A Study of Protein Partitioning in Two Phase Aqueous Solutions of Random and Multi-block Copolymers

by

Waseem Ijaz

A Thesis Presented to the

FACULTY OF THE COLLEGE 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

September, 1995
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MY MOTHER AND GUDYA
ACKNOWLEDGEMENTS

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A novel physical picture is proposed for the interactions between protein and non-ionic block copolymers that enhance the partitioning of proteins in two-phase aqueous polymer system. For the system polyethylene glycol (PEG)/Poly Acrylamide Stryene (PAS)/Water, this novel picture was based on the assumption that in block copolymer (PAS) hydrophobic monomer (stryene) is distributed in the form of blocks which in turn will show better interaction with protein compared with random copolymer (PAS) where hydrophobic monomer is distributed randomly in the polymer chain. Observation showed that the block copolymer (PAS) gives better partitioning of protein, bovine serum albumin (BSA), than the random copolymer. The presence of strong hydrophobic group (stryene) in the block copolymer repels the hydrophilic protein towards PEG rich top phase. The measurements were performed at four different pH values. The simultaneous effect of pH, polymer structure and concentration on the partition coefficient of protein BSA is analysed. Hydrophobically modified water soluble copolymers of acrylamide stryene were synthesized by micellar and homogeneous copolymerization methods.
ملخص

الاسم: وسام اعجاز
العنوان: دراسة توزيع البروتين بين طريقي المخلية المائية لبوليميرات عشوائية وبوليميرات مجمعة
التخصص: هندسة كيميائية
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تم في هذا البحث اقتحام نموذج عن كيفية النثأر التفاعلي بين البروتينات والبوليميرات في محايل البوليميرات المجمعة لاستفادة من هذا النموذج للحصول على فصل أفضل للبروتينات. وتم اختبار هذا النموذج على خليط البرولين جليكون / بولي أكريلاميد - ستيرين / ماء، حيث تعرض وحدات السطحيين لتأثير تفاعل مركز مع البروتينات المفردة من التأثير. وواضحت التجارب أن الفصل في حالة البوليميرات العشوائية، وواضحت النتائج أن الفصل في حالة البوليميرات المجمعة أفضل من الفصل في حالة البوليميرات العشوائية. وتم القياسات عند اربعة قيم للاس الهيدروجيني، وتتم تحليل النتائج بالنسبة لتأثير الاس الهيدروجيني، وتتيح البوليميرات بالبلمرة في المخلية المائية والصودرية،

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Chapter 1

Introduction

Progress in technology based on biochemistry and cell biology depends to a great extent on the development of efficient separation methods. This holds both for soluble substances such as proteins and nucleic acids and for suspended particles, such as cell organelles and whole cells. Much interest is today devoted to the study of complex particles obtained by disintegration of cells or cell organelles, such as mitochondria, chloroplasts or various cellular membranes. These procedures yield very complicated mixtures of particles differing in size, form and chemical composition. The particles are also very fragile; they may aggregate, dissociate or generally change their state with time. This is also true for suspension of whole cells and organelles. In many fields of biological research there is pressing need for mild and efficient fractionation methods.

Since a multitude of components are present in mixtures like those mentioned, one can not expect to solve a separation problem by one type of method alone. It is necessary to combine different methods which utilize different properties of particles. Centrifugation methods, for example, which separate according to the size and density of particles, should be complemented by methods in which other properties, such as surface properties comprise the separation parameters. One of these methods is distribution in a liquid-liquid two phase system. This thesis deals with the application of liquid-liquid distribution techniques to macromolecules and
particles using special aqueous phase systems obtained by mixing aqueous solutions of two suitably different polymers. Both phases in these systems are aqueous and therefore, suitable for particles and macromolecules from biological materials. The particles distribute according to their molecular properties.

Isolation and purification of biopolymers and particles on a large scale is also of increasing importance in industry. There is a need not only for mild and efficient separation methods but also for such methods which can be applied economically to many types of materials.

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

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

1.1

where $C_t$ and $C_b$ are the concentrations of the partitioned substances in moles per litre of top and bottom phase, respectively. Ideally, the partition coefficient is independent of the volume ratio of the two phases, the partitioned substance, and temperature.

The choice of a suitable phase system is the key step in all partition work. Special problems arise when a phase system has to be selected for biogenic particles and macromolecules. The phase system should be mild, that is, consideration must be given to water content, ionic composition, osmotic pressure, ability to elute out substance from the particle's denaturing effect.
Several of the factors above rule out most of the conventional phase systems containing an organic solvent. These are unsuitable both because of the denaturing effect of organic solvents and because, in a water-organic system, biogenic particles and macromolecules almost always segregate completely to the aqueous phase, thereby precluding separation. In addition, the liquid-liquid interface of the conventional phase systems has a rather strong interfacial tension which might damage fragile cell structure. Aqueous-aqueous systems have been used to overcome the above deficiencies. They consist essentially of two immiscible aqueous solutions of different polymers. It is a general phenomena that mixtures of solutions of unlike polymers in a given solvent result in a phase separation. A mixture of aqueous solution of polymers results in a phase with a water content in the range between 85-99 percent. They are very mild towards various biological activities. The interfacial tension is extremely low, between 0.0001-0.1 dynes/cm compared with 1-20 dyne/cm for a conventional system. In fact, experience thus far is that polymers seem to stabilize, rather than damage the particle structures and the biological activities.

The mechanism governing partition is largely unknown. Qualitatively it can be described as follows. When a particle is suspended in a phase it interacts with the surrounding molecules in a complicated manner. Various bonds such as hydrogen bonds, ionic and hydrophobic interactions are probably involved together with other week forces. Their relative contributions are difficult to estimate. However, their net effect is likely to be different in the two phases (Abbott and Hatton, 1988).

Economic analysis shows that purification is often the most important aspect of biomolecular production and processing. This is particularly true of protein
processing which because of the complexity of the starting material, often requires many steps to reach purity levels required for medical and food applications.

The main unit operation which appears in laboratory-scale protein purifications aimed at very pure products is chromatography. Frequent use is made of ion-exchange, hydrophobic, and biospecific (affinity) chromatography, primarily because the processes are effective and relatively simple. Except for a few systems, there is no real need to improve on chromatography at small scale, and the current trend is towards increasing rather than decreasing use of this method in the laboratory.

At large scale, however, it is not clear that chromatography is the best separation process available. There are two main difficulties with chromatography which lead to process bottle-necks. First, chromatography is inherently discontinuous and, although various types of equipment have been proposed which could make chromatographic processes more or less continuous, they all lack simplicity, which is a measure of the usefulness of a process industrially. The best chromatography systems, even at large scale are still discontinuous.

The second problem with chromatography is the mass transfer rate. Protein diffusion in free solution is inherently slow because of the large size of these molecules, but in resins the diffusivity is even smaller because of hindrance set up by the resin matrix material. One method to alleviate slow mass transfer in resin is to use smaller particles, but this does not work well at large scale because large columns will have unacceptably large pressure drops. For these and other reasons not mentioned, much recent research has been directed at the possible use of aqueous two phase partition (ATPP) to replace chromatographic steps in protein
purification schemes. ATPP is analogous to chromatography in many respects but may offer significant advantages over those purification methods once processes using it are properly designed. ATPP involves the use of two liquid polymer solutions as a partitioning system for proteins. The liquid-liquid nature of these systems offers the possibility that separations using ATPP may be designed to be continuous without using complex or unusual equipment. But even discontinuous ATPP processes have an advantage over chromatography because they are easily scaled. In addition, since, there is no solid phase, intimate mixing of two phases is possible and, hence, interphase transport is rapid. Little time is required to bring most two phase systems to equilibrium. Another benefit which may be important is that the phases are compatible with almost all known proteins. The presence of polymers can even stabilize some biomolecules (Goddard and Hannan, 1977; Kula et al., 1982).
Chapter 2

Literature Review

2.1 Studies on Polymer Choice

The starting point for any ATPP system is the selection of the polymers used to generate the phases. Phase separation is seen with almost any combination of two chemically distinct polymers in a single solvent and is seen in both organic and aqueous solutions. Flory (1942) and Huggins (1942) developed the basic theory to describe this phenomena for organic systems. Albertsson (1971, 1986) demonstrated phase separation behavior in a large number of aqueous systems.

Interestingly, despite the number of potentially useful systems for protein partition, only a few polymer mixtures have been studied as ATPP systems. In particular, almost all reported partition systems use polyethylene glycol (PEG) and dextran as the phase forming materials. Aside from some practical advantages, it is rarely made clear why any PEG and dextran were chosen in any particular study. The main problem with dextran is that it is very expensive.

Here we will examine the factors which must be considered when one chooses the polymers for an ATPP system.
2.1.1 Phase Forming Characteristics

At the core of ATPP system is the phase forming characteristics of the polymers involved. Generally one considers a two phase system generated by two soluble polymers and a single solvent. Multiphase systems have also been examined (Albertsson, 1986) as have single polymer systems (Kula et al., 1982, Albertsson, 1986), but only two-polymer-one-solvent system will be considered here.

Flory-Huggins theory is sufficient to qualitatively describe the thermodynamics which leads to phase separation. Phase separation is caused by the fact that polymer solutions have small entropy of mixing so that positive enthalpic effects involving the interaction of the segments of the polymer chains leads to phase separation. Put simply, polymers prefer to self-associate rather than mix with other polymer molecules, so two phases of different polymer composition are formed in mixtures. Detailed descriptions of the considerations which lead to the Flory-Huggins theory can be found in the original work or the reviews published on this aspect (Iliopoulos et al., 1989; Piculilel and Nilsson, 1991).

2.1.2 Effects of Polymer Structure

Aside form the effect that polymer structure has on the phase envelope, there is also more direct implication of the structure for the partition coefficient of a protein. These need to be taken into account in polymer selection.

One choice which must be made is the molecular weight of the polymer. Polymers are available in many different molecular weights. When the molecular weights of the two phase-forming polymers are dissimilar, there is a driving force for proteins
to partition towards the phase with smaller molecular weight. The larger the difference in the molecular weight the stronger the effect (Albertsson, 1986).

A second consideration is the type of polymer based on the relative hydrophobicity (Albertsson, 1986; Zaslavsky and Rogozhim, 1984a and b). Since hydrophobic effects have an impact on the protein partition through interactions between the polymer and protein more strongly, hydrophobic polymers should be expected to enhance partition due to this effect.

A third consideration is whether or not the polymer should be electrically charged and what the sign of the charge should be. The specific structure of the polymer is also important in that it impacts on the ability to make derivatives. Derivatization can be used to change the character of the polymer or to attach ligands.

2.1.3 Density and Viscosity

The density and viscosity of the polymer solutions used in ATPP systems are of major importance in the design of the process for separation since they have impact on the rate of phase separation.

The combination of high molecular weight to promote equilibrium phase separation and lower molecular weight to promote low viscosity and high separation rate, results in an interesting trade off. While increase in molecular weight increases the viscosity of polymer solution, and slow separation rate. lower concentration of high molecular weight polymer may be used to achieve the phase separation. Hence, higher molecular weight polymers will often render systems with improved behavior from a processing viewpoint (Kula et al., 1982).
2.1.4 Cost, Recovery and End Use

In the final analysis the choice of polymers for the ATPP system depends on the cost, recovery prospects and the end use criteria. Inexpensive polymers are favored for economic reasons, but polymers which can be recovered at high yield need not be as inexpensive as those which cannot. The economics of ATPP have been analysed for some particular systems and the analysis shows that systems which minimize the use of dextran relative to PEG are more economical (Kroner et al., 1984). This is primarily because PEG costs only about 1 to 2% of the cost of dextran. Since PEG is such an extremely inexpensive polymer, it is widely used. The ability to recover the polymers will greatly affect process economics and can, if other things are favourable, make other polymers economical to use.

A final consideration for some products is biocompatibility of the polymers. Of the common polymers, only PEG and dextran are fully approved for injectables (Kula et al., 1982; Brooks et al., 1985). For products which will ultimately be ingested or injected, this may be an overriding factor in polymer choice.

2.2 Phase Behavior of Aqueous Polymer Mixture

There is a considerable current interest in the properties of the aqueous mixtures of polymers. A number of studies have drawn attention to phase separation phenomena (Goddard and Hannan, 1977; Wormath, 1991), which display a rich and interesting behavior between aqueous mixtures of dissimilar polymers. Picullee and Lindman (1992) studied the effects of non-ionic and ionic polymers in phase separation and their findings are given below. Some related works reported in the
literature are also cited here (Wormath, 1991; Karlstrom et al., 1990; Thalberg et al., 1991).

Polymer/polymer/solvent mixture results in two main types of liquid-liquid phase separation phenomena. In the first type both the separating phases contain polymers of a comparable total concentration. But the polymer composition is strongly asymmetrical, so that each phase is enriched in one of the polymers. This kind of phase separation, which is often called "polymer incompatibility" will, here, be referred to as a segregative phase separation. The second type of phase separation is when a phase concentrated in both polymers separates form a phase which essentially contains only solvent. This phenomena is often referred to as "Complex Coacervation" or simply associative phase separation.

For mixtures of non-ionic polymers in aqueous solutions, just as in the simple non polar solvents, segregation is the rule for mixtures of nonionic polymers (Albertsson, 1986).

This system is widely used for the partitioning of macromolecules and cell particles in biochemical research (Albertsson, 1986). The tendency towards segregation becomes stronger with increasing molecular weight of either of the polymeric components.

A polyelectrolyte is a polymer which, upon dissolving, dissociates into one polyion and \(n\) counterions, where \(n\) may be of the same order of magnitude as the degree of polymerization of the polymer. The dissociation of the counterions has a profound effect on the phase behavior of the polyelectrolyte solution, since it gives a very large contribution to the entropy of mixing. One consequence of the large
entropic drive towards mixing is the greatly enhanced solubility of a
polyelectrolyte. The release of the counterions may render soluble an intrinsically
insoluble polymer. In the following, we shall see that polyelectrolytic effects
greatly influence the phase behaviour of aqueous polymer mixture.

Consider the phase behavior of an aqueous mixture of polyelectrolyte and a
nonionic polymer. A phase separation by association or segregation would,
therefore, lead to the creation of a phase which has an enhanced concentration of
the counterions, the phase separation of a nonionic/ionic mixture is strongly
disfavored compared to that of the nonionic/nonionic mixture.

When salt is added to a nonionic/ionic polymer mixture, the conditions change
dramatically, since it then becomes possible to create two phases which differ in
the polymer concentration while still having similar concentrations of all small
ions. Consequently, the polyelectrolytic effects discussed here should disappear
largely or entirely upon addition of salt (Ilipoulos et al., 1989).

The tendency towards increasing miscibility, which is found for an intrinsically
segregating nonionic/ionic polymer mixture, should largely disappear in the
mixture of similarly charged polyelectrolytes. This is because, in the latter case, it
is possible for the polyions to segregate into separate phases while an even
distribution of the counterions is still maintained. Therefore, a mixture of similarly
charged polyelectrolytes is expected to behave more like a mixture of nonionic

The difference in the electrical potential of the two phases and their relative
hydrophobicity (determined by the polymer content) play important roles in the
partition of proteins between the phases. However, such analyses do not take into
account the specific surface features of the molecules. For example, the net charge of the protein may not fully explain partition behavior since the type of amino acids contributing to, and the location of the amino acids within the molecule may be important. Likewise, the location of hydrophobic groups on the protein surface may also contribute to the apparent hydrophobicity. The specific volume of the molecule may also be important in partition.

Mixtures of two oppositely charged polyelectrolytes and their phase separation has been extensively studied (Smid and Fish, 1988). The dominating feature of these systems is a strong tendency towards association of the oppositely charged polyions, which results in an associative phase. Upon addition of salt, however, there is often an increase in miscibility, and in many cases the system becomes entirely miscible above a certain salt concentration. In the absence of salt, the mixtures give rise to an associative phase separation. When the salt is added, the mixture eventually forms a single phase; but when still more salt is added, a segregative phase separation occurs. In this case, it seems that polyion pair, in the absence of electrostatic effects, is intrinsically segregating (Albertsson, 1986).

### 2.3 Recent Developments in Micellar Polymers

The importance of water-soluble polymers is increasingly growing, equally for technical applications, for biological/medical purposes, and for environmental aspects. The aqueous-solution properties are not well understood yet, in particular, when charged polymers are involved.

A particularly interesting class of water-soluble polymers are the "micellar polymers" or "polysoaps" (Strauss and Jackson, 1951; Bekturov and Bakauova, 1986). Known since the early 1950s, but neglected for a while - e.g. in favor of
polymeric vesicles - polysoaps experience a revival in recent years. Capable of self-organization due to hydrophobic interactions, but still isotropically soluble, they stand right between classical, homogenously dissolved polymers and extensively self-organized but phase-separated systems, such as monolayers and vesicles of polymer lipids (Ringsdorf et al., 1988). Towards both extremes there are gradual transitions: Towards the homogeneously dissolved polymers we find water-soluble polymers modified by a small number of hydrophobic groups acting as thickeners due to intermolecular aggregations (Magny et al., 1991); towards monolayers and vesicles we find polymeric lyotropic liquid crystals due to superstructures created from surfactant aggregates (Anton et al., 1993).

Polysoaps can be visualized as a large number of surfactant structures linked by a polymer backbone. Aqueous solutions of such polymers are characterized by unusually low viscosities, and by high solubilization capacities. These properties are attributed to the intermolecular aggregation of the surfactant side chains providing hydrophobic microdomains in isotropic aqueous solution. Such a behavior resembles the micelle formation of low molecular weight surfactants in water. Hence the names “polysopas” or “micellar” polymers were coined and the hydrophobic aggregates formed in polysoaps are referred to as “polymeric micelles”.

A particular feature of polysoaps seems to be their intramolecular hydrophobic aggregation which is explained by the close proximity of the surfactant side chains within one macromolecule. Due to the intramolecular aggregation, an equivalent to the “critical micelle concentration” (cms) of low-molecular-weight surfactants is generally missing. This makes a major difference to low-molecular-weight surfactants and amphilic block copolymers which both aggregates intermolecularly
(behaving in many respects like "oversize" surfactants, the latter often being called "macrosurfactants"). Occasionally, there are reports on cms's of polymers which form their molecular structure which could be classified as polysoaps (Tanchuk and Pop, 1978; Arai et al., 1991). But apparently in these cases, low oligomers or chemically ill-characterized (and thus disputable) structures are involved, this makes it difficult to decide whether the reported cms's are real.

2.4 Effects of Protein Structure

Proteins are generally tight folded molecules which sample only a few of the many possible conformational states. Free energy calculations argue that the three-dimensional structure of proteins is relatively fixed, and other evidence indicates that the X-ray structures are similar to structures adopted by proteins in solution. For these reasons a static model of protein structure is generally suitable, and one can think of a protein as a "particle" with a fixed shape including a solvent accessible exterior and an inaccessible interior.

This static molecule model can be used to show which amino acids are exposed on the surface of a molecule, where charged groups are located, and the exact structure and molecular volume of proteins. Analyses indicate that the molecular surface is primarily comprised of hydrophobic amino acid side chains and the hydrophilic portions of the main carbon chain. Most charged groups are located on the surface, or, if buried in the interior, participate in ionic bridges within the molecule. However, there are a significant number of hydrophobic side chains exposed on the molecular surface, and these undoubtedly give the surface a hydrophobic character (Carlson, 1988).
2.4.1 Hydrophobic Character

Although no experiments have been conducted which directly relate partition behavior to protein surface properties, it is clear that surface hydrophobicity has an influence on protein partition. Surface hydrophobicity has been implicated in chromatographic behavior and in solubility of proteins (Zaslavsky and Rogozhin, 1978; & Zaslavsky and Rogozhin, 1982). Retention of proteins on reverse phase chromatography resins is correlated to the strength of the hydrophobic bonds which can form between the protein surface and the resin phase. Similarly, hydrophobic protein-protein interactions appear to be enhanced in some solutions, causing precipitation of the molecules when the salt concentration in a solution is increased (Melander and Horvath, 1977).

2.4.2 Ionic Group Location, Hydration Properties and Dielectric Effects

Besides the hydrophobic character of protein surfaces, the other major determinant of partition behavior is the location and state of the ionic groups of the molecule. Ionic surface groups contribute to partition in a general way by determining the overall (net) charge of the molecule. They also may contribute in a specific way to partition by interacting directly with the ionic species in the phase system or by changing the nature of the potential field around the molecule. The solubility (i.e., the activity coefficient of the protein) is a complex function of these ionic effects and depends on both the first moment (net charge) and higher moments of the ionic nature of the molecule (Kirkwood, 1968).
Closely related to these ionic effects are the "hydration" characteristics of the phases, which have been suggested as determinant in partition behavior (Zaslavsky et al. 1982). Hydration affects the ability of a given phase to accept charged species and dissipate the charge. This effect is similar to the effect of a change in the dielectric strength of the media on the solubility of proteins (Kirkwood, 1968). Zaslavsky et al. (1982) have used hydration properties to correlate partition coefficients, but their conclusions have been criticized because they also downplayed the ionic effects and stated that the potential differences between phases have little to do with partition of ionic species (Walter and Anderson, 1981).

Albertsson (1960) is the pioneer of the work related to protein partitioning in mixtures of dissimilar polymers. He studied many polymer systems which separated into two or more phases upon mixing. He first applied the aqueous two phase system of PEG and K₃PO₄ for partitioning of plant cells in 1955. Albertsson found that this was a very efficient separation system. Later he studied different systems mostly with dextran as one of the two polymers.

In 1992, Hatton and co-workers studied different aspects of protein partitioning in aqueous polymer systems. Their work is related to the effect of molecular weight of the polymers on protein partitioning and they found that partitioning of proteins is enhanced with increase in molecular weight of one of the two polymers. They also studied the protein-polymer interaction, effect of conformation of proteins (spherical and ellipsoidal) and found that these factors play significant part in the partitioning of the proteins.
Forciniti and co-workers (1992) studied the effect of pH on the protein partitioning. They found that the dependence of partition coefficient on the pH and on the polymer molecular weight is a strong function of the kind of protein employed.

The main problem with this work is that until now almost all of the work is carried out with dextran as one of the two polymers. Dextran has certain limitations. First, it is very expensive as compared to other polymers. Secondly, it is not easily recoverable. Therefore, there is a strong need of the replacement of dextran with some cheap polymers which also possess acceptable characteristics, i.e. compatibility and phase forming characteristics.

Partrikios and co-workers (1992) replaced dextran with synthetic polyampholytes for protein partitioning in two phase systems. They used poly-vinyl alcohol as the second polymer. They found interesting results. Although their random copolymer affected partitioning, the partitioning of the protein was not efficient in this system.

2.5 Studies on Phase Behavior

In aqueous two-phase polymer systems, the distribution of the polymers between the phases is governed to a large extent by their molecular weight. For economic reasons, the polymers used for biological separations in aqueous two-phase systems are usually polydisperse. Koningsveld and Staverman (1968) and later Kang and Sandler (1988) showed that the phase behavior of aqueous two-phase systems is affected by the degree of the polydispersity of the polymers. One effect of polydispersity in the phase-forming polymers in aqueous two-phase systems is
that the transition from a two-phase dispersion to one-phase solution is not sharp (Albertsson, 1986).

The binodal curve for aqueous two-phase polymer systems can be constructed by the titration method (Albertsson, 1986). In this method, a turbid two-phase mixture with known concentration of two polymers is quantitatively diluted with the solvent (water) until a homogeneous solution is obtained. The final composition of the two polymers calculated corresponds to one point on the binodal curve. After obtaining the first point, a concentrated solution of one of the polymers is quantitatively added to the solution to again obtain a turbid dispersion. The new concentrations of the two phase-forming polymers are determined, and dilution with solvent (water) is repeated to obtain a second point on the binodal curve. This procedure is continued till a sufficient number of points for the construction of the binodal curve are obtained. This method is useful when the turbid-clear transition is relatively sharp, as happens when the polymers are monodisperse. For polydisperse polymers, however, the turbid-to-clear transition is gradual and difficult to determine precisely.

2.5.1 Effect of Temperature

Despite the fact that aqueous two-phase partitioning has been used extensively for the laboratory separation of biologically active materials, there is not an accurate way to predict the partitioning of polymers and biomolecules in these systems. A fundamental understanding of biomolecule partitioning and polymer phase equilibrium in aqueous two-phase polymer systems could lead to improvements in the design and optimization of large-scale purification processes. The effect of temperature on the relevant polymer-polymer-water-phase equilibrium is
important. Increasing the temperature of the PEG-dextran-water two-phase system proceduces an increase in PEG concentration in the top phase and a decrease of dextran concentration in the lower phase. PEG becomes more hydrophobic at high temperatures, and changes in the PEG-water interactions drive water from the PEG-rich phase to the dextran-rich phase (Hartounian and Sandler, 1993).

2.6 Electrostatic Potential and Protein Partitioning

Recent experiments have shown that protein partitioning in aqueous two-phase systems is strongly influenced by the presence of strong electrolytes, with different electrolytes producing different effects (Johansson, 1974; Reitherman et al., 1973; King et al., 1988). Experiments on poly(ethylene golcyol)/dextran two-phase systems indicate that salts with polyvalent anions, such as sulfate and phosphate, partition preferentially into the dextran-rich bottom phase, while salts of halides partition almost evenly (Brooks et al., 1984). Through capillary-electrode experiments, Brooks et al. (1984) found that the presence of electrolyte in a two-phase system induces an interfacial-electrostatic-potential difference and that the magnitude of this electrostatic-potential difference rises with increasing valence on the anion. King et al. (1988) found that the magnitude of the interfacial electrostatic potential difference is directly proportional to the partition coefficient of the added electrolyte. It is therefore widely believed that the effect of a salt on the partitioning of a protein is due in large part to the electrostatic-potential gradient which develops as a result of the presence of the salt. In essence, a protein responds to the electric field in proportion to the sign and magnitude of the density of its surface charge. This idea was suggested first by Albertsson (1986), who developed a theory based on simple thermodynamic arguments relating the
interfacial-electrostatic-potential difference to the "chemical" activity coefficient of the ions of the added electrolyte.

The Albertsson model (1986) suggests that interfacial electrostatic potential differences are created by a difference in the relative chemical affinities of the ions of the added electrolyte for the two liquid phases. Analysis of the Albertsson model raises two subtle questions:
1. What is the role of the electrostatic potential in thermodynamics?
2. How is the electrostatic-potential difference defined in Albertson's model related to the electrostatic-potential difference measured by Brooks et al. (1984) and others?


2.7 Interactions Between Protein and Polymers

The physics controlling the interactions between globular colloidal particles and flexible chain macromolecules is reflected in such diverse phenomena as the complexation of polymers and micelles (Tokiwa and Tsujii, 1973; Shirahama, 1974; Cabane, 1977), the polymeric stabilization of flocculation of gold sols, ceramic particles and other colloidal dispersions (Woodhead, 1986), and the stabilization, aggregation and precipitation of proteins (Ingham, 1977). Novel physical pictures were proposed for the interactions between globular proteins and flexible nonionic polymers that are responsible for the observed partitioning of
proteins in two-phase aqueous polymer systems by Abbott et al. (1991). For the system polyethylene oxide-dextran-water, these novel physical pictures were based on the observation that a transition occurs in the nature of the top PEO-rich phase, from the dilute to the semidilute polymer solution regimes, with increasing PEO molecular weight. Through a statistical thermodynamic framework, it was possible to discriminate between the novel physical picture on the basis of the predicted qualitative trends. In systems containing high molecular weight PEO, the proteins interact with an entangled polymer network rather than with identifiable polymer coils, and the protein partition coefficient becomes independent of the PEO molecular weight.

2.8 Studies on Polymer Synthesis

Hydrophobically associating polymers consist of a water-soluble polymer with a small number of hydrophobic groups (Evani and Rose, 1987). In aqueous solution, above a certain polymer concentration, intermolecular hydrophobic interactions lead to the formation of polymolecular associations. As a consequence, these copolymers exhibit thickening properties equivalent to those observed for higher molecular weight homopolymers.

In the past few years, there has been an increasing interest in the synthesis and properties of hydrophobically modified water-soluble polymers. The preparation of such materials can be carried out, as for any copolymer synthesis, either by chemical modification of a preformed polymer or by copolymerization of the appropriate monomers or by a combination of both methods. The first synthesis route has mainly been applied to cellulose derivatives (Landoll, 1982) to poly(oxyethylene) which leads to the so-called HEUR thickeners (Schaller, 1985;
Glass, 1986). Hydrophobically modified ethoxylated urethane polymers and more recently to poly(acrylic acid) (Wang et al. 1988). The copolymerization processes concern essentially acrylamide based copolymers (Bock et al. 1986; 1987; Valint et al., 1987). In this case, some difficulties in the copolymer synthesis arise from the insolubility of the hydrophobic monomer in water. To overcome this problem, it is proposed to use an aqueous surfactant solution which ensures solubilization of the hydrophobe within micelles. The “micellar” process was shown to be well suited for the preparation of copolymers with improved thickening properties (King and Constien, 1986; Constien, 1986). However, although many publications deal with copolymers synthesised by the micellar technique the specific feature of this process has rarely been recognized. An important point which was only recently examined is how the copolymer microstructure is affected by the presence of the surfactant micelles during the synthesis. In the earlier papers, a random structure was implicitly admitted (Bock et al. 1986; 1987; Valint et al., 1987) and in some papers the problem of structure of the copolymers was sometimes just mentioned (King and Constien, 1986; Flynn and Goodwin, 1989). The formation of a blocky structure was first suggested by Peer (1987). Direct experimental evidence of this blocky structure was reported by Thomas et al. (1990) and McCormick et al. (1989) from the photophysical studies on polyacrylamide derivatives containing either styryl or pyrenyl groups as hydrophobes. Thus it was shown that samples prepared under micellar conditions exhibit a stronger excimer to monomer fluorescence ratio than samples prepared in homogeneous solutions. As excimer formation is favored when fluorescent chromophores are in close proximity, the photophysical behavior of the copolymers prepared using the micellar process was consistent with the presence of adjoining hydrophobic units along the backbone. Using the same photophysical technique on naphthalene-labeled polyacrylamide, McCormick and co-workers further showed that the
length of the hydrophobic blocks was directly related to the hydrophobe-to-surfactant ratio. Hill et al. (1993) presented results of a more detailed investigation on synthesis-structure-property relationship for acrylamide based copolymers. N-4-ethylphenylacrylamide was chosen as the hydrophobic comonomer because its hydrophobicity is not too high and its UV activity allows the accurate determination of the copolymer composition (Hill et al. 1991; Valint et al., 1990).

Earlier studies have demonstrated the applicability of micellar polymerization for controlling particle sizes of polystyrene latex (Turro and Kuo, 1987; Thomas et al., 1989). In a study, Dowling and Thomas (1990) presented results of micellar polymerization of styrene carried out in an aqueous solution of acrylamide to form the water-soluble copolymers. This technique offered the advantage of being a one-step copolymerization allowing isolation of the hydrophobic styrene monomers and hydrophilic acrylamide monomers during the reaction.

2.9 Difference Between Random and Block Copolymers

Utilization of block instead of random polyampholytes in these applications may have a dramatic effect on the performance of the separation processes, as the properties of these block copolymers are expected to be different form those of random. For instance, in displacement chromatography, it is expected to have enhanced performance because the block architecture will strengthen the driving force for separation which is the electrostatic interaction between the polyampholyte and the chromatography column. The localization of the similar charges within a block will generate an electric field which will be stronger than that of the random copolymer. Another implication of the strengthening of the
electrostatic interaction by block copolymer architecture is that, at the isoelectric point of the polymer, the inter polyampholyte attractions will be stronger, leading to a more extensive precipitation. The ability to precipitate the polymer is very critical in industrial applications because it will facilitate recycling (Patrikios, 1992; Abbot and Hatton, 1992).

Based on these studies, it is intended to synthesize a block copolymer which is expected to give a better partitioning of proteins as compared to the random copolymer. For this purpose, a novel micellar polymerization will be utilized in the one step synthesis of water soluble block copolymers of acrylamide and styrene and in the control of styrene block sizes in the predominantly acrylamide polymer chain (Anton, 1993; Dowling, 1990). It is expected that the hydrophobic part in the block copolymer of acrylamide styrene will partition the hydrophobic proteins better than the random copolymer of acrylamide styrene.

2.10 Studies of Correlations of Polymer and Protein Partitioning

Diamond and Hsu (1990) developed correlations for polymer partitioning in aqueous two phase systems and protein partitioning based on Flory-Huggins theory.

Brooks et al. (1985) suggested the use of Flory-Huggins theory to describe protein partitioning in aqueous two phase systems. Albertsson et al. (1987) also worked on the same theory.
Baskir et al. (1989) have modified the theory of Scheutjens and Fleer (1979, 1980) to predict protein partitioning while King et al. (1988) extended the solution theory of Edmond and Ogston (1968). The advantages and disadvantages of the above models have been discussed in detail by Baskir et al. (1989).

Among all the correlations developed, that of Diamond and Hsu (1990) is the simplest relationship.

2.11 Analysis of the Literature

The analysis of the literature reveals that aqueous two phase partitioning (ATPP) is the most efficient method for protein purification as compared with other available methods like chromatography. The main advantages of ATPP are as follows:

- As proteins are sensitive to the environment, use of aqueous polymer solution even sometimes stabilizes the proteins and almost all the polymers are compatible with proteins.
- This system has very low interfacial tension as compared with polymer solutions in organic solvents.
- It is very economical, the only cost is that of polymers. By utilizing cheaper polymers this can be improved even more.
- ATPP can easily be scaled up. Both continuous and discontinuous processes are practicable.
- ATPP is simply a liquid-liquid extraction process, well understood by chemical engineers.
- Almost all reported partition systems have used dextran as one of the two polymers, as phase forming materials. However, it has some limitations. It is expensive and not easily recoverable.
• Surprisingly, very few attempts have been made to replace dextran. Random copolymers were used once instead of dextran but it was not very effective in selectivity for protein purification.

• Considerable work has been done to study the factors affecting the protein partitioning like molecular weight, relative hydrophobicity, pH, polymer-protein interactions, temperature, shape of protein molecule etc.

After the analysis of the literature it was concluded that the replacement of dextran with another polymer was inevitable, to make ATPP economical and effective. Therefore, in this thesis attempts have been made to look for the replacement with the following characteristics.

1) The polymer should have phase forming characteristics.

2) It should be relatively cheap.

3) It should be very effective in selectivity for protein purification.

As mentioned earlier that the random copolymer was not very effective in selectivity for protein purification due to the random distribution of the monomer into the main polymer chain, It is, therefore, hoped that if the hydrophobic monomer is incorporated into the backbone of the polymer chain, it would show better selectivity for protein partitioning. In order to confirm our hypothesis, both block and random copolymers of acrylamide styrene have been synthesized. Detailed procedure for the synthesis of polymers is explained in Chapter 3. Experiments showed that acrylamide styrene gives phase separation with the following polymers:

   I. Polyethylene glycol,
   II. Polyvinyl alcohol,
   III. Poly pyrrolidone,
IV. Urithmized polyvinyl alcohol.
Chapter 3

Synthesis of the Polymers

3.1 Introduction

Copolymerization of a hydrophobic monomer with a hydrophilic monomer can result in an amphilic polymer, the specific nature of which can be controlled via polymerization parameters. The dual hydrophilic/hydrophobic nature provides these materials with unique solubilization characteristics and modifies physical properties of the bulk polymer. Water soluble amphilic polymers are of particular interest as a chemical system due to the presence of microdomains that may impart unusual properties to a given system. In this thesis, the synthesis is described on acrylamide-styrene copolymers that provide hydrophobic sites in aqueous solutions.

The usual way for preparing polyacrylamide is a free radical polymerization reaction in aqueous solutions. However, in the present case, the experimental conditions of the copolymerization are necessarily different, since the hydrophobic monomer is insoluble in water. In this work two different polymerization processes are used.

i) *Micellar Copolymerization*: For the production of block copolymer of acrylamide-styrene.

ii) *Homogeneous Copolymerization*: For the production of random copolymer of acrylamide-styrene.
3.1.1 Micellar Copolymerization

In this process, initially reported by Evani (1984) and Turner et al. (1985), the hydrophobic monomer (styrene) is solubilized within the surfactant micelles, whereas acrylamide is dissolved together with the potassium per-sulphate initiator in the aqueous continuous medium (Figure 3.1). The surfactant used in this study was sodium dodecyl sulphate (SDS) at concentrations between four and twenty times its critical micelle concentration (cms). The reaction mixture is optically transparent. Below are outlined the major differences between this process and the more conventional polymerizations carried out in the presence of a surfactant, i.e. emulsion or microemulsion processes.

i) In an aqueous emulsion polymerization, the amount of surfactant is low with respect to that of the hydrophobic monomer. On the contrary, in the present micellar process, the surfactant over hydrophobe ratio is quite high (typically in the range of 15/1 to 70/1 by weight.)

ii) A direct emulsion (co)polymerization implies a low water solubility of the monomer, i.e. the monomers are essentially located in the dispersed phase (large monomer droplets and small micelles). The situation is quite different for the micellar copolymerization. Since the major part of the monomeric species, i.e. acrylamide is soluble in the aqueous continuous phase; the dihydrophobic monomer located within the micelles represents only a very small fraction of the total monomer feed (≈ 2 - 7% by weight).
Fig. 3.1 Micellar copolymerization [18].
iii) In the micellar process, the two monomers are segregated into two distinct phases due to their very different solubilities.

iv) In the micellar process, the polymerization reaction occurs in both the continuous phase and the dispersed phase. Therefore, although this process was called *micellar copolymerization*, it involves, in fact, a combination of a micellar polymerization and a solution polymerization.

### 3.1.2 Homogenous Copolymerization

The use of another solvent instead of water is the simplest way to overcome the problem of the insolubilities of the hydrophobic monomer in the aqueous medium. There are few common solvents for the two monomers, however, particularly formamide is used. In the choice of the organic (co)solvents, there is another constraint due to the possibility of chain transfer reactions leading to a lowering of molecular weight. The composition of the mixture was selected as 90% water and 10% formamide.

### 3.2 Preparation of Copolymers of Acrylamide-Styrene:

#### 3.2.1 Materials

Acrylamide was used as received from Flucka AG, Chemische Fabrik. Styrene from the same company was vacuum distilled prior to use (procedure outlined below). Sodium dodecyl sulfate (SDS) and Potassium persulfate were purchased from BDH Limited Pool, England. Formamide was purchased from Flucka AG, Chemische.
Deionized water was used exclusively for all aqueous solutions. Methanol was obtained from BDH Limited, Pool, England.

3.2.2 Methods

Distillation of Styrene:

First dissolve enough NaOH in styrene to neutralize the inhibitor which was present in styrene. Separation is done by separating funnel. Then distilled under vacuum. The sample withdrawn was purged with nitrogen gas and stored in the freezer.

3.2.1 Micellar Copolymerization: Polymer Synthesis

The experimental procedure used for the micellar process is as follows:

Reaction mixtures are provided in Table 3.1. Aqueous solutions of acrylamide were placed in 500 ml erlenmeyer flasks covered with septum caps and were degassed by gentle bubbling with nitrogen for 30 minutes while stirring. The sodium dodecyle sulphate was added followed by injection of styrene with a syringe into the reaction mixture and stirring was continued until a homogenous solution was obtained (within 0.5 - 1 hrs). Then the flask containing reaction mixture was placed into the temperature controlled oil bath at 50°C under magnetic stirring. Polymerization was initiated by the injection of potassium persulphate solution with a syringe. The reaction time is given in Table 3.1.

The polymers were precipitated by slowly pouring the solutions into a constantly stirred six times excess of methanol. After filtration, each polymer was washed
Table 3.1  Composition of the synthesized polymer

<table>
<thead>
<tr>
<th>Copolymer (Sample Code)</th>
<th>[Styrene]a</th>
<th>[Acrylamide]b</th>
<th>[Sodium Dodecyl Sulphate]c</th>
<th>[Potassium Persulfate]d</th>
<th>[Formamide]e</th>
<th>Reaction time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(AM/Sty)9</td>
<td>2.25</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>4.8</td>
</tr>
<tr>
<td>P(AM/Sty)52</td>
<td>2.25</td>
<td>5</td>
<td>2</td>
<td>8</td>
<td>-</td>
<td>1.5</td>
</tr>
<tr>
<td>P(AM/Sty)57</td>
<td>1.7</td>
<td>5</td>
<td>2</td>
<td>8</td>
<td>-</td>
<td>1.5</td>
</tr>
<tr>
<td>P(AM/Sty)56</td>
<td>1.12</td>
<td>5</td>
<td>2</td>
<td>8</td>
<td>-</td>
<td>1.5</td>
</tr>
<tr>
<td>P(AM/Sty)30</td>
<td>2.25</td>
<td>5</td>
<td>-</td>
<td>1</td>
<td>10</td>
<td>48</td>
</tr>
<tr>
<td>P(AM/Sty)77</td>
<td>1.12</td>
<td>5</td>
<td>-</td>
<td>1</td>
<td>10</td>
<td>1.5</td>
</tr>
</tbody>
</table>

a  molepercent in the feed = [Sty]/[Sty] + [AM]
b  Monomer concentration in wt%
c  Weight percent based on volume of water
d  Weight percent relative to monomer feed
e  Percent by volume of water
repeatedly in methanol to remove all traces of water, surfactant and residual monomer before filtering it was dried under vacuum at 50°C for 2 days. Then the polymer was crushed and dried again for few hours.

3.2.2 Homogenous Copolymerization: Polymer Synthesis

The procedure for this method of synthesis, was analogous to that described in the previous section. In the homogenous process, it was important to dissolve first styrene monomer in formamide before adding the aqueous acrylamide solution.

3.3 Polymer Characterization

3.3.1 Elemental Analysis

Elemental analysis to determine carbon, hydrogen and nitrogen content of the copolymers was conducted at the central analytical laboratories of K.F.U.P.M. Research Institute. The analytical data obtained are given below:
Table 3.2: Elemental analysis of polymers

<table>
<thead>
<tr>
<th>Copolymers</th>
<th>%C</th>
<th>%H</th>
<th>%N</th>
<th>%S</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(AM/Sty)9</td>
<td>49.3</td>
<td>7.8</td>
<td>18.4</td>
<td>ND</td>
</tr>
<tr>
<td>P(AM/Sty)52</td>
<td>49.9</td>
<td>7.8</td>
<td>18.1</td>
<td>ND</td>
</tr>
<tr>
<td>P(AM/Sty)56</td>
<td>49.8</td>
<td>7.7</td>
<td>18.1</td>
<td>ND</td>
</tr>
<tr>
<td>P(AM/Sty)77</td>
<td>48.9</td>
<td>7.6</td>
<td>18.2</td>
<td>ND</td>
</tr>
<tr>
<td>P(AM/Sty)87</td>
<td>43.8</td>
<td>6.9</td>
<td>17.4</td>
<td>ND</td>
</tr>
</tbody>
</table>
3.3.2 Molecular Weight of the Polymers

The intrinsic viscosities $[\eta]$ of the copolymers were determined in aqueous solutions at 30°C. Measurements were carried out using $K = 0.005989$ with an Ostwald’s Viscometer at 1.0, 0.5, 0.25 and 0.125 weight %. In these experiments, care must be taken to avoid formation of the copolymer solutions and the solution should be properly filtered, which would otherwise result in erroneous flow time. Each reading is taken three times and the average value is used for the calculation of molecular weight of the copolymers.

Polymer solutions were prepared by dissolving the sample in pure water at room temperature at appropriate weight/weight concentration. Prior to measurements, the solutions were kept for one more day to eliminate air bubbles.

The apparent molecular weight of the copolymers are estimated from the following relationship:

$$\frac{[\eta]}{100} \, cm^3 / g = 6.8 \times 10^{-4} \, M_w^{0.66} \quad (3.1)$$

assuming the copolymers behave similar to the homopolymer, polyacrylamide.

3.3.2.1 Calculation of Molecular Weight

Sample: P(AM/Sty)56 Block PAS

Since, $[\eta] = 6.8 \times 10^{-4} \, M_w^{0.66}$
from graph 1, $[\eta] = 1.74$

Hence, $M_w = 1.456 \times 10^5$

Sample: P(AM/Sty)77 Random PAS

Since, $[\eta] = 6.8 \times 10^{-4} M_w^{0.66}$
from graph 1, $[\eta] = 1.78$

Hence, $M_w = 1.507 \times 10^5$

Sample: P(AM/Sty)9 Block PAS

Since, $[\eta] = 6.8 \times 10^{-4} M_w^{0.66}$
from graph 1, $[\eta] = 2.0$

Hence, $M_w = 1.798 \times 10^5$
Table 3.3  Intrinsic viscosities and molecular weights of the polymers at 30°C

t₀ = Time flow of solvent, water = 137.68 sec

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>% w/v</th>
<th>time (avg) sec</th>
<th>( \eta_{sp} = \frac{(t - t₀)}{t₀} )</th>
<th>( \eta_{red} = \frac{\eta_{sp}}{\text{Con}} )</th>
<th>Intrinsic Viscosity</th>
<th>Apparent Mol. Wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(AM/Sty)56</td>
<td>1.0</td>
<td>536.3</td>
<td>2.90</td>
<td>2.90</td>
<td>1.74</td>
<td>1.456 x 10</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>294.23</td>
<td>1.14</td>
<td>2.28</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.237</td>
<td>203.51</td>
<td>0.478</td>
<td>2.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>170.16</td>
<td>0.235</td>
<td>1.88</td>
<td></td>
<td></td>
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<tr>
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</table>
3.4 Results and Discussion

Two different methods were used for the synthesis of the acrylamide styrene copolymer. In micellar copolymerization, hydrophobic monomer (styrene) was incorporated into the main chain of acrylamide in the form of blocks. Attempts were made to vary the amount of styrene block copolymers as shown in Table 3.1. It was found that by introducing more than 1.7 mole percent of styrene, the resulted block copolymer became turbid in aqueous solutions which in turn causes problem for the determination of phase diagram. Block copolymer showed foaming characteristics when dissolved in water which supports the blocky nature of the polymer.

Homogeneous copolymerization was used for the synthesis of random acrylamide styrene copolymers.

Attempts were made to reduce the molecular weight as shown in Table 3.1. It was found that by increasing the amount of initiator, (potassium per sulfate) from 1 weight percent relative to the monomer to 8 weight percent relative to monomer feed, the reduction in molecular weight was not significant as shown in Table 3.3 and Figs. 3.2-3.4 in agreement with the free radical polymerization rate equations that predict \( M_w \propto 1/\sqrt{I} \), where I is the initiator concentration. However, chain transfer agent can be employed for reduction of molecular weight of the polyacrylamide styrene. Apparent molecular weights were determined by intrinsic viscosities of the polymers. After the synthesis of acrylamide-styrene copolymers, the study of phase behavior of PAS with PEG was carried out as explained in chapter 4.
Figure 3.2. Intrinsic Viscosity of Polyacrylamidestyrene (Block) at 30°C.

Intrinsic Viscosity = 1.74
Figure 3.3. Intrinsic Viscosity of Polyacrylamide-styrene (Random) at 30 c
Figure 3.4. Intrinsic Viscosity of High Molecular Weight PAS (Block) at 30 °C.
Chapter 4

Phase Behavior of Polymers

4.1 Introduction

The study of phase diagram is an important step for this work. The binodal curve distinguishes between single and two phase regions and provides information about the concentration of both the polymers required for phase separation, which in turn helps determine whether the system is economical or not. It also shows the effect of molecular weight on phase separation. In addition, it is helpful in determining the top and bottom phase concentrations of both polymers. Phase diagrams may be constructed in many different ways; only the ones relevant to this study will be described here.

In a mixture of two polymers and water, a two phase system will only arise when the constituents are present in a certain range of proportions. The constituents compositions at which phase separation occurs may be represented in a phase diagram. Figures 4.1 and 4.2 show such a diagram for a system of polymer P polymer Q solvent. In figure 4.1 the concentration of polymer P is plotted as the abscissa and the concentration of polymer Q as the ordinate. The concentrations are expressed as weight per cent. The curve separating the two areas is called a binodial. All mixtures which have compositions represented by points above the line give rise to phase separation, while mixtures represented by points below the lines do not. Thus a
composition represented by point A in Figure 4.1 gives a two-phase system, while the composition represented by point D gives a homogeneous solution.

In order to describe the two-phase system in more detail, one has also to account for the compositions of the two phases which are in equilibrium. Suppose that point A in figure (4.2) represents the composition of the total system (percentages of polymers P and Q per total weight mixture). The compositions of the bottom and top phases obtained with this system will, then, be represented by points B and C respectively. In the same way, the system with the total composition of A' will have a bottom phase composition of B' and top composition of C'. Like all other points representing the composition of pure phases, points C, B, C', and B' lie on the binodial. Pairs of points like B and C are called nodes and the lines joining them are called tie lines. Point A, representing the total composition, lies on the tie line joining B and C. Any total composition represented by points on the same tie line will give rise to phase systems with the same phase compositions, but with different volumes of the two phases. If composition is expressed in per cent weight per weight (w/w), the weight ratio of bottom phase/top phase is equal to the ratio between the lines AC and AB.

4.1.1 The Binodial Curve

A binodal may be determined experimentally using the turbidity method. The procedure are:

1. A few grams of a concentrated solution of one polymer (P) are put into a test tube. A solution of known concentration of the other polymer (Q) is then added dropwise to the test tube. First a homogeneous mixture is obtained. After a certain amount of polymer Q has been added, one further drop will cause turbidity and, thus, a two-phase system will arise. The
Fig. 4.1 By mixing two polymers, P and Q in water, phase separation occurs above concentrations of the two polymers. Thus, mixtures represented by points above the curved line, such as point A, give two liquid phases, while mixtures represented by points below the curved line, such as point D, give one liquid phase. The curved line is called a binodial. [3]
Fig. 4.2 The lines, for example the lines BC or B'C', connect the points representing the composition of two phases in equilibrium. Thus, point B represents the composition of the bottom phase, and point C the composition of the top phase of a system with a total composition represented by point A. K is the critical point. [3]
composition of this mixture is noted. Upon addition of one gram of water the mixture becomes clear again. More solution of polymer Q is then added dropwise until turbidity and two-phase system is again obtained. The composition of this mixture is noted and more water is then added to a one phase system and so on. In this way a series of compositions, close to the binodial are obtained and if the concentration of polymer P is plotted against that of Q for these compositions, a line as that in Figure 4.1 is obtained.

2. The compositions of the phases of a number of different systems are determined and a curve. Then the binodial, is drawn through the points representing these compositions.

4.2 Experimental Method

Random and block copolymers of acrylamide and styrene were prepared by the methods previously discussed. Molecular weights of both random and block polymers were determined through intrinsic viscosities. Polyethylene Glycol (PEG) with average molecular weight 35000 and 10000 were obtained from Merck-Schuchardt.

The samples were prepared with concentration varying from 20% PEG (35000) and PAS (both random and block) 2% to 5% PEG (35000) and 10% PAS. Similarly 30% PEG (10000) and 2% PAS to 5% PEG (10000) and 10% PAS.

After each sample was prepared, the PEG solution and deionized water were taken in to two different 10 ml burrettes. Known quantity of PAS was taken to a flask and PEG solution was added dropwise until the transparent system turned turbid. Then water is added dropwise until the system becomes transparent again. At this point, the
final composition of the two polymers calculated corresponds to one point on the binodal curve. After obtaining the first point, concentrated solution of PEG was added again to obtain turbid dispersion and dilution with water was repeated to obtain a second point on the binodal curve. This procedure was continued until sufficient number of points for the construction of the binodal curve was obtained. The experiments were carried out at room temperature.
4.2.1 Binodals of the Ammonium sulfate & Polyethylene Glycol System at 20°C

Ammonium sulfate/polyethylene glycol/water system was studied and the data was matched with the data available in the literature (Albertsson, 1971) for the confirmation of the experimental procedure for phase diagram study.

Slight variation in the experimental data was due to experimental temperature difference. The results are presented in Table 4.0

Table 4.0 Comparision of Experimental data with data from Literature.

<table>
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<tr>
<th>No. of Observations</th>
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<th>Data of Albertsson at 20°C</th>
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<td>PEG % w/w</td>
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</table>
4.3 Correlation of Polymer Partitioning in Aqueous Two-Phase Systems

In order to facilitate the use of aqueous two phase systems and provide a basis for selecting a system for biomolecule purification, a sound thermodynamic theory is needed for the prediction and correlation of phase equilibrium behaviour. Several theoretical models have been proposed for the thermodynamic behaviour of aqueous two-phase systems and protein partitioning. Brooks et al. (1985) and Albertsson et al. (1986) have shown that the lattice model of Flory (1942) and Huggins (1942) could be used to qualitatively predict protein partitioning trends.

The Flory-Huggins theory, though simplistic in nature, is the classical approach for describing the thermodynamics of phase separation in polymer systems. According to this theory, the polymers are linear (monodisperse), long chained random coils, while the solvent is monomeric. The geometry of the polymer and solvent are considered essentially identical. The polymer and solvent molecules exist in the form of a single lattice scheme, each cell of which may be occupied by either a solvent molecule or the segment of a linear polymer. The entropy change of mixing is a combinatorial term reflecting the variety of ways of arranging the polymers and solvent in the lattice, while the heat of mixing represents the energy change associated with the formation of contacts between unlike neighbour in the lattice.

In order to apply Flory-Huggins theory of polymer solution thermodynamics to aqueous two-phase systems, several fundamental assumptions must be made. These include 1) The liquid lattice model provides an adequate representation of an aqueous solution, 2) An ideal entropy of mixing is suitable, 3) Deviations from ideal solution behaviour can be simply accounted for in terms of (enthalpic) solute-solvent and
solute-solute interaction parameters, and 4) The polymer chains are fully flexible.

Inherent in the first three assumptions is that molecular interactions are almost exclusively of the van der Waals type. However, aqueous two-phase systems will undoubtedly possess, among others, hydrogen bonding and some ionic interactions.

### 4.3.1 Theoretical Development of the Partition Expression

The Gibbs free energy of mixing at constant temperature and pressure for a multicomponent system can be expressed as,

\[
\Delta G_m = \left[ \sum_{i<j} n_i v_j \chi_{ij} + \sum n_i \ln v_i \right]
\] (4.1)

The chemical potential for species i can be calculated from the partial differential:

\[
\mu_i - \mu_i^o = N_A \left[ \frac{\partial \Delta G_m}{\partial n_i} \right]_{n_j, T, P}
\] (4.2)

where \(\mu_i\) is the chemical potential of species i, \(\mu_i^o\) is the chemical potential in the standard state taken as pure component species at the temperature and pressure of the mixture, \(N_A\) is Avogadro's number; \(T\) is the absolute temperature and \(P\) refers to pressure. At equilibrium, the following conditions must be satisfied,

\[
\mu_i' = \mu_i''
\] (4.3)

where single and double prime superscripts refer to the bottom and top phases, respectively.
\[ N_A \left[ \frac{\partial \Delta G_m}{\partial n_i} \right]_{n_j, T, P} = N_A \left[ \frac{\partial \Delta G_m^*}{\partial n_i} \right]_{n_j, T, P} \quad (4.4) \]

For a three component system, composed of water (o), polymer (1) and Polymer (2), the Gibbs free energy of mixing based on Eq. (4.1) can be expressed as:

\[
\Delta G_m = kT(n_o v_1 \chi_{o1} + n_o v_2 \chi_{o2} + n_i v_2 \chi_{12} + n_o \ln v_o
+ n_i \ln v_1 + n_2 \ln v_2) \quad (4.5)
\]

The subscripts 1 and 2 refer to PEG and PAS respectively, for the PEG/PAS/water systems. It should be noted in this system, PAS is enriched in the bottom phase and PEG in the top phase. For this reason, PAS is given the subscript 2 in the PEG/PAS/water system. With the substitution of \( \Delta G_m \) into Eq. (4.4), a series of three equations is obtained, one for each component. For polymers (1) and (2), the result of the above substitution will yield, upon rearrangement, the following expression for the partition coefficients, \( K_1 \) and \( K_2 \).

\[
\ln(K_1) = m_1 (v_2^* - v_o^*) + (v_1^* - v_1) + \frac{m_1}{m_2} (v_2^* - v_2^*) - \chi_{1o} (v_o^{*2} - v_o^*)
+ (-\chi_{1o} + m_1 \chi_{o2} - \chi_{12}) (v_o^* v_2^* - v_o v_2) - \chi_{12} (v_2^{*2} - v_2^2) \quad (4.6)
\]

and

\[
\ln(K_2) = m_2 (v_o^* - v_o^*) + (v_2^* - v_2) + \frac{m_1}{m_2} (v_1^* - v_1) - \chi_{2o} (v_o^{*2} - v_o^*)
+ (-\chi_{2o} + m_2 \chi_{o1} - \chi_{12}) (v_o v_1^* - v_o v_1) - \chi_{12} (v_1^{*2} - v_1^2) \quad (4.7)
\]
The following simplifying assumptions are made (Diamond and Hsu, 1990): 1) A proportionality factor, $\alpha_i$, exists between the volume and weight fraction difference between the phases for species $i$, 2) The parameter, $\phi$ which is defined as,

$$\phi = \frac{w_2 - w_1'}{w_1 - w_1'}$$  \hspace{1cm} (4.8)$$

has been shown to be constant for aqueous two-phase systems and can be introduced into the partition expression, and 3) Second order terms containing $V_1$ and $V_2$ can be neglected. This assumption is based on the fact that polymer concentrations (i.e., species 1 and 2) are typically low, on the order of 0.5 to 15.0% (w/w), in comparison with water which typically has a concentration between 85.0% and 99.0% (w/w).

With these assumptions, equations (4.6) and (4.7) become,

$$\ln(K_1) = \alpha_1 \left( \frac{1}{m_1} - 1 + 2\chi_{o1} \right) + \alpha_2 \phi \left( \frac{1}{m_2} - 1 + \chi_{o1} + \chi_{o2} - \chi_{i2} \right) \left( w_2' - w_1' \right)$$  \hspace{1cm} (4.9)$$

and

$$\ln(K_2) = \alpha_2 \phi \left( \frac{1}{m_2} - 1 + 2\chi_{o2} \right) + \alpha_1 \left( \frac{1}{m_1} - 1 + \chi_{o2} + \chi_{o1} - \chi_{i2} \right) \left( w_1'' - w_1' \right)$$  \hspace{1cm} (4.10)$$

The above linear semilogarithmic relationships can be simplified by defining their slopes as follows,

$$A_i = \alpha_1 \left( \frac{1}{m_1} - 1 + 2\chi_{o1} \right) + \alpha_2 \phi \left( \frac{1}{m_2} - 1 + \chi_{o1} + \chi_{o2} - \chi_{i2} \right)$$  \hspace{1cm} (4.11)$$
and

\[ A_2 = m_2 \left[ \alpha_2 \phi \left( \frac{1}{m_2} - 1 + 2\chi_{o2} \right) + \alpha_1 \frac{1}{m_1} - 1 + \chi_{o2} + \chi_{o1} - \chi_{12} \right] \]  \hfill (4.12)

Equations (4.9) and (4.10) then become,

\[ \ln (K_1) = A_1 \left( w_1^* - w_1' \right) \]  \hfill (4.13)

and

\[ \ln (K_2) = A_2 \left( w_1^* - w_1' \right) \]  \hfill (4.14)

where \( A_1 \) and \( A_2 \) are a function of the polymer molecular weights and the interactions between the polymers and water. Equations (4.13) and (4.14) provide a means for correlating phase equilibrium data for aqueous polymer systems.

### 4.4 Results and Discussion

The binodal curve for PAS/PEG/water system is determined using a relatively simple continuous dilution method. The curve show typical behavior of such systems. The copolymer concentration is low.

It is also found that by changing the molecular weight of one of the two polymers, the binodal curve shifts either downwards or upwards depending upon the increase or decrease in molecular weight respectively. In this work, two different molecular weights of PEG were used. It was found that with PEG- 35000, the binodal curve shifts downward compared with PEG-10000 as shown in figures 4.3 and 4.4. This
implied that small concentration of PEG-35000 is required for phase separation compared with PEG-10000 in PAS/PEG/Water system. Another interesting finding was that both block and random copolymers of acrylamide styrene, with same molecular weight, exhibit almost identical phase diagram with PEG-35000 as shown in Figs. 4.3 and 4.5 and Tables 4.1 and 4.3. The reason is that polymer structure has little effect on phase behaviour.

To check the consistency of the binodal curve PAS/PEG/water system, correlation developed by Diamond and Hsu (1990) based on the Flory-Huggins theory, is employed. These results are shown in Figures 4.6-4.8 and Tables 4.4 - 4.6. In Figure 4.6 and 4.7, the effect of PEG molecular weight on phase separation is presented. These figures revealed that the slope of PAS becomes more negative as PEG molecular weight increases from 10000 to 35000 where slope is a function of polymer molecular weight and the interaction between the polymers and water.

From the results discussed above, it is concluded that both PAS(Block)/PEG(35000)/water and PAS(Random)/PEG (35000)/water systems showed satisfactory phase separation characteristics. Therefore, these systems are used for protein partitioning studies as explained in Chapter 5.
Table: 4.1  Phase behaviour of PEG 35000 and PAS (Block) at 23°C.

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<th>Observation No.</th>
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<th>PEG, % w/w</th>
<th>Water, % w/w</th>
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Table 4.2  Phase Behaviour of PEG 10000 and PAS (Block) at 23°C.

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Table: 4.3  Phase Behaviour of PEG 35000 and PAS (Random) at 23°C.

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<td>95.3</td>
</tr>
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<td>2.09</td>
<td>2.19</td>
<td>95.76</td>
</tr>
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<td>13</td>
<td>1.54</td>
<td>2.3</td>
<td>96.16</td>
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<td>14</td>
<td>0.99</td>
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<td>3.62</td>
<td>96.35</td>
</tr>
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<td>18</td>
<td>0.016</td>
<td>4.27</td>
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<tr>
<td>19</td>
<td>0.009</td>
<td>5.19</td>
<td>94.80</td>
</tr>
</tbody>
</table>
Table 4.4  Phase diagram data of PEG-10000/PAS (Block)/water at 23°C.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Top phase % w/w</th>
<th>Bottom phase % w/w</th>
<th>Partition coefficient $K = C_t / C_b$</th>
<th>ln K</th>
<th>Difference in weight frac. $((w_i - w_j) \times 100)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAS (Block)</td>
<td>0.001</td>
<td>0.08</td>
<td>0.0125</td>
<td>-4.38</td>
<td>7.98</td>
</tr>
<tr>
<td>PEG 10000</td>
<td>0.0456</td>
<td>0.0093</td>
<td>4.903</td>
<td>1.59</td>
<td>3.63</td>
</tr>
<tr>
<td>PAS (Block)</td>
<td>0.0019</td>
<td>0.07</td>
<td>0.027</td>
<td>-3.60</td>
<td>6.82</td>
</tr>
<tr>
<td>PEG 10000</td>
<td>0.041</td>
<td>0.011</td>
<td>3.727</td>
<td>1.31</td>
<td>3.00</td>
</tr>
<tr>
<td>PAS (Block)</td>
<td>0.0059</td>
<td>0.05</td>
<td>0.118</td>
<td>-2.14</td>
<td>4.41</td>
</tr>
<tr>
<td>PEG 10000</td>
<td>0.037</td>
<td>0.0146</td>
<td>2.53</td>
<td>0.93</td>
<td>2.24</td>
</tr>
<tr>
<td>PAS (Block)</td>
<td>0.0118</td>
<td>0.04</td>
<td>0.295</td>
<td>-1.2</td>
<td>2.81</td>
</tr>
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<td>PEG 10000</td>
<td>0.0333</td>
<td>0.018</td>
<td>1.85</td>
<td>0.615</td>
<td>1.53</td>
</tr>
</tbody>
</table>
### Table 4.5  Phase diagram data of PEG-35000/PAS (Block)/water at 23°C.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Top phase % w/w</th>
<th>Bottom phase % w/w</th>
<th>Partition Coefficient $K = C_r / C_h$</th>
<th>In K</th>
<th>Difference in weight frac. $(w_r - w_h) \times 100$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAS (Block)</td>
<td>0.00014</td>
<td>0.08</td>
<td>0.00175</td>
<td>-6.35</td>
<td>7.98</td>
</tr>
<tr>
<td>PEG 35000</td>
<td>0.0425</td>
<td>0.013</td>
<td>4.126</td>
<td>1.417</td>
<td>3.22</td>
</tr>
<tr>
<td>PAS (Block)</td>
<td>0.00028</td>
<td>0.07</td>
<td>0.004</td>
<td>-5.52</td>
<td>6.97</td>
</tr>
<tr>
<td>PEG 35000</td>
<td>0.036</td>
<td>0.0113</td>
<td>3.18</td>
<td>1.158</td>
<td>2.47</td>
</tr>
<tr>
<td>PAS (Block)</td>
<td>0.0006</td>
<td>0.06</td>
<td>0.01</td>
<td>-4.60</td>
<td>5.94</td>
</tr>
<tr>
<td>PEG 35000</td>
<td>0.0319</td>
<td>0.0198</td>
<td>1.611</td>
<td>0.476</td>
<td>1.21</td>
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<tr>
<td>PAS (Block)</td>
<td>0.0024</td>
<td>0.03</td>
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<td>-2.51</td>
<td>2.76</td>
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<tr>
<td>PEG 35000</td>
<td>0.029</td>
<td>0.02</td>
<td>1.45</td>
<td>0.371</td>
<td>0.9</td>
</tr>
</tbody>
</table>
Table 4.6  Phase diagram data of PEG 35000/PAS (Random)/water at 23°C.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Top phase concentration % w/w</th>
<th>Bottom phase concentration % w/w</th>
<th>Partition Coefficient $K = C_t / C_b$</th>
<th>$\ln K$</th>
<th>Difference in weight frac. $\left(\frac{(w_i^t - w_i^b) \times 100}{w_i^t}\right)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAS (Block)</td>
<td>0.00016</td>
<td>0.08</td>
<td>0.002</td>
<td>-6.21</td>
<td>7.98</td>
</tr>
<tr>
<td>PEG 35000</td>
<td>0.0427</td>
<td>0.0101</td>
<td>4.227</td>
<td>1.44</td>
<td>3.26</td>
</tr>
<tr>
<td>PAS (Block)</td>
<td>0.0003</td>
<td>0.07</td>
<td>0.00428</td>
<td>-5.45</td>
<td>6.97</td>
</tr>
<tr>
<td>PEG 35000</td>
<td>0.0362</td>
<td>0.0117</td>
<td>3.094</td>
<td>1.13</td>
<td>2.46</td>
</tr>
<tr>
<td>PAS (Block)</td>
<td>0.00062</td>
<td>0.06</td>
<td>0.0103</td>
<td>-4.57</td>
<td>5.93</td>
</tr>
<tr>
<td>PEG 35000</td>
<td>0.0318</td>
<td>0.0195</td>
<td>1.630</td>
<td>0.474</td>
<td>1.20</td>
</tr>
<tr>
<td>PAS (Block)</td>
<td>0.0026</td>
<td>0.03</td>
<td>0.086</td>
<td>-2.44</td>
<td>2.75</td>
</tr>
<tr>
<td>PEG 35000</td>
<td>0.029</td>
<td>0.0198</td>
<td>1.464</td>
<td>0.381</td>
<td>0.92</td>
</tr>
</tbody>
</table>
Figure 4.3. Phase Diagram of PEG (35000)/PAS (Block)/water system
Figure 4.4. Phase Diagram of $\text{PEC (100000) / PAS (Block)}$ water system.
Figure 4.5. Phase Diagram of PEG (35000)/PAS (Random)/water system
Figure 4.6. Phase Diagram: Correlation of PEG 35000 / PAS (Block) / Water system
Figure 4.7. Phase Diagram Correlation of PEG(10000) / PAS (Block) / Watersystem
Figure 4.8. Phase Diagram Correlation of PEG(35000) / PAS (Random) / Water system
Fig. 4.9 Correlation of PEG/Dextran Water Phase Diagram Data at 22°C [16].
Chapter 5

Protein Partitioning

5.1 Introduction

Following twenty years of rapid progress in molecular biology, chemical engineering is now addressing many important and challenging issues in biotechnology at the process-scale. For example, bioprocess purification and protein refolding are two challenging areas where there is a strong need for the development of innovative and efficient large-scale processes. One approach to the purification of biomolecules which has received considerable attention is the extraction of proteins in two-phase aqueous polymer systems [(Albertsson 1986); Walter and Anderson, (1981); Baskir et al. (1989); Abbott and Hatton (1988); Abbott et al (1993)]. In particular, these systems appear attractive because of the large scale and continuous operability of the process. Other merits include the friendly aqueous environment which is provided for the labile protein products and the fast phase equilibration. On the other hand, a significant disadvantage of the process is the difficulty of protein recovery and polymer recycling. To solve these problems, it is likely that the design and synthesis of new molecules to mediate and improve protein separation and purification will be necessary. Synthetic polymers and surfactants with varying chemical compositions and chain lengths and different affinity ligands are some examples of areas where much progress has been made.
Interest in the partitioning of proteins in two-phase aqueous polymer systems stems from the unique ability of these polymer solutions to provide water-based, yet immiscible, liquid phases for the purification of proteins using liquid-liquid extraction techniques. Because each of the two coexisting phases contains predominantly water, water-soluble proteins maintain their native conformations and biological activity when purified in these systems. In general, it is well known that the partitioning of proteins in two-phase aqueous polymer systems reflects a large number of factors, such as polymer concentration and molecular weight, pH, salt type and concentration. These are often coupled. Indeed, the coupling of these factors has elucidated the underlying molecular-level mechanisms of protein partitioning which is a formidable task (Abbot and Hatton, 1993).

Liquid-liquid extraction offers an attractive alternative to traditional methods for the separation of biomaterials since it may be operated continuously (rather than in batch mode) and may be scaled up relatively easily. One promising extraction method is the two-phase aqueous polymer separation technique, first introduced by Albertsson in the late 1950s. In this technique, aqueous solutions of water-soluble polymers, such as poly(ethylene oxide) (PEO) and dextran are mixed together to produce a thermodynamically stable, two-phase system. The concentration of each "phase-forming" polymer can be as low as 5% w/w or less. In such systems both phases are primarily aqueous with the remainder of each phase being composed primarily of one of the phase forming polymers. Since both phases of the two-phase aqueous polymer system are primarily aqueous, either phase provides a mild, protective environment for biomaterial particles. In addition, the interfacial tension between the phases is usually small, typically 0.0001 to 0.1 dyn/cm², so that minimal stress is imposed on particles as they pass through the interfacial region during separation. Consequently,
one can achieve purification of enzymes, for example, with very little loss of the enzyme activity (Albertsson, 1971, Kula et al., 1982).

5.2 Materials and Experimental Methods

Materials

Polyethylene glycol (PEG) with a molecular weight of 35000 of Merck-Schuchardt was used. Polyacrylamide styrene (PAS), both random and block, having same molecular weights were synthesized. Serum albumin was purchased from BDH Limited, Pool, England. Sodium phosphate was purchased from J.T. Baker Chemical Co.

Methods

All two-phase systems contained PEG, PAS and water. A solution of 0.01 M sodium phosphate was used to control pH at 7.0. All polymer solutions were prepared by weight. All two phase systems were prepared in duplicate for both random and block PAS. Into one of the resulting two-phase systems, known quantity of protein solution was added. The protein free two-phase system served as the reference solution in the spectrophotometer for the measurement of protein concentration. The resulting polymer solutions were gently centrifuged for 15 mins and then equilibrated for 48 hrs at 23°C. In order to determine the concentrations of proteins in each of the coexisting phases, samples from each phases of the solution were separated using a syringe or small pipet. First, without disturbing the fragile liquid-liquid interface between the two phases, a sample of the top PEG-rich solution phase was carefully collected. Following the collection of the top phase sample, the remainder of the top
phase was sucked from the interfacial region using a pasteur pipet. The interfacial sample, which typically contained a mixture of top and bottom phases, was then discarded. The remaining solution, namely the bottom PAS rich phase, was withdrawn and then prepared for the measurement of protein concentration as follows.

Due to high viscosities of the polymer solutions which were withdrawn from each of the phases, it was necessary to dilute the samples prior to the measurement of the protein absorbance. If the samples were not diluted, streaks appeared in the polymer solutions as they were pipetted into spectrophotometric cuvettes, which in turn scattered light during the measurement of the protein absorbance. The absorbance of bovine serum albumin was measured at 282 nm using a Perkin-Elmer Lambda 5, UV/VIS spectrophotometer, utilizing the corresponding protein free two-phase system as a reference.

5.3 Effects of pH on Protein Partitioning

When two aqueous solutions of incompatible polymers such as polyethylene glycol (PEG) and polyacrylamide styrene (PAS) are mixed above critical concentrations, a liquid-liquid phase separation occurs. Proteins or enzymes added to the resulting two-phase mixture tend to partition unequally between the two phases or between the phases and the interface, thus allowing for the extraction of a particular protein. The protein partition coefficient, $K_p$, defined as the ratio between the concentration of protein in the top and the bottom phases depends, among other factors, on the pH of the system. Hence an investigation was made to determine the effect of pH on protein partitioning.
Materials

The materials were the same as described in section 5.2 except that 0.1 M HCl purchased from J.T. Baker Chemical Co. was used.

Method

The system was prepared exactly the same way as described in section 5.2 except that the amount of buffer was varied in order to adjust pH. The buffer used was 0.1 M HCl. The pH was measured with a microelectrode. The rest of the procedure for protein concentration measurement is the same as discussed in section 5.2.

5.4 Correlation for Protein Partitioning

Several theoretical models have been proposed for the thermodynamic behaviour of aqueous two-phase systems and protein partitioning. Brooks et al. (1985) and Albertsson et al. (1987) have shown that the lattice model of Flory (1941) and Huggins (1941) could be used to qualitatively predict protein partition trends.

Brooks et al. (1985) were the first to suggest the use of Flory-Huggins theory to describe protein partitioning in aqueous two-phase systems. The relationship they presented gave a successful qualitative description of protein partitioning, but was not applied quantitatively. In order to apply Flory-Huggins theory of polymer solution thermodynamics to aqueous two-phase systems, several fundamental assumptions must be made including:

1) The liquid lattice model provides an adequate representation of an aqueous solution.
2) An ideal entropy of mixing is suitable.

3) Deviations from ideal solution behaviour can simply be accounted for in terms of (enthalpic) solute-solvent and solute-solute interaction parameters.

4) The polymer chains are fully flexible.

Inherent in the first three assumptions is that molecular interactions are almost exclusively of the van der Waals type. However, aqueous two-phase systems will undoubtedly possess, among others, hydrogen bonding and some ionic interactions. In order to help alleviate some of the problems associated with the first three assumptions, we utilize the non-ionic phase forming polymers. The fourth assumption presents a limitation to the description of protein structure. Many proteins tend to be globular in nature with their structures being held together by intrachain covalent bonding, hydrogen bonding, ionic interactions, and hydrophobic interactions (Lehninger, 1982). Proteins, however, are not rigid spheres and their chains still possess some degree of flexibility. Despite the above assumptions, it will be shown that the Flory-Huggins theory can be modified and used as a simple approach to describe the partition phenomena in aqueous two-phase systems.

The chemical potential for species $i$ in an aqueous two phase polymer system may be expressed as (Albertsson, 1986),

$$\mu_i - \mu_i^o = N_A \left( \frac{\partial \Delta G_m}{\partial n_i} \right)_{n_j, T, P} + z_i F \Psi$$  \hspace{1cm} (5.1)

where

$\mu_i =$ the chemical potential of species $i$
\[ \mu_i^o = \text{the chemical potential in the standard state taken as pure component species at}
\]
\[ \text{the temperature and pressure of the mixture} \]
\[ N_A = \text{Avogadro's number} \]
\[ T = \text{the absolute temperature and} \]
\[ P = \text{pressure.} \]
\[ z_b = \text{net charge of species} \]
\[ F = \text{Faraday constant} \]
\[ \psi = \text{electrostatic potential} \]

Many researchers (Johansson, 1974; Zaslavsky et al., 1982; Brooks et al. 1984; King et al. 1988; Albertsson, 1986) have found that the addition of salts to aqueous two-phase systems induces an electrostatic potential difference between the phases. For this reason, the electrostatic potential term is present in the chemical potential expression. Flory's (1953) expression for the Gibbs free energy of mixing in a multicomponent polymer solution can be inserted into Eq. (5.1), which can then be substituted into the equilibrium relations:

\[ \mu_i^* = \mu_i^\prime \]

(4.2)

If the following simplifying assumptions are made 1) the biomolecule concentration is small relative to that of the polymers and water 2) A proportionality factor, \( \alpha_i \), exists between the volume and weight fraction difference between the phases for species I, and 3) the parameter, \( \phi \) which is defined as,

\[ \phi = \frac{(w_2^* - w_2^\prime)}{(w_1^* - w_1^\prime)} \]

(5.2)
has been shown to be constant for aqueous two-phase systems and can be introduced into the partition expression, one can obtain an expression for the partition coefficient of a biomolecule (component 3) in the system composed of water (component 0), PEG (component 1) and PAS (component 2). The partition coefficient expression is,

\[
\ln K_1 = m_3 \left\{ \frac{\alpha_1}{m_1} + \frac{\alpha_2}{m_2} \phi \left( \chi_{03} - 1 \right) - \alpha_1 \chi_{13} 
- \alpha_2 \phi \chi_{23} + \alpha_1 \chi_{01} + \alpha_2 \phi \chi_{02} \right\} (w_i^\ast - w_i^1) 
+ (\chi_{03} \alpha_2^2 \phi^2 - \chi_{01} \alpha_1^2) (w_i^\ast - w_i^1)^2 
- 2 \chi_{01} \alpha_1^2 w_i^1 (w_i^\ast - w_i^1) - 2 \chi_{02} \alpha_2^2 w_i^2 (w_i^\ast - w_i^1) + \\
(\chi_{12} - \chi_{01} - \chi_{02}) \alpha_1 \alpha_2 (w_i^\ast w_i^2 - w_i^1 w_i^2) \right\} + \frac{z_b F \Delta \psi}{RT} 
\]

(5.3)

where

\[
K = \frac{w_3^\ast}{w_3} \quad \text{with} \quad w_i \text{ the weight fraction of species } i,
\]

\[
m_i = \text{molar volume ratio of species } i \text{ to that of water}
\]

\[
R = \text{gas law constant}
\]

\[
\chi_{ij} = \text{Flory-Huggins interaction parameters for species } i \text{ and } j
\]

\[
\Delta \psi = \text{electrostatic potential difference between the phases}
\]

After further simplifying Eq. 5.3, one get,

\[
\ln K = (A^* + \frac{z_b F g}{RT})(w_i^\ast - w_i^1) + (b + \frac{z_b F h}{RT})(w_i^\ast - w_i^1)^2 
\]

(5.4)

At a constant pH, temperature and pressure, the charge \( z_b \) of the biomolecule will be constant and the terms, \( \frac{z_b F g}{RT} \) and \( \frac{z_b F h}{RT} \) may be incorporated with \( A^* \) and \( b \), respectively. The following relationship is then obtained when Eq. (5.4) is divided by \(( w_i^\ast - w_i^1) \):
\[
\ln K = A^{**} + b^* (w_i^* - w_i^1) \quad (5.5)
\]

where
\[
A^{**} = A^* + \frac{z_b F_g}{RT} \quad (5.6)
\]

and
\[
b^{**} = b^* + \frac{z_b F_h}{RT} \quad (5.7)
\]

Equation (5.5) represents a simple second order semilogarithmic relationship for correlating protein partitioning in aqueous two-phase systems, where the intercept $A^{**}$ is a function of the molecular weight of the protein and phase forming polymer, the protein-water, protein-polymer, polymer-water interaction parameters, pH and concentration. Similarly, the slope $b^*$ is a function of the molecular weight of the protein, the polymer-water interaction parameters, pH and concentration. It is quite interesting that $A^{**}$ and $b^*$ contain $\chi_{01}$ and $\chi_{02}$ which represent the interactions between water and PEG and water and PAS respectively.

### 5.4 Results and Discussion

The PAS/PEG/water system was used for the partitioning of the protein bovine serum albumin (BSA). The partioning of the BSA was studied in block and random copolymers of acrylamide styrene. It is found that at pH = 7.0, block copolymer showed much better selectivity for protein partitioning compared to random copolymer. as shown in Table 5.1. The important factors involved in the
partitioning of protein were the difference in molecular weights of the two phases and the hydrophobic interactions.

It is concluded that in block copolymers the hydrophobic monomer (styrene) is concentrated in the form of blocks which in turn exert more interaction with hydrophilic protein (BSA) as compared with random copolymers where hydrophobic monomer (styrene) was distributed randomly into the polymer chain. Therefore, protein molecules were repelled towards PEG rich top phase. It was also found that the partitioning coefficient of BSA increased by increasing the concentration of PAS as shown in Table 5.1. The shifting of protein molecules was from higher molecular weight PAS phase towards lower molecular weight PEG phase. All studies were carried out at pH = 7.0.

The consistency of the partitioning of protein was checked by using the correlation developed by Diamond and Hsu (1990) which is based on Flory-Huggins theory. Figures 5.2 and 5.3 show that the data lies on a straight line. The intercept is a function of protein and phase forming polymer molecular weights and the protein-water, protein-polymer, polymer-water interaction parameters. Slope is a function of protein molecular weight and polymer-water interaction.

The effect of pH on the partitioning of protein was also studied. At the isoelectric point, I.P, of BSA (4.7), protein carried equal charges that implied that the hydrophobic repulsion became negligible as compared with hydrophilic attraction between hydrophilic main acrylamide chain and the hydrophilic protein. Therefore, at pH 4.7, the net effect was shifting of BSA towards PAS rich bottom phase as shown in Table 5.4 and Figure 5.4. This results was in agreement with the fact that in the absence of styrene, BSA is present in the polyacrylamide rich phase. At pH
2.7, there was slight increase in the partition coefficient as shown in Fig. 5.4. It was due to the fact that BSA has five isomeric forms, depending on the pH (forms E,F,B, N and A). Below a pH of 4.0, albumin becomes fully uncoiled (form E) (Forciniti, 1991). This resulted in the exposure of some hydrophobic parts of BSA that caused a slight increase in the partition coefficient.
Figure 5.1. Protein (BSA) calibration curve at 282 nm.
Figure 5.2. Correlation of Protein (BSA) Partitioning in System of PEG 35000 / PAS (Block) / Water
Figure 5.3. Correlation of protein (BSA) Partitioning in PEG (35000) / PAS (Random) / Water system
Figure 5.4. Effect of pH on the partitioning of protein (BSA)
Fig. 5.5 Correlation of PEG/Dextran Water. Data by [17].
Table 5.1  Protein (BSA) partitioning in PAS(Random)/PEG-35000/water and PAS(Block)/PEG-35000/water systems

<table>
<thead>
<tr>
<th>Total System</th>
<th>Protein Partitioning in PAS(Block)/PEG(35000)/Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAS % w/w</td>
<td>PEG % w/w</td>
</tr>
<tr>
<td>3.88</td>
<td>3.11</td>
</tr>
<tr>
<td>2.333</td>
<td>2.916</td>
</tr>
<tr>
<td>3.997</td>
<td>4.977</td>
</tr>
<tr>
<td>3.889</td>
<td>7.778</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bottom Phase</th>
<th>Top Phase</th>
<th>Protein Partitioning in PAS(Random)/PEG 35000/Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAS % w/w</td>
<td>PEG % w/w</td>
<td>Protein % w/w</td>
</tr>
<tr>
<td>8.77</td>
<td>0.95</td>
<td>0.0023</td>
</tr>
<tr>
<td>9.2</td>
<td>0.90</td>
<td>0.0013</td>
</tr>
<tr>
<td>12.4</td>
<td>0.5</td>
<td>0.0008</td>
</tr>
<tr>
<td>15.21</td>
<td>0.05</td>
<td>0.0006</td>
</tr>
</tbody>
</table>
Table 5.2  Protein partitioning correlation for PAS(Random)/PEG-35000 /water System

<table>
<thead>
<tr>
<th>No.</th>
<th>Conc. of protein in top phase % w/w</th>
<th>Conc. of protein in bottom phase % w/w</th>
<th>Protein Mass in top phase, grams</th>
<th>Protein Mass in bottom phase, grams</th>
<th>Partition Coeff. K</th>
<th>difference in weight fraction ((w_i - w_j)\times100)</th>
<th>ln (K)/((w_i - w_j)\times100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.164</td>
<td>0.167</td>
<td>0.0227</td>
<td>0.0232</td>
<td>0.982</td>
<td>0.05</td>
<td>-0.36</td>
</tr>
<tr>
<td>2</td>
<td>0.161</td>
<td>0.168</td>
<td>0.0223</td>
<td>0.0236</td>
<td>0.958</td>
<td>0.13</td>
<td>-0.367</td>
</tr>
<tr>
<td>3</td>
<td>0.166</td>
<td>0.18</td>
<td>0.022</td>
<td>0.0239</td>
<td>0.922</td>
<td>0.20</td>
<td>-0.39</td>
</tr>
<tr>
<td>4</td>
<td>0.162</td>
<td>0.212</td>
<td>0.0199</td>
<td>0.261</td>
<td>0.76</td>
<td>0.62</td>
<td>-0.43</td>
</tr>
</tbody>
</table>
Table 5.3  Protein Partitioning for PAS(Block)/PEG-35000/water

<table>
<thead>
<tr>
<th>No.</th>
<th>Conc. of protein in top phase % w/w</th>
<th>Conc. of protein in bottom phase % w/w</th>
<th>Protein mass of protein in top phase</th>
<th>Protein mass in bottom phase</th>
<th>Partition Coeff. K</th>
<th>difference in weight fraction (w'_1 - w'_2) x 100</th>
<th>In (K)/(w'_1 - w'_2) x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.192</td>
<td>0.162</td>
<td>0.025</td>
<td>0.021</td>
<td>1.185</td>
<td>0.4</td>
<td>0.425</td>
</tr>
<tr>
<td>2</td>
<td>0.262</td>
<td>0.09</td>
<td>0.034</td>
<td>0.0117</td>
<td>2.91</td>
<td>2.23</td>
<td>0.48</td>
</tr>
<tr>
<td>3</td>
<td>0.251</td>
<td>0.058</td>
<td>0.037</td>
<td>0.0088</td>
<td>4.32</td>
<td>2.81</td>
<td>0.52</td>
</tr>
<tr>
<td>4</td>
<td>0.226</td>
<td>0.038</td>
<td>0.0395</td>
<td>0.0067</td>
<td>5.92</td>
<td>3.28</td>
<td>0.54</td>
</tr>
</tbody>
</table>
Table 5.4  Protein Partitioning: effect of pH

<table>
<thead>
<tr>
<th></th>
<th>Total System</th>
<th>Top Phase</th>
<th>Bottom Phase</th>
<th>pH of the system</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PAS %w/w</strong></td>
<td>3.997</td>
<td>0.001</td>
<td>92.9</td>
<td>87.1</td>
</tr>
<tr>
<td><strong>PEG %w/w</strong></td>
<td>4.977</td>
<td>7.1</td>
<td>12.4</td>
<td>2.7 (Rand)</td>
</tr>
<tr>
<td><strong>Protein %w/w</strong></td>
<td>0.51</td>
<td>0.0009</td>
<td>0.5</td>
<td>2.7 (Block)</td>
</tr>
<tr>
<td><strong>Water %w/w</strong></td>
<td>90.516</td>
<td>0.0008</td>
<td>0.0041</td>
<td>4.7 (Bock)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.0007</td>
<td>0.0043</td>
<td>6.7 (Block)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.0027</td>
<td>0.00435</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.00237</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Top phase absorbance</th>
<th>Bottom phase absorbance</th>
<th>Top phase protein conc.</th>
<th>Bottom phase protein conc.</th>
<th>Protein mass of protein in top phase</th>
<th>Protein Mass in bottom phase</th>
<th>Total protein mass</th>
<th>Partition coefficient K</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.465</td>
<td>1.514</td>
<td>0.064</td>
<td>0.282</td>
<td>0.0085</td>
<td>0.0375</td>
<td>0.046</td>
<td>0.227</td>
</tr>
<tr>
<td>0.460</td>
<td>1.742</td>
<td>0.063</td>
<td>0.327</td>
<td>0.0074</td>
<td>0.0387</td>
<td>0.046</td>
<td>0.1926</td>
</tr>
<tr>
<td>0.447</td>
<td>1.792</td>
<td>0.059</td>
<td>0.342</td>
<td>0.0068</td>
<td>0.0392</td>
<td>0.046</td>
<td>0.1725</td>
</tr>
<tr>
<td>0.998</td>
<td>0.896</td>
<td>0.173</td>
<td>0.151</td>
<td>0.0246</td>
<td>0.0214</td>
<td>0.046</td>
<td>1.145</td>
</tr>
</tbody>
</table>
Chapter 6

Conclusions and Recommendations

6.1 Summary and Conclusions

A novel physical picture is proposed for the interactions between protein and non-ionic block copolymers that enhance the partitioning of proteins in two-phase aqueous polymer system. For the system polyethylene glycol (PEG)/Poly Acrylamide Stryene (PAS)/Water, this novel picture was based on the assumption that in block copolymer (PAS) hydrophobic monomer (stryene) is distributed in the form of blocks which in turn will show better interaction with protein compared with random copolymer (PAS) where hydrophobic monomer is distributed randomly in the polymer chain. Observation showed that the block copolymer (PAS) gives better partitioning of protein, bovine serum albumin (BSA), than the random copolymer. The presence of strong hydrophobic group (stryene) in the block copolymer repells the hydrophilic protein towards PEG rich top phase. The measurements were performed at four different pH values. The simultaneous effect of pH, polymer structure and concentration on the partition coefficient of protein BSA is analysed. Hydrophobically modified water soluble copolymers of acrylamide stryene were synthesized by micellar and homogeneous copolymerization methods.
The results of this thesis have clearly demonstrated that polymer structure has little effect on phase behavior. In both random and multiblock copolymers of acrylamide-styrene, the effect of incorporation of hydrophobic units (styrene) along the backbone of long acrylamide chain is very small. Therefore, the interaction between PEG-random copolymer and PEG-block copolymer of acrylamide styrene is almost identical, which is revealed by their phase diagram.

However, the polymer structure effects protein partitioning. The results presented are based on novel physical picture of the interactions of globular proteins and flexible non-ionic polymers which influence the partition of proteins in two-phase aqueous polymer system. In particular, this study described that in block copolymer of acrylamide styrene the hydrophobic units are distributed in the form of blocks along the polymer chain which implies the localization of the hydrophobic units, which showed better hydrophobic repulsion for hydrophobic globular protein (BSA) as compared with random copolymer of acrylamide styrene where hydrophobic units (styrene) are distributed randomly along the backbone of long acrylamide chain. Therefore, hydrophobic blocks of styrene repelled hydrophilic protein (BSA) molecules towards PEG rich top phase.

The phase behavior of the system PEG/ PAS/ water is used for phase separation. Molecular weight of PEG used is 35000 while that of PAS is $1.4 \times 10^5$. It is found that protein (BSA) molecules move from higher molecular weight PAS rich bottom phase towards lower molecular weight PEG rich phase.

The effect of pH on the partition behavior of protein (BSA) is also studied. At three different values of pH, it is found that partition coefficient has strong dependence on pH of the system. The partition coefficient of BSA (I.P. = 4.7) has
minimum value close to its isoelectric point where protein carries equal positive and negative charges. Therefore, at I.P., the hydrophobic repulsion becomes negligible, which causes the accumulation of most of the protein molecules in PAS rich bottom phase. It is found that partition coefficient increases by increasing the pH of the system. There is a discrepancy noted in the partition coefficient behavior at pH = 2.7. The slight increase in K value is due to the fact that BSA present in five isomeric forms depending upon the pH of the system. Below pH = 4.0, the protein molecule unfolded which resulted in the exposure of some hydrophobic sites which causes the slight increase in the partition coefficient.

6.2 Recommendations

It is hoped that the experimental results presented in this thesis will serve as a guideline for the future development of polymer systems for protein partitioning studies. Future possible directions include:

Varying the type of monomers in the blocks which show attractive interactions with the protein molecules. For this purpose, the molecular weight of PEG should be higher than the block copolymer so that the difference in molecular weight can help in driving the protein molecules from higher molecular weight PEG rich phase towards the lower molecular weight block copolymer rich phase.

As far as precipitation and recycling of the polymer is concerned, charged groups can be incorporated into the polymer chain which will help in this regard.

As proteins are present in very complex mixtures, so to separate a specific protein from the mixture, it should be kept in mind that the system which is going to be utilized for the separation of protein should not show the same affinity for the
other proteins as well. Therefore, the partition of different proteins can be studied with PEG/PAS/water system.

As mentioned earlier that acrylamide styrene copolymer showed phase separation with polyethylene glycol, poly vinyl alcohol, poly pyrrolidone, urithenized polyvinyl alcohol. Therefore, it is suggested that similar studies of PEG with one of the above mentioned polymers can also be done.
References


