Protein Partitioning in Polymer/Polymer Aqueous Two-Phase Systems

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To Sofia and Francisco

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Abstract

The main purpose of the present work is to contribute for a better understanding of the mechanisms that govern biomolecules partitioning in polymer/polymer aqueous two-phase systems (ATPSs).

Ten phase diagrams formed by all pair combinations of Dextran (Dex-75), Ficoll-70, polyethylene glycol (PEG-8000), hydroxypropyl starch (PES-100), and Ucon50HB5100 (a random copolymer of ethylene glycol and propylene glycol) all containing 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.4, at 23 ^{0}C were experimentally obtained.

Distribution coefficients of several biomolecules (six amino acids, two peptides, thirteen proteins and five monosaccharides) were obtained in all the ATPSs. The solvatochromic solvent parameters characterizing the solvent polarity (π^*), solvent hydrogen-bond donor acidity (b*), and solvent hydrogen-bond acceptor basicity (a*) of aqueous media were also measured in the coexisting phases of the above ten different polymer/polymer aqueous two-phase systems.

It was found that for six out of the ten ATPSs the distribution coefficients of the proteins were correlated according to the Collander equation. Moreover, the intercept and slope of this equation were described in terms of independently obtained physico-chemical properties of the phases.

A linear free energy relationship (LFER) was developed to describe the partitioning behavior of all the biomolecules in the ten ATPSs. The new methodology may be used to determine solute descriptors for biological molecules and predict their partitioning behavior in previously characterized ATPSs. It is the first time, to the best of our knowledge, that protein descriptors are experimentally obtained.

Resumo

O principal objectivo do presente trabalho consiste em contribuir para uma melhor compreensão dos mecanismos que governam a partição de biomoléculas em sistemas de duas fases aquosas (SDFA) compostos por dois polímeros.

Determinaram-se experimentalmente dez diagramas de fases para todas as combinações binárias dos polímeros dextrano, PEG, Ficoll, Ucon 50-HB5100 (Ucon), um copolímero aleatório composto por 50% de óxido de etileno (OE) e 50% de óxido de propileno (OP) (percentagens mássicas) e reppal PES todos contendo NaCl 0,15 M em tampão fosfato 0,01 M, pH 7,4, a 23 ^{0}C.

Determinou-se experimentalmente a partição de diversas biomoléculas (seis aminoácidos, dois péptidos, treze proteínas e cinco monossacáridos) nos dez SDFA. Caracterizaram-se adicionalmente as fases em equilíbrio dos sistemas em termos de polaridade, capacidade do solvente em participar como dador e aceitador de pontes de hidrogénio, usando o método solvatocrómico.

Verificou-se que os coeficientes de partição das proteínas em seis dos dez SDFAs estavam correlacionados através da equação de Collander. Além disso, o declive e a ordenada na origem desta equação foram descritos em termos das propriedades físico-químicas das fases.

Desenvolveu-se um modelo linear, derivado das propriedades físico-químicas das fases, para descrever a partição de todas as biomoléculas nos dez SDFA. Esta nova metodologia pode ser usada para obter descriptores para biomoléculas e prever a sua partição em SDFA previamente caracterizados. É a primeira vez que são experimentalmente obtidos descriptores de proteínas.

Résumé

Le principal objectif du travail présent consiste en contribuer pour une meilleur compréhension des mécanismes qui gouvernent la distribution des biomolecules dans systèmes biphasiques aqueux de deux polymères (SBA).

Dix diagrammes de phases formés par toutes les combinaisons binaire de Dextran (Dex-75), Ficoll-70, polyéthylène glycol (PEG 8000), reppal PES (PES-100), et Ucon50HB5100 (un copolymère aléatoire de éthylène glycol et propylène glycol) tout contenant NaCl 0.15 M dans tampon du phosphate 0.01 M, pH 7.4, à 23 ^{0}C ont été obtenus expérimentalement.

Coefficients de partage de diverses biomolecules (six acides aminés, deux peptides, treize protéines et cinq monosaccharides) on été obtenu dans tout l'SBA. Le paramètres solvatochromiques qui caractérisent la polarité du solvant (π*), la capacité du solvant à participer comme donneurs de liaisons hydrogène (b*), et accepteur de liaisons hydrogène (a*) a aussi été mesuré dans les coexistant phases des dix systèmes biphasiques aqueux de deux polymères.

Il a été trouvé que pour six hors du dix SBA les coefficients de partage des protéines ont été corrélées par l'équation de Collander. De plus, les paramètres de cette équation ont été décrits pour les propriétés physico-chimiques des phases obtenues de mode indépendant.

Une modèle linéaire a été développée, dérivé des propriétés physico-chimiques des phases, pour décrire le partage de tout le biomolecules dans les dix SBA. La nouvelle méthodologie peut être utilisée pour déterminer des descripteurs pour les molécules biologiques et prédire leur partage dans SBA précédemment caractérisé. C'est la première fois, au meilleur de notre connaissance, que les descripteurs de protéines sont obtenus expérimentalement.

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1. Introduction

1.1 Relevance and Motivation

The aqueous two-phase systems (ATPSs) technology has been object of intense research in the last few decades. Indeed, the particular advantages of these systems, such as to provide innocuous environment for biomolecules, being a so-called “green” technology, of low cost, easy to scale-up, extremely sensitivity towards solute surface properties, among others, make them attractive not only for separation purposes but also for bioanalytical applications.

Despite all their advantages, the ATPSs have faced enormous scepticism at industrial level. There are many reasons for their underuse in extraction technology for biopharmaceutical processing, including fears over quality control of the phase forming components, difficulties in removing and recycling the phase-forming components, interference with further downstream processing steps, as well as the conservative nature of the industry which limits any company's desire to take on the risk of taking an ATPS containing process through Food and Drug Administration (FDA) approval.

At bioanalytical application level, however, probably the primary reason for the current underutilization of the ATPSs extraction technology is the lack of a generalized theory for biomolecules partitioning in ATPSs, which still makes the design of optimal extraction conditions a lengthy and laborious endeavor.

There are several theoretical models describing phase separation in aqueous mixtures of polymers and/or polymer-inorganic salt and partitioning of solutes in such systems but they all fail to meet the main requirement of any practical theoretical model – they cannot predict partition behavior of any given protein in different ATPSs from a limited set of experimental

data and the knowledge of only the physico-chemical properties of the components in the mixture.

1.2 Objectives

The major objective of the present thesis is to contribute for a better understanding of the mechanisms governing biomolecules partitioning in ATPSs, with special emphasis on proteins.

The present work is divided in 8 Chapters. In Chapter 2 a brief bibliographic review is undertaken, with particular focus on the theory of solute partitioning in ATPSs. Thus, phase diagrams representing ATPSs are presented first with the outline of some experimental techniques for their determination, and the major factors that influence phase compositions are discussed. The parameters that affect solute partitioning in these systems are also briefly summarized. The most well established and used theories found in the literature to explain solute partitioning are briefly presented. The Chapter ends with the major conclusions from the background art which lead to the motivation of the present work.

In Chapter 3 phase diagrams experimentally obtained during this work for ten ATPSs formed by all pair combinations of Dextran (Dex-75), Ficoll-70, polyethylene glycol (PEG-8000), hydroxypropyl starch (PES-100), and Ucon50HB5100 (a random copolymer of ethylene glycol and propylene glycol), all containing 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.4, at 23 ^{0}C are presented. Partition coefficients of a series of dinitrophenylated (DNP) amino acids (DNP-glycine, DNP-alanine, DNP-norvaline, DNP-norleucine, DNP-DL-α-amino-n-octanoic acid) with aliphatic side-chains are studied in all the aqueous two-phase systems at particular polymer concentrations. Free energies of transfer of a methylene group between the coexisting phases, $\Delta G(CH_2)$, are determined as measures of the difference between the hydrophobic character of the phases. The solvatochromic solvent parameters characterizing the solvent polarity (π^*), solvent hydrogen-bond donor acidity (b*), and solvent hydrogen-bond acceptor basicity (a*) of

aqueous media are also measured in the coexisting phases of the above ten different aqueous polymer/polymer two-phase systems. Furthermore, partition coefficients of tryptophan (Trp) and its di- and tri-peptides and a set of p-nitrophenyl (NP)-monosaccharides are measured in all these two-phase systems, and the data obtained are compared with the $\Delta G(CH_2)$ values obtained in the systems. It is established that for eight, out of the ten, two-phase systems of different polymer compositions the partition coefficients for Trp peptides correlate well with the $\Delta G(CH_2)$ values. Similar correlations for NP-monosaccharides are valid for seven out of the ten two-phase systems. These observations indicate that the difference between the hydrophobic character of the coexisting phases represented by the $\Delta G(CH_2)$ value can be used as solvent descriptor in ATPSs of different polymer compositions.

In Chapter 4 distribution coefficients measured for a variety of proteins in the ATPSs Dex-PEG and Dex-Ucon, both containing 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.4, at 23 ^{0}C are given. Distribution coefficients of some selected solutes have also been measured in the above two-phase systems at three different polymer concentrations for each system. The distribution coefficients for all the proteins examined in the ATPSs are in very satisfactory agreement with the so-called Collander equation.

In Chapter 5 distribution coefficients of randomly selected proteins (Chicken egg lysozyme, bovine α-chymotrypsinogen A, bovine hemoglobin, horse heart cytochrome c, bovine ribonuclease B, bovine ribonuclease A, bovine trypsinogen, human hemoglobin, horse myoglobin, bovine β-lactoglobulin, human γ-globulin, bovine γ-globulin, porcine lipase, and human transferrin), measured in ATPSs formed by different combinations of Dex, Ficoll-70, PEG, PES, and Ucon at particular polymer concentrations, all containing 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.4, are presented. Most of the proteins in the PEG-Ucon system precipitate at the interface. In the other ATPSs, namely, PES-PEG, PES-Ucon, Ficoll-PEG, Ficoll-Ucon, and in Dex-PEG and Dex-Ucon described earlier (Chapter 4) the distribution coefficients for the proteins are correlated according to the solvent regression equation: $\ln K_i = a_{io} \ln K_o + b_{io}$, where K_i and K_o are the distribution coefficients for any protein in the i-th and o-th two-phase

systems. Coefficients a_{io} and b_{io} are constants, the values of which depend upon the particular compositions of the two-phase systems under comparison.

In Chapter 6 the analysis of the literature data shows that the solvatochromic solvent parameters are linearly correlated with the corresponding solvent-specific coefficients in the Abraham's linear solvation equation. The solvatochromic solvent descriptors together with the solvent features of the phases derived from the partitioning of a homologous series of dinitrophenylated (DNP) amino acids are used in a linear free energy relationship to calculate the solute descriptors from the partition coefficients of five p-nitrophenylated-monosaccharides, 5 dinitrophenylated (DNP) amino acids, tryptophan, and its di- and tri-peptides in all ATPSs. Three descriptors of the solvent properties of the phases, solvent polarity, π*, solvent hydrogen-bond donor acidity, b*, and coefficient c (derived from partitioning of DNP-amino acids) can describe adequately the partitioning of the solutes. The combined methodology described in this Chapter may be used to determine solute descriptors for biological molecules and to predict their partitioning behavior in previously characterized ATPSs.

In Chapter 7 distribution coefficients of the same selected proteins measured in Dextran-Ficoll, PES-Dextran and PES-Ficoll aqueous two-phase systems (ATPSs), at particular polymer concentrations, all containing 0.15M NaCl in 0.01M phosphate buffer, pH 7.4, are presented. The experimental data presented in this Chapter is used together with data from previous Chapters in a Linear Free Energy Relationship (LFER) to describe partitioning of the proteins in nine different ATPSs. Four descriptors of the solvent properties of the phases, solvent polarity, π*, solvent hydrogen-bond donor acidity, b*, solvent hydrogen-bond acceptor basicity, a*, and coefficient c (derived from partitioning of DNP-amino acids) can describe adequately the partitioning of the proteins. It is the first time, to the best of our knowledge, that protein descriptors are experimentally obtained.

Finally in Chapter 8 the major conclusions of this work are summarized, and future work is also addressed.

2. State of the Art

2.1 Introduction

Beijerink [1] was the first researcher to report on aqueous two-phase systems (ATPSs) when he found that an aqueous mixture of gelatine and starch could form two aqueous-based phases. It took around half a century before these systems became popular with the efforts of Albertsson [2], who applied them for separation processes.

Aqueous two-phase systems arise in aqueous mixtures of different water-soluble polymers or a single polymer and a specific salt. When two certain polymers, e.g., Dextran (Dex) and poly(ethylene glycol) (PEG), are mixed in water above certain concentrations, the mixture separates into two immiscible aqueous phases. There is a clear interfacial boundary separating two distinct aqueous-based phases, each preferentially rich with one of the polymers. The aqueous solvent in both phases was demonstrated to provide media suitable for biological products [3-6]. These systems are unique because each of the phases contains over 80% water on a molal basis and yet the phases are immiscible and differ in their solvent properties [6,7]. Therefore, these systems can be used for differential distribution of solutes and particles.

Extraction in ATPSs has been clearly demonstrated as an efficient method for large scale recovery and purification of biological products [3-5, 8, 9]. Low cost, high capacity and easy scale-up are clear advantages of this technology. Partitioning in ATPSs may also be used as a bioanalytical tool for characterization of protein surface properties [6,10], changes in protein structure [11], conformation [12], ligand binding [3-5], among others. Successful application of partitioning in ATPSs requires the understanding of the mechanisms of solute distribution and properties of the systems at the molecular level.

Understanding the mechanisms of solute partitioning in ATPSs is important for both separation and bioanalytical applications, although for slightly different reasons. When ATPSs are used for separation purposes it is important to predict optimal extraction conditions for a target product from a particular source and/or to design optimized strategies for the search of such optimal conditions. Thus, understanding the role of different factors, such as pH, type and concentration of salt additives, type of phase forming components, etc., affecting distribution of the product and contaminants is much more important from a practical viewpoint than understanding why the product partitions into a certain phase. For analytical applications the comprehension of the partitioning mechanisms is crucial since it defines the information provided by a change in the analyte partition behavior under altered partition conditions or as a response to a change in the analyte properties.

Before discussing the mechanisms that govern solute partitioning in ATPSs attention to the systems where these solutes are to be partitioned should be addressed.

2.2 Phase Diagrams

The composition of aqueous polymer two-phase systems is usually conveniently represented on a rectangular phase diagram as illustrated in figure 2.1.

In this figure, for a given system the total composition is represented by A, and that of the two phases by A’ and A’’. The composition is commonly specified in weight percents but any convenient units may be used. The vertical axis is usually used for the polymer which is enriched in the top phase. The points A’ and A’’ are called nodes and the straight line connecting them is the tie-line. The tie-lines are often parallel [3]. With decreasing feed composition the tie-line length decreases and eventually becomes zero at the critical point, designated by C in figure 2.1. The curve passing through the nodes and the critical point is the binodal. All systems with initial composition above the binodal (like A) split into two liquid phases, all the points below the binodal (like D) give a homogeneous system.

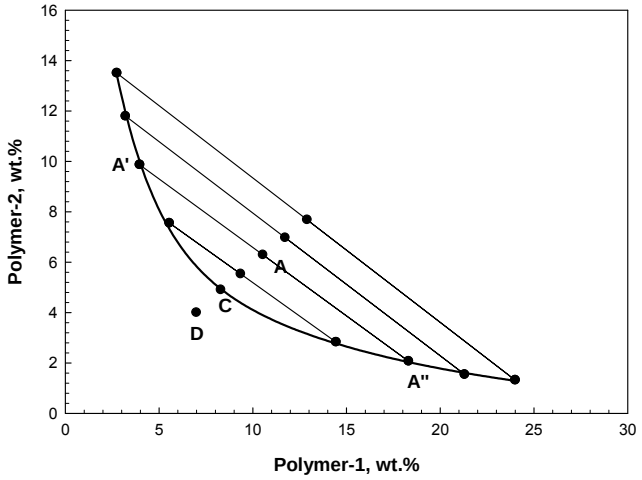

Figure 2.1 Rectangular phase diagram for polymer-1 : polymer-2 : water system

One way to obtain experimentally a phase diagram is to determine compositions of the coexisting phases for several initial different compositions within the heterogeneous region of the diagram. The methods and experimental procedures for quantifying the components were extensively described before [4,13]. For polymers, the most used techniques are polarimetry, refractive index and freeze drying. Another method to experimentally obtain a phase diagram, worthy of mention, is the cloud point determination. In this method aqueous polymer (or salt) stock solutions are dropwise added to the other polymer stock solution and the composition of the mixture at which turbidity appears corresponds to the transition from homogeneous to the two-phase region. This is the so-called cloud point.

It was found empirically [14] that the tie-line length (TLL) can be used as a single numerical measure of the compositions of the two phases, for different initial compositions of the system.

$$TLL = [(\Delta P_1)^2 + (\Delta P_2)^2]^{1/2} \qquad (2.1)$$

where ΔP_1 and ΔP_2 are the differences between the polymer concentrations in the coexisting phases.

The tie-line slope (TLS) is also an important characteristic of a phase diagram [15]. The TLS is calculated according to:

$$TLS = \Delta P_1/\Delta P_2 \qquad (2.2)$$

Albertsson was the first researcher to report that the tie-line slope (TLS) is usually constant for a given ATPS [3].

The factors that can be manipulated to change the equilibrium compositions were extensively reported [3, 4, 6] and include the molecular weight of phase-forming polymers, temperature and addition of inorganic salts. Thus, only very briefly theses properties will be presented here.

In general, the higher the molecular weight of a phase forming polymer, the lower the concentration required for phase separation.

As a general rule, and contrary to polymer-salt aqueous-two-phase systems, the higher the temperature the higher the concentrations of phase-forming polymers required to achieve phase separation.

It was initially stated by Albertsson [3, pp. 34] that "The systems containing nonionic polymers are hardly affected by the addition of, for example, 0.1-1 M sucrose or NaCl…It is only at higher salt concentration that an effect on the phases system may be observed". This view was only contested some years later [6]. Zaslavsky and co-workers [6] studied the effect of several salts on Dex-PEG, Dex-PVP, Dex-PVA and Dex-Ficoll. Their observations and conclusions were astonishing: "The water-structure-breaking salts generally elevate the binodal of an aqueous two-polymer two-phase system similarly to the temperature increase or the urea addition, while the water-structure-making salts depress the binodal of the system. In other words, when the water structure in the mixture of two phase polymers is disrupted by a given factor (temperature increase, addition of urea or water-structure-breaking salts) the threshold amounts of the polymers required for phase separation in the mixture increase. If the factor

(temperature decrease, addition of water-structure-making salt) enhances the water structure in the mixture the threshold amounts of the polymers are reduced."[6, pp. 115-116].

Obviously the chemical nature of the polymers is of paramount importance for phase separation, but the lack of experimental data does not allow to state general rules yet.

It should be noted that the above outlined general rules are applied to systems composed of two non-ionic polymers.

2.2.1 Theoretical Approaches of Phase Separation

The most well established and used theoretical line of reasoning found in the literature in regard to phase separation in ATPSs is based on the view that the macromolecular nature of phase-forming polymers is of primary importance for phase separation, while water as a solvent play a secondary role [6]. These models are typically based on the Flory-Huggins theory [16,17], on lattice models e.g. UNIQUAC [18,19] or on osmotic virial expansion methods [20-23]. The limitations and advantages of these models have been widely discussed in the literature [6, 24-27].

Around fifteen years ago, Zaslavsky [6] advanced a different way to look into ATPSs. According to his observation the unique character of water as a medium plays the primary role in phase separation. The phase forming polymers are viewed in this approach as agents perturbing the water structure. In other words, ATPSs are viewed as two immiscible solvents of aqueous nature, in which the water structure, different in each phase, has been modified by the presence of the polymers or salts engaged in its formation.

It is well established that water molecules interact strongly with each other in a characteristic way [28] and this mustbe incorporated into any model that intends to describe the behavior of these complex systems.

The role of water in phase separation can be better illustrated by referring to figure 2.2 [6, pp. 142]. In this figure the X-ray diffraction data for polyvinyl alcohol (PVA), dextran and their dry mixture are presented. It is clear from this figure that the peaks specific for the individual polymers disappear in their dry 1:1 mixture. This result shows that, in the absence of water, this pair of polymers is totally compatible [6].

It should be noted that the polymers in question, in the presence of water, separate at 3.05 % (w/w) Dex and 2.45 % (w/w) PVA. This means, in terms of the models that attribute the phase separation majorly to the repulsive interactions of the polymers, that these polymers are strongly incompatible.

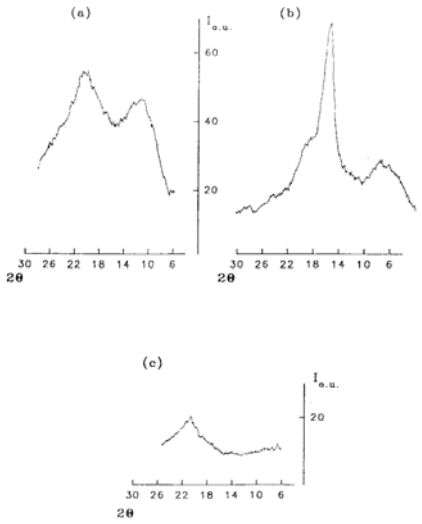

Figure 2.2 Diffractograms of Dextran (a); polyvinyl alcohol (b); and 1:1 mixture of dextran and polyvinyl alcohol (c). Adapted from [6, pp. 142]

Any model or theory intending to describe phase separation in this particular Dex-PVA system, cannot neglect this experimental observation. This result, in particular, is a clear evidence that water plays an essential role in phase separation.

A similar concept has been advanced by Treffry et al. [29]. In their model “the polymer molecules are surrounded by shells of water molecules structured according to the steric

arrangement of bonding sites on the polymer which may or may not match structure in the bulk solvent. Like shells may merge producing micro-aggregates (microphases). When unlike shells are forced together structural discontinuity leads to turbidity and phase separation" [29]. This concept was further developed using the so-called binodal model [24, 30-32] based on the "statistical geometry" theory suggested by Bernal [33, 34]. In this approach the so-called effective excluded volume, V_f, was shown to be the only parameter required to fit experimental water-PEG-Dex phase diagrams [24, 30-32] and PEG-salt ATPSs [35].

2.3 Solute Partitioning

By adding a solute to an ATPS it will distribute more or less evenly between the phases. Its distribution is quantified by the partition coefficient, K, usually defined as the ratio of the solute concentration in the top phase to that in the bottom phase. The commonly used procedures to obtain the partition coefficient as well as the analytical techniques usually employed in the partition technique were reviewed elsewhere [4, 13]. However, it should be stressed out that if the partition coefficient is to be explored to study thermodynamic relationships or to understand the mechanisms of the partition process an internal check on the possibility of solute self-association or dissociation, direct solute-polymer interactions, etc., should be carried out [6]. The way to achieve this internal check was discussed previously [6] and will be presented here only very briefly.

According to this method, four to five different amounts of a solute stock solution are introduced into separately prepared systems in order to achieve the same final system compositions. To assure the reproducibility of the measurements the experiments are repeated two to four times with each amount of the solute. It is also advisable to use a separate blank assay to avoid or correct the eventual error caused by interference of the phase polymers with the analytical technique [6]. It should be noted that in the present work all the partitioning experiments were carried out using this methodology.

The partition coefficient for a given solute is then calculated by the standard linear regression analysis of the equation:

$$C(solute)_t = a + K.C(solute)_b \qquad (2.3)$$

where C(solute) is the solute concentration (usually in mg/mL); subscripts t and b refer to the top and bottom phases, respectively.

Figure 2.3 presents typical data obtained in the present work using this methodology. Figure 2.4 presents typical data where deviations from linearity are observed.

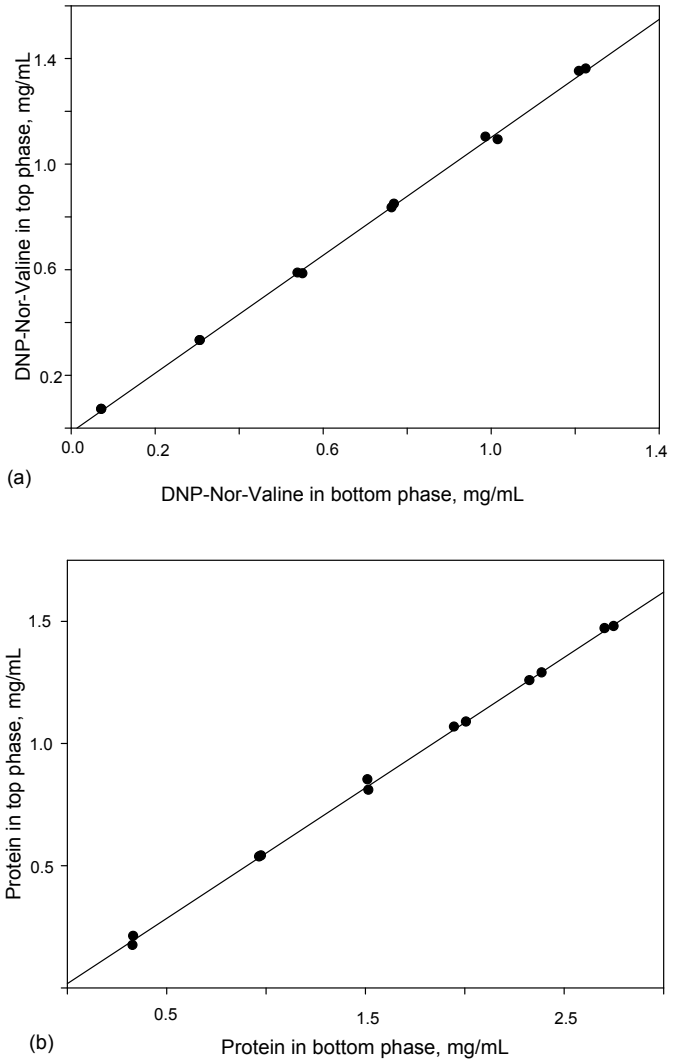

Figure 2.3 (a) Concentrations of sodium salt of DNP-Nor-Valine in the two phases of the ATPS with the following composition: 12.4% Dex-75; 6,1% PEG-8000; 0.15 M NaCl; 0.01 M NaPB, pH 7.4, under varied total concentration of the solute in the system; (b) Concentrations of Ribonuclease B in the two phases of the ATPS with the following composition: 12.0% Dex-75; 6.0% Ficoll-70; 0.15 M NaCl; 0.01 M NaPB, pH 7.4, under varied total concentration of the solute in the system.

It is an experimental fact know that the dimerization of lysozyme occurs at alkaline pH, and this is the most likely reason for the observed deviation of the concentration dependence from the linearity (figure 2.4 adapted from [6]).

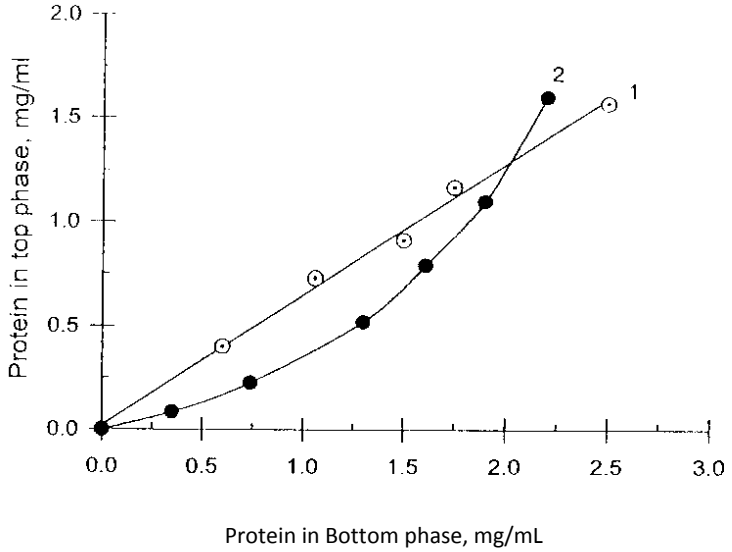

Figure 2.4 Concentrations of egg white lysozyme in the two phases of aqueous Dex-Ficoll two-phase system containing (1) 0.11 mol/kg sodium phosphate buffer, pH 7.4 and (2) 0.01 mol/kg universal buffer, pH 4.4 under varied total concentration of the protein in the system (adapted from [6]).

It should also be mentioned that for the majority of the partitioning data in ATPSs reported in the literature, the method employed does not include a check for eventual aggregation of the solute. Although it may not be necessary for separation purposes, it is crucial when the partitioning data is to be used for thermodynamic developments. The differences in the methodologies used may be an explanation for some disagreements found in the literature [6].

The factors that affect solute partitioning are well known and include type, molecular weight and concentration of phase polymers, type and concentration of additives, pH, temperature, among others. A detailed analysis of the effects of these factors was discussed in detail elsewhere [3,4,6]. However, some of them are controversy in the literature, and since

they are of particular relevance for the present work, they will be briefly discussed here. It should, however, be stressed out that the majority of the factors that affect solute partitioning are hardly independent, making any analysis at most approximate. For example, the change in pH of the mixture cannot be separated of the effect of salt addition, and a change in temperature will certainly affect the equilibrium compositions of the polymers.

a) Effect of pH on solute partitioning

On one hand, any change in the pH is equivalent to a salt composition change of the medium. On the other hand, the proteins (which are the solutes for which such dependence is commonly studied) are known to contain a huge number of acidic and basic groups with different acid dissociation constants (pK_a) values. Besides, the pH changes may alter not only the solute net charge, but may also induce conformational changes in the structure of a given protein, as well as aggregation effects. Thus any interpretation of the pH influence on protein partitioning should take into consideration both the changes on the properties of the aqueous media induced by different salt compositions on the phases, and the eventual pH-induced changes in the solute, resulting in different solute-solvent interactions [6].

It should be noted that the pH-effect on protein partitioning is usually attributed to the so-called interfacial electrostatic potential difference between the phases [4]. This phenomenon is due to the unevenly distribution of salts between the two phases of an ATPS. The misconception frequently encountered in the literature, is that this partitioning creates “charged phases”, “charge sensitive” phases, etc. under the influence of which the charged proteins will partition. However, it is a thermodynamic requirement that electroneutrality of both phases is maintained [36]. This means that the transfer of a charged ion from one phase to the other may only occur if electrically equivalent amounts of counter-ion are also transferred, implying that the electrical interfacial work terms (for hypothetically separated ions) cancel out. Obviously, the difference in salt concentrations between phases will originate different dielectric properties in the aqueous media of the phases. This will in turn translate in different

electrostatic ion-ion and ion-dipole solute-solvent interactions, leading to high dependence of the charged proteins on the type/concentration of salt added to the system.

Another controversial point worth of mention is that several literature data regarding the pH influence on protein partitioning are based on the empirical relationship observed in some cases between the net charge Z and partition coefficient k of a solute [37, 38]:

$$\ln K = \ln K(0) + \gamma Z \qquad (2.4)$$

where K(0) is the partition coefficient of the solute at the pH corresponding to its isoelectric point (pI); and γ is a factor which depends on the polymer composition, salt additives and temperature.

The above equation (2.4) was deeply analyzed elsewhere [6, pp. 244-254]. It was particularly noticed that there are several data in the literature for which the linear correlation described by equation 2.4 is not obeyed.

b) Effect of solute size

There are several attempts on the literature on ATPSs that try to ascertain the influence of the solute molecular size to its partitioning behavior [3, 4 and references cited therein]. The general accepted view, as initially stated by Albertsson [3], of the effect under question, is that small solutes such as amino acids generally distribute evenly in ATPSs and that large solutes, such as proteins, tend to partitioning more one-side than small ones. The trend in question is illustrated by the graph reproduced from [4] in figure 2.5.

Nevertheless, the same authors [39] present data on sixteen different hemoproteins where the partitioning behavior is independent of the protein molecular weight. This question was deeply analyzed by Zaslavsky [6, pp. 254-260]. Probably, the influence of the molecular weight is difficult to separate from the effect of the solute chemical structure. This fact is

illustrated in figure 2.6, which shows the results obtained with the partitioning of different β—1,4-glucomannanes from different plant sources [6, 40].

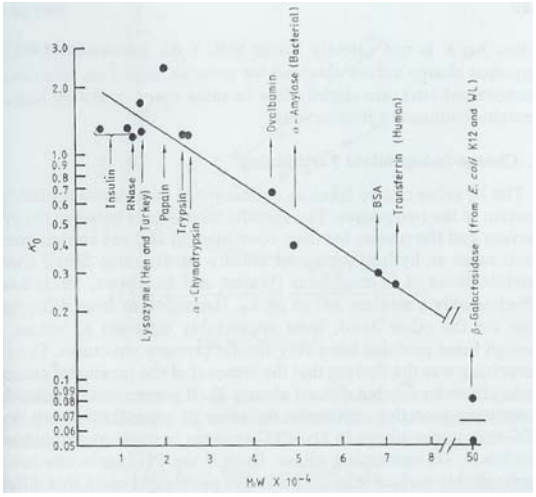

Figure 2.5 Relationship between K at the isoelectric point, and molecular weight (Mw) of some proteins. System: 7% Dex, 4.4% PEG-6000, 0.1 M NaCl or 0.05 M Na_2SO_4 and 0.01 M phosphate buffer or glycine buffer at 20 ^{0}C. (adapted from [4]).

The data presented in figure 2.6 clearly shows that the partitioning of polysaccharides in ATPSs does not follow the pattern consistent with the assumption about the direct effect of the solute molecular weight on its partitioning behavior.

As Zaslavsky suggested [6, 41, 42] the only type of solutes that allow to explore the effect of the solute molecular weight is probably the synthetic homopolymers, since an increase in the solute size is not accompanied by a change in its chemical structure.

Zaslavsky and co-authors [6] analyzed the partitioning behavior of some polymers in Dex-Ficoll ATPS containing 0.15 mol/kg NaCl in 0.01 mol/kg sodium phosphate buffer, pH 7.4 as a function of its molecular weight (figure 2.7).

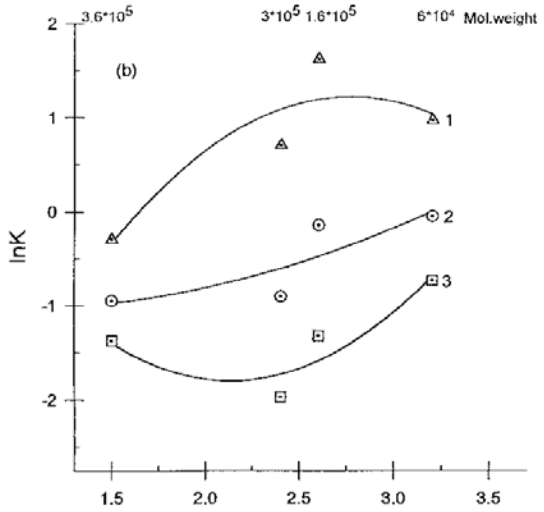

Figure 2.6 Partition coefficients of β-1,4-glucomannanes as function of the mannose/glucose ratio in the polysaccharide structure in aqueous Dex-Ficoll two-phase systems containing (1) 0.11 mol/kg sodium phosphate buffer, pH 7.4; (2) 0.09 mol/kg NaCl in 0.05 mol/kg sodium phosphate buffer, pH 7.4; and (3) 0.15 mol/kg NaCl in 0.01 mol/kg sodium phosphate buffer, pH 7.4 (adapted from [6]).

The data presented in figure 2.7 shows that (1) for PEG the partitioning behavior is independent of the solute size for the range of molecular weights studied (1,500 to 40000); (2) for PVP the higher the molecular weight (from 5,000 to 180,000) the less one-sided it is its partitioning behavior; and (3) for the polyacrylamides the higher the molecular weight the more one-sided it is its partitioning behavior. On the analysis of the previous it seems that “the chemical structure of a solute and not the solute size is the primary factor governing the solute partitioning behavior in a given aqueous two-phase systems” [6, pp. 260].

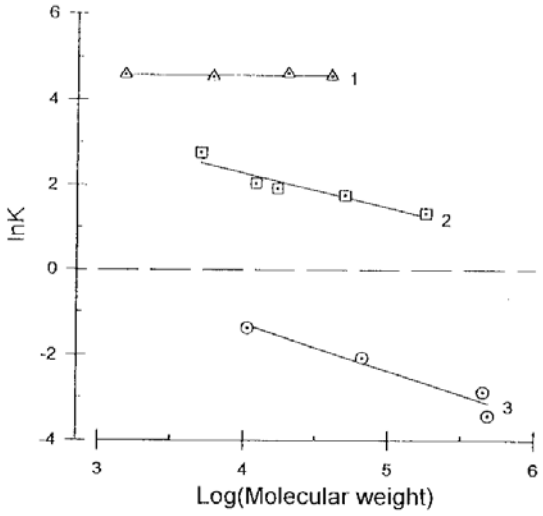

Figure 2.7 Partition coefficients of different molecular weight fractions of PEG (1), polyvinylpyrrolidone (PVP) (2), and polyacrylamide (3) in aqueous Dex-7O---Ficoll-400 two-phase system containing 0.15 mol/kg NaCl in 0.01 mol/kg sodium phosphate buffer, pH 7.4 as functions of the molecular weight of the polymer fraction being partitioned (adapted from [6]).

2.3.1 Theoretical Approaches for Solute Partitioning

There are essentially two lines of reasoning in the literature [6] in regard to the mechanisms of solute partitioning in ATPSs. The underlying concept of the first one is based on solute interactions with the phase-forming components being the driving force for partitioning, while the specific features of water as a solvent in the coexisting phases plays a secondary role. According to the models based on this approach, water is considered as a solvent whose properties are taken into account by coefficients representing deviations from the behavior of ideal solutions defined according to the Lewis-Randall standard state. These models are typically based on the Flory-Huggins theory [16,43-44] or on osmotic virial expansion methods [20-22, 45-50].

One important difference of ATPSs from organic solvent-water biphasic systems is that while a single equilibrium composition exists for any solvent-water system, a variety of quantitatively different compositions exists for an ATPS formed by a given pair of polymers or a single polymer and inorganic salt. The physico-chemical properties in ATPSs (dielectric,

solvatochromic, potentiometric as well as studies of partitioning of homologous series of monofunctional aliphatic compounds [51-55]) were demonstrated to depend on the composition of the equilibrium phases. This composition-dependent physico-chemical properties of aqueous media is not taken into account in the above theoretical approaches. On the other hand, the fact that that the solvent features of the aqueous media in the phases at equilibrium are different is ignored [6].

The underlying concept for the other line of reasoning [6] is that polymers and salts engaged in the formation of an ATPS serve as components that are essentially neutral to the solute being partitioned and are important only in regard to their effects on the solvent features of the aqueous media in the coexisting phases. (It should be mentioned that this concept is clearly inapplicable to the ATPS containing charged polymers or polymers carrying ligands for the so-called affinity partitioning.) This approach is based on the experimental evidence indicating that (a) the solvent features of aqueous media in the coexisting phases of ATPS are different, and (b) there are clear similarities between partitioning of solutes in ATPS and in water-organic solvent systems [6,56-60].

If this concept holds true, then it is possible to describe partitioning in ATPSs using the empirical and semi-empirical approaches frequently used in water-organic solvent biphasic systems.

2.3.2 Collander Equation

About sixty years ago Collander [61] showed that the solute partitioning in different water-organic solvent biphasic systems can be correlated according to the following equation, now universally known as the Collander or solvent regression equation:

$$\log P_i = a_i \log P_o + b_i \qquad (2.6)$$

where a and b are constants; P are partition coefficients in water-organic solvent for any given solute and subscripts “i” and “o” denote the systems under comparison.

Even in his first publication on the subject [61], Collander realized that the parameters a and b could vary, depending both on the type of solutes being compared and on the solvent system.

Later, Leo and Hansch [62-63] found that if the solvent systems being compared were similar, e.g. various alcohols, one equation could fit all the experimental data points. However, for different solvent systems the occurrence of usually three (donor, acceptor, and neutral species) different Collander-type relationships has been suggested. It is also interesting to point out that the intercept on the Collander equation was shown to be correlated with the water solubility in the corresponding organic solvents [62].

The applicability of this relationship to ATPSs for the partition of biomolecules was explored earlier with a limited set of systems formed by Dextran and Ficoll or PEG [6, pp.268-276], and for small organic neutral compounds in systems formed by PEG and various inorganic salts [56-57]. It was particularly found in the case of polymer/polymer ATPSs that the slope and intercept on the Collander equation could be described by independently obtained physico-chemical information assessed from partitioning of homologous series of dinitrophenylated (DNP) amino acids [64].

2.3.3 Abraham's Solvation Equation

The Linear Solvation Energy Relationship (LSER), based on the generalized solvation equation of Abraham [65-67], is probably the most widely used semi-empirical approach for treatments of solute partitioning in water-organic solvent systems and it was recently reported to be applicable to partitioning of solutes in ATPSs [55,57,68,69]. The LSER is based on the empirical determination of the Gibbs energy based descriptors by multiple regressions of chromatographic data obtained from a variety of processes and is represented by the following equation:

$$\log P = vV + rR_2 + sS + aA + bB + z \qquad (2.5)$$

where P is a partitioning property of a solute between two bulk phases of interest; and v, r, s, a, b, and z are fitted coefficients characteristic of a given two-phase system. It should be noted that the solute descriptors V, S, A and B are usually referred to as V_x, π_2^H, $\sum\alpha_2^H$, and $\sum\beta_2^H$, respectively, in older notation. The solute descriptors are defined as follows: Vx is a group-contributable solute volume [70] which accounts for both the solvent cavitation energy and part of the solute-solvent London dispersion interaction; R_2 is the "excess molar refraction" of a solute [67] (the rR_2 term is intended to capture solute-solvent interactions which involve an induced dipole (polarization) on the solute beyond what is accounted by the vV term [71]); parameter S (π_2^H in older notation) is a polarity/polarizability descriptor believed to reflect the interactions associated with both induced and stable polarity of the solute [71]; A and B ($\sum\alpha_2^H$, and $\sum\beta_2^H$ in older notation) refer to the total hydrogen bond donating and accepting capacities of the solute, respectively (or the overall solute hydrogen bond acidity and overall solute hydrogen bond basicity). Finally, the fitted regression constant term, z, depends on the standard states and units of the partitioning property, P [71]. It must be noted that physical explanation of A, B, and S solute descriptors relies on the assumed linear separate contributions of the underlying processes that they are intended to represent [71].

Typically, V and R_2 can be calculated from the structure – V is a simple sum of atom and bond contributions [70], and R_2 is readily obtained from the refractive index or summed from fragment values. The other solute descriptors, S, A, and B may be found experimentally or calculated [65-67]. Once the descriptors for the solutes are known, the partition coefficients for these solutes in a given two-phase system are determined and regression analysis is used to establish the two-phase system-specific coefficients (v, r, s, a, b, and z) [66,67].

The system-specific coefficients can be interpreted as follows [67]. The coefficient r shows the difference between the tendencies of the coexisting phases to interact with solutes through π- and n-electronic pairs. The coefficient s represents the difference between the tendencies of the coexisting phases to interact with dipolar/polarizable solutes. The coefficient a denotes the difference between the hydrogen bond basicity of the phases and coefficient b

characterizes the difference between the hydrogen bond acidity of the phases. The coefficient v is a combination of an exoergic dispersion term and endoergic cavity term.

The approach above described was used by Huddleston et al. [55,57,68,69] to analyze the solvent properties of PEG-salt and Dex-PEG ATPSs. The particular challenge when using this approach to polymer/polymer ATPSs, such as Dex-PEG, is that the partition coefficients of solutes with known solute descriptor values vary within a very limited range, e.g., from 1.054 to 1.690 in the particular Dex-PEG system used by Moody et al. [55]. Therefore, the accuracy of the multiple linear regression analysis performed over such a limited range of the partition coefficients values may be debatable. There are two additional drawbacks if we intend to use this approach for the partitioning of biomolecules in ATPSs. First, the Abraham's methodology does not take into account ion-ion and ion-dipole interactions and second, it is very difficult to obtain Abrahams descriptors for biomolecules [72].

2.4 Conclusions

The aqueous two-phase systems (ATPSs) extraction technique is a relatively recent technology. However, due to their particular advantages, the ATPSs have been object of intense research in the last decades, and have shown to be suitable for several applications. Particularly interesting in this regard are the bioanalytical applications. Their extreme sensitivity towards solute surface properties, and the fact that both phases are aqueous in nature, makes them a potential powerful bioanalytical tool. But before these systems can be utilized on a routine basis in any laboratory, it is first necessary to understand the mechanisms of solute distribution in the systems and properties of the systems at the molecular level.

About fifteen years ago Zaslavsky [6] gave a great contribution in this regard. In his view, ATPSs are seen as two immiscible solvents of aqueous nature, in which the water structure has been modified by the presence of the polymers or salts engaged in its formation. The polymers and salts forming components of an ATPS serve as constituents that are essentially neutral to

the solute being partitioned and are important only in regard to their effects on the solvent features of the aqueous media in the coexisting phases.

One important consequence of this theory is that it should be possible to describe partitioning in ATPSs employing the empirical and semi-empirical approaches used in water-organic solvent biphasic systems.

This is the starting point of this work. First, it will be checked if the Collander equation is observed in ATPSs. The solutes that are under study here are biomolecules, with particular emphasis on proteins. It is also intended to contribute to a better understanding of the biomolecules interactions with aqueous media. But before carrying out the partitioning experiments it is important to have a physico-chemical characterization of ATPSs where the solutes are to be partitioned. This is the content of the next Chapter.

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3. Physico-Chemical Characterization of the Systems

3.1 Introduction

An aqueous two-phase system occurs when different water-soluble polymers or a single polymer and a specific salt are mixed in water above certain concentrations. The system separates into two distinct aqueous-based phases, each preferentially rich with one of the polymers. The ATPSs have been proved to be suitable for differential distribution of biological products [1-4].

Besides its application for the large scale recovery and purification of biological products [1-3,6-7], partitioning in ATPSs may also be used as a bioanalytical tool for characterization of protein surface properties [4,8], changes in protein structure [9], conformation [10], ligand binding [1-3], etc. However, for the successful application of partitioning in ATPSs it is important to have an understanding of the mechanisms of solute distribution in the systems and properties of the systems at molecular level.

From several studies reported in the literature [4-5,8-9,11-15] it seems clear that distribution of low molecular weight solutes as well as biomacromolecules in ATPSs is governed by the differences in the solvent features of aqueous media in the coexisting phases of a particular two-phase system. So far, however, the solvent properties of ATPSs were examined in the systems formed by only two pairs of polymers, Dex-PEG and Dex-Ficoll, [4,11] and those formed by PEG and inorganic salts [5,12-16].

One of the solvent features important for the characterization of ATPSs of different polymer and salt compositions is the free energy of transfer of a methylene group between the coexisting phases [4,5,11-23]. Another approach to quantify solvent features of coexisting

phases in two-phase systems is the solvatochromic method developed by Kamlet et al. [24], which is based on using different solvatochromic probes to quantify solvent polarity/polarizability, s* (or π*), solvent hydrogen bond basicity, a*, and solvent hydrogen bond acidity, b*. This approach was used successfully by Huddleston et al. [25] to characterize the solvent features of PEG-salt ATPSs.

In this Chapter phase diagrams measured for all possible pair combinations of dextran, PEG, Ficoll, Ucon 50-HB5100 (Ucon - a random copolymer of 50% ethylene oxide and 50% propylene oxide), and hydroxypropyl starch (Reppal PES100) all containing 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.4, at 23 ^{0}C, are presented. Distribution coefficients of a series of sodium salts of DNP-amino acids with aliphatic side-chains are studied in all the aqueous two-phase systems at particular polymer concentrations. These data is used to obtain the free energy of transfer of a methylene group between the coexisting phases as a measure of the relative hydrophobic character of aqueous media in the phases of the aqueous two-phase systems. In addition, distribution coefficients for a series of tryptophan and its di- and tri-peptides and a set of p-nitrophenyl-monosaccharides are studied in all the systems. Moreover solvatochromic studies are carried out in the ten ATPSs.

3.2 Experimental

3.2.1. Materials

3.2.1.1. Polymers. All polymers were used without further purification. Dextran 75 (lot 115195), average molecular weight (Mw) ≅ 75 000 was purchased from USB (Cleveland, OH, USA). Polyethylene glycol 8000 (lot 69H00341), Mw = 8000 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ucon 50-HB-5100 (lot SJ1955S3D2), Mw = 3930 was purchased from Dow Chemical (Midland, MI, USA). Ficoll 70 (lot 302970), Mw ≅ 70 000 was purchased from GE Healthcare Biosciences AB (Uppsala, Sweden). Reppal PES-100 (lot D702-09/01), Mw ≅ 100 000 was purchased from REPPE AB (Växjö, Sweden).

3.2.1.2. Amino acids. Dinitrophenylated (DNP) amino acids - DNP-glycine, DNP-alanine, DNP-norvaline, DNP-norleucine, DNP-DL-α-amino-n-octanoic acid, and L-tryptophan were purchased from Sigma. The sodium salts of the DNP-amino acids were prepared by titration.

3.2.1.3. Peptides. Di-tryptophan and tri-tryptophan were purchased from Bachem Bioscience (King of Prussia, PA, USA).

3.2.1.4. Monosaccharides. Nitrophenylated (NP) monosaccharides – 4-NP-β-D-galactopyranoside, 4-NP-β-D-fucopyranoside, 4-NP-β-D-glucopyranoside, and 4-NP-α-D-mannopyranoside were purchased from USB. 4-NP-α-D-glucopyranoside was purchased from Sigma.

3.2.1.5. Solvatochromic Dyes. The solvatochromic probes 4-nitrophenol (reagent grade, >98%) and 4-nitroanisole (>97%, GC) were both supplied by Aldrich, Milwaukee, WI, USA. Reichardt's carboxylated betaine dye was kindly provided by Professor C. Reichardt (Philipps University, Marburg, Germany).

3.2.1.6. Others. o-Phthaldialdehyde (OPA) reagent solution (complete) was purchased from Sigma. All salts and other chemicals used were of analytical-reagent grade.

3.2.2. Methods

3.2.2.1. Phase Diagrams. The systems were prepared by adding appropriate amounts of the aqueous stock ca. 42% (w/w) Dex-75, 50% (w/w) PEG-8000, 70% (w/w) Ucon 50-HB-5100, 48% (w/w) Ficoll-70, or 33% (w/w) PES-100 solution into a 50.0 mL separatory funnel. Appropriate amounts of 3.0 M NaCl, and 1.0 M sodium phosphate buffer, pH = 7.4 were added in order to give the required ionic and polymer composition. Water was finally added to obtain a 25 g amount of final system. After vigorous mixing the systems were allowed to settle for 24 h at room temperature (23 ^{0}C). Samples from both phases were collected for characterization. A

pipette was used to remove the top phase, while the bottom phase was removed through the drain of the separatory funnel.

Dex, Ficoll or PES concentrations were measured by polarimetry (polarimeter AA-1000, Optical Activity, Ramsey, UK). Since Dex, Ficoll and PES are very hygroscopic, the stock solution concentration was determined gravimetrically following freeze-drying of aliquots of pre-determined weights. Concentrations of PEG or Ucon were assayed by refractive index measurements with an ABBEMAT refractometer (Kernchen, Hannover, Germany), taking into consideration the contributions of salts and the other polymers. Chloride was determined by the argentometric method with a relative error < 2.0%. For details see reference 26 pp. 4-67:4-68, and the total phosphorus by the ascorbic acid method with a relative error < 5.0 %. For details see reference 26 pp. 4-146:4-147. The refractive index measurements were performed at 25 °C. The relative uncertainty in polymer concentration determination was < 5 %. All gravimetric measurements were performed with Adam Equipment analytical balance model AAA 250L with a 0.2 mg uncertainty. The PEG-Ucon binodal was determined by the cloud-point method [2] and the tie-lines were assigned from the relationship between the mass phase ratio and system composition using mathematical methods according to procedure presented elsewhere [12].

3.2.2.2. Partitioning.

(a) Phase systems. A mixture of polymers was prepared by dispensing appropriate amounts of the aqueous stock polymer solutions into a 1.2 mL microtube using a Hamilton (Reno, NV, USA) ML-4000 four-probe liquid-handling workstation. Appropriate amounts of stock buffer solutions were added to give the required ionic and polymer composition of the final system with total volume of 0.5 mL. For simplicity, only the polymer compositions of the coexisting phases in the ATPSs used for partitioning of solutes in this study are listed in Table 3.1 together with the corresponding tie-line length (TLL) and slope of tie-line (STL) values. All systems have salt composition of 0.15 M NaCl in 0.01 M sodium phosphate buffer (NaPB), pH 7.4.

(b) Partitioning experiments. An automated instrument for performing aqueous two-phase partitioning, Automated Signature Workstation, ASW (Analiza, Cleveland, OH, USA) was used for the partition experiments. The ASW system is based on the liquid-handling workstation ML-4000 (Hamilton) integrated with a FL600 fluorescence microplate reader (Bio-Tek Instruments, Winooski, VT, USA) and a UV-Vis microplate spectrophotometer (SpectraMax Plus384, Molecular Devices, Sunnyvale, CA, USA). Solutions of all compounds were prepared in water at concentrations of 1–5 mg/mL. Varied amounts (e.g. 0, 15, 30, 45, 60, and 75 µL) of a given compound solution and the corresponding amounts (e.g. 100, 85, 70, 55, 40 and 25 µL) of water were added to a set of the same polymer/buffer mixtures. The systems were vortexed in a Multi-pulse vortexer and centrifuged for 30-60 min. at 1160 g in a refrigerated centrifuge (Jouan, BR4i, Thermo Fisher Scientific, Waltham, MA, USA) to hasten phase settling. The upper phase in each system was removed, the interface discarded, and aliquots of 20 to 70 µL from the upper and lower phases were withdrawn in duplicate for analysis. Peptides and tryptophan samples were combined with 250 µL of o-phthaldialdehyde reagent solution (complete) in microplate wells. After moderate shaking for 2 min. at room temperature, fluorescence was determined with a fluorescence plate reader with a 360 nm excitation filter and a 460 nm emission filter, and with a 100-125 sensitivity setting. Aliquots from both phases in the partition experiments with DNP-amino acids and NP-carbohydrates were diluted with water up to 320 µL in microplate wells. Following moderate shaking for 20 min in an incubator (Vortemp 56EVC, Labnet International, Edison, NJ, USA) at room temperature (23 ^{0}C), optical absorbance was measured at 362 nm and 302 nm for DNP-amino acids and carbohydrates, respectively, with a UV-Vis plate reader. In all measurements the correspondingly diluted pure phases were used as blank solutions. The partition coefficient, K, is defined as the ratio of the sample concentration in the upper phase (mg/mL) to the sample concentration in the lower phase (mg/mL). The K value for each solute was determined as the slope of the concentration in the upper phase plotted as a function of the concentration in the bottom phase averaged over the results obtained from two to four partition experiments carried out at the specific ionic composition of the system. The deviation from the average K value (average of at least 12 independent assays) was always less than 5% and in most cases lower than 2%.

Table 3.1. Polymer compositions[a] of the phases in the aqueous two-phase systems used for partitioning, tie-line lengths (TLL)[b], and average slopes of tie-lines $(STL)_{av}$[c]

No	Poly. 1	Poly. 2	Total composition		Top phase		Bottom phase		TLL[b]	STL_{av}[c]
			Poly. 1	Poly. 2	Poly. 1	Poly. 2	Poly. 1	Poly. 2		
1	Dextran	Ficoll	12.9	18.1	3.2	28.3	21.6	9.0	26.6	-1.05 ± 0.02
2	Dextran	PEG	12.4	6.1	0.3	13.0	22.4	0.5	25.4	-0.566 ± 0.007
3	Dextran	Ucon	12.4	10.1	0.2	18.3	26.5	0.6	31.7	-0.682 ± 0.008
4	PES	Dextran	17.3	12.4	5.3	21.7	31.4	1.9	32.7	-0.74 ± 0.01
5	PEG	Ucon	15.0	30.0	0.4	50.3	35.5	1.6	60.0	-1.41 ± 0.02
6	Ficoll	PEG	15.1	7.9	9.6	11.7	24.0	1.8	17.5	-0.684 ± 0.003
7	Ficoll	Ucon	13.0	9.9	2.9	16.4	24.5	2.5	25.7	-0.638 ± 0.008
8	PES	Ficoll	17.3	14.9	10.3	20.2	25.4	7.8	19.5	-0.822 ± 0.008
9	PES	PEG	15.2	7.0	3. 7	12.3	29.6	0.4	28.5	-0.467 ± 0.007
10	PES	Ucon	12.9	7.7	2.8	13.5	24.0	1.3	24.5	-0.55 ± 0.02

[a] – polymer concentrations are given in % (w/w); [b] – Tie-Line Length (TLL) is calculated as $TLL = (\Delta_{Polymer\text{-}1}^{2} + (\Delta_{Polymer\text{-}2}^{2})^{1/2}$, where $\Delta_{Polymer}$ is the difference between the given polymer concentrations in the coexisting phases; [c] – average slope of tie-line (STL) is calculated as the mean of slopes of tie lines determined as the ratio $STL = \Delta_{Polymer\text{-}2}/\Delta_{Polymer\text{-}1}$ for four different polymer compositions of each particular two-phase system.

3.2.2.3. Solvatochromic Studies. The ATPSs of the compositions listed in Table 3.1 were prepared as mentioned. The phases were separated and used for solvatochromic analysis. The solvatochromic probes 4-nitroanisole, 4-nitrophenol and Reichardt's carboxylated betaine were used to measure the polarity/polarizability π*, H-bond acceptor (HBA) basicities a*, and H-bond donor (HBD) acidities b* in both phases of each particular ATPS. Three to four concentrations of each probe were prepared for each analysis in order to check for aggregation effects by adding 10 to 40 µL of solvatochromic probe stock solution to 1.1 mL of the ATPS phase sample. 20 µL of NaOH (1M) were added to the samples containing the Reichardt's carboxylated betaine to ensure a basic pH. The samples were mixed thoroughly in a vortex mixer and then scanned in a UV-Vis spectrophotometer (Thermo Electron Corp., model UV1). A scan of the phase (blank) was performed first to build the base-line, and then the different mixtures were scanned in the

UV-Vis spectrophotometer in triplicate or more. The maximum wavelength was the average between these scans. Average standard deviations of all measured wavelengths were always below 1.0 nm and in most cases < 0.7 nm for all probes. 20 µL of water was added to the systems to prevent clouding. A few drops of HCl (1 M) were added to the samples containing the 4-nitrophenol, in order to eliminate charge transfer bands that were observed in some systems.

Determination of the solvent polarity π^*

π^* was determined from the wave number ($\nu_{(1)}$) of the longest wavelength absorption band of the 4-nitroanisole dye using the relationship given by Marcus [27]:

$$\pi^* = 0.427(34.12 - \nu_{(1)}) \qquad (3.1)$$

Determination of the solvent hydrogen-bond acceptor basicity a*

a* values were determined from the wave number ($\nu_{(2)}$) of the longest wavelength absorption band of the 4-nitrophenol dye using the relationship [27]:

$$a^* = 0.346(35.045 - \nu_{(2)}) - 0.547\pi^* \qquad (3.2)$$

Determination of the solvent hydrogen-bond donor acidity b*

b* values were determined from the longest wavelength absorption band of Reichardt's betaine dye using the relationship [27]:

$$b^* = 0.0649E_T(30) - 2.03 - 0.72\pi^* \qquad (3.3)$$

The $E_T(30)$ value was obtained directly from the wavelength absorption band of the probe as

$$E_T(30) = 28591/\lambda \qquad (3.4)$$

where λ is the wavelength in nm.

The solvatochromic parameters obtained in the phases of the ATPSs under analysis are presented in Table 3.2. The differences between the values found for the top phases and those for the corresponding bottom phases are also given.

Table 3.2. Solvatochromic solvent parameters characterizing solvent polarity (π*), solvent hydrogen-bond donor acidity (b*), and solvent hydrogen-bond acceptor basicity (a*), and the differences[a], Δπ*, Δb*, and Δa*, between these characteristics of the media in the coexisting phases of the aqueous two-phase systems.

#	Polymer 1[b]	Polymer 2[b]	Top phase			Bottom phase			Δπ*[a]	Δ b*[a]	Δa*[a]
			π*	b*	a*	π*	b*	a*			
1	Dextran	Ficoll	1.188	0.984	0.633	1.150	1.048	0.678	0.038	-0.064	-0.045
2	Dextran	PEG	1.099	1.078	0.632	1.167	1.096	0.624	-0.068	-0.018	0.008
3	Dextran	Ucon	1.112	0.882	0.659	1.179	1.023	0.597	-0.068	-0.141	0.062
4	PES	Dextran	1.275	0.995	0.596	1.163	0.997	0.681	0.113	-0.002	-0.084
5	PEG	Ucon	1.035	0.628	0.757	1.158	0.766	0.697	-0.123	-0.138	0.060
6	Ficoll	PEG	1.116	0.999	0.612	1.167	0.976	0.634	-0.051	0.023	-0.022
7	Ficoll	Ucon	1.146	0.850	0.667	1.031	1.063	0.644	0.115	-0.213	0.023
8	PES	Ficoll	1.137	1.050	0.668	1.196	0.996	0.628	-0.059	0.054	0.040
9	PES	PEG	1.116	1.032	0.595	1.175	0.962	0.697	-0.059	0.069	-0.102
10	PES	Ucon	1.133	0.640	0.904	1.209	0.634	0.962	-0.076	-0.059	0.006

[a] All differences were calculated between values measured for the top phases and those measured for the bottom phases; [b] Polymer 1 – predominant polymer in the bottom phase; Polymer 2 – predominant polymer in the top phase (polymer composition of each phase – see Table 3.1)

3.3 Results and Discussion

Phase diagrams for ATPSs formed by Dextran with PEG, Ucon, Ficoll, and PES are presented in figure 3.1. The phase diagram for each ATPS was obtained by analysis of polymer composition of coexisting phases at four different polymer concentrations as indicated above. For clarity, however, only particular compositions used for partitioning experiments are denoted in figure 3.1 with the tie-lines. If we assume that the polymer concentration required for phase separation in an aqueous mixture with Dextran is indicative of the polymer effect on the water structure, we might conclude from the data in figure 3.1 that the effect of a polymer on the water structure has the following trend: Ucon ≈ PEG > PES > Ficoll.

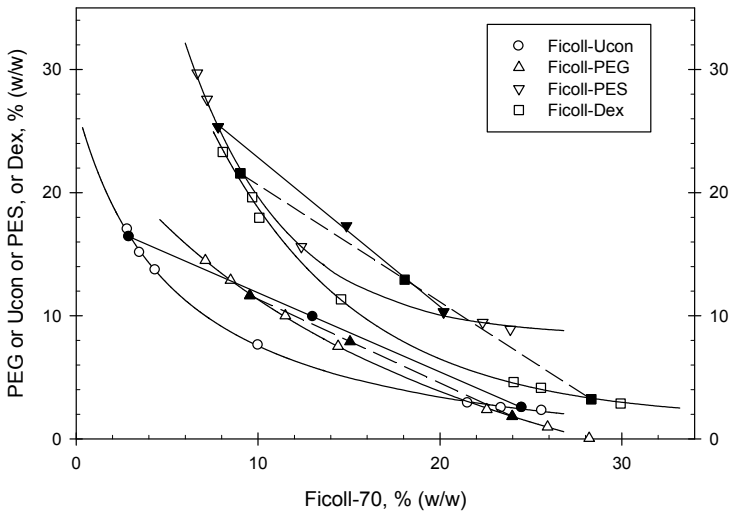

Figure 3.1 Phase diagrams for Dex-75-PEG-8000, Dex-75-Ucon50HB5100, Dex-75-Ficoll-70, and Dex-75-PES-100 ATPSs at 23°C. Tie-lines are shown for particular compositions used in partition experiments.

Phase diagrams for the ATPSs formed by Ficoll with Dextran, PEG, Ucon, and PES are presented in figure 3.2 in the same way as described above. From these data we can conclude that the effect of a polymer on the water structure has the following trend: Ucon > PEG > Dex > PES.

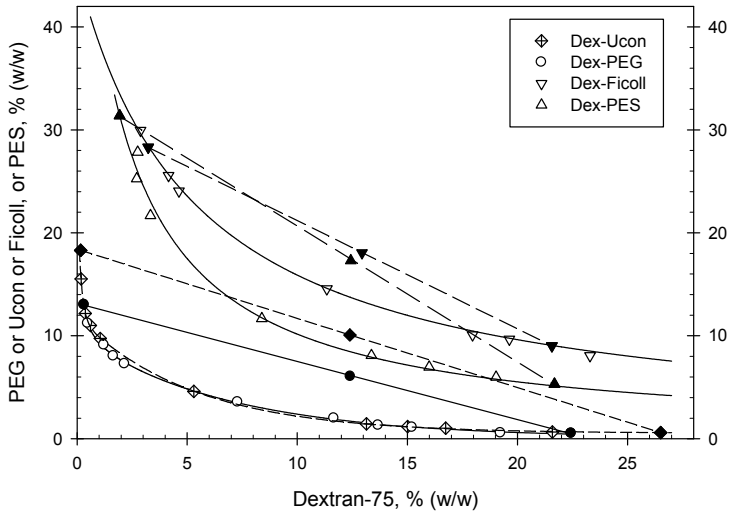

Figure 3.2 Phase diagrams for Ficoll-70-Ucon50HB5100, Ficoll-70-PES-100, Ficoll-70-PEG-8000, and Ficoll-70-Dex-75 (same as in figure 3.1) ATPSs at 23°C. Tie-lines are shown for particular compositions used in partition experiments.

Phase diagrams for the ATPSs formed by PES and either PEG or Ucon are shown in figure 3.3. Phase diagrams for the systems formed by PES with Dex and with Ficoll are also plotted in figures 3.1 and 3.2, respectively. It follows from all these data that in the presence of PES the effect of a polymer on the water structure decreases in the series: PEG >/< Ucon > Dex > Ficoll (where >/< denotes the observation that while Ucon seems to display stronger structural effect in the presence of relatively low PES concentrations in water, PEG appears to affect the water structure in a more pronounced manner once the PES concentration is above ~7.5 % (w/w)).

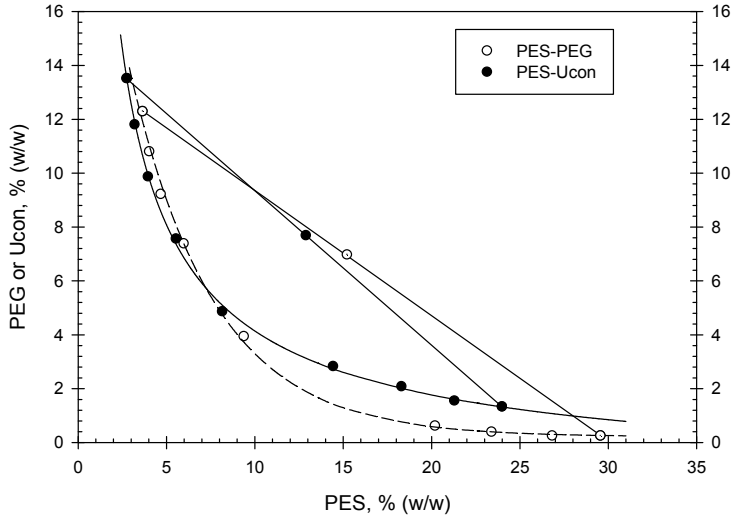

Figure 3.3 Phase diagrams for PES-100-PEG-8000 and PES-100-Ucon50HB5100 ATPSs at 23^{o}C. Tie-lines are shown for particular compositions used in partition experiments.

Phase diagrams for the ATPSs formed by PEG with Dextran, Ficoll, Ucon, and PES are presented in figure 3.4. From these data it follows that the effect of a polymer on the water structure decreases in the series: Dex > PES > Ficoll > Ucon.

From similar analysis of the ATPSs formed by Ucon with Dextran, Ficoll, PEG, and PES, it follows that the effect of a polymer on the water structure decreases in the series: Dex > PES > Ficoll > PEG. If we classify all the polymers used in the present work according to their chemical nature, i.e., as polysaccharides (Dex, Ficoll and PES) and poly(alkylene glycols) (PEG and Ucon),

the series observed seem to agree with the above assumption that the polymer concentration required for phase separation in an aqueous mixture with another polymer is indicative of the polymer effect on the water structure. It seems reasonable to expect that the effects of polysaccharides on the water structure would be relatively similar, same as those of poly(alkylene glycols). Thus in the presence of, e.g., Dex, it is not surprising that PES and Ficoll aqueous solutions will give rise to phase separation at higher concentrations than PEG or Ucon aqueous solutions, resulting in the obtained sequence: Ucon ≈ PEG > PES > Ficoll. Also, the extremely high compatibility of PEG and Ucon aqueous solutions is in agreement with this view due to the structural similarity between these polymers.

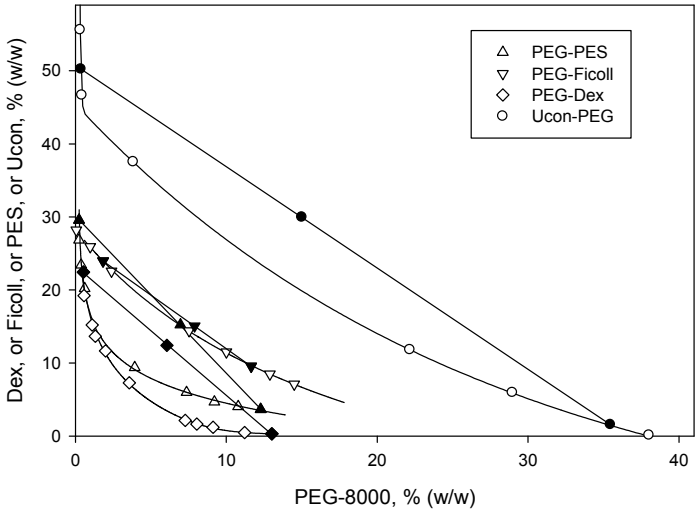

Figure 3.4 Phase diagrams for PEG-8000-Ucon50HB5100, PEG-8000-PES-100, PEG-8000-Ficoll-70, and PEG-8000-Dex-75 ATPSs at 23^{o}C. Tie-lines are shown for particular compositions used in partition experiments.

Typical experimental partitioning data obtained for sodium salts of DNP-amino acids in different ATPSs are plotted in figure 3.5, and the linear curves observed may be described as:

$$\ln K^{(i)}_{DNP\text{-}AA} = C^{(i)} + E^{(i)} N_C \qquad (3.5)$$

where K_{DNP-AA} is the partition coefficient of a DNP-amino acid with aliphatic side-chain; superscript (i) denotes the particular i-th ATPS used for the partition experiments; N_C is the equivalent number of CH_2 groups in the aliphatic side-chain of a given DNP-amino acid; E is an average ln K increment per CH_2 group; C represents the total contribution of the non-alkyl part of the structure of a DNP-amino acid into ln K_{DNP-AA}.

The $E^{(i)}$ and $C^{(i)}$ values obtained in the ATPSs examined in this essay are listed in Table 3.3. It should be noted here that the partition coefficient of a DNP-amino acid, K_{DNP-AA}, was determined in each ATPS as the ratio of the solute concentration in the top phase to that in the bottom phase. Therefore, the values of coefficient E listed in Table 3.3 may be positive or negative depending on what particular phase (upper or lower) is more hydrophobic than the other phase at equilibrium. As the free energy of transfer of a solute between the coexisting phases is described by:

$$\Delta G = -RT\ln K \qquad (3.6),$$

where R is the universal gas constant and T is the absolute temperature, it follows that

$$\Delta G(CH_2) = -RTE \qquad (3.7),$$

where $\Delta G(CH_2)$ is the free energy of transfer of a methylene group from one coexisting phase to another. The $\Delta G(CH_2)$ values calculated from the experimental data with Eqs. 3.5-3.7 are listed in Table 3.3. In this Table and elsewhere, r^2 is the correlation coefficient; F the ratio of variance and SD the standard deviation obtained by standard statistical analysis from the linear regression using 95% confidence intervals. It should be noted that in some cases not statistical significant parameters were observed and were purposely included in order to treat all the data in the same way for a better comparison.

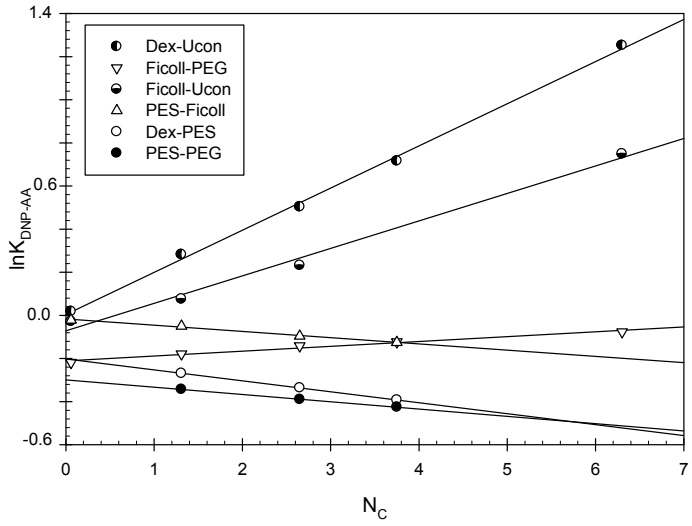

Figure 3.5. Distribution coefficients of sodium salts of DNP-amino acids with aliphatic side-chains as functions of side-chain lengths in different ATPSs as indicated.

Table 3.3. Results of partitioning of sodium salts of DNP-amino acids with aliphatic side-chains (Gly, Ala, NVal, NLeu, α-amino-n-octanoic acid)[a]

No	Poly. 1*	Poly. 2*	C[a]	E[a]	r^2	F ($x10^{-3}$)	SD ($x\ 10^{3)}$	$\Delta G(CH_2)$ (cal/mol)
1	Dextran	Ficoll	0.125 ± 0.005	0.044 ± 0.001	0.997	1.00	6.6	-26.1 ± 0.6
2	Dextran	PEG	-0.089 ± 0.007	0.062 ± 0.002	0.997	0.63	9.1	-37 ± 1
3	Dextran	Ucon	0.00 ± 0.02	0.196 ± 0.005	0.998	1.6	22	-116 ± 3
4	PES	Dextran	-0.203 ± 0.0002	-0.051 ± 0.0001	1.000	104	0.3	+30.20 ± 0.06
5	PEG	Ucon	1.402 ± 0.007	0.245 ± 0.003	1.000	6.09	8.4	-144 ± 11
6	Ficoll	PEG	-0.211 ± 0.007	0.022 ± 0.002	0.978	0.13	9.4	-13 ± 1
7	Ficoll	Ucon	-0.07 ± 0.03	0.127 ± 0.008	0.991	0.23	4.0	-75 ± 5
8	PES	Ficoll	-0.017 ± 0.004	-0.029 ± 0.002	0.995	0.36	4.2	+17 ± 1
9	PES	PEG	-0.300 ± 0.0007	-0.034 ± 0.0003	1.000	5.1	0.8	+20.1 ± 0.2
10	PES	Ucon	-0.27 ± 0.02	0.08 ± 0.01	0.974	0.04	24.7	-49 ± 8

[a] – partitioning of sodium salts of DNP amino acids is described by Eq. 3.5; coefficients C and E are calculated from the experimental data using Eq. 3.5; * Polymer 1 – predominant polymer in the bottom phase; Polymer 2 – predominant polymer in the top phase.

According to the $\Delta G(CH_2)$ values presented in Table 3.3, the relative hydrophobic character of the phases increases in the following series: Dex-rich phase < Ficoll-rich phase < PEG-rich phase < PES-rich phase < Ucon-rich phase. The particular position of each phase in this series may vary, however, depending upon the particular composition of the phase and that of the reference phase indicating that the particular compositions of the phases under comparison

affect the difference between the hydrophobic characters of the phases. In other words, we may have a Dex-PEG composition in which $\Delta G(CH_2)$ is higher than in a particular Dex-Ucon ATPS. However, when we measure the same quantity in the system PEG-Ucon, the Ucon rich phase will always have more affinity for a CH_2 group. It should also be noticed here that the tie-line length (TLL), which is commonly used as a convenient measure of the divergence of the properties of the phases for an ATPSs of different initial feed polymer concentration is not valid for comparison of the data obtained in the systems formed by different polymers. It seems that the difference between the hydrophobic characters of the coexisting phases may serve as a better general measure for the ATPSs comparison. In order to test this assumption, however, we need to consider the data obtained in the partition experiments with Trp and its di- and tri-peptides and monosaccharides.

Typical experimental data obtained for the series of homooligopeptides of Trp in different ATPSs are plotted in figure 3.6, and the linear curves observed may be described as:

$$\ln K^{(i)} = a^{(i)} + \Delta \ln K^{(i)}_{Trp} n \qquad (3.8)$$

where $K^{(i)}$ is the partition coefficient of a peptide or free Trp in the i-th ATPS; n is the number of Trp residues in the solute molecule, $a^{(i)}$ is a constant, and $\Delta K^{(i)}_{Trp}$ is the slope representing the total contribution of a n-th Trp residue and newly formed peptide bond into the partition coefficient of a homooligopeptide (Table 3.4).

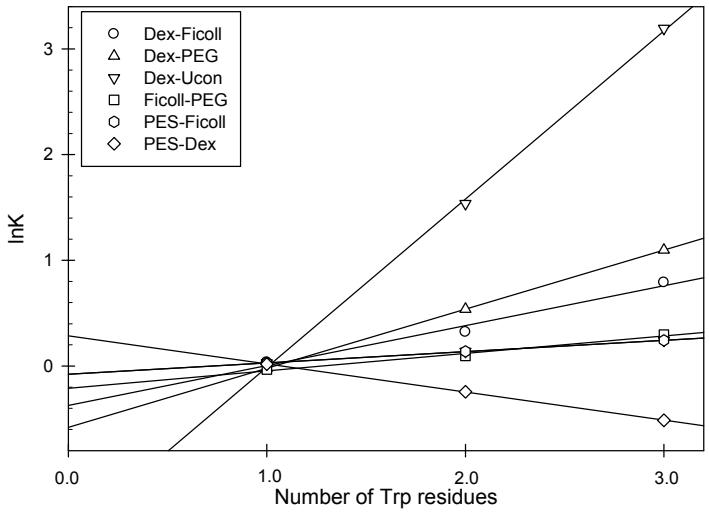

Figure 3.6. Distribution coefficients of tryptophan and its di- and tri-peptides as functions of the number of Trp residues in the molecule in different ATPSs.

Table 3.4. Results of partitioning of series of homooligopeptides of tryptophan[a] in the aqueous two-phase systems indicated

No	Poly. 1*	Poly. 2*	a[b]	$\Delta \ln K_{Trp}$[b]	r^2	F	SD
1	Dextran	Ficoll	-0.4 ± 0.1	0.38 ± 0.05	0.981	52	0.07
2	Dextran	PEG	-0.579 ± 0.0001	0.559 ± 0.0001	1.000	>9000	<0.001
3	Dextran	Ucon	-1.60 ± 0.08	1.59 ± 0.04	0.999	1710	0.05
4	PES	Dextran	0.285 ± 0.006	-0.266 ± 0.003	1.000	9152	0.004
5	PEG	Ucon	-2.677 ± 0.0002	1.842 ± 0.0001	1.000	>9000	0.0002
6	Ficoll	PEG	-0.21 ± 0.05	0.17 ± 0.02	0.984	62	0.03
7	Ficoll	Ucon	-1.438 ± 0.0002	0.936 ± 0.0001	1.000	>9000	0.0001
8	PES	Ficoll	-0.077 ± 0.003	0.107 ± 0.002	1.000	4883	0.002
9	PES	PEG	0.44 ± 0.09	0.35 ± 0.04	0.987	77	0.06
10	PES	Ucon	-0.533 ± 0.002	0.504 ± 0.001	1.000	>9000	0.002

[a] — partitioning of homooligopeptides of Trp described by Eq. 3.8; coefficients a and $\Delta \ln K_{Trp}$ were determined from experimental data using Eq. 3.8; * Polymer 1 – predominant polymer in the bottom phase; Polymer 2 – predominant polymer in the top phase.

The $E^{(i)}$ and $\Delta \ln K^{(i)}_{Trp}$ values obtained in the ATPSs examined are listed in Tables 3.3 and 3.4. For eight out of the ten ATPSs used in this work there is a clear correlation between the $\Delta \ln K^{(i)}_{Trp}$ and $E^{(i)}$ values. It should be mentioned that when both $\Delta \ln K^{(i)}_{Trp}$ and $E^{(i)}$ values are negative, as observed in the case of Dextran-PES two-phase system, the signs may be reversed, since this means only that the K values should be determined as the ratios of concentrations of a solute in the bottom (more hydrophobic) PES-rich phase to those in the top Dex-rich phase

for both series of DNP-amino acids and Trp peptides. The correlation is presented in figure 3.7, and it is described as:

$$\Delta \ln K^{(i)}_{Trp} = 0.04(\pm 0.04) + 7.4(\pm 0.3)\, E^{(i)} \qquad (3.9)$$

$$N = 8;\ r^2 = 0.9879$$

where N is the number of different ATPS, and r^2 is the correlation coefficient.

This correlation seems to indicate that the difference between the relative hydrophobic characters of the coexisting phases represented by coefficient $E^{(i)}$ (or the corresponding $\Delta G(CH_2)$ value) is a general measure enabling comparison of the partitioning of solutes in ATPSs formed by different polymers.

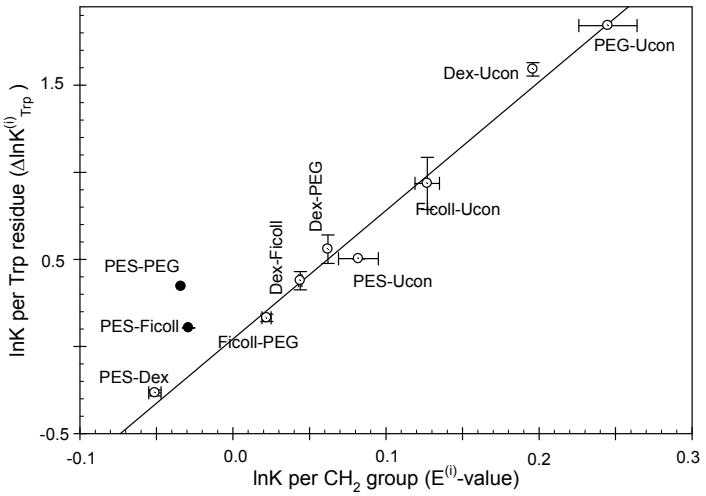

Figure 3.7. Contribution of a Trp residue into lnK of a homooligopeptide versus coefficient E (contribution of a CH_2 group into ln K for DNP-amino acids with aliphatic side-chains) in different ATPSs. Filled symbols denote the ATPSs not fitting the correlation.

However, two ATPSs, formed by PES and PEG or PES and Ficoll do not fit the above correlation. While in all other ATPSs, partitioning of Trp and its peptides follows the same trend as displayed by DNP-amino acids with aliphatic side-chains, in these two systems Trp and its

homooligopeptides distribute into the phase opposite to the one displaying higher affinity for a CH_2 group.

Typical experimental data obtained for p-nitrophenyl-monosaccharides in different ATPSs are plotted in figure 3.8, and the linear curves observed may be described as:

$$\ln K_j^{(i)} = a_j + b_j E^{(i)} \qquad (3.10)$$

where $K_j^{(i)}$ is the partition coefficient of a j-th p-nitrophenyl-monosaccharide in the i-th ATPS; a_j and b_j are constant coefficients, and $E^{(i)}$ is as defined above, i.e., the measure of the difference between the relative hydrophobic characters of the coexisting phases in the i-th ATPS.

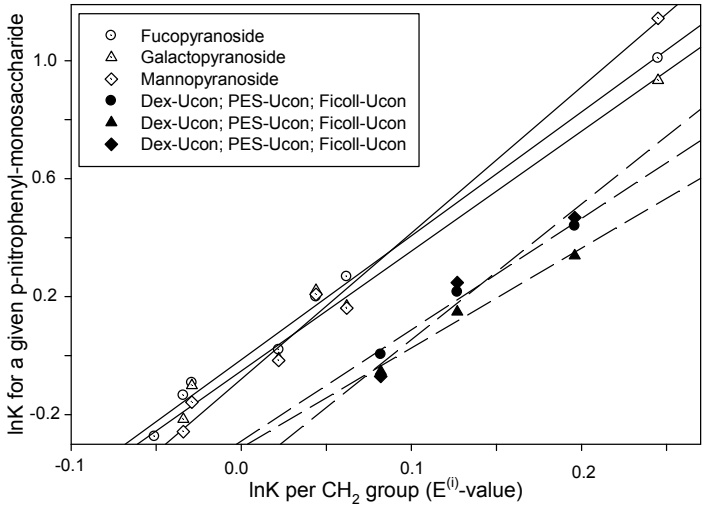

Figure 3.8. Partition coefficients of 4-nitrophenyl-β-D-fucopyranoside, 4-nitrophenyl-β-D-galactopyranoside, and 4-nitrophenyl-α-D-mannopyranoside versus coefficient E (contribution of a CH_2 group into lnK for DNP-amino acids with aliphatic side-chains) in different ATPSs. Filled symbols denote the aqueous Dex-Ucon, PES-Ucon, and Ficoll-Ucon two-phase systems not fitting the correlation.

The coefficients a_j and b_j obtained for all the monosaccharides studied are listed in Table 3.5. For seven out of the ten ATPSs examined there is a clear correlation between the $\ln K_j^{(i)}$ and $E^{(i)}$ values. It should be mentioned that when the $\ln K_j^{(i)}$ for each monosaccharide and $E^{(i)}$ values are negative, as observed in the case of PES-Ficoll two-phase system, the signs may be reversed, since this means that the K values could be determined as the ratios of concentrations of a solute in the bottom (more hydrophobic) PES-rich phase to those in the top

Ficoll-rich phase for both series of DNP-amino acids and monosaccharides under study. It should be noticed that partition coefficients for each monosaccharide studied do not fit the correlation described by Eq. 3.10 in three ATPSs – Dex-Ucon, PES-Ucon, and Ficoll-Ucon. The partition coefficients for the monosaccharides in these systems follow the same trends as can be seen from figure 3.8 but they are down-shifted from the correlations observed in the other ATPSs.

Table 3.5 Results of partitioning of p-nitrophenyl-monosaccharides[a] in the aqueous two-phase systems

Carbohydrate	A	b	N	r^2	F	SD
Fucopyranoside	-0.01 ± 0.02	4.2 ± 0.2	7	0.9915	581	0.04
Galactopyranoside	-0.05 ± 0.02	4.1 ± 0.2	7	0.9807	254	0.06
β-Glucopyranoside	-0.02 ± 0.02	3.9 ± 0.2	7	0.9888	440	0.05
α-Glucopyranoside	-0.02 ± 0.01	3.9 ± 0.1	7	0.99311	721	0.04
α-Mannopyranoside	-0.08 ± 0.02	5.0 ± 0.2	7	0.9893	462	0.06

[a] – partitioning of each p-nitrophenyl-monosaccharide is described by Eq. 3.10; coefficients a and b are calculated from the experimental data using Eq. 3.10; N – number of ATPSs (see in Table 3.11); partition coefficients for each monosaccharide in Dex-Ucon, PES-Ucon, and Ficoll-Ucon did not fit the correlations (see text)..

More experimental studies are clearly necessary, but the unavoidable conclusion from this observation seems to be that the difference between the hydrophobic characters of the coexisting phases represented by the coefficient E or $\Delta G(CH_2)$ value alone cannot be used as a universal measure for comparison of the ATPSs of different polymer compositions. Properties of the phases are likely to be too complex to be characterized by a single measure. Further studies to gain better insight into this issue will be discussed in the next Chapters.

Analysis of the solvent-specific coefficients determined experimentally for the ATPSs under analysis (Tables 3.1 and 3.2) shows that there is a strong linear correlation between coefficient E and the difference between the solvent polarity $\Delta\pi^*$ and the solvent hydrogen-bond donor acidity Δb^* of the coexisting phases. This correlation may be described as:

$$E = 0.00(\pm 0.01) - 1.1(\pm 0.1)\Delta b^* - 0.7(\pm 0.1)\Delta\pi^* \qquad (3.11)$$

$$n = 10,\ r^2 = 0.9508,\ SD = 0.03;\ F = 67.6$$

The consequences of this equation will be discussed on Chapters 6 and 7.

3.4 Conclusions

Partition coefficients of a series of DNP-amino acids, tryptophan and its di- and tri-peptides and a set of NP-monosaccharides were measured in ten aqueous two-phase systems formed by all possible pair combinations of Dex-75, Ficoll-70, PEG-8000, PES-100, and Ucon50HB5100, all containing 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.4, at 23 ^{0}C. The data obtained were compared with the $\Delta G(CH_2)$ values determined in all the systems. For eight out of the ten two-phase systems of different polymer compositions the partition coefficients for Trp peptides correlate well with the $\Delta G(CH_2)$-values. Similar correlations for NP-monosaccharides are valid for seven out of the ten two-phase systems. These observations indicate that the difference between the hydrophobic characters of the coexisting phases represented by the $\Delta G(CH_2)$ value is an adequate descriptor of the solvent properties of the phases. However, we conclude that this parameter alone cannot be used as a single universal measure for comparison of the ATPSs of different polymer compositions. It is suggested that additional solvent features of the aqueous media in the coexisting phases must be used to describe biomolecules partitioning in ATPSs.

The solvatochromic solvent parameters characterizing the solvent polarity (π^*), solvent hydrogen-bond donor acidity (b*), and solvent hydrogen-bond acceptor basicity (a*) of aqueous media were also measured in the coexisting phases of the above ten different aqueous polymer/polymer two-phase systems (ATPSs). Analysis of these parameters shows that there is a strong linear correlation between coefficient E and the difference between the solvent polarity $\Delta\pi^*$ and the solvent hydrogen-bond donor acidity Δb* of the coexisting phases.

3.5. References

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4. Correlations between distribution coefficients of various biomolecules in different polymer/polymer aqueous two-phase systems

4.1 Introduction

One of the current line of reasoning in the literature [1] in regard to the mechanisms of solute partitioning in ATPSs is that polymers and salts engaged in formation of an ATPS serve as components that are essentially neutral to the solute being partitioned and are important only in regard to their effects on the solvent features of the aqueous media in the coexisting phases. (It should be mentioned that this concept is clearly inapplicable to the ATPSs containing charged polymers or polymers carrying ligands for the so-called affinity partitioning.) This approach is based on the experimental evidence indicating that (a) the solvent features of aqueous media in the coexisting phases of ATPSs are different [1,2], and (b) there are clear similarities between partitioning of solutes in ATPSs and in water-organic solvent systems [1-6].

One of the important features of organic solvent-water biphasic systems is the linear relationship between the logarithms of the distribution coefficients of solutes of the same chemical nature in different solvent biphasic systems [1, pp.100-103]. The applicability of this relationship to ATPSs for the partition of biomolecules was explored earlier with a limited set of systems formed by Dextran and Ficoll or PEG [1, pp.268-276], and for small organic neutral compounds in systems formed by PEG and various inorganic salts [2,3]. It seems reasonable to expect that the analysis of relationships between distribution coefficients of various biomolecules in different ATPSs may provide information useful for practical purposes as well as for better insight into the mechanisms of partitioning.

In this Chapter, we examine the distribution coefficients for a variety of proteins and low molecular weight solutes in ATPSs formed by Dex and PEG and by Dex and Ucon, both containing 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.4, at 23^0C.

4.2. Experimental

4.2.1. Materials

4.2.1.1. Proteins. Chicken egg lysozyme (#L-6876), bovine α-chymotrypsinogen A (#C-4879), bovine hemoglobin (#H-2500), horse heart cytochrome c (#C-7752), bovine ribonuclease B (#R-7884), bovine ribonuclease A (#R-5000), bovine trypsinogen (#T-1143), human hemoglobin (#H-7379), horse myoglobin (#M-0630), bovine β-lactoglobulin (#L-3908), and human γ-globulin (#G-4386) were purchased from Sigma. Porcine lipase (#18480), human transferrin (#22508), and bovine γ-globulin (#16021) were purchased from USB. Numbers in parenthesis denote the catalog ID numbers for the particular products in the corresponding catalogs.

4.2.1.2 Others. All polymers, amino acids, peptides, monosaccharides and o-Phthaldialdehyde (OPA) reagent solution (complete) used were the same as previously described (Chapter 3).

4.2.2. Methods

4.2.2.1. Phase Diagrams. The systems were prepared as described in Chapter 3.

4.2.2.2. Partitioning. The partitioning measurements were carried out as described in Chapter 3. The protein samples were treated in an analogous manner to that of peptides (see Chapter 3). The deviation from the average K-value was always less than 5%, and in most cases lower than 2%.

4.3. Results and Discussion

Phase Diagrams

Figure 4.1 presents the phase diagrams of both Dex-75-PEG-8000 and Dex75-Ucon50HB5100 two-phase systems. The corresponding polymer compositions are given in Table 4.1. In this Table numbers I-VI correspond to the systems used for the partitioning experiments. It can be seen from comparison of the phase diagrams represented in figure 4.1 that while the binodal curves for both ATPSs are so close that they almost coincide, the slopes of the tie-lines are constant, for each system, and very different for the two systems. Albertsson was the first researcher to report that while the tie-line slope (TLS) is usually constant for a given ATPS there are exceptions [7].

Taking into account the definitions of tie-line length (TLL) and tie-line slope (TLS) (equations 2.1 and 2.2, respectively) and combining the Equations we can obtain the following linear relationship:

$$TLL = [1 + (1/TLS)^2]^{1/2} \Delta P_1 \qquad (4.1).$$

where the symbols have the same meaning as before.

Equation 4.1 is a simple way to confirm/reject the assumption of the TLS being constant. The experimental data are plotted in figure 4.2 and the slopes of both experimental linear curves in the graph are 2.03 ± 0.02 for Dex-PEG system and 1.78 ± 0.02 for Dex-Ucon system. These values agree very well with those calculated from the above equation (using the experimentally obtained values of TLS, for each system –Table 4.1) – 2.030 and 1.775, respectively.

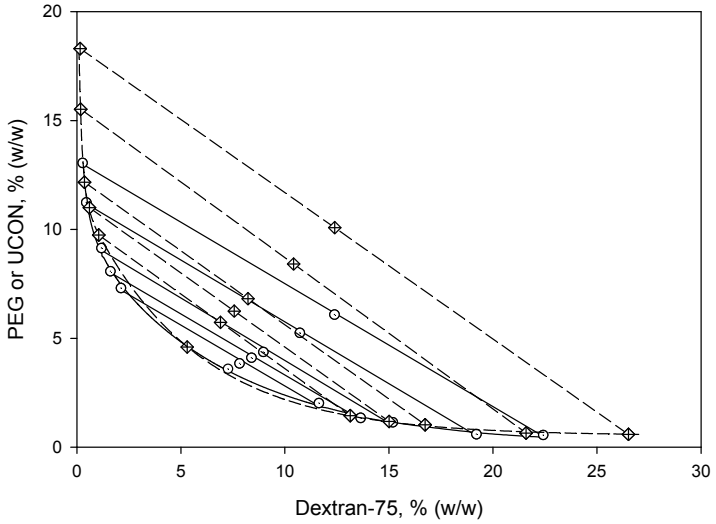

Figure 4.1. Phase diagrams of Dex75-PEG8000 (—) and Dex75-Ucon50HB5100 (---) ATPSs at 23^0C.

Table 4.1. Phase compositions for Dextran-75-PEG-8000 and Dextran-75-Ucon-50-HB-5100 two-phase systems (total ionic composition: 0.15M NaCl in 0.01 M Na-phosphate buffer, pH 7.4)

Dextran-75-PEG-8000-0.15M NaCl-0.01 M Na-phosphate buffer (NaPB), pH 7.4								
System	Total composition		Top Phase			Bottom Phase		
	Dextran, %(w/w)	PEG, %(w/w)	Dextran, %(w/w)	PEG, %(w/w)	NaPB, M	Dextran, %(w/w)	PEG, %(w/w)	NaPB, mM
I	12.4	6.1	0.3	13.0	4.8	22.4	0.5	12.5
II	10.8	5.2	0.5	11.2	5.9	19.2	0.6	13.0
III	9.0	4.3	1.2	9.1	6.5	15.2	1.1	11.4
	8.4	4.1	1.6	8.1	6.9	13.7	1.3	11.1
	7.9	3.8	2.2	7.3	7.0	11.7	2	10.6
DP	7.3	3.6						
Dextran-75-Ucon-50HB5100-0.15M NaCl-0.01 M Na-phosphate buffer (NaPB), pH 7.4								
	Total composition		Top Phase			Bottom Phase		
	Dextran, %(w/w)	Ucon, %(w/w)	Dextran, %(w/w)	Ucon, %(w/w)	NaPB, M	Dextran, %(w/w)	Ucon, %(w/w)	NaPB, M
IV	12.39	10.1	0.2	18.3	3.2	26.5	0.6	13.2
V	10.42	8.4	0.2	15.5	3.9	21.6	0.7	12.4
VI	8.23	6.8	0.4	12.2	5.1	16.7	1.0	12.7
	7.57	6.3	0.6	11.0	5.7	15.0	1.2	10.9
	6.90	5.7	1.1	9.7	6.3	13.1	1.5	10.6
DP	5.30	4.6						

DP – disruption point

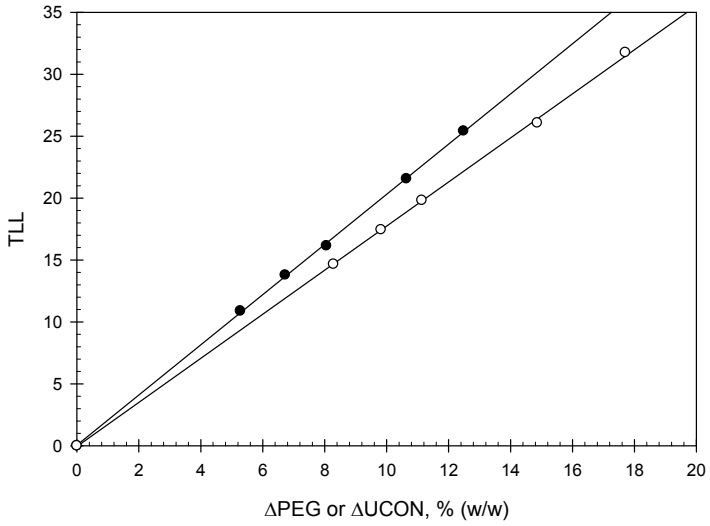

Figure 4.2. Tie Line Length (TLL) in aqueous Dextran-PEG and Dextran-Ucon two-phase systems as a function of ΔP. Δ denotes the difference between the polymer weight fractions (P) in the coexisting phases. P is PEG (•) or Ucon (o).

Partitioning of Solutes at Different Polymer Concentrations within a Given ATPS

Partition coefficients for all the solutes examined in the present work in ATPSs of different polymer composition and polymer concentrations are listed in Table 4.2. Figure 4.3 presents the logarithms of the distribution coefficients (defined as concentration in the top phase divided by concentration in the bottom phase) for solutes examined in systems I, II and III (Dex-PEG system with different polymer concentrations) as a function of the TLL. Similar data obtained for the aqueous Dex-Ucon two-phase system (systems IV, V and VI) are presented in Figure 4.4. All the experimental observed curves are essentially linear, in agreement with earlier observations in the literature [1, pp. 181-184, 11]. These experimental data may be generally described as:

$$\ln K_j = \alpha_{ji} \, TLL \qquad (4.2)$$

where α_{ji} is a constant characterizing the effect of the tie-line length of a given system on the distribution coefficient K_j of the solute j in that system.

Table 4.2. Partition coefficients of the proteins and other compounds in the aqueous two-phase systems Dextran-75-PEG-8000 and Dextran-75-Ucon-50-HB-5100 at the indicated compositions

System	I[a]	II[a]	III[a]	IV[b]	V[b]	VI[b]
Solute	Partition coefficient, K					
RNAse A	0.489	0.559	0.686	0.247	0.355	0.537
RNAse B	0.455			0.265		
Chymotrypsinogen	2.71			1.78		
Trypsinogen	0.89			0.702		
Lysozyme	2.36	1.68	1.458	2.95	2.69	2.200
Hb Bovine	0.074			0.053		
Hb Human	0.131			0.117		
Lactoglobulin	0.071	0.119	0.2445	0.033	0.037	0.115
Transferrin	0.0084			0.0015	0.0039	0.0279
Myoglobin	0.161	0.251	0.379	0.080	0.136	0.250
Bovine γ-Globulin	0.024			0.007		
Human γ-Globulin	0.043			0.014		
Cytochrome c	0.29			0.12		
Lipase	0.716	0.733	0.773	0.658	0.649	0.761
DNP-Gly	0.873	0.905	0.944	1.018	1.127	1.072
DNP-Ala	0.985			1.326		
DNP-NVal	1.085			1.654		
DNP-NLeu	1.164	1.108	1.088	2.046	1.795	1.513
DNP-AO*	1.348			3.496		
Trp	0.980			1.011		
Di-Trp	1.714			2.816		
Tri-Trp	3.981			24.35		
Fucopyranoside**	1.307			1.552		
Galactopyranoside**	1.184			1.403		
β-Glucopyranoside**	1.268			1.538		
α-Glucopyranoside**	1.231			1.587		
α-Mannopyranoside**	1.175			1.597		

System compositions: [a] I – 12.4% Dex; 6.1% PEG; II - 10.8% Dex; 5.2% PEG; III – 9.0% Dex; 4.3% PEG; [b] IV - 12.4% Dex; 10.1% Ucon; V - 10.4% Dex; 8.4% Ucon; VI - 8.2% Dex; 6.8% Ucon; * dinitrophenyl-aminooctanoic acid; ** - p-nitophenyl-derivatives of carbohydrates indicated.

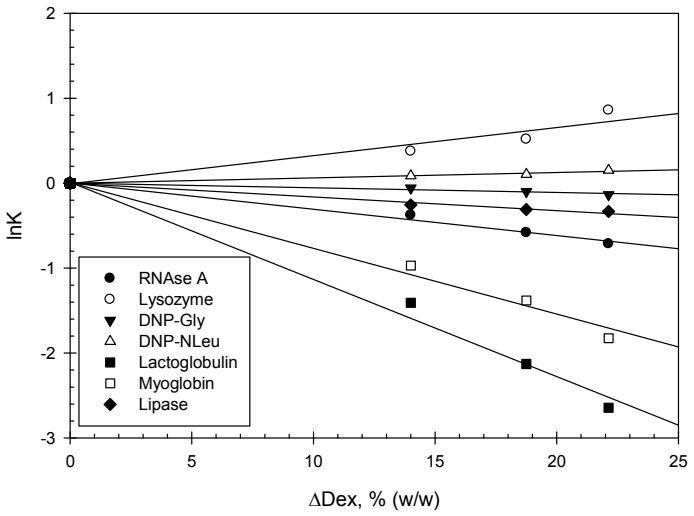

Figure 4.3. Logarithms of distribution coefficients for several solutes examined in aqueous Dex-PEG system with different polymer concentrations (systems I, II and III, Table 4.1) as functions of the TLL.

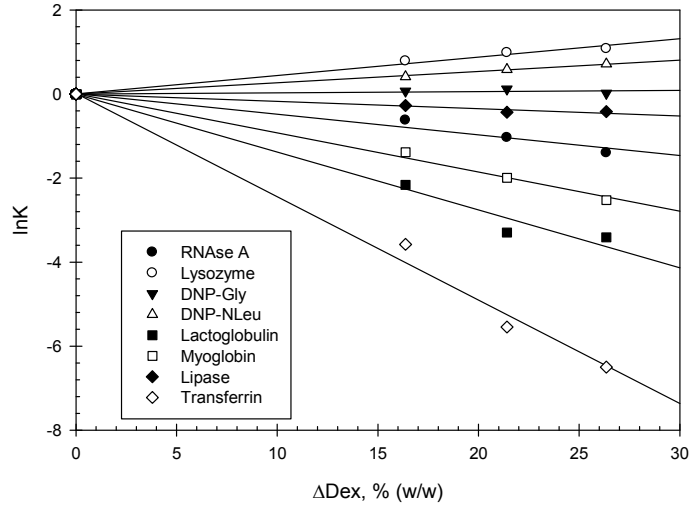

Figure 4.4. Logarithms of distribution coefficients for several solutes examined in aqueous Dex-Ucon system with different polymer concentrations (systems IV, V and VI, Table 1) as functions of the TLL.

For two different tie-lines, within a given ATPS, it follows from Eq. 4.2 that:

$$\ln K_j^1 = \left(\frac{TLL^1}{TLL^2}\right) \ln K_j^2 \qquad (4.2a)$$

where the superscripts refer to different tie-lines (1 and 2) of the same ATPS and the other symbols have the same meaning as before. Figure 4.5 depicts a plot of experimental lnK data versus the calculated values based on Eq. 4.2a for solutes partitioned in the ATPSs studied in the present Chapter as well as for those found in the literature (Table 4.3). The results plotted in figure 4.5 may be expressed by:

$$\ln K_{cal} = 0.02\ (\pm 0.02) + 0.99(\pm 0.02) \ln K_{exp} \qquad (4.3)$$

$$N = 45;\ r^2 = 0.9855,$$

where K_{calc} is the partition coefficient calculated according to Eq. 4.4a, K_{exp} is the experimental partition coefficient, N is the number of solutes, and r^2 is the correlation coefficient.

Equation 4.2a may be considered as a simplified version of the solvent regression equation (so-called Collander equation [14]):

$$\ln K_i = a_{ij} \ln K_j + b_{ij} \qquad (4.4)$$

where K_i and K_j are partition coefficients for any given solute in the i-th and j-th two-phase systems; a_{ij} and b_{ij} are constants, the values of which depend upon the particular composition of the i-th and j-th two-phase systems under comparison. For a given ATPS at different polymer

concentrations corresponding to different tie-lines the intercept in the Collander equation should be equal to zero in agreement with the experimental data reported in Table 4.3.

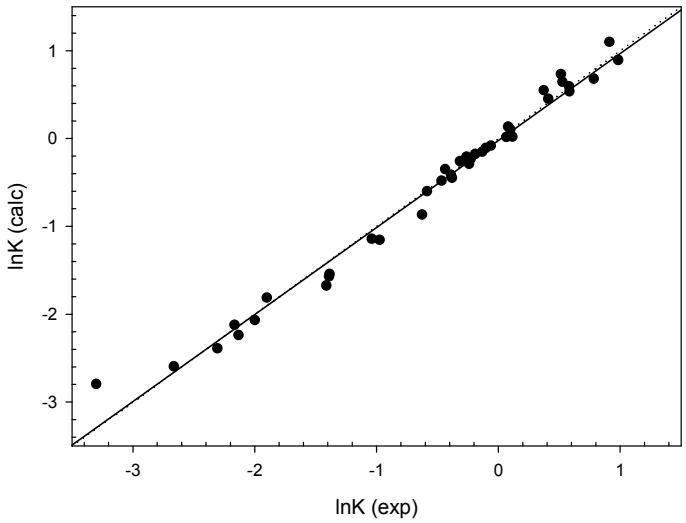

Figure 4.5. Comparison of experimental lnK data and predicted values based on Eq. 4.4a. The data is from the present work and from the literature [27, 28, 29]. Theoretical (…) and experimental (-) lines.

Partitioning of Solutes in ATPSs Formed by Different Components

Analysis of the α_{ji} values found for all the solutes in the aqueous Dex-PEG and Dex-Ucon two-phase systems indicate that the α_{ji} values observed in the two ATPSs under consideration are linearly correlated. This relationship may be represented as:

$$\alpha_{ji}^{\text{Dex-Ucon}} = 0.004(\pm 0.004) + 1.18(\pm 0.08)\ \alpha_{ji}^{\text{Dex-PEG}} \qquad (4.5)$$

$$N = 7;\ r^2 = 0.9782,$$

where N is the number of solutes, and r^2 is the correlation coefficient.

Table 4.3. Comparison of equation coefficients in two different tie-lines within a given ATPS indicated as calculated from the data reported here as well as from the literature $\ln K_i = a*\ln K_o + b$; r^2 – correlation coefficient; N – number of solutes examined (all polymer concentrations in %wt.).

System "i"		System "0"		Coefficient a	Coefficient b	N	r^2	Ref
Dex-75	**PEG-8000**	**Dex-75**	**PEG-8000**					
System III from Table 1		System I from Table 1		0.520 ± 0.005	-0.022 ± 0.009	7	0.9959	Present work
Dex-75	**PEG-8000**	**Dex-75**	**PEG-8000**					
System II from Table 1		System I from Table 1		0.762 ± 0.007	-0.05 ± 0.01	7	0.9961	Present work
Dex-75	**Ucon-50HB5100**	**Dex-75**	**Ucon-50HB5100**					
System VI from Table 1		System IV from Table 1		0.58 ± 0.01	0.04 ± 0.03	8	0.9925	Present work
Dex-75	**Ucon-50HB5100**	**Dex-75**	**Ucon-50HB5100**					
System V from Table 1		System IV from Table 1		0.87 ± 0.02	0.02 ± 0.04	8	0.9942	Present work
Dex-40	**PEG-4000**	**Dex-40**	**PEG-4000**					
7.00%	10.02%	7.50%	10.50%	0.87 ± 0.02	-0.03 ± 0.02	10	0.9976	[8,9][a]
Dex-500	**EO30PO70**	**Dex-500**	**EO30PO70**					
7.1%	6.8%	9.0%	9.0%	0.62 ± 0.03	-0.03 ± 0.01	27	0.9590	[10][b]
Dex-500	**Ucon 50-HB-5100**	**Dex-500**	**Ucon 50-HB-5100**					
5.1%	4.4%	9.2%	4.6%	0.59 ± 0.06	0.05 ± 0.06	5	0.9576	[11][c]
PEG-8000	**K-phosphate buffer**	**PEG-8000**	**K-phosphate buffer**					
ΔPEG[d] = 0.171 %		ΔPEG[d] = 0.257 %		0.71 ± 0.02	-0.01 ± 0.05	11	0.9908	[12][d]
PEG-8000	**$MgSO_4$**	**PEG-8000**	**$MgSO_4$**					
0.109%	0.60 M	0.101%	1.40 M	0.341 ± 0.008	0.04 ± 0.01	66	0.9689	[13][e]

[a] solutes: amino acids; peptides; proteins; [b] 0.05 M K_2SO_4 in 0.005 M sodium phosphate buffer, pH 7.0; solutes: proteins (7) and amino acids (20); [c] 0.01 M sodium acetate buffer, pH 5.0; solutes: peptides, proteins; [d] Composition of the systems not reported explicitly; pH ~5.5; ΔPEG – reported difference between PEG concentrations in the coexisting phases; solutes: proteins (3); peptides (6); amino acid; ethanol; [e] solutes: di- and tri-peptides (37), amino acids (14), and organic nonionic compounds (15).

The correlation described by Eq. 4.5 suggests that the distribution coefficients for the solutes examined in the two different ATPSs are also correlated according to the Collander linear equation (Eq. 4.4).

Figure 4.6 presents the logarithms of distribution coefficients for all the solutes examined in Dex-Ucon system (system IV, Table 4.1) plotted against the logarithms of distribution coefficients of the same solutes in Dex-PEG system (system I, Table 4.1). There is a linear correlation between distribution coefficients of all the proteins examined in the two systems under study:

$$\ln K_j^{\text{Dex-Ucon}} = -0.2(\pm 0.1) + 1.24(\pm 0.05)\ln K_j^{\text{Dex-PEG}} \qquad (4.6)$$

$$N = 14;\ r^2 = 0.9780,$$

where $K_j^{\text{Dex-Ucon}}$ is the distribution coefficient of protein j in Dex-Ucon system (system IV, Table 4.1), $K_j^{\text{Dex-PEG}}$ is the distribution coefficient of protein j in Dex-PEG system (system I, Table 4.1), N is the number of proteins; r^2 is the correlation coefficient.

Analysis of the data from the literature confirms the results presented here suggesting that the general linear relationship between the logarithms of the distribution coefficients in different systems holds for any ATPS. The parameters for these equations are listed in Table 4.4.

Table 4.4. Comparison of equation coefficients for different ATPSs indicated as calculated from the data reported here as well from the literature; r^2 – correlation coefficient; N – number of solutes examined.

System "i"		System "0"		Coefficient a	Coefficient b	N	r^2	Ref
Dex-75	**Ucon-50HB5100**	**Dex-75**	**PEG-8000**					
System IV from Table 1		System I from Table 1		1.24 ± 0.05	-0.2 ±0.1	14	0.9780	Present work
Dex-40	**PEG-8000**	**Dex-70**	**PEG-8000**					
8.0%	6.0%	8.0%	6.0%	0.99 ± 0.08	-0.3 ± 0.2	9	0.9571	[15][a]
Dex-70	**PEG-8000**	**Dex-220**	**PEG-8000**					
8.0%	6.0%	8.0%	6.0%	0.9 ± 0.1	-0.7 ± 0.2	8	0.9118	[15][a]
Dex-220	**PEG-8000**	**Dex-500**	**PEG-8000**					
8.0%	6.0%	8.0%	6.0%	1.10 ± 0.07	-0.2 ± 0.1	8	0.9592	[15][a]
Dex-500	**PEG-8000**	**Dex-2000**	**PEG-8000**					
8.0%	6.0%	8.0%	6.0%	0.86 ± 0.07	-0.4 ± 0.1	9	0.9598	[15][a]

[a] 0.01 M sodium phosphate buffer, pH 6.8; solutes: protein

A similar correlation was previously observed for the partitioning of different solutes (amino acids, glycosides, nucleotides, proteins and nucleic acids) in the system Dex-PEG and Dex-Ficoll [1, pp.270-276]. It was concluded [1] that the correlation implies either that the solute-phase polymer interactions are identical for all the different solutes examined, or that

the solute-polymer specific interactions do not occur and/or do not affect the solute partitioning in these systems. The last assumption appears to be more plausible due to purely probabilistic reasons. Thus, the major conclusions from the above equation 4.6 are: (i) the proteins randomly selected for analysis in this study do not interact with the phase-forming polymers; and (ii) the distribution coefficient for any protein determined in one of the two systems may be used to predict the distribution coefficient for the same protein in the other system.

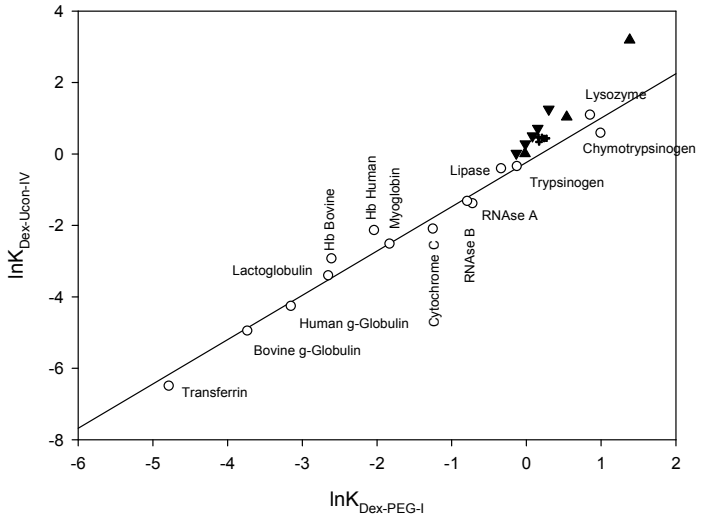

Figure 4.6. Logarithms of distribution coefficients for different solutes (o - proteins; ▼ – DNP-amino acids; ▲ – tryptophan, di-tryptophan and tri-tryptophan; and + - NP-monossacharides) examined in system IV (Dex-Ucon system, Table 4.1) plotted against logarithms of distribution coefficients of the same solutes in system I (Dex-PEG system, Table 4.1).

The data plotted in figure 4.6 also indicate that there is a correlation for the distribution coefficients of examined DNP-amino acids different from the one described by Eq. 4.6, and a different one for the homooligopeptides of tryptophan (tryptophan, di-tryptophan and tri-tryptophan). We did not examine these correlations because of the limited number of compounds included in each series. There seems to be no correlation for the distribution coefficients of nitrophenylated monosaccharides. The lack of correlation between the distribution coefficients for monosaccharides may be because all the K-values for these compounds are very close to unit in both systems.

It has been previously suggested [1, pp.270-276] that the physical meaning of the slope of Eq.4.6 (coefficient a in Eq. 4.4) is the ratio of the differences between the hydrophobic character of the coexisting phases in the ATPSs under comparison, while the intercept in Eq.4.6 (coefficient b – Eq. 4.4) represents the difference between the polar and electrostatic interactions of the solutes with the aqueous media in the coexisting phases in the systems under comparison. The data obtained for DNP-amino acids were used to estimate these parameters, as described in detail in [1, pp.162-196]. The experimental data obtained for DNP-amino acids are plotted in figure 4.7, and the linear trends observed may be described as:

$$\ln K_{DNP\text{-}AA} = C + E\, N_C \qquad (4.7)$$

where $K_{DNP\text{-}AA}$ is the distribution coefficient of a DNP-amino acid with aliphatic side-chain; N_C is the equivalent number of CH_2 groups in the aliphatic side-chain of a given DNP-amino acid; E is an average lnK increment per CH_2 group; C represents the total contribution of the non-alkyl part of the structure of a DNP-amino acid into lnK. The E values are 0.196 ± 0.005 in Dex-Ucon system IV and 0.062 ± 0.002 in the Dex-PEG system I, while C values are 0.00 ± 0.02 in Dex-Ucon system IV and -0.089 ± 0.007 in the Dex-PEG system I. The difference between the hydrophobic character of the coexisting phases in the Dex-Ucon system being around 3 times larger than that in the Dex-PEG system seems intuitively reasonable, since Ucon should be more hydrophobic than PEG due to the presence of propylene oxide monomers in its structure, and hence should have a more pronounced effect on the properties of the aqueous media in the corresponding phase of a given ATPS.

Since distribution coefficients of homologous series of compounds in two different two-phase systems are described by Eq. 4.7 (with different E and C values depending on the particular two-phase systems under comparison), and N_C for any given DNP-amino acid is constant, it follows that:

$$\ln K^{I}_{DNP\text{-}AA} = a\ln K^{II}_{DNP\text{-}AA} + b = (E^{I}/E^{II})\ln K^{II}_{DNP\text{-}AA} + C^{I} - (E^{I}/E^{II})\, C^{II} \qquad (4.8)$$

where superscripts I and II denote two different two-phase systems under comparison.

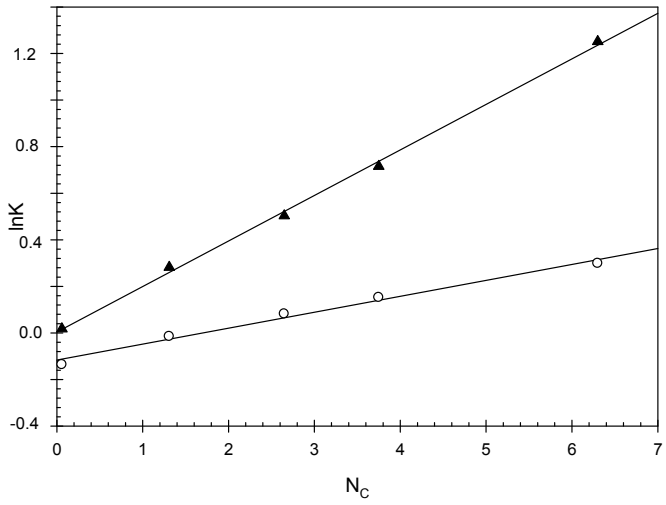

Figure 4.7. Distribution coefficients of sodium salts of DNP-amino acids with aliphatic side chains as functions of side chain length. O – Dex-PEG system I; ▲ - Dex-Ucon system IV.

It clearly follows from Eq. 4.8 that coefficient a = (E^{I}/E^{II}), and coefficient b = $C^{I} - (E^{I}/E^{II})*C^{II}$. From the data obtained in this study it is obvious that the value of coefficient a in Eq. 4.6 does not agree with the one estimated from the analysis of the distribution coefficients of DNP-amino acids. It also follows from the different slopes of the correlation between K-values for the proteins and for DNP-amino acids in the two ATPSs under comparison (figure 4.6). This fact seems to indicate that the previous assumption [1] was incorrect, and that describing all the solute-solvent interactions with just two descriptors derived from partitioning of DNP-amino acids in aqueous two-phase systems is an oversimplification.

The major conclusion presented here, particularly that proteins do not interact with the phase-forming polymers, is at this stage only valid for a limited set of systems. Unfortunately,

there are very few studies of the same proteins partitioned in ATPSs formed by polymers of different chemical nature, and even then the purity and other characteristics of the protein preparations used by different authors may differ significantly. It should also be mentioned that the correlation observed here is valid only for ATPSs with the same ionic composition. Therefore it is early at this stage to draw any general conclusion in regard to the existence of a linear correlation between the logarithms of the distribution coefficients of different proteins in ATPSs formed by different polymers, and more experimental work is still needed. These issues will be further addressed in the next Chapter.

4.4. Conclusions

Distribution coefficients for several proteins were measured in the aqueous two-phase systems (ATPSs) Dextran-PEG and Dextran-Ucon50HB5100 (a random copolymer of ethylene glycol and propylene glycol) both containing 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.4, at 23 ^{0}C. Distribution coefficients of some selected solutes were also measured in the above two-phase systems at three different polymer concentrations for each system. It was established that the distribution coefficients for all of the proteins examined in the ATPSs under study are interrelated according to the so-called Collander linear equation.

Thus, the major conclusions of the present Chapter are: (i) the proteins randomly selected for analysis in this study do not interact with the phase-forming polymers; and (ii) the distribution coefficient for any protein determined in one of the two systems may be used to predict the distribution coefficient for the same protein in the other system.

4.5 References

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5. "On the Collander equation": Protein partitioning in polymer/polymer aqueous two-phase systems

5.1 Introduction

One important consequence of the similarity between partitioning of solutes in ATPS and in water-organic solvent systems is the possible application of the Collander equation [1]. This equation describes a linear correlation between distribution coefficients of solutes in different biphasic systems. It was established in the previous Chapter that the distribution coefficients for different randomly selected proteins in the Dex-PEG and Dex-Ucon ATPSs are correlated according to the so-called Collander equation or solvent regression equation. We explore in the present work the applicability of the solvent regression equation to ATPSs formed by different paired combinations of Dextran (Dex-75), Ficoll-70, polyethylene glycol (PEG-8000), hydroxypropyl starch (PES-100), and Ucon50HB5100 at particular polymer concentrations, all containing 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.4.

5.2. Experimental

5.2.1. Materials

All polymers, amino acids, peptides, proteins, monosaccharides, o-Phthaldialdehyde (OPA) reagent solution (complete), and solvatochromic dyes used were the same as previously described (Chapter 3, pp. 30-31).

5.2.2. Methods

5.2.2.1. Phase Diagrams. The systems were prepared as described in Chapter 3 (pp. 31-32).

5.2.2.2. Partitioning. The partitioning measurements were carried out as described in Chapters 3 (pp. 32-33) and 4 (pp. 51).

5.2.2.3. Electrophoresis

All proteins (Chicken egg lysozyme, bovine α-chymotrypsinogen A, bovine hemoglobin, horse heart cytochrome c, bovine ribonuclease B, bovine ribonuclease A, bovine trypsinogen, human hemoglobin, horse myoglobin, bovine β-lactoglobulin, human γ-globulin, bovine γ-globulin, porcine lipase, and human transferrin) were characterized by SDS-PAGE electrophoresis in a microfluidic chip using Bioanalyzer 2100 (Agilent Technologies, USA) under non-reduced conditions. All the proteins except both gamma-globulins were observed as single bands in the electrophoregrams. These two proteins displayed a series of overlapping bands and were judged to be heterogeneous.

5.3. Results and Discussion

Protein Partitioning

In the previous Chapter we showed that distribution coefficients for randomly selected proteins in Dex-PEG and Dex-Ucon ATPSs are correlated according to the Collander equation [1] or solvent regression equation [2-5] (equation 4.4).

The distribution coefficients for all the proteins studied in the ATPSs under consideration (Chicken egg lysozyme, bovine α-chymotrypsinogen A, bovine hemoglobin, horse heart cytochrome c, bovine ribonuclease B, bovine ribonuclease A, bovine trypsinogen, human hemoglobin, horse myoglobin, bovine β-lactoglobulin, human γ-globulin, bovine γ-globulin, porcine lipase, and human transferrin) are presented in Table 5.1. It should be noted that on the PEG-UCON system most of the proteins either partially or completely precipitated and their distribution behavior could not be studied. ATPS formed by these two polymers at relatively

high concentrations which served as precipitating agents is not suitable for partitioning of hydrophilic proteins and is not further considered in this work. In all the other ATPSs listed in Table 5.1 the protein distribution coefficients are clearly correlated according to Eq.4.4. Figure 5.1 presents typical data, where logarithms of the distribution coefficients in different ATPSs are plotted versus those in the Dex-PEG system. It should be mentioned that the molecular weights of the proteins studied varied from ~14,000 (ribonuclease, lysozyme) to ~77,000 (transferrin), with isoelectric points (pI) from 5.2 (lipase, lactoglobulin) to 11.4 (lysozyme) (Table 5.1). Gamma-globulins studied previously in Dex-PEG and Dex-Ucon systems were not examined here, since the protein preparations were highly heterogeneous.

Table 5.1. Partition coefficients, K, for the proteins examined in the ATPSs indicated

Protein	$M_{w,}$ kD	pI[a]	Dex-PEG	Dex-Ucon	PES-PEG	PES-Ucon	Ficoll-PEG	Ficoll-Ucon	PEG-Ucon
RNAse A	13.7	9.6	0.489	0.247	0.604	0.506	0.466	0.245	~0.014*
RNAse B	~15.0	9.5	0.455	0.265	0.703	0.625	0.440	0.237	-
Chymotrypsinogen	~25.7	9.0	2.712	1.780	2.977	1.798	1.042	0.638	0.0098
Trypsinogen	23.7	9.3	0.886	0.702	0.967	0.779	0.580	0.345	0.015
Lysozyme	14.3	11.4	2.357	2.952	1.287	1.279	0.913	0.995	0.036
Hemoglobin	64.5	6.8	0.074	0.053	0.148	0.208	0.094	0.052	*
Hemoglobin	64.5	6.8	0.131	0.117	0.216	0.282	0.153	0.074	*
Lactoglobulin	18.4	5.2	0.071	0.033	0.213	0.176	0.112	0.044	*
Transferrin	77.0	5.7	0.0084	0.0015	0.0516	0.0422	0.0189	0.0035	*
Myoglobin	17.6	7.3	0.161	0.080	0.340	0.310	0.258	0.154	0.065
Lipase	48	5.2	0.716	0.658	0.751	0.775	0.733	0.634	0.358
γ-Globulin human[b]	~160	~6.8	0.043	0.014	-	-	-	-	-
γ-Globulin bovine[b]	~180	~6.5	0.024	0.007	-	-	-	-	-
Cytochrome c	12.4	10.0	0.288	0.122	-	-	-	-	-

[a] – pI – isoelectric point; [b] - heterogeneous preparations; * - protein precipitation

All the data presented in Table 5.1 were processed according to Eq.4.4. The coefficients a_{io} and b_{io} and the corresponding correlation coefficients, r^2, and number of proteins examined in a given pair of ATPSs, N, are presented in Table 5.2, with different ATPSs used as the reference system. As expected, the linear correlations shift depending on the ATPS used as a reference. The observed correlations indicate that neither the charge nor the molecular weight of a protein are the sole factor governing its partition behavior.

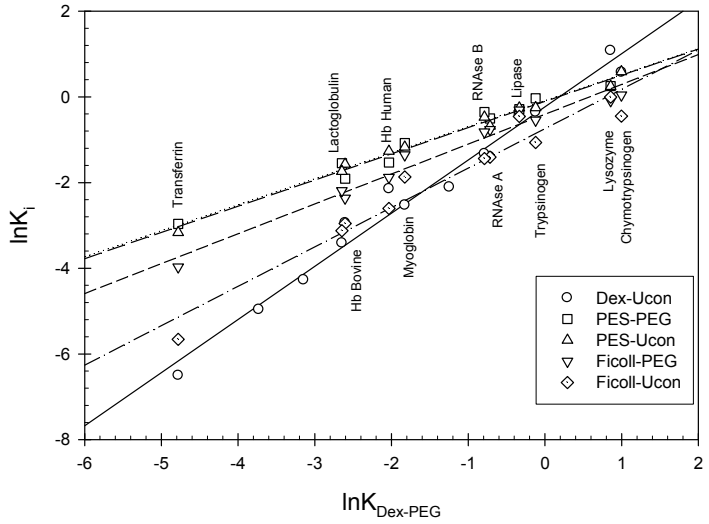

Figure 5.1. Natural logarithms of distribution coefficients for proteins indicated in ATPSs Dex-Ucon, Ficoll-Ucon, Ficoll-PEG, PES-Ucon, and PES-PEG versus natural logarithms of distribution coefficients for the same proteins in Dex-PEG system.

Physico-chemical characterization of the phases

One current approach used to characterize the properties of coexisting phases in a given ATPS is derived from partitioning a homologous series of solutes in the system (Chapter 3, equation 3.5) [6-19]).

It was previously observed (Chapter 3) that the difference in the relative hydrophobic character of the coexisting phases, represented by coefficient E, cannot serve as a single measure of the phase properties governing the partitioning of a solute in ATPSs. The relationships between coefficients a_{io} (Table 5.2) and coefficients E and C in the reference ATPS were examined in order to explore if coefficients E and C in Eq.3.5 or a combination of the two may characterize the properties of the ATPS which influence the partitioning of proteins. The typical data observed when PES-PEG system is the reference system are presented in figure 5.2.

Table 5.2. Coefficients a_{io} and b_{io} in solvent regression equation (eq.4.4) with different ATPS used as a reference system (N – number of proteins; r^2 – regression coefficient)

ATPS	Reference	a_{io}	b_{io}	N	r^2
Dex-PEG	Dex-PEG	1.000	0.000		
Dex-Ucon		1.24 ± 0.05	-0.2 ± 0.1	14	0.9780
PES-PEG		0.61 ± 0.03	-0.09 ± 0.07	11	0.9739
PES-Ucon		0.61 ± 0.02	-0.11 ± 0.04	11	0.9890
Ficoll-PEG		0.70 ± 0.04	-0.41 ± 0.09	11	0.9677
Ficoll-Ucon		0.92 ± 0.07	-0.7 ± 0.2	11	0.9465
Dex-PEG		0.79 ± 0.03	0.2 ± 0.1	14	0.9780
Dex-Ucon	Dex-Ucon	1.000	0.000		
PES-PEG		0.46 ± 0.04	-0.1 ± 0.1	11	0.9186
PES-Ucon		0.49 ± 0.02	-0.02 ± 0.07	11	0.9746
Ficoll-PEG		0.56 ± 0.04	-0.3 ± 0.1	11	0.9451
Ficoll-Ucon		0.75 ± 0.05	-0.6 ± 0.1	11	0.9595
Dex-PEG		1.60 ± 0.09	0.1 ± 0.1	11	0.9739
Dex-Ucon		2.0 ± 0.2	-0.0 ± 0.3	11	0.9186
PES-PEG	PES-PEG	1.000	0.000		
PES-Ucon		1.00 ± 0.07	-0.1± 0.1	11	0.9608
Ficoll-PEG		1.18 ± 0.07	-0.26 ± 0.09	11	0.9738
Ficoll-Ucon		1.6 ± 0.1	-0.5 ± 0.2	11	0.9387
Dex-PEG		1.62 ± 0.06	0.16 ± 0.08	11	0.9890
Dex-Ucon		2.0 ± 0.1	-0.0 ± 0.1	11	0.9746
PES-PEG		1.00 ± 0.06	0.03± 0.08	11	0.9608
PES-Ucon	PES-Ucon	1.000	0.000		
Ficoll-PEG		1.13 ± 0.07	-0.29 ± 0.09	11	0.9679
Ficoll-Ucon		1.5 ± 0.1	-0.6 ± 0.2	11	0.9510
Dex-PEG		1.39 ± 0.09	0.5 ± 0.2	11	0.9677
Dex-Ucon		1.7 ± 0.1	0.4 ± 0.2	11	0.9451
PES-PEG		0.83 ± 0.05	0.19 ± 0.08	11	0.9738
PES-Ucon		0.85 ± 0.05	0.22 ± 0.09	11	0.9679
Ficoll-PEG	Ficoll-PEG	1.000	0.000		
Ficoll-Ucon		1.32 ± 0.06	-0.2 ± 0.1	11	0.9808
Dex-PEG		1.03 ± 0.08	0.7 ± 0.2	11	0.9465
Dex-Ucon		1.28 ± 0.09	0.7 ± 0.2	11	0.9595
PES-PEG		0.59 ± 0.05	0.2 ± 0.1	11	0.9387
PES-Ucon		0.63 ± 0.05	0.3 ± 0.1	11	0.9510
Ficoll-PEG		0.74 ± 0.04	0.13 ± 0.09	11	0.9808
Ficoll-Ucon	Ficoll-Ucon	1.000	0.000		

All the coefficients a_{io} listed in Table 5.2 are correlated to the ratios of the coefficients E_i/E_o and C_i/C_o (coefficients E_i and C_i are listed in Table 3.3, and the subscript o denotes the particular reference system) according to the following equation:

$$1/a_{io} = \alpha_o + \beta_o * E_i/E_o + \gamma_o * C_i/C_o \qquad (5.1)$$

where α_o, β_o, and γ_o are coefficients dependent upon the ATPS used as a reference. The values of these coefficients are presented in Table 5.3 together with the corresponding correlation coefficients.

It is important to highlight that the slope of the solvent regression equation (Eq.4.4) for various proteins is correlated with coefficients E_i and C_i which were determined from separate experiments with the homologous series of DNP-amino acids [18-19, Chapter 3] and used here to characterize the differences between the solvent properties of the aqueous media in the coexisting phases of ATPSs. Coefficient a_{io} in Eq.5.1 may be simplified:

$$a_{io} = E_o C_o/(\alpha_o E_o C_o + \beta_o E_i C_o + \gamma_o E_o C_i) \qquad (5.2)$$

where all the parameters are as defined previously. This result strongly suggests that both E and C can be used as solvent descriptors in polymer/polymer ATPSs.

It was suggested earlier that coefficients a_{io} and b_{io} in the solvent regression equation may be represented as [5]:

$$a_{io} = E_i/E_o \text{ and } b_{io} = E_i(C_i/E_i - C_o/E_o) \qquad (5.3).$$

Using the solvent regression equation to compare the distribution coefficients of proteins and other solutes in ATPSs formed by pairs of polymers of the same chemical nature but different molecular weights [6, pp.268-276] allowed to confirm this suggestion.

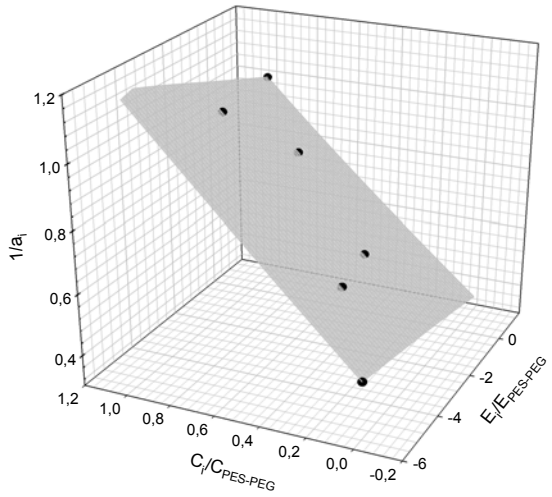

Figure 5.2. 3-D plot of coefficient a_{io} in the solvent regression equation (Eq.4.4) as a function of the ratios of coefficients E_i/E_o and C_i/C_o in Eq.3.5. System PES-PEG was used as the reference system (with coefficients C_o and E_o) and E_i and C_i are coefficients in Eq.3.5 for the systems Dex-PEG, Dex-Ucon, PES-PEG, PES-Ucon, and Ficoll-Ucon.

Table 5.3. Coefficients α, β, and γ in Eq.5.1 with different ATPSs used as a reference system (r^2 – correlation coefficient; N = 6 – number of ATPSs used in analysis)

Reference ATPS	α_o	β_o	γ_o	r^2
Dex-PEG	0.68 ± 0.08	0.05 ± 0.03	0.30 ± 0.03	0.9903
Dex-Ucon	0.91 ± 0.09	0.1 ± 0.1	-0.017 ± 0.001	0.9932
PES-PEG	0.36 ± 0.02	-0.028 ± 0.004	0.68 ± 0.03	0.9980
PES-Ucon	0.42 ± 0.05	0.04 ± 0.02	0.54 ± 0.05	0.9911
Ficoll-PEG	0.48 ± 0.04	0.013 ± 0.005	0.52 ± 0.03	0.9963
Ficoll-Ucon	0.67 ± 0.07	0.07 ± 0.05	0.24 ± 0.02	0.9933

It follows from the data obtained for proteins in this study as well as from previously reported data (Chapter 3) for homooligopeptides and monosaccharides that Eq. 5.3 does not hold for the comparison of ATPSs formed by pairs of polymers of different chemical nature.

This fact may be explained qualitatively as the result of different effects of phase-forming polymers on the solvent features of the aqueous media in the coexisting phases of ATPSs. Separation of the various types of protein-water molecular interactions into just two classes of interactions involving nonpolar and polar groups implied by Eq. 5.3 is obviously an oversimplification. Molecular interactions of water with DNP-amino acids used for the

characterization of the solvent features of coexisting aqueous phases in a given ATPS are unlikely to completely represent the much more complex variety of molecular interactions involved in protein-water interactions. This likely explains the fact that no reliable correlation between the coefficient b_{io} and coefficients C and E could be found.

Another current approach used to characterize the properties of coexisting phases in a given ATPS is derived from the solvatochromic method developed by Kamlet et al. [20]. The relationships between coefficients b_{io} (Table 5.2) and coefficients Δa^*, Δb^* and $\Delta\pi^*$ (Chapter 3) were examined in order to explore if these coefficients or a combination of them (along with E and C) can explain the relative roles of different protein-water molecular interactions in protein partitioning in ATPSs.

All the coefficients b_{io} listed in Table 5.2 are correlated to the coefficients C_i, Δa_i^*, Δb_i^* and $\Delta\pi_i^*$ (coefficient C_i and Δa_i^*, Δb_i^* and $\Delta\pi_i^*$are listed in Tables 3.3 and 3.2, respectively) according to the following general equation:

$$b_{io} = z_o + z_1 \Delta\pi_i^* + z_2(1/\Delta b_i^*) + z_3(1/\Delta a_i^*) + z_4 C_i \qquad (5.4)$$

where z_o, z_1, z_2, z_3 and z_4 are coefficients dependent upon the ATPS used as a reference. It should be noted that for a given ATPS used as reference not all the parameters are statistically significant and the equation can be simplified. The values of these coefficients are presented in Table 5.4 together with the corresponding statistics.

Table 5.4. Coefficients z_o, z_1, z_2, z_3 and z_4 in Eq.5.4 with different ATPSs used as a reference system (r^2 – correlation coefficient; F - ratio of variance; SD - standard deviation; –N = 6 – number of ATPS used in analysis)

Reference ATPS	z_o	z_1	z_2	z_3	z_4	r^2	F	SD
Dex-PEG	-0.44±0.06	-2.6±0.5	-0.007±0.002	-0.0013±0.0008	-0.8±0.4	0.985	16.4	0.07
Dex-Ucon	-0.23±0.01	-3.1±0.1	-0.0057±0.0005	-0.0007±0.0002	*	0.998	321.7	0.02
PES-PEG	-0.17±0.04	-2.6±0.4	-0.005±0.002	0.0013±0.0007	*	0.955	14.3	0.07
PES-Ucon	-0.24±0.04	-3.2±0.6	-0.003±0.001	*	*	0.936	21.8	0.09
Ficoll-PEG	0.14±0.03	-2.7±0.3	-0.007±0.001	0.0016±0.0005	*	0.982	35.4	0.06
Ficoll-Ucon	0.45±0.04	-3.1±0.4	-0.0031±0.0008	*	1.5±0.2	0.986	46.8	0.06

* - not statistically significant

From the analysis of the data presented in Table 5.4, it seems that Δa_i*and Δb_i* are similar for the ATPS under comparison, and essentially all the H-bonds accepting and donating interactions are quite similar for all the ATPS studied. $\Delta\pi_i$* seems to be much more important and its contribution different in the ATPS under comparison. The absence of a given descriptor means either that it does not seriously contribute or that its contribution is about the same in the systems under comparison.

Finally the fact that the intercept is not zero, probably indicates that there are some additional factors involved in the differences between the systems that are not described by these descriptors. This equation will be further discussed on Chapter 7.

Nevertheless, it is important to highlight that both the slope and the intercept of the solvent regression equation (Eq.4.4) for various proteins is correlated with parameters which were determined from separate experiments and used here to characterize the differences between the solvent properties of the aqueous media in the coexisting phases of ATPSs.

Finally, it should also be mentioned that three out of the ten ATPSs, Dex-Ficoll, Dex-PES, and Ficoll-PES displayed distribution behavior of proteins different from that observed in this study. Protein partitioning in these three systems and the solvent features of the phases in these systems as compared to those of the ATPSs examined here are the subject of the next Chapters.

5.4. Conclusions

Distribution coefficients of randomly selected proteins (Chicken egg lysozyme, bovine α-chymotrypsinogen A, bovine hemoglobin, horse heart cytochrome c, bovine ribonuclease B, bovine ribonuclease A, bovine trypsinogen, human hemoglobin, horse myoglobin, bovine β-lactoglobulin, human γ-globulin, bovine γ-globulin, porcine lipase, and human transferrin) were measured in aqueous two-phase systems (ATPSs) formed by different pair combinations of Dextran-75 (Dex), Ficoll-70, polyethylene glycol-8000 (PEG), hydroxypropyl starch-100 (PES),

and Ucon50HB5100 at specific polymer concentrations, all containing 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.4. In the PEG-Ucon system most of the proteins precipitated at the interface. In PES-PEG, PES-Ucon, Ficoll-PEG, Ficoll-Ucon, Dex-PEG and Dex-Ucon ATPSs the distribution coefficients for the proteins were correlated according to the Collander equation. Moreover the slope and intercept of this equation were correlated by physico-chemical properties of the coexisting aqueous based phases, with parameters which were determined from separate experiments. These results suggest that the protein-solvent interactions in these systems are similar.

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6. Solvent descriptors in polymer/polymer aqueous two-phase systems

6.1 Introduction

There are several experimental evidence indicating that the solvent features of the aqueous media in the coexisting phases of ATPSs are different [1,2] and that there are clear similarities between partitioning of solutes in ATPSs and in water-organic solvent systems [1-7].

Until now, the solvent properties of ATPSs examined were restricted to a limited number of systems formed by only two pairs of polymers, Dex-PEG and Dex-Ficoll, [1] and those formed by PEG and inorganic salts [2-8]. The free energy of transfer of a methylene group between the coexisting phases, $\Delta G(CH_2)$, has been shown [1-14] to be useful for characterization of ATPSs of different polymer and salt composition. The $\Delta G(CH_2)$ value is derived from the partition of a homologous series of solutes with varied aliphatic alkyl chain length in a given two-phase system (Eq. 3.5)

Equation 3.5 represents a group-contribution approach developed by Leo and Hansch [15-16]. The correlative approach of Collander [17] was also used with limited success [1,3-4,13-14] to predict partitioning behavior of solutes in different ATPSs. These two empirical approaches, while enabling to gain useful information, do not provide insight into the nature of solute-solvent interactions in ATPSs at the molecular scale.

The Linear Solvation Energy Relationship (LSER), based on the generalized solvation equation of Abraham [18-20], was recently reported to be applicable to partitioning of solutes in ATPSs [3-4,21-22]. LSER is based on the empirical determination of the Gibbs energy based descriptors by multiple regression of chromatographic data obtained from a variety of processes and is represented by the following equation:

$$\log P = vV + rR_2 + sS + aA + bB + z \qquad (6.1)$$

where P is a partitioning property of a solute between two bulk phases of interest; and v, r, s, a, b, and z are fitted coefficients characteristic of a given two-phase system. It should be noted that the solute descriptors V, S, A, B, and V are usually referred to as V_x, π_2^H, $\sum\alpha_2^H$, and $\sum\beta_2^H$, respectively, in older notation. The solute descriptors are defined as follows: V_x is a group-contributable solute volume [23] which accounts for both the solvent cavitation energy and part of the solute-solvent London dispersion interaction; R_2 is the "excess molar refraction" of a solute [20] (the rR_2 term is intended to capture solute-solvent interactions which involve an induced dipole (polarization) on the solute beyond what is accounted by the vV term [24]); parameter S (π_2^H in older notation) is a polarity/polarizability descriptor believed to reflect the interactions associated with both induced and stable polarity of the solute [24]; A and B $\sum\alpha_2^H$, and $\sum\beta_2^H$ in older notation) refer to the total hydrogen bond donating and accepting capacities of the solute, respectively (or the overall solute hydrogen bond acidity and overall solute hydrogen bond basicity). Finally, the fitted regression constant term, z, depends on the standard states and units of the partitioning property, P [24]. It must be noted that physical explanation of A, B, and S solute descriptors relies on the assumed linear separability of the underlying processes that they are intended to represent [24].

Typically, V and R_2 can be calculated from the structure – V is a simple sum of atom and bond contributions [23], and R_2 is readily obtained from the refractive index or summed from the fragment values. The other solute descriptors, S, A, and B may be found experimentally or calculated [18-20]. Once the descriptors for the solutes are known, the partition coefficients for these solutes in a given two-phase system are determined and regression analysis is used to establish the two-phase system-specific coefficients (v, r, s, a, b, and z) [19-20].

The system-specific coefficients can be interpreted as follows [20]. The coefficient r shows the difference between the tendencies of the coexisting phases to interact with solutes through π- and n-electronic pairs. The coefficient s represents the difference between the tendencies of the coexisting phases to interact with dipolar/polarizable solutes. The coefficient a denotes the difference between the hydrogen bond basicity of the phases and the coefficient

b characterizes the difference between the hydrogen bond acidity of the phases. The coefficient v is a combination of an exoergic dispersion term and endoergic cavity term.

The approach described above was used by Huddleston et al. [3-4,21-22] to analyze the solvent properties of PEG-salt and Dex-PEG ATPSs. The particular challenge when using this approach to polymer/polymer ATPSs, such as Dex-PEG, is that the partition coefficients of solutes with known solute descriptor values vary within a very limited range, e.g., from 1.054 to 1.690 in the particular Dex-PEG system used by Moody et al. [3]. Therefore, the accuracy of the multiple linear regression analysis performed over such a limited range of the partition coefficients values may be debatable.

Another approach to quantify solvent features of coexisting phases in two-phase systems is the solvatochromic method developed by Kamlet et al. [25], which is based on using different solvatochromic probes to quantify solvent polarity/polarizability, s* (or π*), solvent hydrogen bond basicity, a*, and solvent hydrogen bond acidity, b* (asterisks are used here to denote properties quantified by solvatochromic measurements as opposed to the above approach). This approach was used successfully by Huddleston et al. [26] to characterize the solvent features of PEG-salt ATPSs. As mentioned in [26], the limitations of this approach involve not accounting for the role of the molar refractivity of a solute and the contribution of the free energy of cavity formation. In the present study we tried to compensate these limitations by combining the solvatochromic approach with the group-contribution approach.

In summary, the two main ways to characterize the solvent parameters in Eq. (6.1) are:

1. Use of a series of solutes with known descriptors, perform partitioning experiments, and calculate solvent parameters.

2. Use solvatochromic probes to directly determine the solvent parameters.

An obvious extension of the above two methods is to combine their benefits. The final procedure could be depicted in the following mathematical description:

$$\begin{pmatrix} 1 & R_{21} & \pi_{21}^{H} & \Sigma\alpha_{21}^{H} & \Sigma\beta_{21}^{H} & V_{x1} \\ 1 & R_{22} & \pi_{22}^{H} & \Sigma\alpha_{22}^{H} & \Sigma\beta_{22}^{H} & V_{x2} \\ 1 & R_{23} & \pi_{23}^{H} & \Sigma\alpha_{23}^{H} & \Sigma\beta_{23}^{H} & V_{x3} \\ \vdots & \vdots & \vdots & \vdots & \vdots & \vdots \\ 1 & R_{2n} & \pi_{2n}^{H} & \Sigma\alpha_{2n}^{H} & \Sigma\beta_{2n}^{H} & V_{xn} \end{pmatrix} \begin{pmatrix} z \\ r \\ s \\ b \\ a \\ \mathrm{v} \end{pmatrix} = \begin{pmatrix} \log SP_1 \\ \log SP_2 \\ \log SP_3 \\ \vdots \\ \log SP_n \end{pmatrix} \qquad (6.2)$$

In general, the above system represents an over-determined system of equations, and the unknown solvent coefficients are determined using least squares techniques. In our approach, some of the solvent coefficients are independently obtained from a separate set of experiments and the others are calculated using the above general description, with the known coefficients replacing the unknown ones in the parameter vector.

In the present Chapter we explore the applicability of the combination of partitioning of a homologous series of compounds and solvatochromic analysis of solvent features of the coexisting phases in ATPSs formed by different pair combinations of Dextran-75 (Dex-75), Ficoll-70, polyethylene glycol (PEG-8000), hydroxypropyl starch (PES-100), and Ucon50HB5100 (a random copolymer of ethylene glycol and propylene glycol) at particular polymer concentrations, all containing 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.4, at 23 ^{0}C.

6.2. Experimental

6.2.1. Materials

6.2.1.1. Polymers. All polymers, amino acids, peptides, monosaccharides, o-Phthaldialdehyde (OPA) reagent solution (complete), and solvatochromic dyes used were the same as previously described (Chapter 3).

6.2.2. Methods

6.2.2.1. Solvatochromic Studies. The solvatochromic studies were carried out as described in Chapter 3 (pp. 34-35).

6.3 Results and Discussion

The solvatochromic parameters obtained in the phases of the ATPSs under analysis are presented in Table 3.2. The differences between the values found for the top phases and those for the corresponding bottom phases are also presented there.

It is suggested that the linear solvation equation, LSER, of Abraham (Eq.6.1) may be expressed as:

$$\ln K = vV + rR_2 + \Delta\pi^*S + \Delta a^*A + \Delta b^*B + z \qquad (6.3)$$

where all the terms are as defined above with the only difference being that solvatochromically derived coefficients Δπ *, Δa*, and Δb* are used instead of the coefficients s, a, and b in Eq.6.1.

In order to test the assumption that coefficients Δπ *, Δa*, and Δb* are similar to coefficients s, a, and b in the original Abraham equation (Eq.6.1) we compared values for these coefficients reported in the literature for organic solvent-water two-phase systems.

It was shown previously by Moody et al. [3] that the coefficient r representing the difference between the tendencies of the coexisting phases to interact with solutes through π- and n-electronic pairs might be neglected in the Dex-PEG ATPS. Here and we assumed that it is possible to extend this approximation to all the ATPSs examined in our study.

In order to estimate if the replacement of Abraham's solvent-specific coefficients s, a, and b in Eq.6.1 with the solvatochromic solvent descriptors Δπ *, Δa*, and Δb* in Eq.6.3 is valid, we examined the values reported in the literature for organic solvents [20,27]. Table 6.1

presents the π*, a*, and b* Kamlet parameters obtained from solvatochromic studies [27] and the s, a and b coefficients obtained with Abraham's approach for several solvents [20].

Table 6.1. Solvent polarity (π*), solvent hydrogen-bond donor acidity (b*), and solvent hydrogen-bond acceptor basicity (a*) obtained from solvatochromic studies [27] and coefficients s, b and a obtained with Abraham's solvation equation [20]

Solvent system	π*	b*	a*	Reference	s	b	a	Reference
n-hexane	-0.04	0.00	0.00	[27]	-1.723	-3.599	-4.764	[20]
n-heptane	-0.08	0.00	0.00	[27]	-2.061	-3.317	-4.733	[20]
n-octane	0.01	0.00	0.00	[27]	-1.647	-3.480	-5.067	[20]
n-decane	0.03	0.00	0.00	[27]	-1.734	-3.435	-5.078	[20]
benzene	0.59	0.10	0.00	[27]	-0.588	-3.099	-4.625	[20]
toluene	0.54	0.11	0.00	[27]	-0.720	-3.010	-4.824	[20]
methanol	0.60	0.66	0.98	[27]	-0.671	0.080	-3.389	[20]
ethanol	0.54	0.75	0.86	[27]	-0.959	0.186	-3.645	[20]
n-propanol	0.52	0.90	0.84	[27]	-1.098	0.389	-3.893	[20]
n-butanol	0.47	0.84	0.84	[27]	-1.177	0.096	-3.919	[20]
n-pentanol	0.40	0.86	0.84	[27]	-1.294	0.208	-3.908	[20]
n-hexanol	0.40	0.84	0.80	[27]	-1.153	0.083	-4.057	[20]
n-octanol	0.40	0.81	0.77	[27]	-1.048	-0.028	-4.229	[20]
n-decanol	0.45	0.82	0.70	[27]	-1.461	0.063	-4.053	[20]
chlorobenzene	0.71	0.07	0.00	[27]	-0.462	-3.038	-4.769	[20]
acetonitrile	0.75	0.40	0.19	[27]	0.326	-1.566	-4.391	[20]
carbondisulfide	0.61	0.07	0.00	[27]	-0.943	-3.603	-5.818	[20]

Comparison of the solvent hydrogen-bond donor acidity b* and coefficients b values shows an existence of a linear correlation between these coefficients:

$$b = -3.41(\pm 0.05) + 4.21(\pm 0.07)b^* \qquad (6.4)$$

$$N = 10;\ R^2 = 0.9975;\ SD = 0.080;\ F = 3199,$$

where N is the number of solvents (benzene, chlorobenzene, toluene, acetonitrile, n-propanol, n-butanol, n-pentanol, n-hexanol, n-octanol, n-decanol), R^2 – correlation coefficient, SD – standard deviation, and F – ratio of variance. Even though methanol and ethanol are outliers and had to be excluded, the correlation described by Eq.6.4 is sufficiently strong to support the possibility of replacing coefficient b with coefficient b* in Eq.6.3.

Comparison of the solvent hydrogen-bond acceptor basicity a* and coefficient a shows an existence of a linear correlation between these coefficients as well:

$$a = -7.2(\pm 0.1) + 3.9(\pm 0.1)a^* \qquad (6.5)$$

$$N = 6;\ R^2 = 0.9931;\ SD = 0.026;\ F = 571.9,$$

where N is the number of solvents (methanol, n-propanol, n-butanol, n-pentanol, n-hexanol, and n-octanol) and all the other coefficients are as above defined. Ethanol and n-decanol are outliers and had to be excluded. However, the correlation described by Eq.6.5 is sufficiently strong to support the possibility of replacing the coefficient a with the coefficient a* in Eq.6.3 at least in the case of ATPSs under consideration.

Comparison of the solvent polarity π* and coefficient s values shows an existence of linear correlations between these coefficients plotted in figure 6.1 and described as:

$$s = -1.70(\pm 0.03) + 1.77(\pm 0.07)\pi^* \qquad (6.6a)$$

$$N = 8;\ R^2 = 0.9900;\ SD = 0.059;\ F = 594.9$$

for n-hexane, n-octane, n-decane, benzene, toluene, methanol, n-octanol, and chlorobenzene, and

$$s = -1.92(\pm 0.05) + 1.7(\pm 0.1)\pi^* \qquad (6.6b)$$

$$N = 7;\ R^2 = 0.9765;\ SD = 0.064;\ F = 207.4$$

for n-heptane, ethanol, n-propanol, n-butanol, n-pentanol, n-hexanol, and carbondisulfide.

While there is no clear distinction between the two groups of solvents, it is readily apparent that both correlations are essentially parallel to each other. This suggests that ATPSs of the same aqueous nature may be expected to fit a single linear correlation.

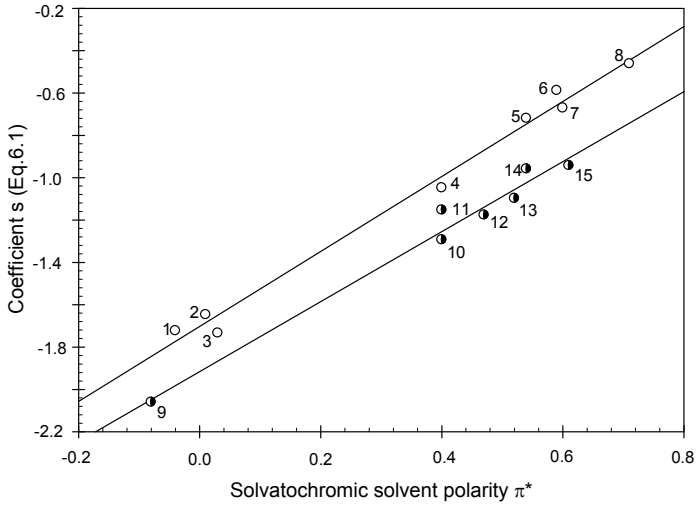

Figure 6.1. Coefficient s in Eq.6.1 representing the difference between the tendencies of the coexisting phases to interact with dipolar/polarizable solutes versus solvatochromically derived solvent polarity/polarizability π* (Table 6.1). Solvents: n-Hexane (1); n-octane (2); n-decane (3); n-octanol (4); toluene (5); benzene (6); methanol (7); chlorobenzene (8); n-heptane (9); n-pentanol (10); n-hexanol (11); n-butanol (12); n-propanol (13); ethanol (14), and carbondisulfide (15).

The results obtained indicate that the replacement of the solvent-specific coefficients a, b, and s in Eq.6.1 with the solvatochromic coefficients Δa*, Δb*, and Δπ* is valid, and while it may affect the constant z it should not affect the values of the solute descriptors in Eq.6.3.

The solvent-specific coefficient v in Eq.6.1 (or Eq.6.3) should also be considered. This coefficient, as mentioned above, is a combination of an exoergic dispersion term and endoergic cavity term. The cavity term in this case represents the endoergic effect of disrupting solvent-solvent bonds, and the exoergic dispersion term represents the solute-solvent London dispersion interactions [24]. In order to examine the possibility of replacing the coefficient v with parameter E (Eq. 3.5), representing the free energy of transfer of a CH_2 group between the coexisting phases, we compared the data reported in the literature [28-32] (Table 6.2).

Table 6.2. Coefficients E (Eq.3.5) representing the free energy of transfer of a CH_2 group between the coexisting phases of organic solvent-water two-phase systems and solvent-specific descriptors for the same systems according to Abraham's solvation equation (Eq.6.1)

System	E	Reference	z	r	s	a	b	v	Reference
Cyclohexane	1.900 ± 0.073	31	0.127	0.816	-1.731	-3.778	-4.905	4.646	32
Hexane	1.704 ± 0.053	31	0.361	0.579	-1.723	-3.599	-4.764	4.344	32
Toluene	1.819 ± 0.202	31	0.015	0.594	-0.781	-2.918	-4.571	4.533	28
CCl_4	1.439 ± 0.117	30,31[a]	0.212	0.602	-1.234	-3.515	-4.528	4.552	29
$CHCl_3$	1.330 ± 0.098	30,31[a]	0.125	0.118	-0.372	-3.390	-3.467	4.521	28
Benzene	1.419 ± 0.112	31	0.017	0.490	-0.604	-3.013	-4.628	4.587	28
Hexadecane	1.284 ± 0.110	31	0.087	0.667	-1.617	-3.587	-4.869	4.443	32
Diethyl ether	1.235 ± 0.170	31	0.256	0.649	-1.130	0.103	-4.998	4.380	29
Octanol	1.226 ± 0.021	31	0.088	0.562	-1.054	0.034	-3.460	3.814	32
Oleyl alcohol	1.141 ± 0.010	30,31[a]	-0.359	-0.270	-0.528	-0.035	-4.042	4.204	28
n-Hexanol	0.989 ± 0.040	30	0.143	0.718	-0.980	0.145	-3.214	3.403	28
n-Pentanol	0.930 ± 0.083	30	0.175	0.575	-0.787	0.020	-2.837	3.249	28

[a] – Average value for those reported in [30] and [31]

Comparison of the coefficients v (Eq.6.1) and coefficients E (Eq.3.5) presented in Table 6.2 shows an existence of linear correlations plotted in figure 6.2 and described as:

$$E = -1.5(\pm0.1) + 0.72(\pm0.03)v \qquad (6.7a)$$

$$N = 6;\ R^2 = 0.9920;\ SD = 0.043;\ F = 498.8$$

for n-hexane, cyclohexane, toluene, n-octanol, n-hexanol and n-pentanol; and

$$E = -2.1(\pm0.5) + 0.8(\pm0.1)v \qquad (6.7b)$$

$$N = 6;\ R^2 = 0.9209;\ SD = 0.036;\ F = 46.5$$

for hexadecane, chloroform, tetrachloromethane, benzene, diethyl ether, and oleyl alcohol.

As for s and π*, there is no clear distinction between the two groups of solvents, it is readily apparent that both correlations are essentially parallel to each other. This suggests, once more, that the ATPSs of the same aqueous nature may be expected to fit a single linear correlation.

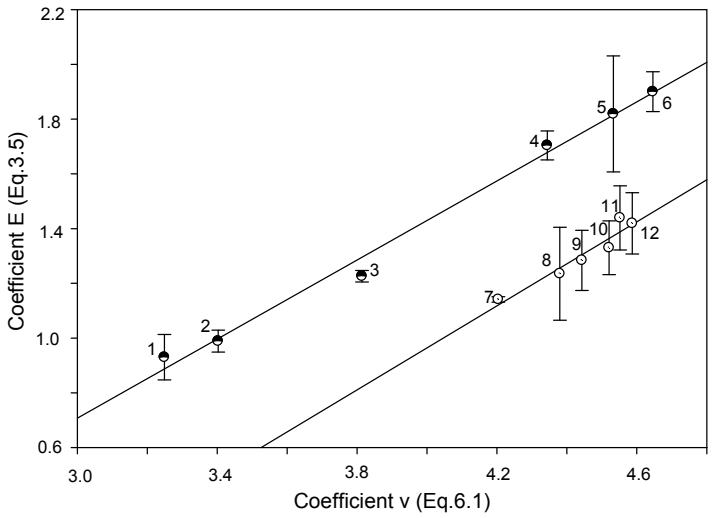

Figure 6.2. Coefficient E in Eq.3.5 representing the free energy of transfer of a CH_2 group from water into organic solvent versus coefficient v in eq.6.1 (Table 6.2). Organic solvents: n-Pentanol (1); n-hexanol (2); n-octanol (3); n-hexane (4); toluene (5); cyclohexane (6); oleyl alcohol (7); diethyl ether (8); n-hexadecane (9); chloroform (10); tetrachloromethane (11); benzene (12).

Based on the above considerations, the linear solvation equation of Abraham (Eq.6.1) when applied to partitioning of solutes in ATPSs may be expressed as:

$$\ln K = EV + \Delta\pi^*S + \Delta a^*A + \Delta b^*B + z \qquad (6.8)$$

where all the terms are as above defined with the solvatochromically derived coefficients $\Delta\pi^*$, Δa^*, and Δb^* used instead of s, a, and b in Eq.6.1, and coefficient E from Eq.3.5 is used instead of coefficient v in Eq.6.1.

Analysis of the solvent-specific coefficients determined experimentally for the ATPSs under analysis (Tables 3.2 and 3.3) shows that there is a strong linear correlation between coefficient E and the difference between the solvent polarity $\Delta\pi^*$ and the solvent hydrogen-bond donor acidity Δb^* of the coexisting phases. This correlation may be described as:

$$E = 0.00(\pm 0.01) - 1.1(\pm 0.1)\Delta b^* - 0.7(\pm 0.1)\Delta\pi^* \qquad (6.9)$$

$n = 10$, $r^2 = 0.9508$, SD = 0.03; F = 67.6

A similar correlation was found for the solvent-specific descriptors reported in the literature [27-32] for organic solvent-water biphasic systems. For the alcohol-water biphasic systems listed in Table 6.3 the correlation between the descriptors is:

$$v = 0.8(\pm 0.1) - 0.70(\pm 0.06)b - 0.5(\pm 0.1)s \qquad (6.10)$$

$n = 15$, $r^2 = 0.9836$, SD = 0.075, F = 359.2.

Analysis of the general applicability of the correlation established for the solvents of similar nature was beyond the scope of the present work. However, it is clear that the correlations described by Eqs. 6.9 and 6.10 indicate that one of the coefficients $\Delta\pi^*$, Δb^*, or E might be sufficient for analysis of the solutes partitioning in ATPSs.

Table 6.3. Coefficients s, b and v in Abraham's solvation equation for water-alcohol two-phase systems

Organic solvent[a]	S	b	v	Reference
Methanol/dry	-0.671	-3.389	3.512	20
Ethanol/dry	-0.959	-3.645	3.928	20
n-Propanol/dry	-1.098	-3.893	4.036	20
n-Butanol/dry	-1.177	-3.919	4.122	20
n-Pentanol/dry	-1.294	-3.908	4.208	20
n-Hexanol/dry	-1.153	-4.057	4.249	20
n-Heptanol/dry	-1.258	-4.155	4.415	20
n-Octanol/dry	-1.048	-4.229	4.219	20
n-Decanol/dry	-1.461	-4.053	4.293	20
Ethyleneglycol/dry	-0.522	-2.492	2.708	20
Isobutanol	-0.693	-2.258	2.776	28
Pentanol	-0.787	-2.837	3.249	28
Hexanol	-0.980	-3.214	3.403	28
Octanol	-1.054	-3.460	3.814	32
Decanol	-0.974	-3.798	3.945	28

[a] /dry denotes the results calculated for hypothetical partition between water and dry solvents. Alcohol-water two-phase systems were used for partition experiments in all other cases.

An important feature of the ATPSs under consideration, and that differentiates them from organic solvent-water systems is the presence of different amounts of phosphate buffer and NaCl in the coexisting phases [1,33-35]. These and other salts are known to affect the

polymer composition of the phases and their solvent properties so much that, for example, the same Dex-PEG systems with different concentrations of salt and/or buffer additives are to be considered as different ATPSs with different solvent properties of the phases [1, pp.155-220]. Different salt compositions of the coexisting phases in a given ATPS create different electrostatic properties of the aqueous media in these phases [1, pp196-208]. The difference between the electrostatic properties translates into different ion-ion, ion-dipole and possibly dipole-dipole solute-solvent interactions in the coexisting phases of an ATPS. LSER (Eq.6.1 or Eq.6.3) is applicable to partitioning of nonionic solutes but an added advantage of ATPSs is the possibility to examine partitioning of ionizable compounds, and hence it appears that an additional descriptor capable of quantifying the difference between the electrostatic properties of the phases is necessary. Zaslavsky [1, pp.208-216] has proposed the use of a contribution of an ionic group into the solute partition coefficient as an empirical measure of the difference in question. The majority of the relevant experimental results were obtained for sodium salts of p-dinitrophenyl-amino acids, i.e. compounds containing a DNP-NH-CH-COO-Na^+ group. The disadvantages of using this particular group, which is voluminous and contains substituted aromatic ring, as a probe of solely electrostatic ion-ion, ion-dipole and dipole-dipole interactions are obvious. Only to a first approximation the free energy of transfer of this group between the coexisting phases of an ATPS can be viewed as a measure of the ability of aqueous media to participate in a particular kind of intermolecular interactions. This parameter is represented by coefficient c in Eq.3.5, and we included it in the modified linear solvation equation as follows:

$$\ln K = z + \Delta\pi^*S + \Delta^*A + \Delta b^*B + cC \qquad (6.11)$$

where coefficient c is the contribution of a DNP-NH-CH-COO-Na+ group into lnK (Eq.3.5), parameter C is the solute descriptor characterizing the ability of a solute to participate in electrostatic ion-ion, ion-dipole, and dipole-dipole interactions with an aqueous media, and all the other terms are as previously defined. Analysis of the data showed that contribution of the coefficient Δa^* (representing the difference between the solvent hydrogen bond basicity of the phases) to the solute partition behavior in the ATPS under conditions employed was not

statistically significant in agreement with the results reported by Moody et al. [3] for an aqueous Dex-PEG two-phase system. Therefore Eq.6.11, was simplified to:

$$\ln K = z - \Delta\pi^{*}S - \Delta b^{*}B + cC \qquad (6.12)$$

where all of the terms are as above defined.

The solute descriptors calculated by multiple linear regression according to Eq.6.12 are presented in Table 6.4, and the partition coefficients ($\ln K_{calc}$) calculated for all the ATPSs employed are listed in Table 6.5 and plotted against the experimental values ($\ln K_{exp}$) in figure 6.3. It should be noted that the partition coefficients for Trp, di-Trp, tri-Trp, and Na salt of DNP-α-amino-n-octanoic acid are not adequately described by Eq.6.12 in some of the ATPSs. The reason is not clear yet.

Table 6.4 Solute descriptors for p-nitrophenyl-monosaccharides, dinitrophenyl-amino acids, tryptophan, di- and tri-tryptophan calculated from the partition coefficients in different ATPS reported in [13] with Eq.6.12 (N – number of ATPS used)

Solute	z	$-\Sigma\beta_2^H$	$-\pi^2_H$	c	N	F	SD	r^2
fucopyranoside	0.02 ± 0.04	2.0 ± 0.4	1.6 ± 0.5	0.42 ± 0.09	10	39.1	0.10	0.9513
galactopyranoside	-0.01 ± 0.04	1.7 ± 0.4	1.3 ± 0.5	0.45 ± 0.09	10	38.8	0.10	0.9510
β-glucopyranoside	0.02 ± 0.04	2.0 ± 0.4	1.4 ± 0.4	0.38 ± 0.08	10	44.5	0.09	0.9570
α-glucopyranoside	0.01 ± 0.03	2.1 ± 0.4	1.5 ± 0.4	0.37 ± 0.07	10	52.5	0.08	0.9633
mannopyranoside	-0.03 ± 0.04	2.4 ± 0.5	1.5 ± 0.5	0.53 ± 0.09	10	52.1	0.10	0.9630
DNP-glycine Na-salt	-0.03 ± 0.02	0.4 ± 0.2	0.0 ± 0.3	1.08 ± 0.05	10	307	0.05	0.9935
DNP-alanine Na-salt	0.00 ± 0.01	1.3 ± 0.1	0.8 ± 0.1	1.00 ± 0.02	10	1350	0.03	0.9985
DNP-norvaline Na-salt	0.03 ± 0.02	2.3 ± 0.2	1.5 ± 0.2	1.16 ± 0.04	10	690	0.05	0.9971
DNP-norleucine Na-salt	0.03 ± 0.03	3.1 ± 0.4	2.1 ± 0.4	1.23 ± 0.08	10	281	0.08	0.9929
DNP-α-AO acid Na-salt	0.04 ± 0.05	6.3 ± 0.4	4.7 ± 0.5	1.27 ± 0.07	6	616	0.06	0.9989
Tryptophan	-0.10 ± 0.05	0.3 ± 0.5	-0.2 ± 0.5	-0.6 ± 0.1	9	18.6	0.9	0.9176
Di-tryptophan	0.18 ± 0.04	2.9 ± 0.5	3.1 ± 0.5	0.1 ± 0.1	8	31.6	0.10	0.9595
Tri-tryptophan	0.31 ± 0.05	8.0 ± 0.5	5.5 ± 0.6	0.6 ± 0.1	7	248	0.10	0.9960

Table 6.5. Partition coefficients, K, experimental (exp) and calculated (calc), for p-nitrophenyl-monosaccharides, dinitrophenyl-amino acids, and tryptophan, di- and tri-tryptophan examined in the ATPSs indicated

Solute		Dex-Ficoll	Dex-PEG	PEG-Ucon	Dex-Ucon	PES-PEG	PES-Ucon	Ficoll-PEG	Ficoll-Ucon	PES-Dex	PES-Ficoll
4-NP-β-D-fuco-pyranoside	K_{exp}	1.22	1.31	2.74	1.55	0.87	1.00	1.02	1.24	0.76	0.91
	K_{calc}	1.15	1.13	2.80	1.50	0.86	1.15	0.97	1.26	0.79	1.00
4-NP-β-D-galacto-pyranoside	K_{exp}	1.25	1.18	2.54	1.40	0.81	0.95	0.99	1.16	0.74	0.90
	K_{calc}	1.11	1.07	2.62	1.37	0.83	1.07	0.92	1.19	0.79	0.97
4-NP-β-D-gluco-pyranoside	K_{exp}	1.22	1.27	2.54	1.54	0.86	1.01	1.00	1.25	0.78	0.92
	K_{calc}	1.15	1.13	2.61	1.48	0.87	1.15	0.97	1.28	0.81	0.99
4-NP-α-D-gluco-pyranoside	K_{exp}	1.18	1.23	2.55	1.59	0.86	1.01	1.02	1.27	0.78	0.92
	K_{calc}	1.14	1.12	2.60	1.50	0.85	1.16	0.96	1.29	0.80	0.98
4-NP-α-D-manno-pyranoside	K_{exp}	1.23	1.18	3.14	1.60	0.77	0.93	0.98	1.28	0.70	0.85
	K_{calc}	1.14	1.07	3.24	1.51	0.77	1.09	0.89	1.30	0.74	0.93
DNP-glycine Na	K_{exp}	1.13	0.87	4.11	1.02	0.62	0.76	0.80	0.97	0.79	0.98
	K_{calc}	1.14	0.88	4.14	1.03	0.68	0.74	0.76	0.98	0.78	0.93
DNP-alanine Na	K_{exp}	1.21	0.99	4.80	1.33	0.71	0.87	0.84	1.08	0.76	0.95
	K_{calc}	1.19	0.99	4.82	1.27	0.72	0.88	0.82	1.11	0.75	0.97
DNP-norvaline Na	K_{exp}	1.28	1.09	7.83	1.65	0.68	0.94	0.87	1.26	0.71	0.91
	K_{calc}	1.30	1.07	7.75	1.59	0.68	0.97	0.82	1.30	0.69	0.97
DNP-norleucine Na	K_{exp}	1.33	1.16	10.13	2.05	0.65	0.94	0.88	1.39	0.68	0.88
	K_{calc}	1.36	1.13	10.13	1.85	0.65	1.05	0.82	1.45	0.64	0.96
DNP-α-amino-n-octanoic acid Na	K_{exp}	1.49	1.35	23.02	3.50	0.42	0.75	0.93	2.12	0.39	0.63
	K_{calc}	1.52	1.43	22.78	3.48	0.61[a]	1.53[a]	0.88	2.10	0.48[a]	0.96[a]
Tryptophan	K_{exp}	1.03	0.98	0.43	1.01	0.93	0.97	1.02	1.02	1.02	1.03
	K_{calc}	0.86[a]	0.94	0.45	0.93	1.03	1.05	1.00	1.02	1.03	0.89
Ditryptophan	K_{exp}	1.38	1.71	2.74	4.64	1.22	1.61	1.10	1.54	0.78	1.15
	K_{calc}	1.28	1.55	2.78	2.21[a]	1.16	1.77	1.30[a]	1.53	0.83	1.23
Tritryptophan	K_{exp}	2.20	3.00	17.27	24.36	1.85	2.66	1.35	3.93	0.60	1.27
	K_{calc}	2.00	2.17[a]	17.79	6.16[a]	0.90[a]	2.83	1.32	3.84	0.66	1.21

[a] – not included in the correlation

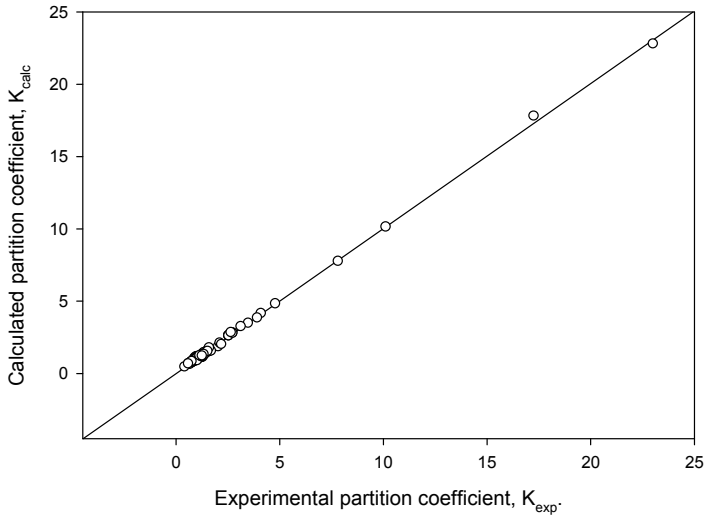

Figure 6.3. Logarithm of the partition coefficient for compounds (listed in Table 6.5) in ten different ATPSs (Table 3.1) versus values predicted by the empirical characteristics of the solvent features of the coexisting phases and the calculated solute descriptors (Table 6.4) utilizing Eq.6.12.

It should also be noted that the overall solute hydrogen-bond basicity descriptor, B, and the solute dipolarity/polarizability descriptor, S, contribute negatively to the logarithm of the partition coefficient value. Similarly negative contributions of the solute polarity/polarizability and hydrogen-bond accepting (HBA) capacity into the logarithm of the solute partition coefficient in octanol/water two-phase system were reported by Marcus [36]. The lack of the volume term is rather surprising. This term was reported by Moody et al. [3] to be the second most significant term (after the solvent hydrogen bond acidity term) in the LFER equation for solutes in the Dex-PEG ATPS. The likely reason seems to be the apparent linear relationship between the volume term and the solvent hydrogen-bond acidity and solvent dipolarity/ polarizability parameters described above by Eqs.6.9 and 6.10. The absence in Eq.6.12 of the vV term might also be due to a very limited number of solutes examined here. Analysis of the proteins partition coefficients studied in the next chapter might help us to resolve this issue.

6.4 Conclusions

We have examined the Kamlet solvatochromic solvent features of aqueous media in the coexisting phases of ten different aqueous polymer/polymer two phase systems of the same overall ionic composition. Analysis of the data reported in the literature demonstrated that each of the three solvatochromic descriptors (solvent polarity/polarizability, solvent hydrogen-bond acidity, and solvent hydrogen-bond basicity) is linearly correlated with the corresponding coefficient in the Abraham's linear free energy solvation equation. Combination of the solvatochromic solvent characteristics of the coexisting phases together with the parameter c derived from partitioning of a homologous series of solutes in ATPSs and used as a characteristic of the differences between the electrostatic ion-ion, ion-dipole, and dipole-dipole solute-solvent interactions in the coexisting phases was used to calculate solute descriptors for a series of p-nitrophenyl-monosaccharides, dinitrophenyl-amino acids, and tryptophan, di- and tri-tryptophan in all the ATPSs employed. The descriptors calculated for the set of compounds are constant for all ten ATPSs. This approach is a reversed variation of the Abraham's approach – the solvent features are determined experimentally, and the solute descriptors are derived from the partition experiments. This approach may be useful for quantification of the solute descriptors for biological macromolecules, such as proteins and nucleic acids, for which ATPSs provide media suitable for separation and analysis.

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7. Protein descriptors from partitioning in polymer/polymer aqueous two-phase systems

7.1 Introduction

In the previous Chapter we tried to compensate the limitations of Abraham's solvation equation [1-3] and the solvatochromic method to the partitioning of biomolecules in ATPSs by combining the solvatochromic approach with the group-contribution approach.

The proposed methodology is described by the following equation:

$$\ln K = z + \Delta\pi^*S + \Delta a^*A + \Delta b^*B + cC + EV \qquad (7.1)$$

where all the terms are as defined previously with the solvatochromically derived coefficients $\Delta\pi^*$, Δa^*, and Δb^* used instead of s, a, and b in Abraham's equation (Eq.6.1) and coefficient E from Eq.3.5 is used instead of coefficient v in Eq.6.1. The new solute descriptor, c, is intended to represent the different ion-ion, ion-dipole and possibly dipole-dipole solute-solvent interactions in the coexisting phases of an ATPS [4]. This approach is a reversed variation of the Abraham's approach – the solvent features are determined experimentally, and the solute descriptors are derived from the partition experiments.

Eq. 7.1 can be simplified to a previously presented expression:

$$\ln K = z + \Delta\pi^*S + \Delta b^*B + cC \qquad (6.12)$$

to describe the partitioning of five nitrophenyl-monosaccharides, five dinitrophenyl-amino acids and tryptophan and its di- and tri-peptides in ten different ATPSs (Chapter 6) . The absence of Δa^* (representing the difference between the solvent hydrogen bond basicity of the

phases) to the solute distribution behavior in the ATPSs under the conditions employed was not statistically significant, in agreement with the results reported by Moody et al. [5] using Abraham´s approach for an aqueous Dex-PEG two-phase system. The lack of the volume term was rather surprising, and it is suggested to be due to the linear relationship between the volume term and the solvent hydrogen-bond acidity and solvent dipolarity/ polarizability parameters, as well as to the very limited number of solutes examined.

In this Chapter we present the distribution coefficient of thirteen randomly selected proteins (Chicken egg lysozyme, bovine α-chymotrypsinogen A, bovine hemoglobin, bovine ribonuclease B, bovine ribonuclease A, bovine trypsinogen, human hemoglobin, horse myoglobin, bovine β-lactoglobulin, human γ-globulin, bovine γ-globulin, porcine lipase, and human transferrin) in the Dextran-Ficoll, PES-Dextran and PES-Ficoll ATPSs, at particular polymer concentrations, all containing 0.15M NaCl in 0.01M phosphate buffer, pH7.4, at 23 ^{0}C. These experimental data along with data reported in previous Chapters are used to extend the methodology to the study of protein's partitioning behavior.

7.2. Experimental

7.2.1. Materials

7.2.1.1. Polymers. All polymers, amino acids, o-Phthaldialdehyde (OPA) reagent solution (complete), and solvatochromic dyes used were the same as previously described (Chapter 3, pp.30-31)).

7.2.2. Methods

7.2.2.1. Phase diagrams. The ATPSs are presented in Chapter 3 (pp.31-32).

7.2.2.2. Solvatochromic Studies. The solvatochromic studies were carried out as described in Chapter 3 (pp.34-35).

7.2.2.3. Partitioning.

The partitioning experiments were carried out as described previously (Chapter 4), and the data is given in Table 7.1.

7.3 Results and Discussion

The partition coefficients for all the proteins in the aqueous Dex-Ficoll, Dex-PES, and Ficoll-PES examined in the present work are listed in Table 7.1. Analysis of the proteins partition coefficients, K, in terms of Eq.7.1 with the solvent parameters presented in Tables 3.1 and 3.2 indicates that Eq.7.1 is reduced to equation 6.11:

$$\ln K = z + \Delta\pi^*S + \Delta a^*A + \Delta b^*B + cC \qquad (6.11)$$

It was found previously (Chapter 6) that partition coefficients for low molecular weight compounds in ATPSs when described in terms of Eq.7.1 did not need the inclusion of the volume term. This observation disagrees with the conclusion of Moody et al. [5], who reported the volume term to be the most significant term in the LFER equation for small solutes in Dex-PEG ATPS. The lack of the volume term in Eq.6.11 for proteins with molecular weights up to ca.180 KDa is rather surprising**.** This term, as mentioned above, is a combination of an exoergic dispersion term and endoergic cavity term. The cavity term in this case represents the endoergic effect of disrupting solvent-solvent bonds, and the exoergic dispersion term represents the solute-solvent London dispersion interactions [6]. Since the most important solvent-solvent interactions in ATPS are hydrogen bonds it seems reasonable to suggest that these interactions are already accounted for by the Δb^*B, Δa^*A, and $\Delta\pi^*S$ terms.

It is also important to emphasize that the set of solvent descriptors $\Delta\pi^*$, Δa^*, and Δb^*, sufficient for the representation of partitioning of nonionic solutes in organic solvent/water biphasic systems, is inadequate for the description of partitioning of ionic solutes in ATPSs,

where an additional descriptor c is needed. Detailed consideration of this descriptor can be found in Chapter 6.

Table 7.1. Experimental distribution coefficients, K, for proteins examined in the ATPSs indicated

Solute/system	Ficoll-Dex	Dex-PES	Ficoll-PES
RNAse A	1.16	1.26	1.37
RNAse B	1.07	1.45	1.44
Chymotrypsinogen	2.02	1.31	1.99
Trypsinogen	1.988	1.07	1.56
Lysozyme	2.31	0.42	1.06
Hemoglobin bovine	1.19	2.20	2.32
Hemoglobin human	1.41	1.73	2.30
Lactoglobulin	0.41	6.02	2.36
Transferrin	0.580	7.15	3.04
Myoglobin	0.69	1.96	1.41
Lipase	0.90	1.27	1.04
γ-Globulin human	5.59	2.37	8.26
γ-Globulin bovine	4.48	3.11	9.0

It should be noted that the three ATPSs that do not fit the so-called Collander equation for protein partition coefficients, particularly, Dex-Ficoll, PES-Ficoll, and PES-Dex are the only ATPSs out of ten systems examined with the solvent descriptors Δb^* and Δa^* having the same sign. It means that in these particular ATPSs in contrast to all the other two-polymer ATPSs examined, both solvent H-bond basicity and solvent H-bond acidity of one phase exceeds those of the coexisting phase. Unfortunately, our current limited understanding of the water structure and solvent properties of aqueous media [7,8] does not allow to propose even a semi-quantitative model for the properties of the media in the phases of ATPSs. It can be concluded, however, that from the point of view of analytical applications, protein partitioning in the three aforementioned ATPSs provide information different from that given by partitioning in the other two-polymer ATPSs in this study.

We explored the relationships between the coefficients a_{io} and b_{io} in the Collander equation (eq. 4.4) and solvent descriptors of the systems under comparison and found that coefficient a_{io} is related to parameters Δa^* and c according to:

$$1/a_{io} = z_{ao} + z_{a1}\Delta a^*_i + z_{a2}c_i \qquad (7.2)$$

where z_{ao}, z_{a1}, z_{a2} are constants, and Δa^*_i and c_i are parameters representing the solvent properties of the ATPSs as above described. The coefficients z_{ao}, z_{a1}, z_{a2} are presented in Table 7.2. It is necessary to emphasize once again that the standard solvent descriptors commonly used for analysis of partitioning of nonionic solutes in solvent biphasic systems are not sufficient, and the descriptor c appears to have an important contribution here.

Table 7.2. Coefficients z_{ao}, z_{a1} and z_{a2} in Eq.7.2 with different ATPSs used as a reference system

Reference ATPS	Z_{a0}	Z_{a1}	Z_{a2}	r^2
Dex-PEG	0.72 ± 0.03	-0.6 ± 0.2	-3.3 ± 0.1	0.9963
Dex-Ucon	0.92 ± 0.04	-0.5 ± 0.2	-4.2 ± 0.2	0.9966
PES-PEG	0.43 ± 0.04	-0.4 ± 0.2	-2.0 ± 0.2	0.9877
PES-Ucon	0.45 ± 0.02	-0.4 ± 0.1	-2.0 ± 0.1	0.9956
Ficoll-PEG	0.52 ± 0.02	-0.4 ± 0.1	-2.3 ± 0.1	0.9965
Ficoll-Ucon	0.71 ± 0.05	-0.4 ± 0.2	-3.2 ± 0.2	0.9932

r^2 – correlation coefficient; N = 6 – number of ATPSs used in analysis.

Analysis of coefficient b_{io} in Collander equation (equation 4.4) indicates that this coefficient is related to the solvent descriptors of the ATPSs under comparison, according to:

$$b_{io} = z_o + z_1\,\Delta\pi_i^* + z_2(1/\Delta b_i^*) + z_3(1/\Delta a_i^*) + z_4C_i \qquad (5.4)$$

where z_{bo}, z_{b1}, z_{b2}, z_{b3}, and z_{b4} are constants, and $\Delta\pi^*_i$, Δa^*_i, Δb^*_i, and c_i are parameters representing the solvent properties of the ATPSs as described above. The coefficients z_{bo}, z_{b1}, z_{b2}, z_{b3}, and z_{b4} are presented in Table 5.4. It should be noted that in all the cases the contributions of the $z_{b2}(1/\Delta a^*_i)$ and $z_{b3}(1/\Delta b^*_i)$ terms are small. The $z_{b1}\Delta\pi^*_i$ term provides the most significant contribution. When Dex-Ucon, PES-PEG, PES-Ucon, and Ficoll-PEG are used as reference systems $z_{b4} = 0$ in Eq.7.3, implying that the contribution of the solvent descriptor c_i is the same in the systems under comparison. When PES-Ucon is used as a reference system coefficient $z_{b3} = 0$ (as well as z_{b4}), indicating lack of contribution of the $z_{b3}(1/\Delta b^*_i)$ term. When Dex-PEG and Ficoll-Ucon are used as the reference systems contributions of the $z_{b4}c_i$ term are quite significant suggesting that these two systems differ from those under comparison in regard to the solute-aqueous media ion-ion and ion-dipole interactions.

For all the proteins examined, solute descriptors calculated from the multiple linear regression analysis are presented in Table 7.3. It is the first time to our knowledge that solute descriptors are experimentally established for proteins. Analysis of the descriptors values listed in Table 7.3 indicates they are interrelated as (figure 7.1):

$$\pi^2_H = 0.3(\pm 0.6) + 0.58(\pm 0.04)B + 0.9(\pm 0.1)A \qquad (7.3)$$

$$N = 13;\ r^2 = 0.9954;\ F = 1089;\ SD = 1.1$$

where N is the number of proteins examined, r^2 is the correlation coefficient, F stands for ratio of variance and SD is standard deviation.

Table 7.3. Solute descriptors for proteins calculated from the distribution coefficients in different ATPSs with eq.6.11

Solute	z	$\Sigma\beta_2^H$	$\Sigma\alpha_2^H$	π^2_H	c	N	F	SD	r^2
RNAse A[a]	0.17 ± 0.08	9.3 ± 0.9	0.3 ± 1.5	6.0 ± 1.0	3.3 ± 0.5	8	44.7	0.14	0.983
RNAse B[a]	0.2 ± 0.2	9.5 ± 1.7	0.3 ± 2.8	6.1 ± 1.8	3.0 ± 0.9	8	13.5	0.25	0.947
Chymotrypsinogen[b]	0.63 ± 0.08	0.9 ± 0.8	-5.2 ± 1.5	-5.4 ± 0.9	1.3 ± 0.4	8	23.1	0.13	0.969
Trypsinogen[a,b]	0.4 ± 0.1	4.9 ± 1.1	-3.1 ± 1.8	0.1 ± 1.2	3.5 ± 0.6	7	17.6	0.16	0.972
Lysozyme	0.2 ± 0.1	-5.6 ±1.1	-7.5 ± 2.0	-9.2 ±1.2	3.1 ± 0.6	9	21.6	0.18	0.956
Hb Bovine[a,c]	0.4 ± 0.2	27.8 ± 3.3	7.1 ± 4.6	24.5 ± 3.6	7.9 ± 1.4	7	24.5	0.41	0.980
Hb Human[a,c]	0.4 ± 0.2	23.2 ± 2.5	4.9 ± 3.5	19.0 ± 2.7	7.3 ± 1.1	7	30.7	0.31	0.984
Lactoglobulin[a,c]	0.3 ± 0.3	33.4 ± 3.5	13.3 ± 4.8	31.3 ± 3.8	3.9 ± 1.5	7	25.9	0.44	0.981
Transferrin[a]	0.3 ± 0.7	43.9 ± 7.5	12.1 ± 12.4	37.6 ± 8.1	10.8 ± 4.0	8	13.1	1.12	0.946
Myoglobin[a]	0.0 ± 0.2	15.9 ± 2.3	5.7 ± 3.8	14.5 ± 2.5	3.1 ± 1.2	8	18.3	0.34	0.961
Human g-Globulin[a]	1.4 ± 0.7	36.7 ± 7.7	2.2 ± 12.8	24.4 ± 8.3	17.9 ± 4.1	8	10.5	1.16	0.934
Lipase[a,c]	0.01 ± 0.03	4.3 ± 0.4	1.5 ± 0.6	4.1 ± 0.5	0.7 ± 0.2	7	29.3	0.05	0.983
Bovine g-Globulin[a,b]	1.5 ± 0.6	41.2 ± 7.0	7.8 ± 11.7	29.1 ± 7.6	15.9 ± 3.9	7	13.6	1.05	0.965

a – outlier: Ucon-PES; b – outlier: PEG-Ficoll; c – outlier: Ucon-Dex; (N – number of ATPSs used)

It seems that the interrelationship described by Eq.7.3 is not unique to proteins. A similar relationship was found to exist for tripeptide derivatives studied by Plass et al. [10]:

$$\pi^2_H = -9.5(\pm 0.97) + 8.3(\pm 0.5)B - 4.8(\pm 0.4)A \qquad (7.4)$$

$$N = 12;\ r^2 = 0.9716;\ F = 153.9;\ SD = 0.28$$

It should be mentioned that the solute descriptors in [10] were calculated from HPLC retention times on a set of five different HPLC columns using Abraham's equation (Eq.6.1). Similar interrelationships for the solute descriptors can also be found for other classes of compounds reported in the literature, but analysis of these data is beyond the scope of the present study.

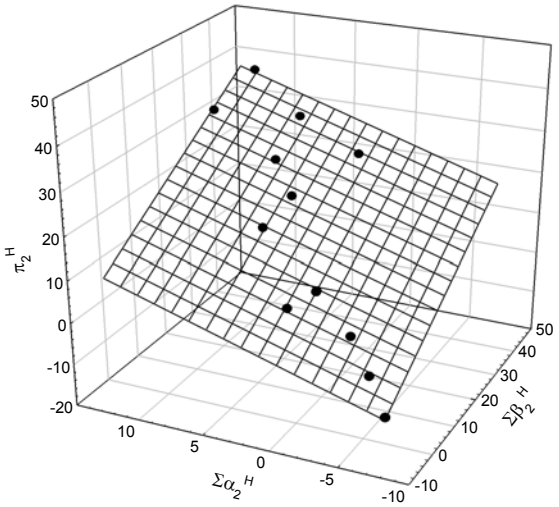

Figure 7.1. Correlation between protein descriptors (Eq. 7.4): π_2^H - polarity/polarizability; $\sum\alpha_2^H$ and $\sum\beta_2^H$ - total hydrogen bond donating and accepting capacities of the solute.

The comparison of the partition coefficients K_{calc} calculated using Eq.6.11 from solvent descriptors (Tables 3.2 and 3.3) and protein solute descriptors (Table 7.3) with the experimental K_{exp} values shows (figure 7.2) that partition coefficients are generally adequately described by Eq.6.11 for all the ATPS except Ucon-PES system. It seems that the protein-solvent interactions as described by Eq.6.11 are not completely accounted for in regard to the protein partitioning in this particular ATPS.

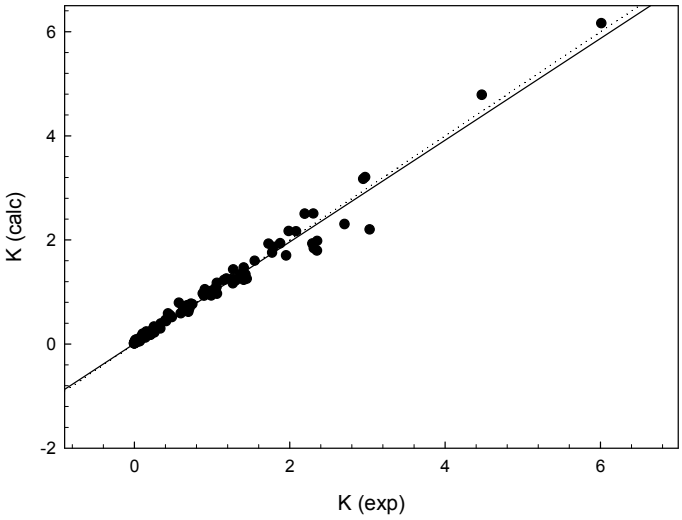

Figure 7.2. Distribution coefficients for proteins in different ATPSs (Table 7.1) versus values predicted by the empirical characteristics of the solvent features of the coexisting phases and the calculated solute descriptors (Table 7.3) utilizing Eq.6.11. Theoretical (…) and experimental (-) lines.

Analysis of the solute descriptors (polarity/polarizability, S, hydrogen bond donor acidity, A, and hydrogen bond acceptor basicity B) for the proteins (Table 7.2), shows no correlation with the protein molecular weight or isoelectric point, pI, as expected. Hydrogen-bond acceptor basicity, B, for all the proteins examined in this study exceeds that of their hydrogen bond donor acidity. Hydrogen bond acidity and basicity values are close (within the experimental error limits) for lysozyme and for trypsinogen.

Comparison of the solute descriptors values for the proteins (Table 7.3) and for tryptophan di- and tri-peptides reported previously (Chapter 6) indicates that in absolute value hydrogen bond basicity and polarity/polarizability for some proteins, such as chymotrypsinogen, trypsinogen, lipase or lysozyme, are of the same order of magnitude as those reported for small peptides. This observation suggests that the same scale of values might be used for solute descriptors to characterize small molecules and biopolymers, namely proteins.

Most of the descriptors used and reported in the literature for analysis of protein-protein interfaces [11] or prediction of protein functional families [12] are based on analysis of the physicochemical properties of amino acid residues. The limitations of these descriptors are widely recognized. The descriptors experimentally determined in this study might be more suitable for protein characterization.

7.4 Conclusions

Partitioning data for thirteen randomly selected proteins in three different ATPSs are presented. These data along with data from previous Chapters were used in a proposed LFER developed to describe the distribution of ionizable solutes in ATPSs. Proteins descriptors were obtained by this methodology. It is the first time to our knowledge that solute descriptors are experimentally established for proteins. Analysis of these descriptors seems to be in agreement with Kauzmann [13] view according to which "there is probably a basic structure more or less common to all proteins".

7.5 References

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8. Conclusions and Future Work

The aqueous two-phase systems (ATPSs) technology has been object of intense research in the last few decades. Indeed, their particular advantages, such as providing an innocuous environment for biomolecules, being a so-called "green" technology, the low cost, its easy scaling-up, extreme sensitivity towards solute surface properties, etc., make them attractive not only for separation purposes but also for bioanalytical applications.

Since the partition coefficient defines the information provided by a change in the analyte distribution behavior under altered partition conditions or as a response to a change in the analyte properties, the comprehension of the partitioning mechanisms is crucial if the ATPSs are to be used for analytical applications. This was the major objective of the present work.

Thus, in Chapter 3, a physico-chemical characterization of several ATPSs was carried out: ten ATPSs formed by all pair combinations of Dextran (Dex-75), Ficoll-70, polyethylene glycol (PEG-8000), hydroxypropyl starch (PES-100), and Ucon50HB5100 (a random copolymer of ethylene glycol and propylene glycol) all containing 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.4, at 23 ^{0}C were obtained. Partition coefficients of a series of dinitrophenylated (DNP) amino acids with aliphatic side-chains were studied in all the aqueous two-phase systems at particular polymer concentrations. Free energies of transfer of a methylene group between the coexisting phases, $\Delta G(CH_2)$, were determined as measures of the difference between the hydrophobic character of the phases. The solvatochromic solvent parameters characterizing the solvent polarity (π^*), solvent hydrogen-bond donor acidity (b*), and solvent hydrogen-bond acceptor basicity (a*) of aqueous media were also measured in the coexisting phases of the above ten different aqueous polymer/polymer two-phase systems. Furthermore, partition coefficients of tryptophan (Trp) and its di- and tri-peptides and a set of p-nitrophenyl (NP)-monosaccharides were measured in all the two-phase systems, and the data obtained compared with the $\Delta G(CH_2)$ values obtained in the systems. It was established that for eight out of ten of two-phase systems of different polymer compositions the partition coefficients for Trp peptides

correlate well with the $\Delta G(CH_2)$ values. Similar correlations for NP-monosaccharides were valid for seven out of ten two-phase systems. These observations indicated that the difference between the hydrophobic characters of the coexisting phases represented by the $\Delta G(CH_2)$ value can be used as solvent descriptor in ATPSs of different polymer compositions.

In Chapter 4 we selected two particular systems to verify the possibility of the application of the Collander equation. Thus, distribution coefficients for a variety of proteins were measured in the ATPSs Dex-PEG and Dex-Ucon both containing 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.4, at 23 ^{0}C. Distribution coefficients of some selected solutes were also measured in the above two-phase systems at three different polymer concentrations for each system. It was established that the distribution coefficients for all the proteins examined in the ATPSs are correlated according to the so-called Collander linear equation.

In Chapter 5 we checked for the applicability of the Collander equation to polymer/polymer ATPSs in more general terms. Thus, distribution coefficients of randomly selected proteins (Chicken egg lysozyme, bovine α-chymotrypsinogen A, bovine hemoglobin, horse heart cytochrome c, bovine ribonuclease B, bovine ribonuclease A, bovine trypsinogen, human hemoglobin, horse myoglobin, bovine β-lactoglobulin, human γ-globulin, bovine γ-globulin, porcine lipase, and human transferrin) were measured in ATPSs formed by different combinations of Dex, Ficoll-70, PEG, PES, and Ucon at particular polymer concentrations, all containing 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.4, at 23 ^{0}C. Most of the proteins in the PEG-Ucon system precipitated at the interface. In the other ATPSs, namely, PES-PEG, PES-Ucon, Ficoll-PEG, Ficoll-Ucon, and in Dex-PEG and Dex-Ucon described earlier (Chapter 4) the distribution coefficients for the proteins were correlated according to the solvent regression equation. Moreover, the coefficients on the Collander equation were correlated with independently obtained physico-chemical properties of the phases.

The major conclusions from Chapters 4 and 5 were that (i) the proteins randomly selected for analysis in this study do not interact with the phase-forming polymers; and (ii) the

distribution coefficient for any protein determined in one of the two systems may be used to predict the distribution coefficient for the same protein in the other systems.

It should also be mentioned that the correlation observed in the previous Chapters was validated only for ATPSs with the same ionic composition. Before draw any general conclusion in regard to the existence of a linear correlation between the logarithms of the distribution coefficients of different proteins in ATPSs more experimental work is still needed. In our opinion valuable information might be obtained by running similar experiments: (i) at different ionic composition and pH of the aqueous media; (ii) on different types of ATPSs, like polymer/salt aqueous two-phase systems; and (iii) with chemically different solutes.

There were however three aqueous two-phase systems, Dex-Ficoll, Dex-PES, and Ficoll-PES, that displayed distribution behavior of proteins different from that observed in Chapters 4 and 5.

The linear solvation energy relationships (LSERs) have contributed to a significant insight into the physical chemical processes governing solute-solvent interactions. LSERs have been shown to accurately predict solvation free energies for a wide range of dilute solutes across different solvent environments. Thus, we explored its applicability to ATPSs.

Accordingly, In Chapter 6 a new LFER was developed to account for the electrostatic ion-ion and ion-dipole solute-solvent interactions present in these systems. First, analysis of the literature data showed that the solvatochromic solvent parameters are linearly correlated with the corresponding solvent-specific coefficients in the Abraham's linear solvation equation. The solvatochromic solvent descriptors together with the solvent features of the phases derived from the partitioning of a homologous series of dinitrophenylated (DNP) amino acids were used in a linear free energy relationship to calculate the solute descriptors from the partition coefficients of five p-nitrophenylated-monosaccharides, 5 dinitrophenylated (DNP) amino acids, tryptophan, and its di- and tri-peptides in all ATPSs. Three descriptors of the solvent properties of the phases, solvent polarity, π^*, solvent hydrogen-bond donor acidity, b*, and coefficient c

(derived from partitioning of DNP-amino acids) could describe adequately the partitioning of the solutes.

In Chapter 7 the previous study was extend to the partitioning behavior of proteins. It was concluded that four descriptors of the solvent properties of the phases, solvent polarity, π*, solvent hydrogen-bond donor acidity, b*, solvent hydrogen-bond acceptor basicity, a*, and coefficient c (derived from partitioning of DNP-amino acids) could describe adequately the partitioning of the proteins. It is the first time, to the best of our knowledge, that protein descriptors are experimentally obtained.

The most important implication of the results obtained in Chapters 6 and 7 is that partitioning in ATPSs when considered in LSER descriptor terms can be used for detailed analysis of solute-solvent interactions for ionizable biomolecules such as proteins.

Although the descriptors experimentally determined in this study might be suitable for protein characterization, there are a lot of open questions to be answered first. The most important questions in our view are: (i) effect of ionic composition and pH of aqueous media on the protein features described by the descriptors, and (ii) effect of type and position of single point mutations in proteins on the protein descriptors under analysis.

It should finally be pointed out that it become obvious from what has been discussed in the present work as well as from literature data, that ATPSs are extremely sensitive towards biomolecules surface properties, making them a powerful bioanalytical tool. In our opinion this peculiarity should be explored in the future, for example in the medical diagnosis field.

Appendix A - Phase Diagrams

The methods of analysis of the polymers and salt compositions of the phases have been given in Chapter 3. The compositions below are given in weight percent (% w/w).

Table A.1. Phase compositions for Dextran-75-Ficoll-70 two-phase system (total ionic composition: 0.15M NaCl in 0.01 M Na-phosphate buffer, pH 7.4, at 23 ^{0}C)

Total composition		Top Phase		Bottom Phase		STL
Dextran, %(w/w)	Ficoll, %(w/w)	Dextran, %(w/w)	Ficoll, %(w/w)	Dextran, %(w/w)	Ficoll, %(w/w)	
13.45	18.83	2.88	29.93	23.30	8.06	-1.071
12.94	18.06	3.23	28.31	21.57	9.03	-1.051
12.41	17.15	4.15	25.55	19.63	9.67	-1.026
11.88	16.55	4.62	24.05	17.96	10.07	-1.048

DP – Disruption point: Dex – 11.34; Ficoll – 15.78; STL_{av} : -1.05 ± 0.02

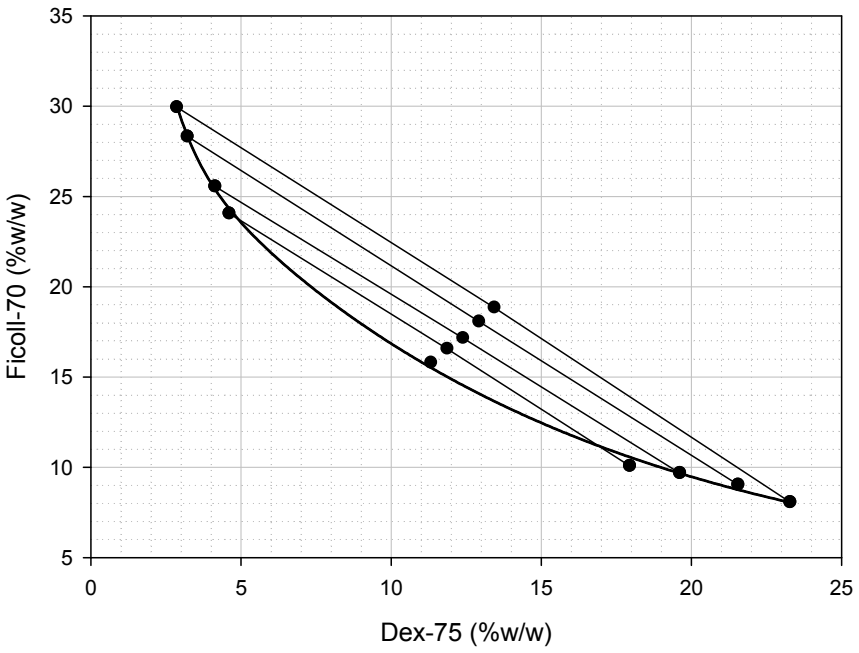

Figure A.1. Phase diagram of Dex75-Ficoll70 ATPS at 23^{0}C.

Table A.2. Phase compositions for Dextran-75-PEG-8000 two-phase system (total ionic composition: 0.15M NaCl in 0.01 M Na-phosphate buffer, pH 7.4, at 23 ^{0}C)

Total composition		Top Phase		Bottom Phase		STL
Dextran, %(w/w)	PEG, %(w/w)	Dextran, %(w/w)	PEG, %(w/w)	Dextran, %(w/w)	PEG, %(w/w)	
12.41	6.06	0.31	13.02	22.44	0.53	-0.565
10.75	5.22	0.48	11.21	19.23	0.57	-0.568
8.99	4.34	1.21	9.17	15.21	1.11	-0.578
8.42	4.08	1.64	8.05	13.67	1.32	-0.561
7.85	3.82	2.15	7.28	11.66	2.00	-0.559

DP – Disruption point: Dex – 7.29; PEG – 3.57; STL_{av} : -0.566 ± 0.007

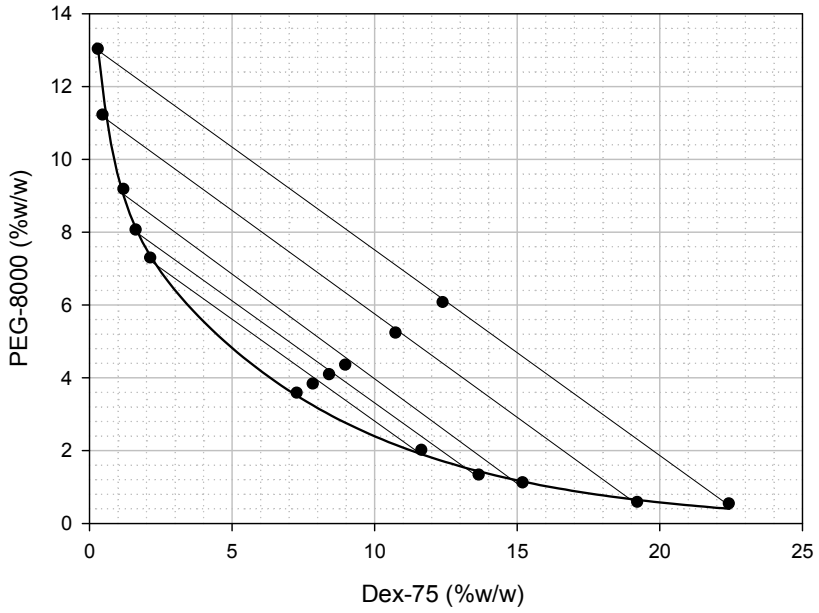

Figure A.2. Phase diagram of Dex75-PEG8000 ATPS at 23^{0}C.

Table A.3. Phase compositions for PEG-8000-Ucon50HB5100 two-phase system (total ionic composition: 0.15M NaCl in 0.01 M Na-phosphate buffer, pH 7.4, at 23 ^{0}C)

Total composition		Top Phase		Bottom Phase		STL
PEG, %(w/w)	Ucon, %(w/w)	PEG, %(w/w)	Ucon, %(w/w)	PEG, %(w/w)	Ucon, %(w/w)	
16.25	32.29	0.32	55.62	38.02	0.12	-1.47
15.00	29.97	0.36	50.25	35.46	1.58	-1.39
13.80	27.58	0.42	46.67	28.96	5.96	-1.43
12.63	25.20	3.83	37.54	22.17	11.82	-1.40

DP – Disruption point: PEG – 11.88; Ucon – 24.10; STL_{av} : -1.41 ± 0.02

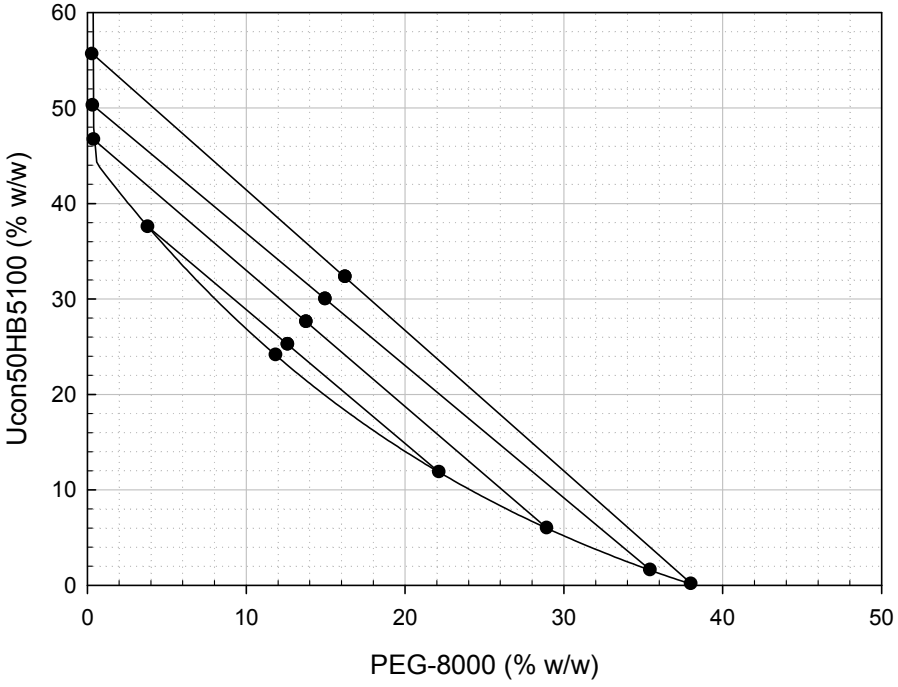

Figure A.3. Phase diagram of PEG8000-Ucon50HB5100 ATPS at 23^{0}C.

Table A.4. Phase compositions for Dextran-75-Ucon50HB5100 two-phase system (total ionic composition: 0.15M NaCl in 0.01 M Na-phosphate buffer, pH 7.4, at 23 ^{0}C)

Total composition		Top Phase		Bottom Phase		STL
Dextran, %(w/w)	Ucon, %(w/w)	Dextran, %(w/w)	Ucon, %(w/w)	Dextran, %(w/w)	Ucon, %(w/w)	
12.39	10.08	0.16	18.30	26.51	0.59	-0.67
10.42	8.41	0.18	15.52	21.59	0.65	-0.69
8.23	6.82	0.37	12.17	16.74	1.03	-0.68
7.57	6.25	0.60	11.00	15.01	1.18	-0.68
6.90	5.73	1.05	9.74	13.14	1.45	-0.69

DP – Disruption point: Dex – 5.30; Ucon – 4.60; STL_{av} : -0.683 ± 0.008

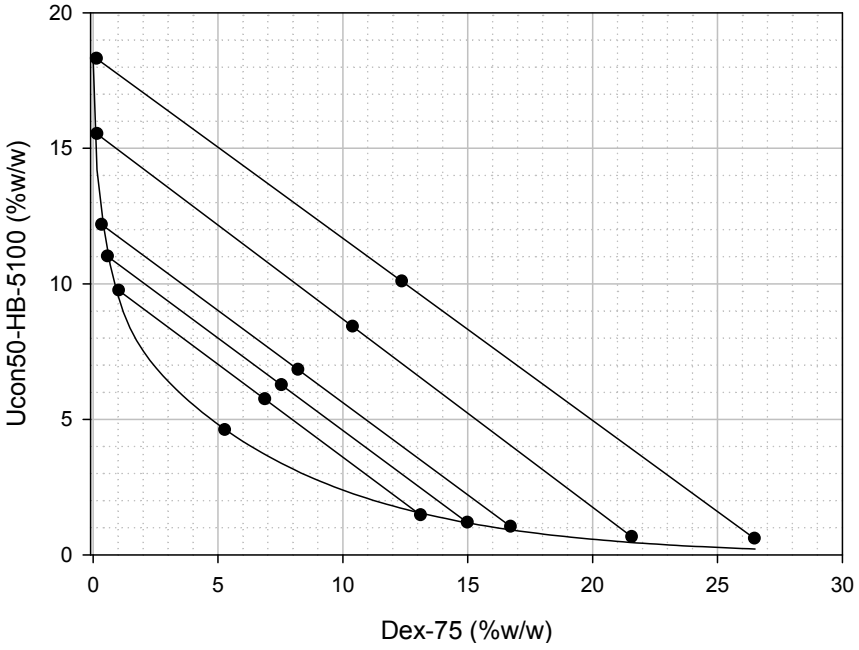

Figure A.4. Phase diagram of Dex75-Ucon50HB5100 ATPS at 23^{0}C.

Table A.5. Phase compositions for PES-100-PEG-8000 two-phase system (total ionic composition: 0.15M NaCl in 0.01 M Na-phosphate buffer, pH 7.4, at 23 ^{0}C)

Total composition		Top Phase		Bottom Phase		STL
PES, %(w/w)	PEG, %(w/w)	PES, %(w/w)	PEG, %(w/w)	PES, %(w/w)	PEG, %(w/w)	
15.24	6.96	3.67	12.28	29.58	0.37	-0.460
13.69	6.33	4.05	10.79	26.85	0.24	-0.463
12.33	5.61	4.70	9.21	23.42	0.38	-0.472
10.86	5.06	6.00	7.37	20.22	0.61	-0.475

DP – Disruption point: PES – 9.40; PEG – 4.29; STL_{av} : -0.467 ± 0.007

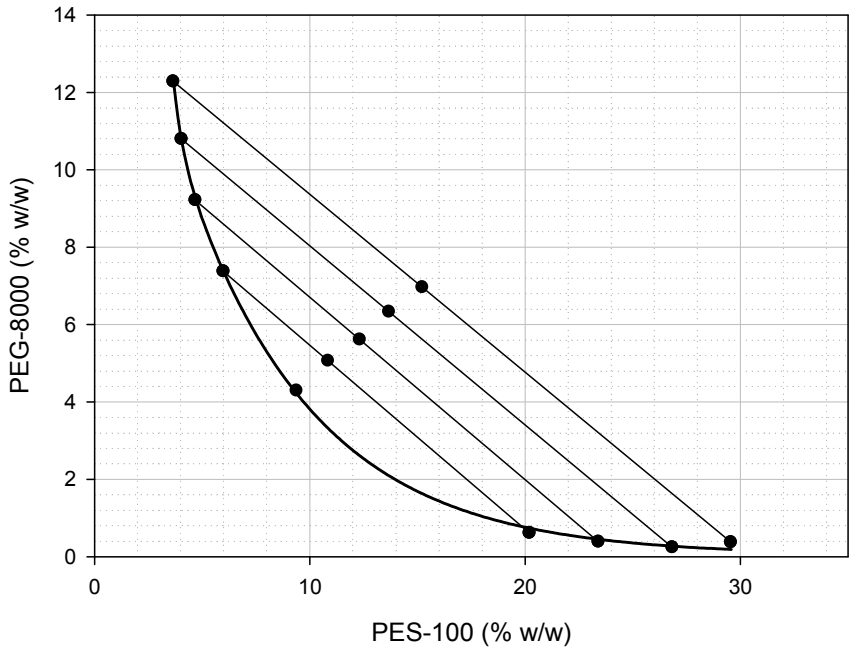

Figure A.5. Phase diagram of PES100-PEG8000 ATPS at 23^{0}C.

Table A.6. Phase compositions for PES-100-Ucon50HB5100 two-phase system (total ionic composition: 0.15M NaCl in 0.01 M Na-phosphate buffer, pH 7.4, at 23 ^{0}C)

Total composition		Top Phase		Bottom Phase		STL
PES, %(w/w)	Ucon, %(w/w)	PES, %(w/w)	Ucon, %(w/w)	PES, %(w/w)	Ucon, %(w/w)	
12.91	7.68	2.76	13.50	24.01	1.32	-0.573
11.73	6.97	3.22	11.79	21.31	1.54	-0.566
10.55	6.29	3.98	9.86	18.32	2.07	-0.543
9.36	5.53	5.56	7.55	14.45	2.82	-0.532

DP – Disruption point: PES – 8.17; Ucon – 4.85; STL_{av} : -0.55 ± 0.02

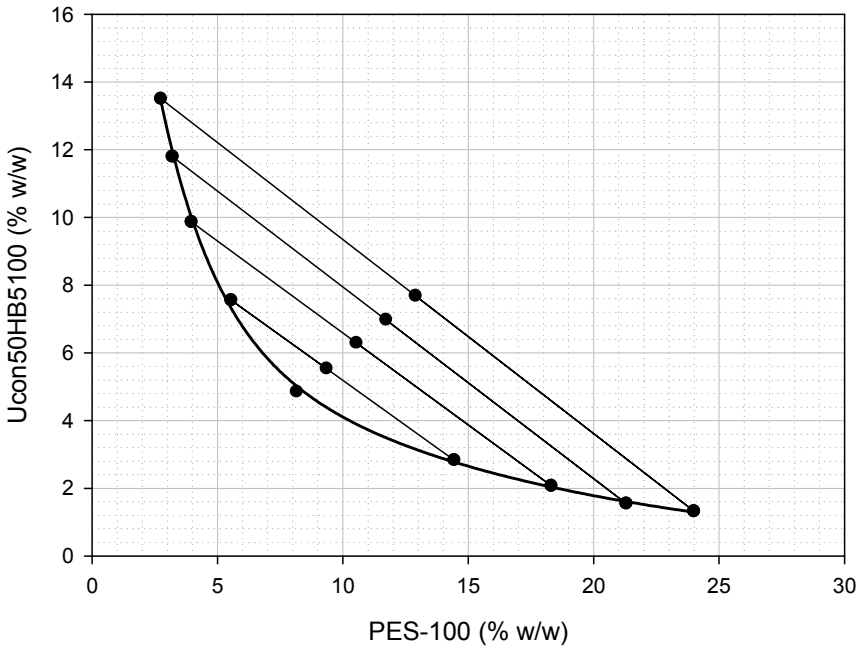

Figure A.6. Phase diagram of PES100-Ucon50HB5100 ATPS at 23^{0}C.

Table A.7. Phase compositions for Ficoll-70-PEG-8000 two-phase system (total ionic composition: 0.15M NaCl in 0.01 M Na-phosphate buffer, pH 7.4, at 23 ^{0}C)

Total composition		Top Phase		Bottom Phase		STL
Ficoll, %(w/w)	PEG, %(w/w)	Ficoll, %(w/w)	PEG, %(w/w)	Ficoll, %(w/w)	PEG, %(w/w)	
15.95	8.45	7.10	14.50	28.20	0.06	-0.684
15.48	8.12	8.49	12.89	25.92	0.98	-0.683
15.06	7.90	9.55	11.65	23.97	1.83	-0.681
14.77	7.76	11.50	10.01	22.57	2.39	-0.688

DP – Disruption point: Ficoll – 13.69; PEG – 7.18; STL_{av} : -0.684 ± 0.003

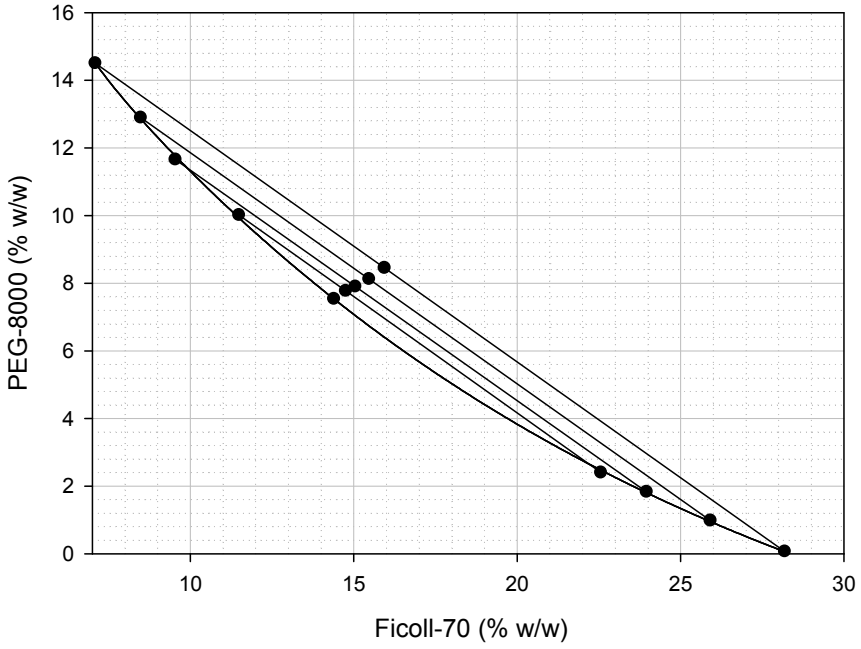

Figure A.7. Phase diagram of Ficoll70-PEG8000 ATPS at 23^{0}C.

Table A.8. Phase compositions for Ficoll-70-Ucon50HB5100 two-phase system (total ionic composition: 0.15M NaCl in 0.01 M Na-phosphate buffer, pH 7.4, at 23 ^{0}C)

Total composition		Top Phase		Bottom Phase		STL
Ficoll, %(w/w)	Ucon, %(w/w)	Ficoll, %(w/w)	Ucon, %(w/w)	Ficoll, %(w/w)	Ucon, %(w/w)	
13.66	10.02	2.82	17.03	25.60	2.31	-0.646
13.01	9.93	2.90	16.42	24.50	2.54	-0.643
12.40	9.49	3.49	15.14	23.36	2.53	-0.635
11.82	9.02	4.35	13.72	21.52	2.92	-0.629

DP – Disruption point: Ficoll – 10.00; Ucon – 7.62; STL_{av} : -0.638 ± 0.008

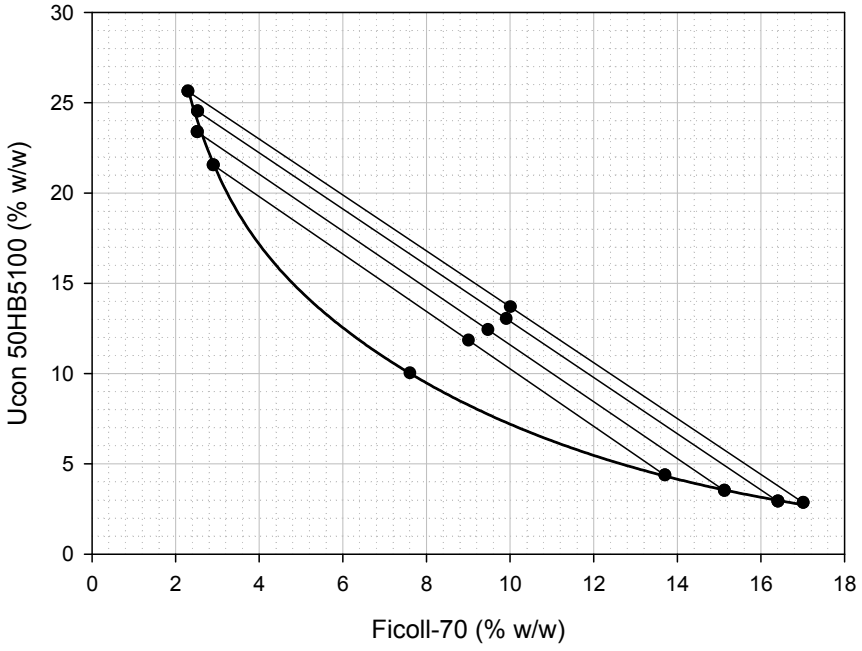

Figure A. 8. Phase diagram of Ficoll70-Ucon50HB5100 ATPS at 23^{0}C.

Table A.9. Phase compositions for PES-100-Dextran-75 two-phase system (total ionic composition: 0.15M NaCl in 0.01 M Na-phosphate buffer, pH 7.4, at 23 ^{0}C)

Total composition		Top Phase		Bottom Phase		STL
PES, %(w/w)	Dextran, %(w/w)	PES, %(w/w)	Dextran, %(w/w)	PES, %(w/w)	Dextran, %(w/w)	
17.30	12.43	5.31	21.68	31.38	1.93	-0.757
15.89	11.42	5.98	19.03	27.84	2.75	-0.744
14.53	10.41	6.97	15.99	25.26	2.71	-0.726
13.13	9.39	8.09	13.37	21.68	3.33	-0.736

DP – Disruption point: PES – 11.68; Dex – 8.39; STL_{av} : - 0.74 ± 0.01

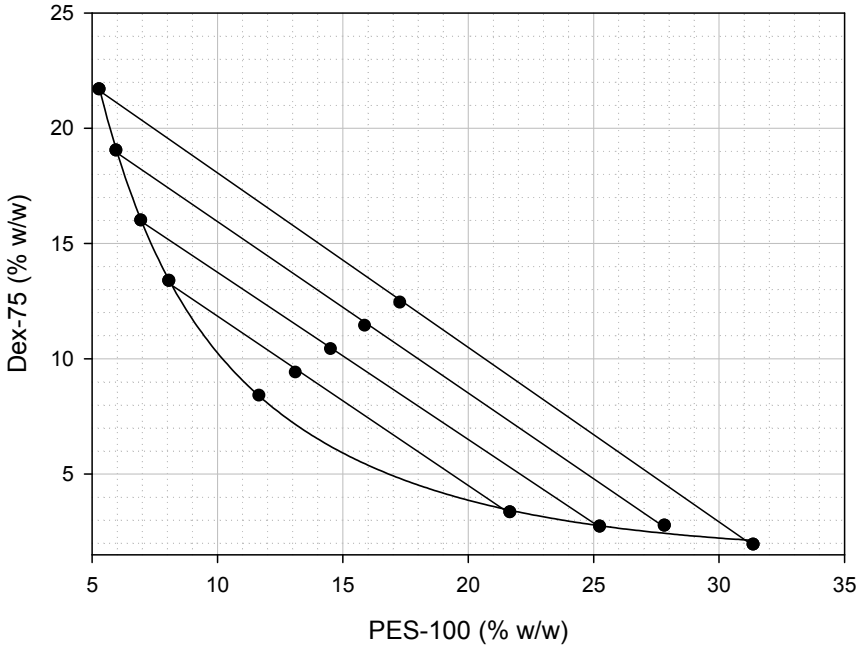

Figure A.9. Phase diagram of PES100-Dex75 ATPS at 23^{0}C.

Table A.10. Phase compositions for PES-100-Ficoll-70 two-phase system (total ionic composition: 0.15M NaCl in 0.01 M Na-phosphate buffer, pH 7.4, at 23 ^{0}C)

Total composition		Top Phase		Bottom Phase		STL
PES, %(w/w)	Ficoll, %(w/w)	PES, %(w/w)	Ficoll, %(w/w)	PES, %(w/w)	Ficoll, %(w/w)	
18.75	16.11	8.90	23.87	29.71	6.66	-0.828
18.12	15.49	9.46	22.35	27.58	7.62	-0.813
17.31	14.86	10.31	20.20	25.35	7.80	-0.826

DP – Disruption point: PES – 14.72; Ficoll – 12.38; STL_{av} : - 0.822 ± 0.008

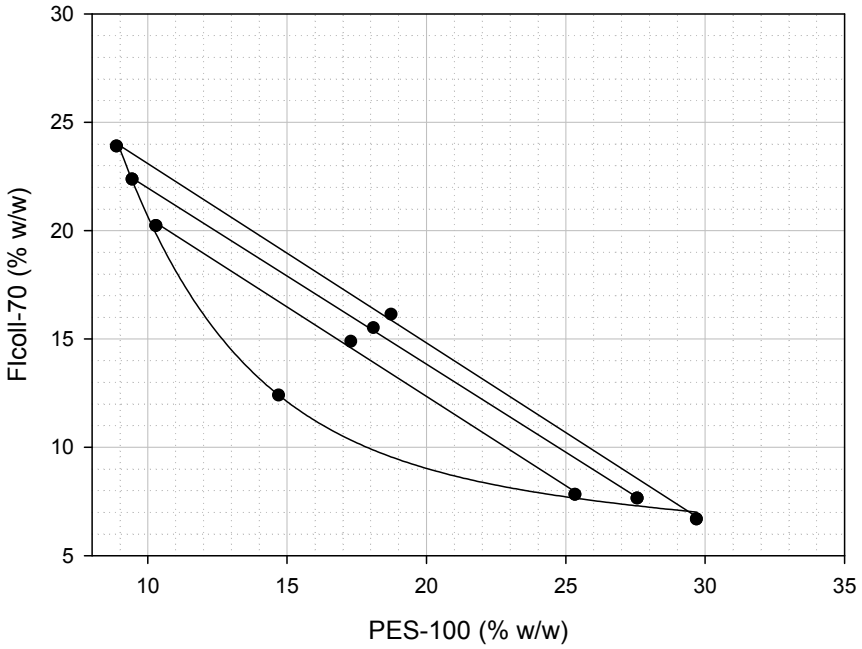

Figure A.10. Phase diagram of PES100-Ficoll70 ATPS at 23^{0}C.