism. Perhaps, this could be due to size exclusion effects between

TABLE 5.1 Protein partitioning in PEG-APE-water system at 296.15 K.

Observation	Total system (% w/w)				Partition coefficient		Separation factor
No	APE	PEG	pH	KCl (N)	K_{BSA}	$K_{Cytocrome\ c}$	($K_{BSA}/K_{cytocrome\ c}$)
1	9.2	3.2	7.37	0.3	0.837	0.402	2.08
2	6.4	2.9	7.37	0.3	0.391	0.321	1.22
3	5.8	2.6	7.37	0.3	0.722	0.438	1.65
4	5.7	2.5	7.37	0.3	0.690	0.452	1.52
5	9.4	5.0	7.41	0.3	0.459	0.193	2.38
6	6.9	3.0	7.41	0.3	0.657	0.366	1.80
7	6.5	2.7	7.41	0.3	0.673	0.424	1.59
8	6.4	2.3	7.41	0.3	0.768	0.536	1.43
9	10.6	8.6	7.89	0.3	0.004	0.006	0.58
10	7.5	5.1	7.89	0.3	0.016	0.062	0.26
11	7.4	4.6	7.89	0.3	0.216	0.092	2.36
12	6.7	4.0	7.89	0.3	0.173	0.155	1.12
13	13.5	6.5	7.37	0.1	1.438	0.243	5.92
14	10.3	2.8	7.37	0.1	2.824	0.316	8.94
15	10.3	2.0	7.37	0.1	4.431	0.626	7.08
16	11.5	10.8	7.89	0.1	0.310	0.006	48.21
17	10.2	12.5	7.89	0.1	4.714	0.345	13.66
18	8.1	10.0	7.89	0.1	0.030	0.299	0.10
19	5.1	6.3	7.89	0.1	0.136	0.247	0.25
20	13.5	6.5	7.41	0.1	0.280	0.047	5.97
21	7.1	3.3	7.41	0.1	0.948	-	-
22	7.2	6.6	7.41	0.1	0.675	-	-
23	6.7	2.9	7.41	0.1	0.921	-	-
24	8.7	3.9	7.41	0.1	-	0.091	-
25	7.9	3.0	7.41	0.1	-	0.180	-
26	7.6	2.5	7.41	0.1	1.003	0.261	3.85

the BSA and the PEG-rich phase. Other factors such as hydrogen bonding and protein self-association may also be important.

A close examination of Table 5.1 reveals that generally the model proteins preferentially partitioned into the bottom (APE-rich) phase. An exception to this was seen in the systems at pH 7.37 and 0.1 KCl. Here, the BSA partitioned to the top phase while the cytochrome c prefers the bottom phase. Presumably, the behavior of the proteins at this condition is predominantly influenced by electrostatic interactions. Even though both BSA and cytochrome c preferentially partitioned into the bottom phase it is important to note that the magnitude of their partition coefficients varies widely in most of the cases. It can be seen from Table 5.1 that the separation factor of BSA over cytochrome c, defined as the ratio of partition coefficients of the two proteins, can be as high as 48 (observation No 16).

Figures 5.4-5.8 show how the partition coefficients of the two proteins vary with tie line lengths. Generally, K values deviate from unity as tie line length increases. However, for the system composed of PEG and APE at 0.1 N KCl and pH 7.37 (shown in Figure 5.5) a contrary behavior was noticed. In that case the partition coefficient of BSA approaches one as the tie line length increases.

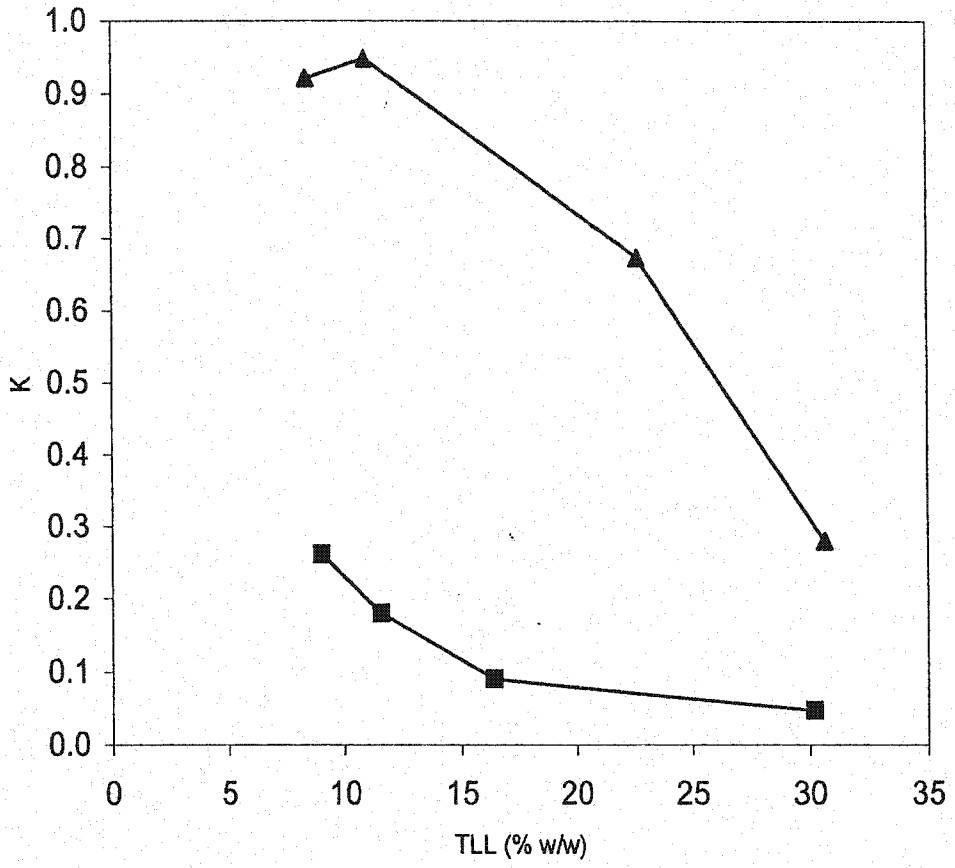


Figure 5.4 Effect of TLL on the partitioning of BSA (▲) and cytochrome c (■) in the system PEG-APE-water at pH 7.41 and 0.1N KCl.

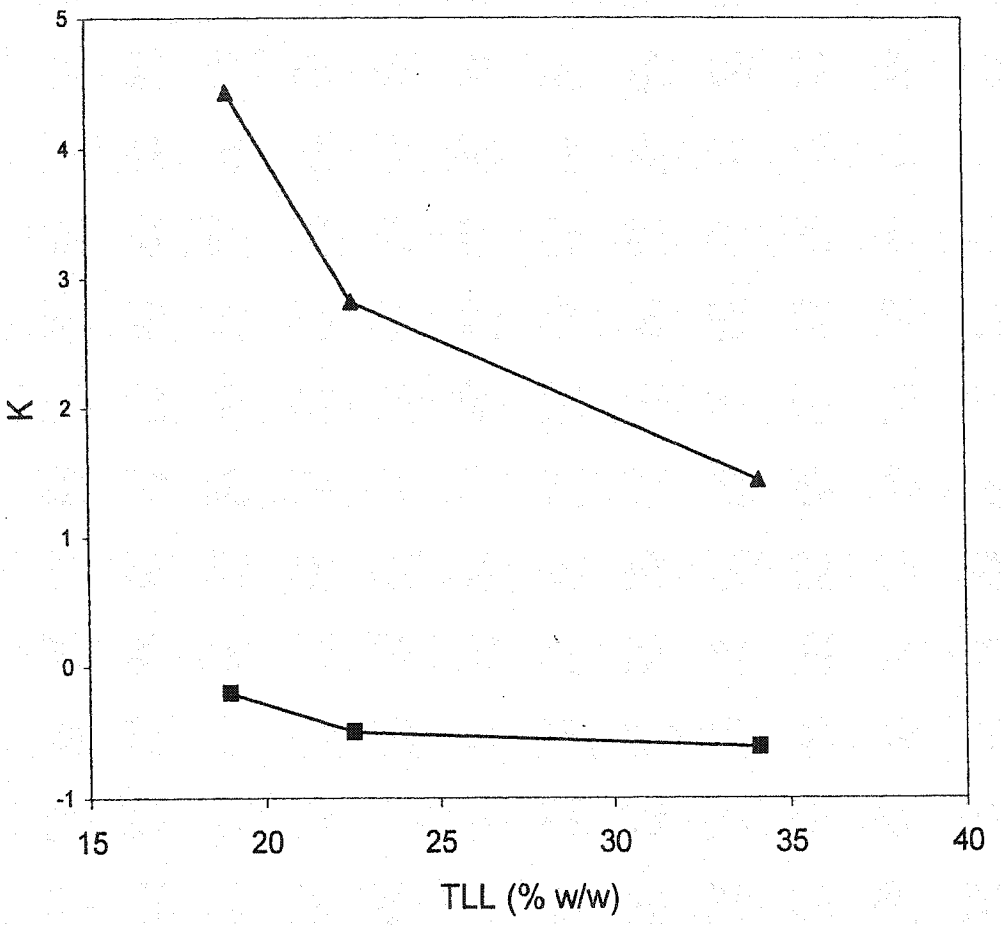


Figure 5.5 Effect of TLL on the partitioning of BSA (▲) and cytochrome c (■) in the system PEG-APE-water at pH 7.37 and 0.1N KCl.

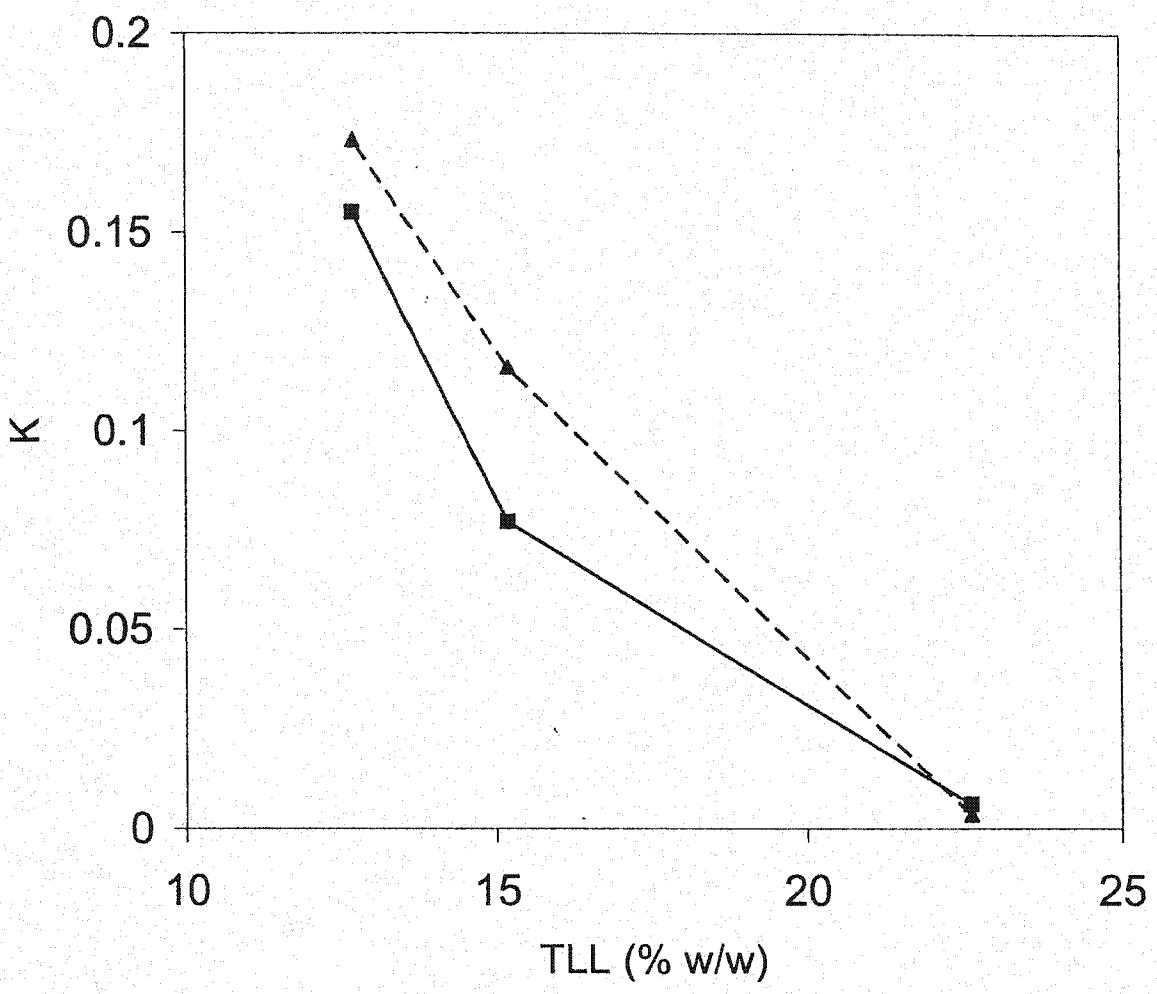


Figure 5.6 Effect of TLL on the partitioning of BSA (▲) and cytochrome c (■) in the system PEG-APE-water at pH 7.89 and 0.3N KCl.

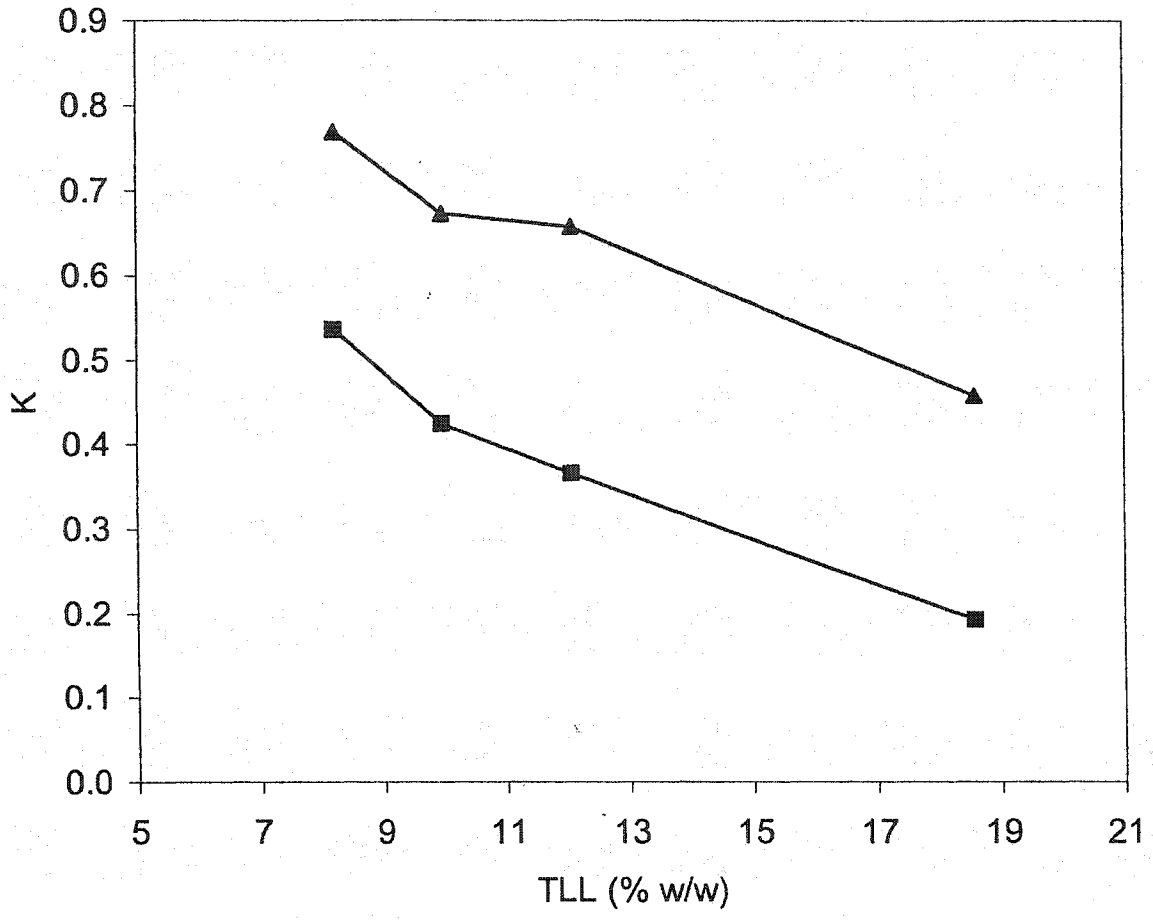


Figure 5.7 Effect of TLL on the partitioning of BSA (▲) and cytochrome c (■) in the system PEG-APE-water at pH 7.41 and 0.3N KCl.

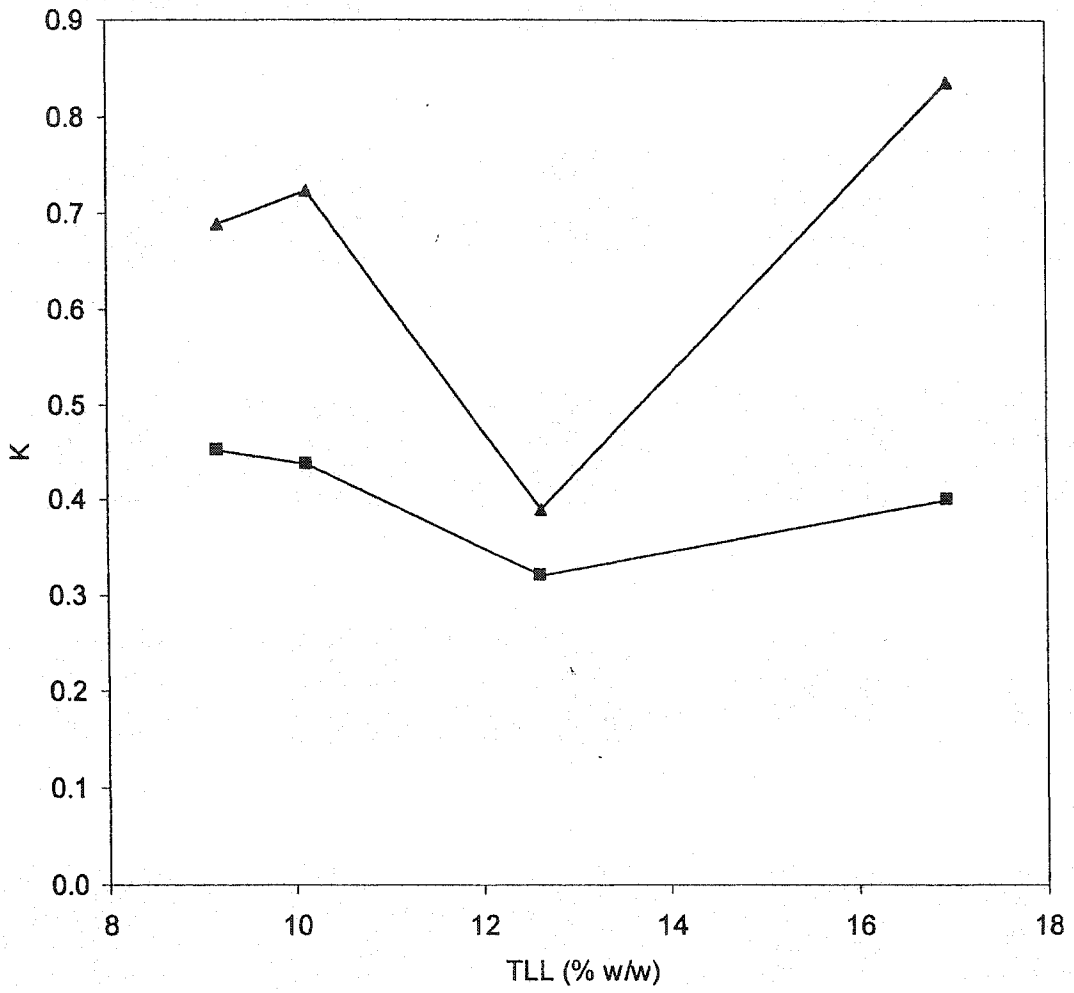


Figure 5.8 Effect of TLL on the partitioning of BSA (▲) and cytochrome c (■) in the system PEG-APE-water at pH 7.37 and 0.3N KCl.

The combined effect of pH and TLL on partitioning coefficients for systems at 0.3N KCl is presented in figures 5.9 and 5.10. For both proteins, pH 7.89 gives the best partitioning coefficient independent of tie line length. Figures 5.11 and 5.12 show that salt concentration has strong influence on partitioning behavior of the model proteins in the aqueous system. At pH 7.41, increase in salt concentration leads to better partitioning of cytochrome c between the phases while the reverse is the case for BSA.

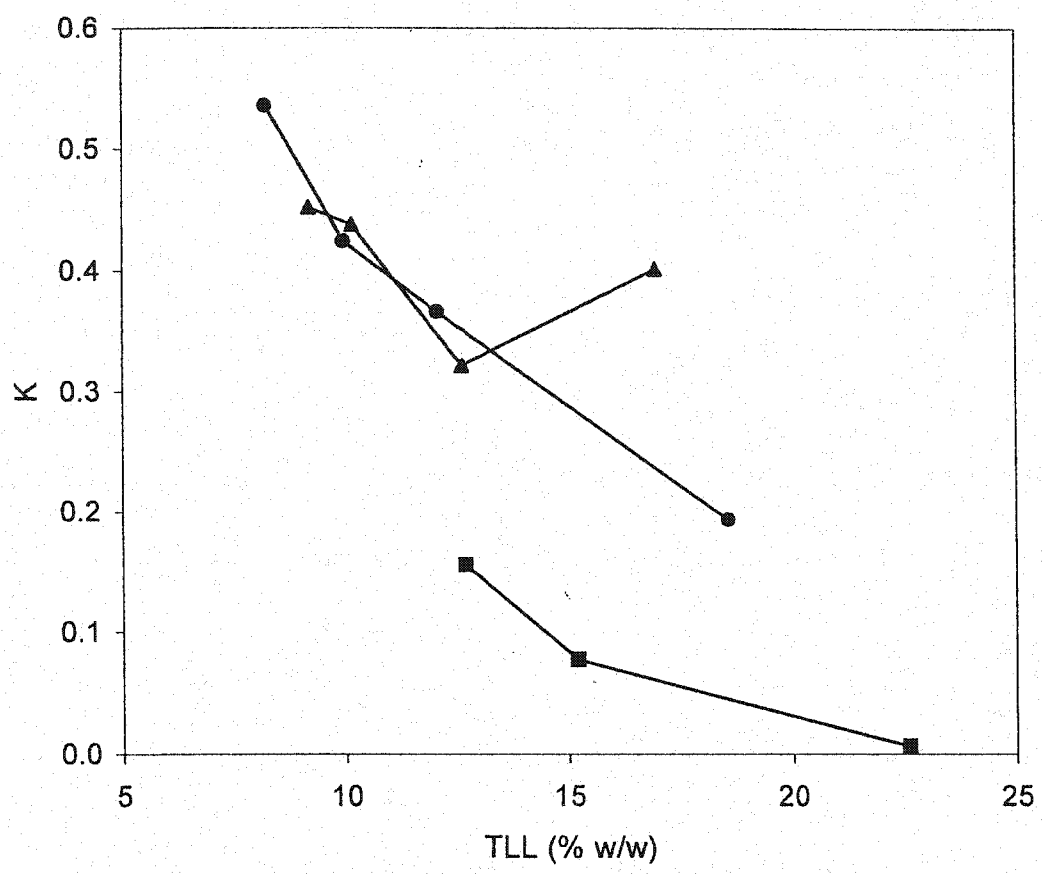


Figure 5.9 Effect of pH on partitioning coefficient of cytochrome c in PEG-APE-water system at 0.3 N KCl. (■) pH 7.89; (●) pH 7.41 (▲) pH 7.37.

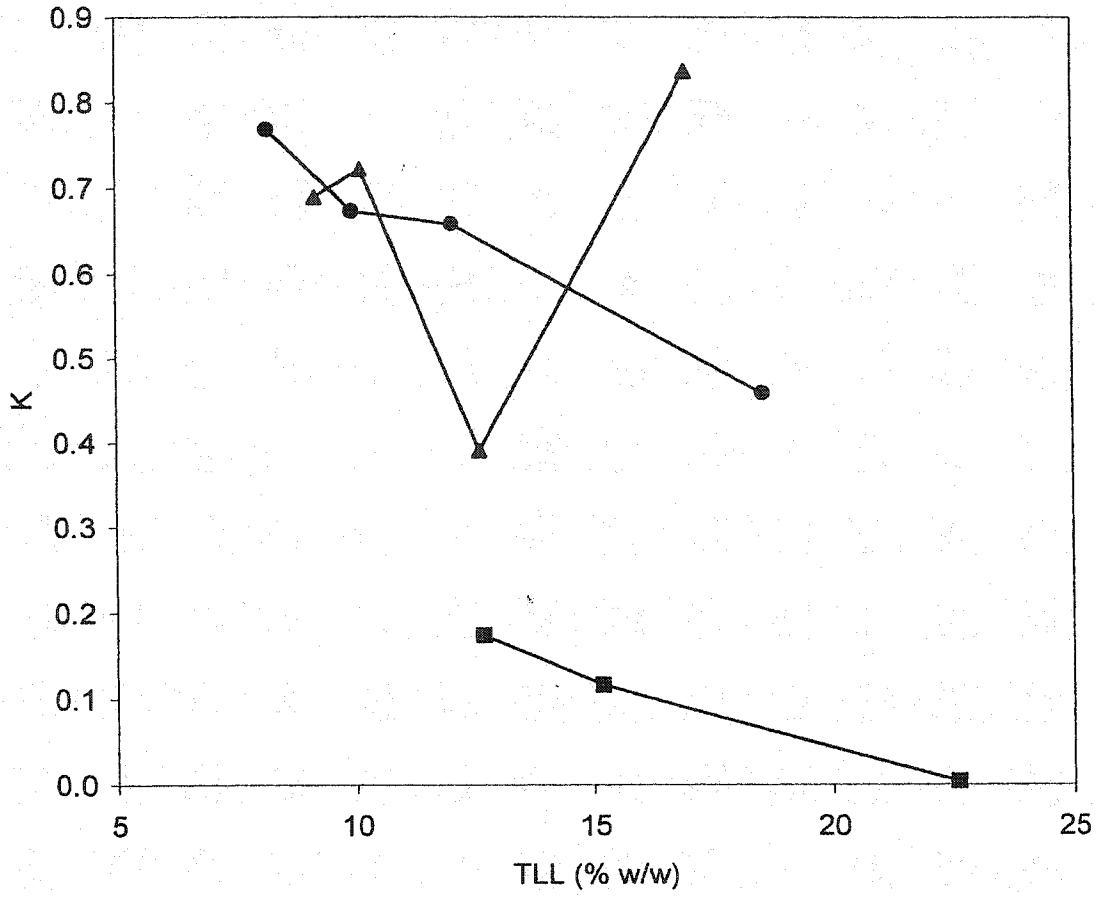


Figure 5.10 Effect of pH on partitioning coefficient of BSA in PEG-APE-water system at 0.3 N KCl. (■) pH 7.89; (●) pH 7.41 (▲) pH 7.37.

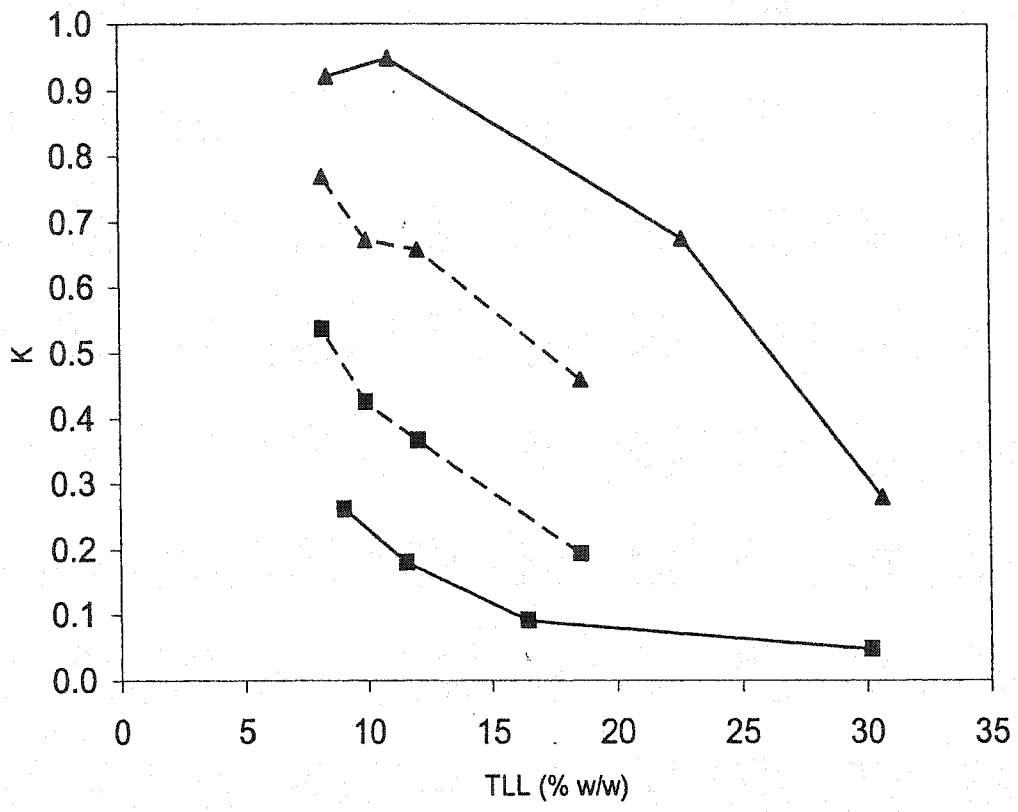


Figure 5.11 Effect of salt concentration on partitioning coefficient of BSA (▲) and cytochrome c (■) in PEG-APE-water system at pH 7.41. KCl concentration: (-----) 0.1N KCl; (——) 0.3N KCl

5.4.1 Correlation of Protein Partitioning Coefficient

Electrochemical potential difference is an important factor affecting the partitioning behavior of proteins in aqueous two-phase systems with added electrolytes. Since one of the phase polymers used in our investigation is charged we strongly believe that electrostatic potential difference would play a significant role in determining the pattern of partitioning behavior of proteins in the systems. Figure 5.12 shows the electrochemical potential difference between the two phases of the PEG-APE-water system as a function of tie line length. Diamond and Hsu model as well as the simplified model developed by Hartounian et al [42] were used to correlate the experimental data of the protein partitioning coefficients. Figures 5.13-5.19 shows some of the correlation results.

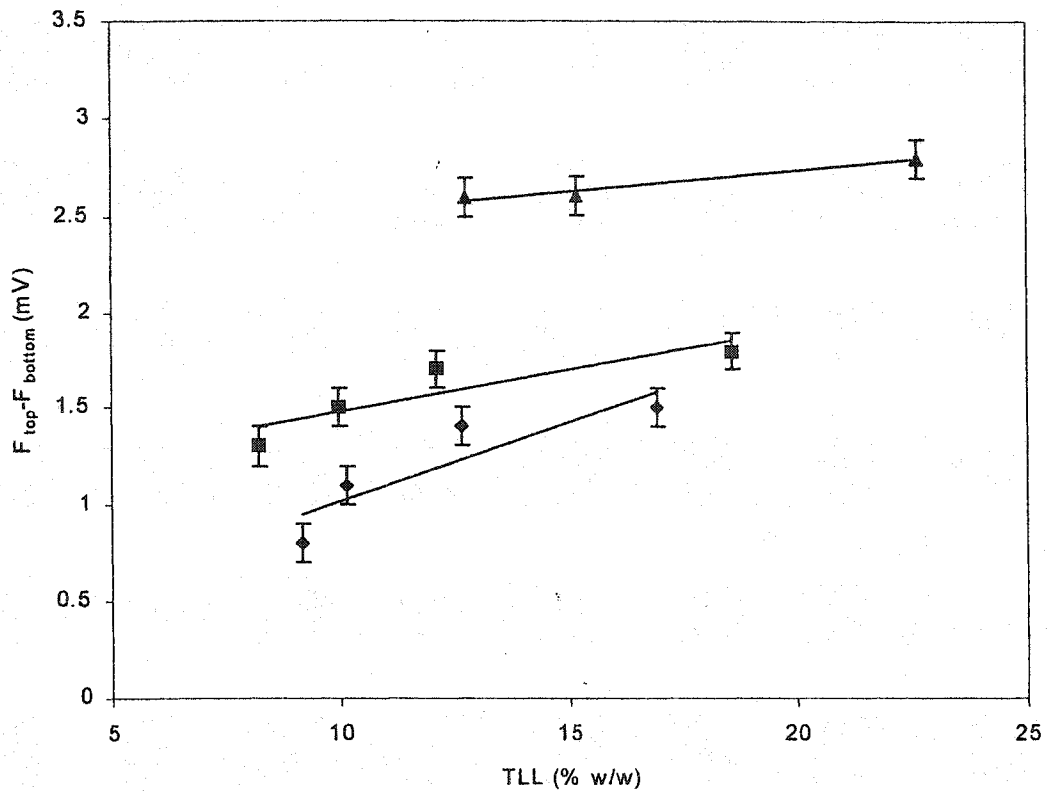


Figure 5.12 Electrochemical potential differences between the two phases of PEG-APE-water system at 0.3 N KCl. (▲) pH 7.89; (■) pH 7.41; (●) pH 7.37.

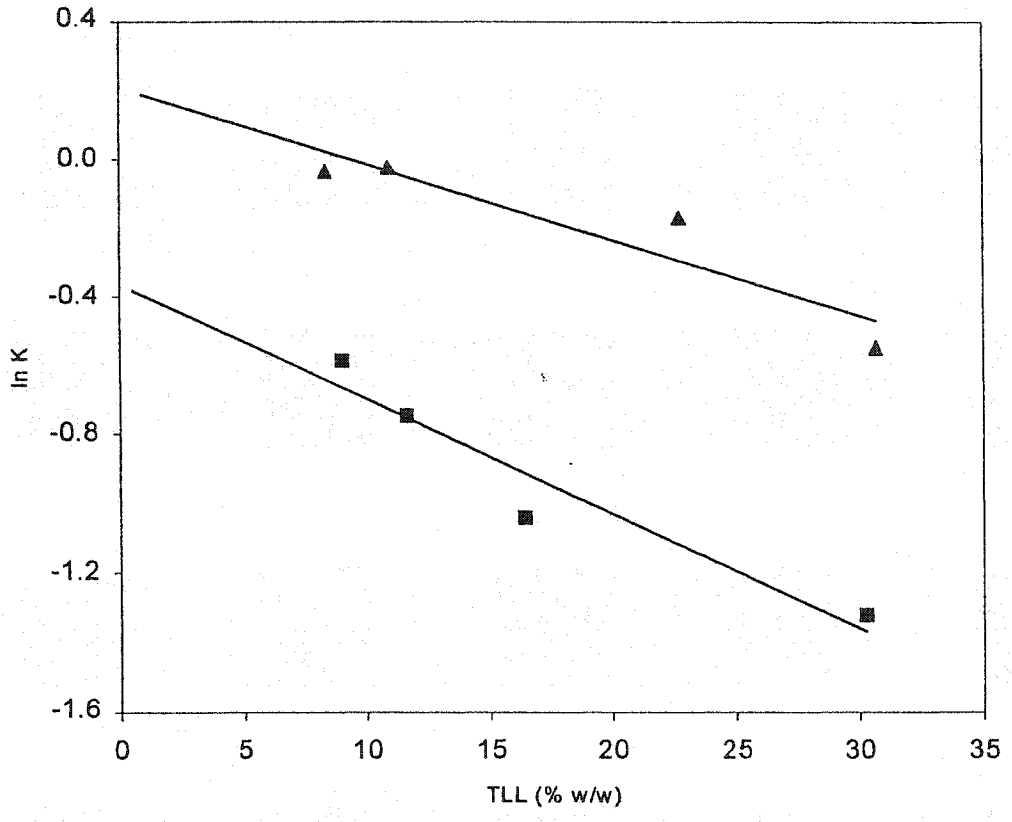


Figure 5.13 Correlation of partitioning coefficient of BSA (▲) and cytochrome c (■) in PEG-APE-water (0.1N KCl) at pH 7.41 using Hartounian *et al* Model [42].

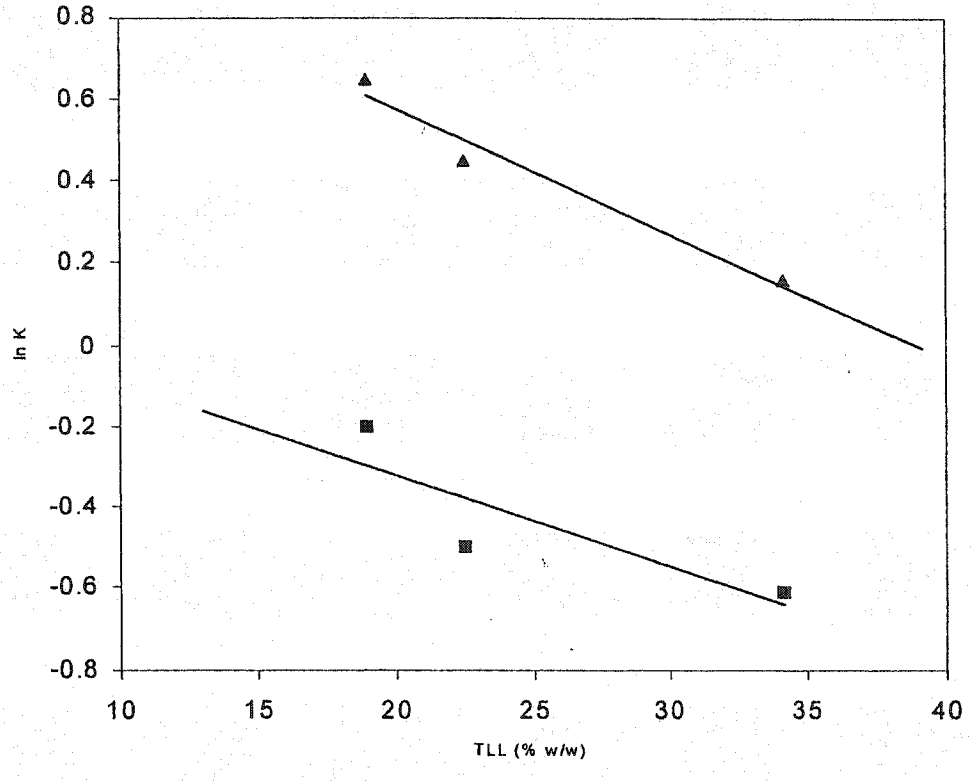


Figure 5.14 Correlation of partitioning coefficient of BSA (▲) and cytochrome c (■) in PEG-APE-water (0.1N KCl) at pH 7.37 using Hartounian *et al* Model [42].

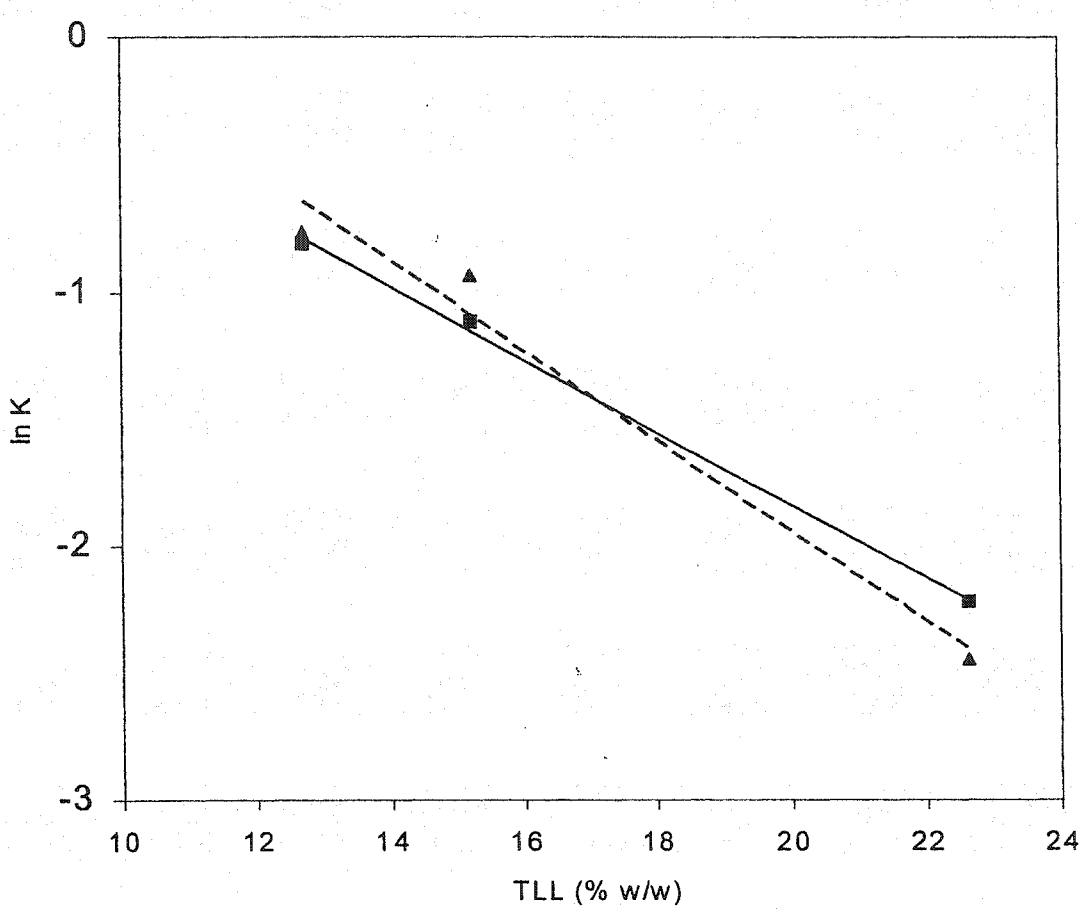


Figure 5.15 Correlation of partitioning coefficient of BSA (▲) and cytochrome c (■) in PEG-APE-water (0.3N KCl) at pH 7.89 using Hartounian *et al* Model. Correlation lines: BSA (---); cytochrome c (—)

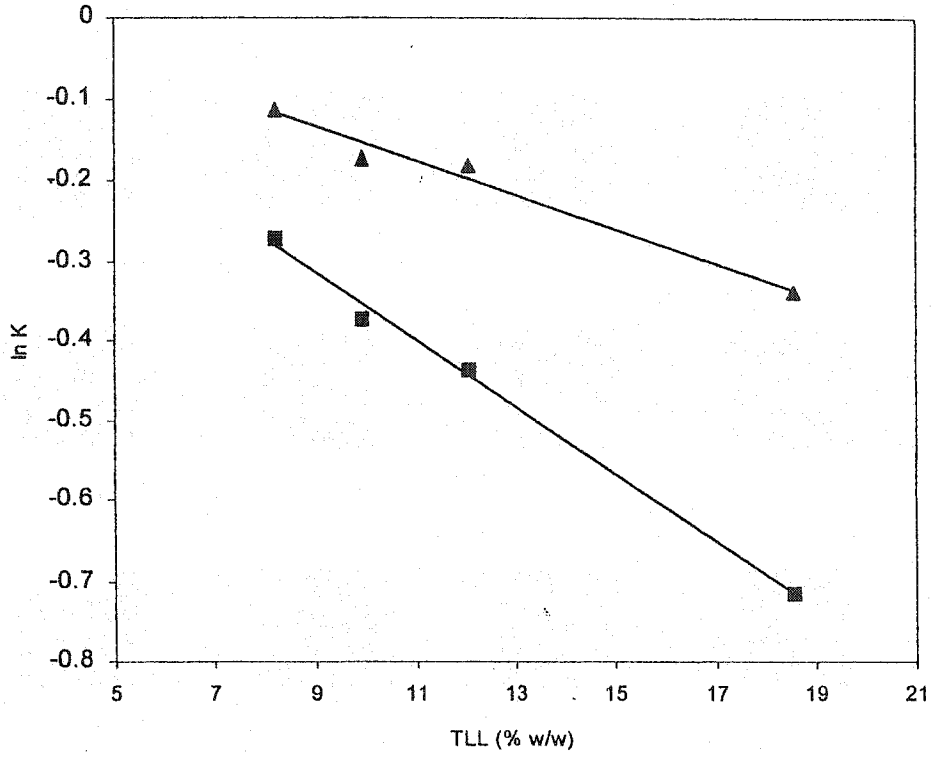


Figure 5.16 Correlation of partitioning coefficient of BSA (▲) and cytochrome c (■) in PEG-APE-water (0.3N KCl) at pH 7.41 using Hartounian *et al* Model [42].

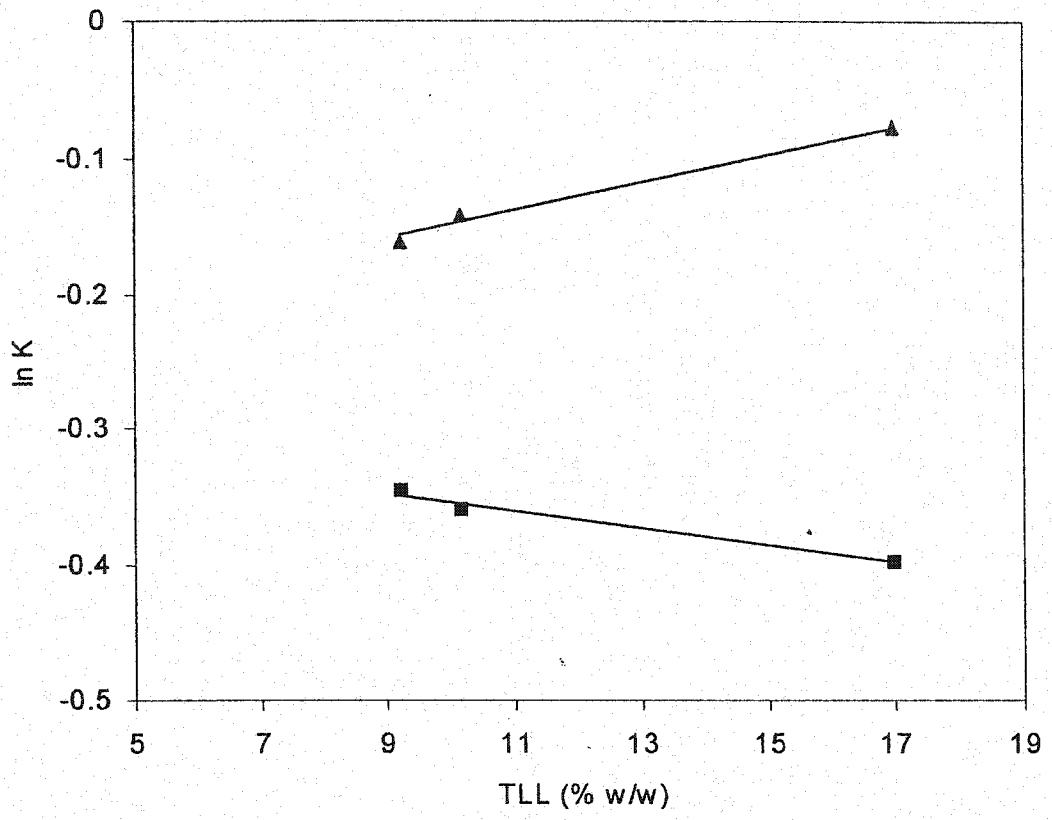


Figure 5.17 Correlation of partitioning coefficient of BSA (▲) and cytochrome c (■) in PEG-APE-water (0.3N KCl) at pH 7.37 using Hartounian *et al* Mode [42].

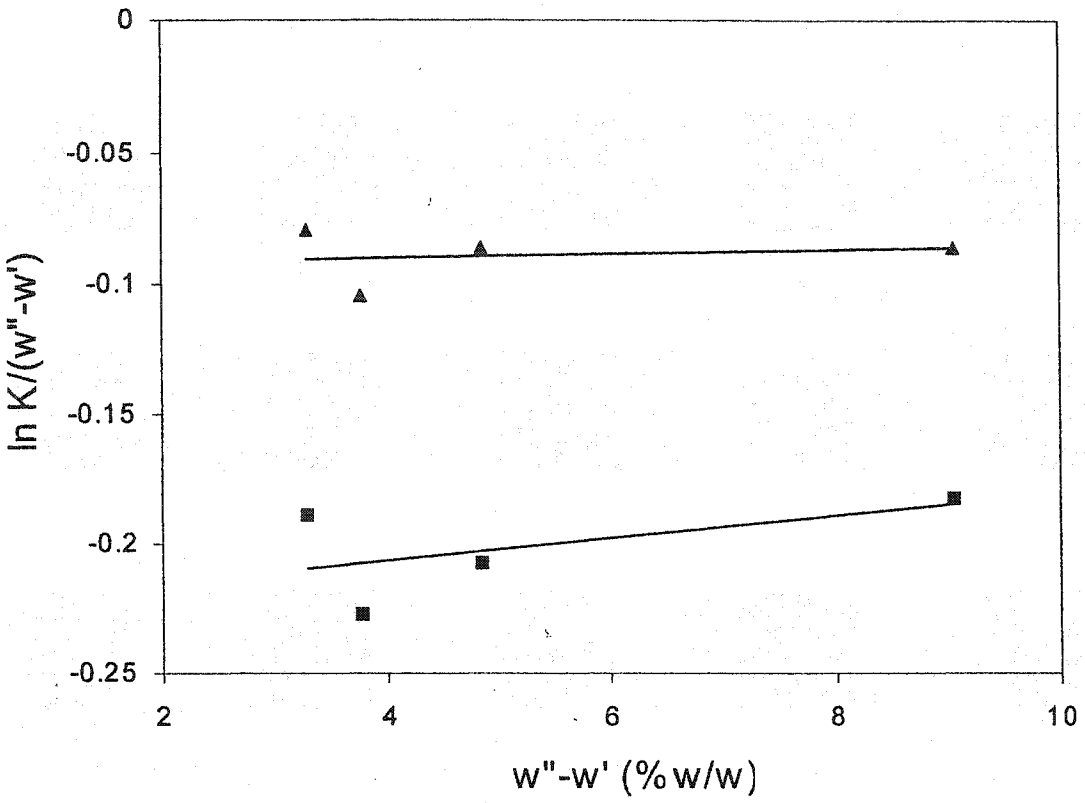


Figure 5.18 Correlation of partitioning coefficient of BSA (▲) and cytochrome c (■) in PEG-APE-water (0.3N KCl) at pH 7.41 using Diamond and Hsu model [21].

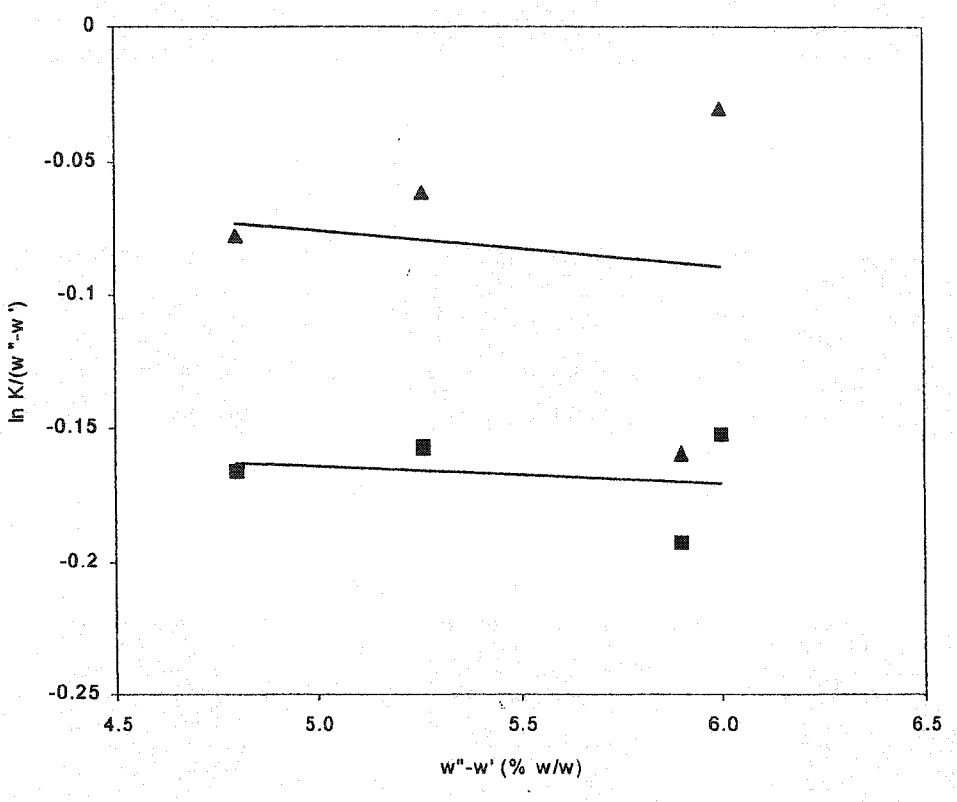


Figure 5.19 Correlation of partitioning coefficient of BSA (▲) and cytochrome c (■) in PEG-APE-water (0.3N KCl) at pH 7.37 using Diamond and Hsu model [21].

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The phase behavior of two-phase aqueous polymer system containing PEG and APE was studied under different conditions of pH. The binodal curve was found to move towards lower concentrations of polymers with increasing pH as a result of decreased solubility of the APE with pH. Electrolytes also have tremendous effect on the phase behavior of the two-phase system. The APE show good promise for protein partitioning because it forms two-phase systems with PEG at low concentrations. Another attractive property of the PEG/APE system is the pH-sensitivity of the APE. The APE can be easily precipitated out of solution after protein partitioning, thus providing a means for recovery of phase polymers.

The effect of pH and salt concentration on the partition behavior of BSA and cytochrome c in aqueous two phase systems of APE and PEG has been studied. Under all the conditions of pH and salt concentrations considered in the study, the positively charged cytochrome c was found to preferentially partition into the negatively charged APE-rich (bottom) phase. Unlike cytochrome c, the behavior of BSA in the system was very

unpredictable. The results show that the best uneven partitioning coefficients and separation factors were obtained at conditions of low salt concentrations and high pH, i.e. 0.1N KCl and pH 7.89 (see Table 5.1). Because of the charged nature of one of the phase polymers as well as the presence of electrolyte in the system, electrochemical potential difference was assumed to play a major role in the partitioning behavior of the model proteins even though there was evidence of other opposing factors.

Correlation studies of both the phase behavior experimental data and the protein partitioning results were conducted. A correlation of type proposed by Diamond and Hsu was found to correlate the phase behavior with acceptable accuracy. The binodal was also correlated accurately with a relationship of the type proposed by Guan *et al.* The protein partitioning was correlated using a simplified model developed by Hartounian *et al.*. Overall, the models employed were found to adequately represent our experimental results, even though they were developed for systems formed by neutral polymers.

6.2 Recommendations

The research conducted during this thesis work is the first protein partitioning studies conducted in a system composed of APE and PEG. Considering the promising results obtained in this preliminary study, it is hoped that further work would be continued in the area. I will like to recommend the following as possible future directions.

1. In order to make the system more selective hydrophobic groups can be introduced into the APE structure. Hopefully, this would increase the number of factors determining partitioning behavior of proteins in the system.
2. Since the APE was found to be stable up to 220 °C [10], it is recommended that its potential as a thermoresponsive polymer should be investigated.
3. The toxicity of this new polymer should be evaluated. Otherwise, this can hamper its application in the industry despite its other promising features.
4. The APE should be fully characterized using techniques such as low-angle laser light scattering technique, vapor pressure osmometry and membrane osmometry. This will provide vital information about the polymer size as well as interaction parameters between it and other polymers or proteins.

NOMENCLATURE

Symbols

c	Solute concentration, percentage weight per weight
F	Faraday constant, 96,490 C
f_{ij0}	volume fraction of unfilled effective volume, dimensionless
G	Degree of separation, dimensionless
k	Boltzman's constant, 1.3805×10^{-23} J K^{-1}
K	Partition coefficient, dimensionless
m_i	Molality of species i, mol kg^{-1}
M_m	Mass-averaged molecular mass, kg mol^{-1}
M_{rms}	Root-mean-square molecular mass, kg mol^{-1}
M_w	Weight-averaged molecular mass, kg mol^{-1}
N_A	Avogadro's constant, 6.023×10^{23} molecules mol^{-1}
n_i	no of molecules of species i
R	Universal gas constant, J mol^{-1} K^{-1}
T	Temperature, K
TLL	Tie line length, percentage weight per weight
V	Volume, m^3
V_{ji0}^*	Effective excluded volume of molecules j in i aqueous solution, kg $kmol^{-1}$
w_i	weight fraction of species i, dimensionless
z_i	net charge of species i, C mol^{-1}

Greek letters

ρ	Density of solvent or solution, kg m^{-3}
μ	Chemical potential, J mol^{-1}
χ_{ij}	Flory-Huggins interaction parameter between components *i* and *j*
ν	Molecular number density, m^{-3}
ψ	Electrostatic potential, J C^{-1}
ΔG_m	Molar Gibbs free energy of mixing, J mol^{-1}
ΔH_m	Molar enthalpy of mixing, J mol^{-1}
ΔS_m	Molar entropy of mixing, J mol^{-1} K^{-1}

Superscripts

'	Bottom phase
"	Top phase

Subscripts

i	component *i*
j	component *j*

REFERENCES

[1] Abbott, N. L., Blanksschtein, D, Hatton, T. A., "On Protein Partitioning in Two-Phase Aqueous Polymer Systems", *Bioseparation,* 1(1990) 191-225.

[2] Abbott, N. L., Blanksschtein, D, Hatton, T. A., *Macromolecules*, 24 (1991) 4334; 25 (1992) 3917, 3932, 5192; 26 (1993) 825.

[3] Abrams, D. S., and Prausnitz, J. M., *AIChE J*, 21, 1 (1975) 116.

[4] Albertsson-A, "Partition of Cell particles and Macromolecules", 1st ed., Wiley Interscience, New York, (1960).

[5] Albertsson-A, "Partition of Cell particles and Macromolecules", 2nd ed., Wiley Interscience, New York, (1971).

[6] Albertsson-A, Johansson, G, Tjernald, F, "Aqueous two-phase Separations", in *Separation Processes in Biotechnology*, Asenjo, J. A (Ed.), Marcel Dekker Inc, New York, (1990).

[7] Albertsson-A, *Nature*, 177,771, (1956).

[8] Ali, S. A., Wazeer, M. I. M, Al-Muallem, H.A, Synthesis of new polyampholytes and their potential use in protein purification, in *The Proceedings of The First Saudi Conference (Part 1:Chemistry)*, KFUPM printing press, (2001) 403-409.

[9] Almeida, M. C., Venancio, A., Teixeira, J. A. and Aires-Barros, M, R., *J. of Chromatography B*, 711 (1998) 151-159.

[10] Al-Muallem, H. A, Wazeer, M. I. M, Ali, S. A., 2002, "Synthesis and solution properties of a new ionic polymer and its behavior in aqueous two-phase polymer systems", *Polymer*, 43, 1041-1050.

[11] Baskir, J. N., Hatton, T. A., and Suter, U. W., "Protein Partitioning in Two-Phase Aqueous Polymer Systems" *Biotechnol. Bioeng.*, 34 (1989)541-558.

[12] Baskir, J., Hatton, T. A., and Suter, U. W., *Macromolecules*, 20 (1987) 1300.

[13] Benge, G.G., "A Comparison of Thermodynamic Models for the Prediction of Phase Behavior in Aqueous Polymer Two-Phase Systems", *M.S. Thesis*, Virginia Tech., Blacksburg, VA, (1986).

[14] Bradford, M. M., "A Rapid and Sensitive Method for the Quantification of Microgram Quantities of Protein Utilizing the Principle of Protein-dye Binding", *Analyt. Biochem.*, 72 (1976), 248-254.

[15] Brooks, D. E., Sharp, K. A. and Fisher, D., "Theoretical Aspects of Partitioning in Aqueous Two-Phase Systems", Academic press, Florida, 1985.

[16] Butler, G. B., and Ingley, F. L., J Am Chem Soc., 73 (1951) 895.

[17] Cabezas Jr, H., "Theory of Phase Formation in Aqueous Two-Phase Systems", *J. of Chromatography B*, 680 (1996) 3-30.

[18] Cabezas, H., Evans, J. D. and Szlag, D. C., *Fluid Phase Equilibria*, 53 (1989) 453-462.

[19] Carlson, A., "Factors Influencing the Use of Aqueous Two-Phase Partition for Protein Purification", Separation Science and Technology, 23 (8 & 9), pp785-817, 1988.

[20] Diamond, A. D., and Hsu, J. T, "Correlation of Polymer Partitioning in Aqueous Two-Phase Systems" *AIChE Symposium Series*, 88, 290 (1992) 105-111.

[21] Diamond, A. D., and Hsu, J. T, "Protein Partitioning in PEG/Dextran Aqueous Two-Phase Systems", *AIChE J*, 37, 7 (1990) 1017-1024.

[22] Dissing, U., and Matiasson, B., "Partition of proteins in polyelectrolyte-neutral polymer aqueous two-phase systems, Bioseparation 4 (1994) 335-342.

[23] Dobry, A and Boyer-Kawenoki, F, *J. Polym. Sci.*, 2 (1947) 90.

[24] Edmond, E and Ogston, A. G., *Biochem. J.*, 117 (1970) 85.

[25] Enfors, S.-O., Kohler, K., and Veide, A., "Combined Use of Extraction and Genetic Engineering for Protein Purification: Recovery of β-galactosidase Fused Proteins" *Bioseparation* 1, (1990), 305-310.

[26] Evers, A. A., Scheutjens, J. M. and Fleer, G. J., *Macromolecules*, 23 (1990) 5221.

[27] Flory, P.J., "Principles of Polymer Chemistry", Cornell University Press, Ithaca, NY (1953).

[28] Flory, P.J., *J. Chem. Phys.*, 10 (1942) 51.

[29] Flory, P.J., *J. Chem. Phys.*, 9 (1941) 660.

[30] Flygare, S., Wilstrom, P., Johnson, G., and Larsson, P. -O., "Magnetic Aqueous Two-Phase Separation in Preparative applications", *Enzyme Microb. Technol.*, 12 (1990) 95-103.

[31] Fortiniti, D. and Hall, C. K., *ACS Symp. Ser.*, 53 (1990) 419.

[32] Fortiniti, D., Hall, C. K. and Kula, M. -R., Chem. Eng. Sci. 47,1 (1992) 165-175.

[33] Gaube, J., Pfennig, A. and Stumpf, M., J., Chem. Eng. Data 38, (1993) 163.

[34] Gaube, J., Pfennig, A. and Stumpf, M., J., *Fluid Phase Equilibria*, 83 (1996) 365.

[35] Guan, Y., Lilley, H. T., and Treffry, T.E., 1993, "A new excluded volume theory and its application to the coexistence curves of aqueous polymer two-phase systems", *Macromolecules*, 26, pp 3971-3979

[36] Guan, Y., Treffry, T.E., and Lilley, H. T., 1994, "Application of a statistical geometrical theory to aqueous polymer two-phase systems", *J. Chromatogr.* A 668, 31-45.

[37] Guggeinheim, E. A., *Proc. R. Soc. London* A 183 (1944) 203.

[38] Gupta, V., Nath, S.,and Chand, S., "Role of water structure on phase separation in polyelectrolyte-polyethylene glycol based aqueous two-phase systems", *Polymer,* 43 (2002) 3387-3390.

[39] Gustafsson, A., Wennerstrom, H. and Tjerneld, F., *Polymer*, 27 (1986) 1768.

[40] Hamad, E. Z., Ijaz, W., Ali, S. A., and Hastaoglu, M. A., "Influence of Polymer Structure on Protein Partitioning in Two-Phase Aqueous Systems", *Biotechnol. Prog.* 12, (1996), 173-177.

[41] Hartman, A., Johansson, G., and Albertsson, P.-A., "Partition of Protein in a Three-Phase System. Eur. J. Biochem., 46, (1974), 75-81.

[42] Hartounian, H., Kaler, E. W., and Sandler, S. I., *Ind. Eng. Chem. Res.*, 33 (1994) 2294-2300.

[43] Hartounian, H., Sandler, S. I., and Kaler, E. W., *Ind. Eng. Chem. Res.*, 33 (1994) 2288; 2294.

[44] Hatti-Kaul, R., "Aqueous Two-Phase Systems: A General Overview", *Methods in Biotechnology, Vol. 11: Aqueous Two-phase Systems. Methods and Protocols.* Humana Press Inc, NJ, p 6, 2000.

[45] Haynes, C. A., Benitez, F. J., Blanch, H. W. and Prausnitz, J. M., *AIChE J*, 39 (1993) 1539.

[46] Haynes, C. A., Beynon R. S., King, R. S., Blanch, H. W. and Prausnitz, J. M., *J. Phys. Chem.*, 93 (1989) 5612.

[47] Haynes, C. A., Blanch, H. W. and Prausnitz, J. M., *Fluid Phase Equilibria*, 53 (1989) 463-474.

[48] Hill, T. L., *J. Chem. Phys.*, 13 (1959) 93.

[49] Hino, T., and Prausnitz, J. M., *Appl. Polm. Sci.* 68 (1998) 2007.

[50] Huddleston, J. G., Griffin, S. T., Zhang, J., Willauer, H. D., and Rogers, R. B.(2000), Metal ion separation in aqueous biphasic systems and with ABEC™ resins. Methods in Biotechnology, vol 11: Aqueous Two Phase Systems: Methods and Protocols, Humana Press Inc, Totowa, NJ. 77-94. J. Chromatogr. B 711, 255-263.

[51] Huddleston, J. G., Willauer, H. D., Boaz, K. R., and Rogers, R. B. (1998), Separation and recovery of food coloring dyes using aqueous biphasic extraction chromatographic resins. J. Chromatogr. B 711, 237-244

[52] Hughes, P and, Lowe, C. R, "Purification of Proteins by Aqueous Two-Phase Partition in Novel Acrylic Co-polymer Systems", *Enzyme Microb. Technol.*, 10 (1988) 115.

[53] Jiang, J., and. Prausnitz, J. M., *J. Phys. Chem. B*, 104 (2000) 7197-7205.

[54] Johansson, G, "Affinity Aqueous Polymer Two-Phase Systems with Liquid Protein (bovine serum albumin) Phase for Partitioning of Enzymes", *J. of Chromatography B*, 680 (1996) 123-130.

[55] Johansson, G, "Affinity Partitioning of Proteins Using aqueous Two-Phase Systems", in *Protein Purification*, 2nd Ed., Janson, J. -C. and Ryden, L., Eds, John Wiley & Sons, NY, 1998.

[56] Johansson, G., "Studies on aqueous dextran-polyethylene glycol two-phase systems containing charged polyethylene glycol I. Partition of Albumin", Biochemica et Biophysica Acta 222 (1970) 381-389.

[57] Jones, K., "A review of Biotechnology and Large Scale Affinity Chromatography", *Chromatographia*, 32 (1991), 469-480.

[58] Kamihiri, M., "Affinity Partitioning Using Magnetic Two-Phase Systems", *Methods in Biotechnology, Vol. 11: Aqueous Two-phase Systems. Methods and Protocols.* Humana Press Inc, NJ, p 381-390, 2000.

[59] Kamihiri, M., Hatti-Kaul, R., and Mattiason B., "Integration of Extraction with Affinity Precipitation", *Methods in Biotechnology, Vol. 11: Aqueous Two-phase Systems. Methods and Protocols.* Humana Press Inc, NJ, p 371-379, 2000.

[60] Kang, C. H. and Sandler, S. I., *Fluid Phase equilibria*, 38 (1987) 245.

[61] Kang, C. H. and Sandler, S. I., *Macromolecules.*, 21 (1988) 3088.

[62] Kaul, A., Pereira, R. A. M., Asenjo, J. A. and Merchuk, J. C., *Biotechnol. Bioeng.* 48 (1995) 246.

[63] Kenkare, P. U., and Hall, C. K., *AIChE J*, 42 (1996) 3508.

[64] King, R. S., "Aqueous two-phase partitioning in Biotechnology", in *Polymer Applications in Biotechnology: Macromolecular Separation & Identification*, Soane, D. S (Ed), Prentice Hall Inc., NJ, (1992) 204-243.

[65] King, R. S., Blanch, H. W. and Prausnitz, J. M., *AIChE J*, 34 (1988) 1585.

[66] Koningsveld, R., Staverman, A.J., *J. Polym. Sci., Polm. Phys. Ed.*, 6 (1968) 367.

[67] Kroner, K. H., Hustedt, H., and Kula, M. -R., "Technical aspects of separation using aqueous two-phase systems in enzyme isolation processes", *Biotechnol. Bioeng.*, 24 (1978) 1015-1045.

[68] Kroner, K. H., Hustedt, H., and Kula, M. -R., *Biotech. Bioeng.*, 24 (1982) 1015.

[69] Kula, M. -R., "Extractive and Purification of Enzymes", Appl. Biochem. Bioeng., Academic Press, New York p71 (1979).

[70] Kula, M. -R., "Liquid-Liquid Extraction of Biopolymers", *Comprehensive Biotechnology*, Vol 2, Pergamon Press Ltd., NY, (1985).

[71] Kula, M. -R., Kroner, K. H., Hustedt, H., and Schutte, H., "Technical aspects of extractive enzyme purification", Ann. N. Y. Acad. Sci., 369 (1981) 341-354.

[72] Landoll, L. M., *J. Polym. Sci.; Polym. Chem.* Ed. 20 (1982) 243.

[73] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., "Protein Measurement with Folin Phenol Reagent", *J. Biol. Chem.*, 193 (1951) 265-275.

[74] Luong, J. H. T., and Nguyen, A. L., *Bio/Technology*, 8 (1990) 306-307.

[75] Malmsten, M., Linse, P. and Zhang, K-W, *Macromolecules*, 126 (1993) 2905.

[76] McMillan, W. J. and Mayer, J. E., *J. Chem. Phys.*, 13 (1945) 276.

[77] Merchuk, J. C., Barbara, A. A. and Asenjo, J. A., *J. of Chromatography B.*, 711 (1988) 285-293.

[78] Mistry, S. L., Kaul, A., Merchuk, J. M., and Asenjo, J. A., *J. of Chromatography A*, 741 (1996) 151.

[79] Munk, P., Introduction to Macromolecular Science, Wiley-Interscience 1989.

[80] Nyugen A. L., Grothe, S and Luong, J. H. T., Applied Microbiol. Biotecnol., 27, s (1988) 341-346.

[81] Ohlemacher, A., Candau, F., Munich, J. P., and Candau, S. J., "Aqueous Solution Properties of Polyampholytes: Effect of the Net Charge Distribution", *Journal of Polymer Science: Part B: Polymer Physics*, 34 (1996) 2747-2757.

[82] Patrickios, C. S., Abbott, N. L., Foss, R. P., and Hatton, T. A., "Synthetic Polyampholytes for Protein Partitioning in Two-Phase Aqueous Polymer Systems", *in New developments in Bioseparation*, Ataai, M. M and Sikdar, S. K (Eds.), AIChE Symposium series, 88, 290 (1990), 80-89.

[83] Patrickios, C. S., Hertler, W. R., Abbott, N. L., and Hatton, T. A., "Diblock, ABC Triblock, and Random Methacrylic Polyampholytes: Synthesis by Group Transfer Polymerization and Solution Behavior", *Macromolecules*, 27, (1994), 930-937.

[84] Patrickios, C. S., Hertler, W.R. and Hatton, T.A., "Phase Behavior of Random and ABC Triblock Methacrylic Polyampholytes with Poly (vinyl alcohol) in Water: Effect of pH and Salt", *Fluid Phase Equilibria*, 108, 1-2 (1995) 243-254.

[85] Peng, Q., Li, Z., Li, Y., *Fluid Phase Equilibria*, 107 (1995) 303.

[86] Peng, Q., Li, Z., Li, Y., *Fluid Phase Equilibria*, 95 (1994) 341.

[87] Persson, J., Johansson, H. -O. and Tjerneld, F., *Ind. Eng. Chem. Res.*, 39 (2000) 2788-2796.

[88] Persson, J., Nystrom, L., Ageland, H. and Tjerneld F., *J. of Chromatography B*, 711 (1998) 97-109.

[89] Pietruszka, N, Galaev, I. Y., Kumar, A., Brzozowski, Z. K., and Matiasson, B., "New Polymers Forming Aqueous Two-Phase Systems", *Biotechnol. Prog.*, 16, (2000) 408-415.

[90] Rastogi, A and Chand, S., *Biotechnol. Techniques*, 3,1 (1989) 33-38.

[91] Raya-Tonetti, G. and Perotti, N. I., "Combination of Extraction and Adsorption for Protein Purification", *Methods in Biotechnology, Vol. 11: Aqueous Two-phase Systems. Methods and Protocols.* Humana Press Inc, NJ, p 363-370, 2000.

[92] Salamanca, M. H., Merchuk, J. C., Andrews, B. A and Asenjo, J. A., J. of Chromatography B, 711 (1998) 319-329.

[93] Salamone J. C. and Rice, W. C., Polyampholytes , *Concise Encyclopedia of Polymer Science and Engineering*, Kroschwitz, J.I.(Ed), John Wiley & Sons, N.Y., p764, 1990.

[94] Sivars, U., Bergfeldt, K., Piculell, L., and Tjerneld F., " Protein Partitioning in Weakly Charged Polymer-Surfactant Aqueous Two-Phase Systems", *J. of Chromatography B*, 680 (1996) 43-53.

[95] Szlag, D. C. and Guiliano, K. A., *Biotechnol. Techniques,* 2, 4 (1988) 277-282.

[96] Tjerneld, F., in *Advances in Separations Using Aqueous Phase Systems in Cell Biology and Biotechnology*, D. Fisher and Sutherland, Eds., Plenum Press, NY, (1988).

[97] Walter, H., Brooks, D. E., and Fisher, D. (eds.), *Partitioning in Aqueous Two-Phase Systems. Theory, Methods, Uses and Applications to Biotechnology.*, Academic press, Florida, 1985.

[98] Wheelwright, S. M., "Protein Purification, Design and Scale-up of Downstream Processes", Hanser Verlag, Munich, 1991.

[99] Wu, Y. -T., Lin., D.-Q., Zhu, Z.-Q. and Mei, L.-H., *Fluid Phase Equilibria*, 67 (1996) 124.

[100] Zhou, F., Kakisaka, K., Ishidao, T., Iwai, Y., Arai, Y. and Faruya T., *J. Chem Eng. Jpn.*, 30 (1997) 349; 31 (1998) 991.