

1. SOLVATOCHROMIC STUDIES IN POLYETHYLENE GLYCOL/SALT AQUEOUS BIPHASIC SYSTEMS

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Aqueous solutions of polymers have excited some recent interest for their ability to solubilize otherwise poorly soluble species without the involvement of organic solvents. Such systems include cloud point extraction, micellar extraction, PEO-PPO co-polymer solutions, and aqueous biphasic systems (ABS). It is well known that the UV-Visible absorption spectra of a wide range of organic compounds show alterations in the position intensity and shape of the absorption bands in solvents of different polarity. Such effects have been used for many years to derive empirical scales (Linear Solvation free energy relationships LSERs) with which to relate the polarity of solvents to such diverse phenomena as chemical reactivity, quantitative structure activity relationships, and solvent extraction. In the latter case scales of solvent polarity have been established for a wide range of systems and the solvatochromic method has also recently been used for the characterization of micellar extraction systems. We report on the use of the wavelength shift of the absorption spectrum of Reichardt's carboxylated pyridinium N-phenoxide betaine dye in a PEG-2000/potassium triphosphate system. The behavior of this dye in both the monophasic region and in the separated phases from within the binodal curve will be discussed. The apparent polarity of the system as reported by the bathochromic shift of the dye will be discussed in the context of conventional solvents used in solvent extraction. The results will also be compared to similar determinations made in micellar and cloud point extraction systems.

2. THE USE OF SMALL ANGLE NEUTRON SCATTERING FOR THE STUDY OF SOLUTIONS OF PROTEINS AND POLYMERS

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Small-angle neutron scattering (SANS) has been successfully used to study biomolecules and polymers in solution. Depending on the experimental conditions, intramolecular or intermolecular information can be obtained. The intramolecular information obtained by using SANS supplements the information obtained by other techniques such as laser light scattering or light spectroscopy. In addition, the intermolecular information obtained using SANS offers direct evidence about the type and strength of the intermolecular interactions in solution, which is not obtainable by light spectroscopy. The general approach to interpret SANS data is as follows. The experimental data, obtained under conditions where intermolecular interactions can be neglected (low protein concentrations, high ionic strength), is fitted using a model for the intramolecular structure factor. Then, the intermolecular structure factor is calculated using, for example, the mean spherical approximation. The intra- and intermolecular structure factors are combined and used to fit SANS data at higher protein concentrations and low ionic strength.

We describe in this paper the use of SANS for the study of solutions of polymers and proteins. We demonstrate that by analyzing the data correctly, SANS yields information that allows the identification of the molecular factors that contribute to the distribution of proteins between two liquid phases. The effect of hydrophobic interactions in partitioning is discussed in terms of molecular theories.

3. A FUNDAMENTAL INVESTIGATION OF PROTEIN PARTITIONING IN TWO-PHASE AQUEOUS MIXED MICELLAR SYSTEMS

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One of the major challenges in the biotechnology industry is the large-scale purification of a desired protein from a fermentation broth containing a wide variety of biomolecules. One possible strategy for addressing this challenge is to utilize novel two-phase aqueous complex-fluid solvents in a liquid-liquid extraction process.

An aqueous solution containing C10E4, a nonionic surfactant belonging to the alkyl poly(ethylene oxide) family, undergoes macroscopic phase separation with an increase in temperature. Although the water content in both phases is greater than or equal to 90 wt%, the top phase is micelle-rich, while the bottom phase is micelle-poor. Our research group has previously investigated the partitioning behavior of water-soluble proteins in this system (Liu, Nikas, Blankschtein, *Biotechnol. Bioeng.* **1996**, 52, 185 and Liu, Kamei, King, Wang, Blankschtein, *J. Chromatogr. B* **1998**, 711). Through an experimental and theoretical investigation, the partitioning behavior of water-soluble proteins in two-phase aqueous nonionic micellar systems was rationalized as being primarily governed by repulsive, steric, excluded-volume interactions between the nonionic micelles and the proteins.

In an effort to improve protein purification, we are currently investigating electrostatic interactions, in addition to excluded-volume interactions, between the micelles and the proteins by utilizing a novel two-phase aqueous mixed (nonionic-ionic) micellar system. This mixed micellar system, which is composed of the nonionic surfactant C10E4 and the anionic surfactant sodium dodecyl sulfate (SDS), phase separates with an increase in temperature. After initially mapping-out the ternary phase diagram for the two-phase aqueous C10E4/SDS mixed micellar system, cytochrome c and catalase were experimentally partitioned along appropriate tie lines. In order to determine the effect of the electrostatic interactions, these same proteins were also partitioned in the two-phase aqueous C10E4 nonionic micellar system (without SDS) at the same excluded-volume conditions. By comparing the partitioning results, we concluded that two-phase aqueous mixed micellar systems could be used to modulate both electrostatic and excluded-volume interactions between the micelles and the proteins. Theoretical studies were also conducted to model the electrostatic interactions for the purpose of predicting protein partition coefficients in the two-phase aqueous C10E4/SDS mixed micellar system.

4. KINETICS OF PHASE SEPARATION UNDER DIFFERENT PROCESS AND DESIGN PARAMETERS IN AQUEOUS TWO-PHASE SYSTEMS

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The exploitation of aqueous two-phase systems (ATPS) for the separation and recovery of desired biomolecules is gaining importance in biotechnology. Such trend is justified in the relatively easily scale-up and the potential of continuous steady-state operation of the ATPS processes. However, most of the research in ATPS have concentrated on the influence of factors affecting the partition behavior of biomolecules. For industrial implementation of ATPS it is necessary to study the aspects of these systems that provide insight into the kinetics of phase separation for the design of appropriate equipment. In the present research a number of process parameters and settlers have been identified to study their relationship with the phase separation in ATPS.

This paper will present a practical study of the influence of selected process parameters on the phase separation of ATPS. The study of the rate of phase separation in different batch settlers will be presented. Correlations using dimensionless numbers to predict the rate of phase separation (as function of time) and the time for total phase separation will be discussed. The impact of solids from different biological suspensions upon the rate of phase separation will be examined. Conclusion will be draw concerning the characterization of the process parameters involved in the phase separation for the design of equipment.

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5. NOVEL TIE-LINE LENGTH CHARACTERIZATION OF AQUEOUS TWO-PHASE SYSTEMS: IMPLICATIONS FOR OPERATIONS WITH COMPLEX PARTICULATE BIO-FEEDSTOCKS

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The characterization of aqueous two-phase systems (ATPS) by the cloud-point method or by compositional analysis of phase-forming components is commonly compromised when a complex feedstock (*e.g.*, whole fermentation broth, cell homogenate or biological extract) is included for fractionation. As a result, the quantitation of the impact of added biological feedstock upon the position of the binodal curve is difficult to establish, particularly in respect of the phase-forming influences of components of that feedstock.

Presentation will be made of a method whereby the equilibrium distribution in selected PEG-phosphate ATPS of a set of tritiated amino acids, characterized by varying degrees of hydrophobicity, can be exploited to define the relative hydrophobicity (RH) and subsequently the tie-line length of that system. This relationship will be demonstrated to hold for ATPS characterized by PEG average molecular weights in the range 300 to 8000 Da.

Addition of biomass to such systems in the form of whole bovine blood (up to 25% w/w) was expected to modify the phase diagram in an undefined manner. Inclusion of the tritiated amino acids in such a loaded system and subsequent analysis of their distribution has facilitated an estimation of an equivalent value of tie-line length (TLL_e). Such values vary markedly with added biomass in so-called sensitive systems (*i.e.*, short, original TLL in clean ATPS), but not with robust systems (longer, original TLL). In the former, partition coefficients for the bulk blood protein were found to vary with added biomass.

The application of this analytical tool will be discussed in the context of the practical exploitation of ATPS. In particular, the harnessing of the biological feedstock to lower the working inventory for added phase-forming polymers and salts components and the combating of feedstock variation offer potential process advantages.

6. MODELING THE PARTITIONING BEHAVIOR OF SMALL ORGANIC MOLECULES IN AQUEOUS BIPHASIC SYSTEMS

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Predicting the partitioning of organic solutes in aqueous biphasic systems (ABS) is essential for the design and evaluation of these systems for use in industrial applications. System parameters have been developed to describe partitioning behavior in a Polyethylene glycol/salt ABS based on the degree of phase divergence of the system as measured by tie line length or difference in PEG composition between the phases. This presentation will discuss our efforts to develop, both a system and solute parameter, that describe partitioning behavior in an ABS independent of solute type, PEG molecular weight, salt type, salt concentration, and temperature at which the system was formed.

This research is supported by the Division of Chemical Sciences, Office of Basic Energy Sciences, Office of Energy Research, U. S. Department of Energy (Grant No. DE-FG02-96ER14673).

7. ANALYTICAL APPLICATIONS OF AQUEOUS TWO-PHASE PARTITIONING

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Aqueous two-phase partitioning is used predominantly as the versatile and inexpensive separation technique for biopolymers. New analytical applications of the technique are coming into use recently. The two applications reviewed in the presentation include:

1. analysis of the relative hydrophobicity of common therapeutical drugs and biopharmaceuticals; and
2. quality control of biopharmaceuticals.

Scientific principles of these applications will be discussed together with advantages and limitations of the partition technique when introduced into the pharmaceutical and biopharmaceutical industry.

8. THERMOSEPARATING WATER/POLYMER SYSTEM: A NOVEL ONE-POLYMER AQUEOUS TWO-PHASE SYSTEM FOR PROTEIN PURIFICATION

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In this study we show that proteins can be partitioned and separated in a novel aqueous two-phase system composed of only one polymer in water solution. This system represents an attractive alternative to traditional phase systems which uses either two polymers (*e.g.*, PEG/dextran) or one polymer in high salt concentration (*e.g.*, PEG/salt). The polymer used is an hydrophobically modified ethylene oxide-propylene oxide random copolymer (HM-EOPO) with myristyl (C14) groups attached at both ends. This polymer is thermoseparating in water, *i.e.*, it forms an aqueous two-phase system with a top phase composed of almost 100 % water and a bottom phase composed of 5-9 % HM-EOPO in water when separated at 17-30°C. The partitioning behavior of three proteins (lysozyme, bovine serum albumin and apolipoprotein A-1) in water/HM-EOPO systems has been studied, as well as the effect of various ions, pH and temperature on protein partitioning. The amphiphilic protein apolipoprotein A-1 was strongly partitioned to the HM-EOPO rich phase within a broad temperature range. The partitioning of BSA could be controlled with pH when NaClO₄ was included in the system: below the pI the protein was partitioned to the HM-EOPO rich phase and above pI to the water phase. Lysozyme was directed to the HM-EOPO rich phase with NaClO₄ and to the water phase with Na-phosphate. The possibility to direct protein partitioning between water and copolymer phases shows that this system can be used for protein separations. This was tested on purification of apolipoprotein A-1 from human plasma and from *E. coli* fermentation solution. Apolipoprotein A-1 could be recovered in the HM-EOPO rich phase and the majority of the contaminating proteins were partitioned to the water phase. By adding a new water/buffer phase at higher pH and with 100 mM NaClO₄, and after raising the temperature for separation, the apolipoprotein A-1 could be back-extracted from the HM-EOPO phase into the new water phase. This novel system has a strong potential for use in biotechnical extractions as it uses only one polymer and can be operated at moderate temperatures and salt concentrations, and the copolymer can be recovered.

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9. SELECTIVE RECOVERY OF PROTEINS USING STIMULI-RESPONSIVE AQUEOUS TWO-PHASE SYSTEMS

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A novel bioprocess based on the stimuli-responsive behaviors of the synthetic polymers and proteins has been developed for the selective recovery of the target protein in aqueous two-phase systems (ATPS). PEG bound to the thermo-reactive hydrophobic head (such as poly(propylene oxide)-phenyl-group (PPO-Ph-group)) was then used as the functional ligands to modify the PEG phase of the ATPS. A monomeric carbonic anhydrase from bovine and a tetrameric β -galactosidase were selected as target proteins.

The change of the surface properties of PPO-Ph-PEG was firstly investigated, where the local hydrophobicity of PPO-Ph-PEG was found to be increased at the temperature of more than 318 K. In the ATPS modified with PPO-Ph-PEG, the partitioning behaviors of the proteins have been investigated with varying the external conditions (*i.e.*, temperature, pH, and denaturant (GuHCl) concentration).

The partition coefficients of the proteins were found to be increased at the specific temperature, where the protein structure was changed to the partly-damaged state (which is called as Molten-Globule state) 1) and the surface of PPO-Ph-PEG was hydrophobic. The refolding of the partly-damaged proteins was also achieved in the ATPS with PPO-Ph-PEG under the various external conditions to recover it with active state. The refolding yield of the proteins was increased and the aggregate-formation was reduced by adding the PPO-Ph-PEG to the ATPS under the stimulus condition. Such stimuli-responsive ligands were found to possess a chaperon-like function, which could assist the protein refolding.

Based on the above results, a possible bioprocess using the ATPS with stimuli-responsive ligand has finally been presented, where (i) a partly-damaged target protein is selectively partitioned to the top-phase by the interaction with the stimuli-responsive ligands and (ii) is recovered as the active state by the ligand-assisted refolding. A bioprocess for the selective recovery of the target can be designed by using the present ATPS, which can be called as stimuli responsive ATPS (SR-ATPS) as well as the conventional SR-ATPS (*i.e.*, thermoseparating ATPS).

1. Yamahara, K. *et al.*, *J. Chem. Eng. Japan* **1998** 31, 795-803.

10. PARTITIONING OF AMINO ACIDS IN AQUEOUS TWO-PHASE SYSTEMS WITH VOLATILE SALTS AS RECYCLABLE PHASE-FORMING SUBSTANCES

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In industrial biotechnological processing, extraction with aqueous two-phase systems has only been applied on a limited scale. One of the main causes is the relatively large amount of waste material, which are mainly auxiliary, phase forming components such as polymers and salts. The development of aqueous two-phase extraction processes that do not have this environmental disadvantage could greatly increase the application of these potentially useful types of separation and purification processes in industry. In previous work, a new approach was presented, namely the use of volatile salts that can easily be recycled.¹

In the present paper, the results of partitioning experiments are described with these aqueous two-phase systems with volatile salts. The model components are seven amino acids, namely l-serine, glycine, l-alanine, l-valine, l-methionine, l-isoleucine, and l-phenylalanine. The partitioning behavior of these components is influenced both by the chemical and physical properties of the amino acids and by the properties of the extraction system. Relevant system properties which have been discussed include the PEG molecular weight, the ratio between total ammonia and carbon dioxide content (which influences pH and ionic strength), and the system composition.

1. van Berlo, M.; Luyben, K. Ch. A. M.; van der Wielen, L. A. M. Poly(ethylene glycol)-salt aqueous two-phase systems with easily recyclable volatile salts, *J. Chromatogr. B* **1998**, *711*, 61-68. (Erratum: *J. Chromatogr. B* **1999**, *724*, 203-204.)

11. AFFINITY PARTITIONING USING AQUEOUS TWO PHASE SYSTEMS FORMED BY THERMOSENSITIVE POLYMERS

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The selective partitioning of proteins between two aqueous phases formed by two incompatible polymers has proven to be an efficient tool of purification of proteins and of some low-molecular-weight substances. The main problem of the method - how to separate the target protein from the phase-forming polymer - has not yet been completely solved. Stimuli-responsive or so called 'smart' polymers provide an elegant solution of this problem. Stimuli-responsive are polymers which undergo fast and reversible transition from hydrophilic and soluble state to hydrophobic and hence insoluble state. These transitions are triggered only by small changes of medium property (pH, temperature, ionic strength, presence of specific chemicals, light, electric or magnetic field).

Copolymers of N-vinylcaprolactam and 1-vinyl imidazole (poly-VI-VCL) and copolymers of N-isopropylacrylamide and 1-vinyl imidazole (poly-VI-NIPAM) undergo the transition from soluble to insoluble state on increasing temperature. The critical temperature, where the transition takes place depends on pH as the protonation of imidazole groups renders the copolymers more hydrophilic hence it undergoes the transition at higher temperatures. The increase in ionic strength reduces electrostatic repulsion of charged units and promotes hydrophobic interactions decreasing the transition temperature. Imidazole groups of copolymers perform as ligands capable of chelating metal ions. Immobilized metal ion, *e.g.*, Cu(II), could interact with histidine groups at the surface of protein molecules and promote polymer-protein interaction.

Poly-VI-VCL and poly-VI-NIPAM have been studied as phase forming polymers in combination with dextrans, starch derivatives and modified polyethyleneoxides. When the copolymers have been loaded with Cu(II), the partition of proteins with surface histidines was strongly favored into the phase formed by Cu(II)-loaded copolymers. Recombinant lactate dehydrogenase (from the thermophilic *Bacillus stearothermophilus*) carrying a tag of six histidine residues was purified 8-fold with 80% yield by partitioning in the system formed by Cu(II)poly-VI-VCL and dextran T70 followed by thermoseparation of the polymer in the presence of EDTA at 45 °C.

12. DIFFUSION OF LYSOZYME IN GELS AND LIQUIDS – A GENERAL APPROACH FOR THE DETERMINATION OF DIFFUSION COEFFICIENTS USING HOLOGRAPHIC LASER INTERFEROMETRY

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A study on diffusion measurements of the protein lysozyme in liquids and agarose gels, at different pH and ionic strengths, has been performed using holographic laser interferometry. The measurements showed that the diffusive flux was very dependent of pH and ionic strength when the protein was not at its isoelectric point or when the charge of the lysozyme molecules were not screened by ions in the solution.

Evaluation of the experimental data with Fick's law, resulted in diffusion coefficients for lysozyme that are strongly dependent on pH and ionic strength. At a high ionic strength the obtained diffusion coefficients agreed well with literature data, independent of pH. However, for a solution with a low salt concentration and at low pH, non-Fickian diffusion was observed. Thus, using Fick's law for evaluation in this case gives abnormally high diffusion coefficients.

Evaluation of the experimental data using a more general transport model, based on chemical potential gradients instead of concentration gradients resulted in lysozyme diffusion coefficients that are independent of pH and ionic strength. The chemical potential was estimated by using the Poisson-Boltzmann equation. The high diffusive flux at low pH and low ionic strength can thus be explained by the repulsive forces between the charged lysozyme molecules. At a high ionic strength this effect is screened by the salt ions

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13. ACID PRECIPITATION OF FOOD PROTEINS USING HIGH-PRESSURE CO₂

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Recovery of food proteins by precipitation from natural feed stocks such as milk and soy bean is generally accompanied by the consumption of substantial amounts of auxiliary compounds, particularly acids, bases or salts. In the precipitation of food proteins, the environmental burden is considerable due to the large volumes that are processed. Also in case of the production of biopharmaceuticals that are expressed in the milk of transgenic animals, the removal of undesired proteins such as casein is an important subject. In conventional processes, mineral acids such as HCl or H₂SO₄ are used, which lead to estimated waste streams of 64 ktons of salt per year for Europe. In this paper, we focus on a relatively novel technique, which is the acid precipitation of food proteins using high-pressure CO₂.

Acidification of aqueous solutions by high-pressure carbon dioxide has been proposed for the recovery of casein (Jordan *et al.*, 1987) as well as for the recovery of lactic acid (Halsema *et al.*, 1998) and other organic acids (Urbas, 1984). A typical range of operating conditions involves pressures up to 60 bars. An attractive feature of CO₂ is the possibility for its easy recovery by lowering pressure and increasing temperature. Also in the preservation of milk and its consequences in cheese making, the use of CO₂ has received considerable attention. We have investigated the possible window of operating conditions by experiment and theory. A relatively simple model was developed that could predict the relation between CO₂-pressure, protein concentration, pH and ionic composition of the aqueous solution as well as precipitate yield. In particular, the differences from conventional precipitation processes in possible process configurations as well as in product composition and morphology will be outlined. An important factor is the mineral content (Ca, phosphates) of the final product which differs from conventional processes. In this contribution, we will also demonstrate the feasibility of various interesting process configurations.

14. RELEASE AND SWELLING DYNAMICS OF HYDROGELS

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Controlled release of drugs from polymer gels or release of aroma components from gel-type foods are important situations where mass transfer is essentially determining product quality. It has been shown that the release of these solutes from soft gels is often accompanied by changes in particle dimensions and structure (Akano, 1998; Lee, 1983). And vice versa, the presence of solutes in the gel seriously affects the swelling dynamics. This may lead to apparently anomalous swelling behavior where the radius of the swelling bead can go through a maximum. A rapid increase is followed by a gradual decrease towards the equilibrium value (Lee, 1983). In other cases, sigmoidal swelling and release profiles were observed. After an initial period with a relatively low release rate, the release kinetics accelerate, to level off when the solute concentration in the gel decreases to zero. The release rate seems to follow the swelling rate of the gel material (Okuyama *et al.*, 1993). In both cases, a Fick-based diffusion model does not apply. In this paper, we aim to describe these combined release and swelling phenomena on the unified basis of Maxwell-Stefan theory.

Recently, a predictive mass transfer model based on Maxwell-Stefan (MS) theory has been proposed, that accounts for the effects of composition dependent diffusion and increasing diffusion path in expanding hydrogel particles (Bisschops *et al.*, 1998). A simplified thermodynamic description for the gel network-solvent system was used, and an approximate function of the frictional (diffusional) interaction of solvent and neutral free polymer chains was employed as an input. The thermodynamic model has essentially a Flory-Huggins basis with an elastic term. The model was shown to be able to predict the expanding diameter as well as intraparticle profiles with a fair accuracy.

In this work, we extended this model for a gel-solvent system to include a third species, the solute, to analyze the impact of a dissolving component on the swelling and release kinetics of the initially dry polymer-solute particles. The MS-diffusivities were predicted on the basis of Free Volume theory (Wesselingh and Bollen, 1997) The model was tested on two sets of experimental data that were available in literature. The first case considers the release of thiamine. HCl from poly (2-hydroxyethyl methacrylate) spheres in water, where loading dependent 'overshoot'-phenomena were observed (Lee, 1983). The second case considers the release of indomethacin from poly (N-propyl-acrylamide-cobutyl methacrylate) hydrogel (Okuyama *et al.*, 1993). This simplified model can indeed predict some of the essential features of the ternary hydrogel+solvent+solute systems.

15. DIFFUSION OF LARGE MOLECULES IN FIBROUS STRUCTURES

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Diffusion and partition coefficients of proteins in agarose gel were measured with size exclusion chromatography. Other data on the diffusion of proteins and random coils in fibrous structures were collected from the literature. We tried to fit the data with Ogston's model and found that the diffusion coefficients depend on the flexibility of the solute and the fibers. This can be accounted for by introducing flexibility parameters for the solute and the fibers. Furthermore we propose that two diffusion regimes can be distinguished, depending on the size of the holes in the fibrous structure. This can also be accounted for in Ogston's model.

16. VARIATION OF PARTITION COEFFICIENT WITH BIOMASS LOADING IN AQUEOUS TWO-PHASE SYSTEMS: IMPACT UPON THE FRACTIONATION AND RECOVERY OF ALBUMIN AND HEMOGLOBIN FROM WHOLE BOVINE BLOOD

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The characterization of aqueous two-phase systems (ATPS) by the cloud-point method or by compositional analysis of phase-forming components is commonly compromised when a complex feedstock (*e.g.*, whole fermentation broth, cell homogenate or biological extract) is included for fractionation. As a result, the quantitation of the impact of added biological feedstock upon the position of the binodal curve is difficult to establish, particularly in respect of the phase-forming influences of components of that feedstock.

A method has been described in an accompanying abstract, whereby the distribution at partition equilibrium of a selection of tritiated amino acids can be exploited to redefine PEG-phosphate ATPS loaded with complex biological feedstocks in terms of an equivalent tie-line length (TLL_e). Presentation will here be made of a study of the variation with added biomass (5 to 25% w/w) of the partition coefficient and consequent quality of recovery of the two dominant proteins in whole bovine blood (serum albumin and hemoglobin). Individual partition coefficients were estimated by laser densitometry of SDS-PAGE analyses of samples taken from the partition of different batches of citrated-blood. In so-called sensitive ATPS (short, original TLL for clean ATPS), partition coefficients for albumin and hemoglobin decreased to different degrees with increasing TLL_e. Mass balances and visual inspection confirmed an insignificant level of interfacial precipitation, even at the highest added biomass. In contrast, changes in individual partition coefficients in robust ATPS (long original TLL) were attributed to in part to visible interfacial precipitation at biomass loadings greater than 15% w/w whole blood.

These findings will be discussed in the context of exploiting determinations of TLL_e to facilitate the intensification of ATPS (*i.e.*, increasing biomass loading) to increase throughput and decrease the inventory of phase-forming chemicals whilst maintaining the quality of separation and recovery for the target product in a crude feedstock.

17. EFFECT OF BIOLOGICAL SUSPENSIONS ON THE POSITION OF THE BINODAL CURVE IN AQUEOUS TWO-PHASE SYSTEMS

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The potential of aqueous two-phase systems (ATPS) comprising mixtures of poly(ethylene glycol) (PEG)-phosphate for the recovery of macromolecules from fermentation broth and biological extracts has been demonstrate. Particularly as an alternative to conventional processes for particle and solute handling. However, the adoption and commercial application of extraction processes exploiting ATPS for the recovery of value products from biological suspensions requires well characterized operating conditions that can be applied to a wide range of two-stage processes and understanding of the process disadvantages attributed to two-phase partitioning.

It has been established that several system parameters (*e.g.*, system pH, molecular mass of PEG, type of salt, etc.) have an impact on the position of the binodal curve. The understanding of such effects have been exploited to select operating conditions for ATPS processes. However, reports which discuss the effect of the biological suspension on the position of the binodal curve are not common. We have shown that the binodal curve moved away from the origin at high concentration of PEG in the presence of whey. Such behavior was attributed to the caused by residual fat of the cheese whey.

This paper will present a study of the influence of biological suspension on the position of the binodal curve in ATPS. Different biological suspensions (*i.e.*, whey, disrupted yeast, *E. coli* homogenate and micellial culture) were evaluated and their impact upon ATPS performance will be examined. In addition, the change in the volume ratio of the biological ATPS (compared with that of the non-biological ATPS) will be discussed. Conclusion will be drawn concerning the impact of the presence of biological suspension upon the position of the binodal in ATPS.

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18. THE USE OF QUANTUM MECHANICAL MODELS (AMSOL) TO PREDICT THE DISTRIBUTION OF ORGANIC MOLECULES IN AQUEOUS BIPHASIC SYSTEMS

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Recently the SM5.4 quantum mechanical solvation model (AMSOL) has been extended so that the free energies of solvation of a solute in almost any organic solvent may be calculated. Using a thermodynamic cycle and a reference solute, we have estimated the distribution of a number of organic solutes in various solvent water systems. Of particular interest is the estimate of Log P in octanol/water systems which is widely used as a reference partitioning system in a range of applications from simple comparison of the relative lipophilicity of different solvent systems to sophisticated quantitative structure activity relationships in, for example, drug design. We will present our methods and the results obtained on the calculation of $K_{o/w}$ of a number of low molecular weight organic solutes. These will be compared to distribution values obtained in PEG – salt ABS. The difficulties inherent in extending the methodology directly to ABS systems will be discussed.

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19. DRIVING FORCES FOR PHASE SEPARATION AND PARTITIONING IN AQUEOUS TWO-PHASE SYSTEMS

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A set of simple analytical equations, derived from Flory-Huggins theory, are used to identify the dominant driving forces for phase separation and solute (*e.g.*, protein) partitioning, in the absence and presence of added electrolyte, in every general class of aqueous two-phase system. The resulting model appears to capture the basic nature of two-phase systems and all trends observed experimentally. Case studies are used to identify fundamental differences in and the magnitudes of enthalpic and entropic contributions to partitioning in polymer/polymer (*e.g.*, PEG/dextran), polymer/salt, and thermoseparating polymer/water (*e.g.*, Ucon/water) two-phase systems. The model provides practitioners with a better understanding of partition systems and industry with a simple, fundamental tool for selecting an appropriate two-phase system for a particular separation.

20. PEPTIDES PARTITIONING IN AQUEOUS DEXTRAN-PEG TWO-PHASE SYSTEM

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Partitioning of glycine, lysine, and aspartic acid and their oligopeptides was examined in aqueous dextran-PEG two-phase system containing either 0.15 M NaCl in 0.01 sodium phosphate buffer at pH 7.3 or 0.11 M sodium phosphate buffer at pH 7.3. Relative hydrophobicity of the amino acid residues and peptide bonds were estimated and expressed in equivalent numbers of methylene groups. Analysis of a series of reversed di-peptides and tri-peptides in terms of relative hydrophobicity showed that the additivity principle holds for the hydrophobicity of short peptides. While the relative hydrophobicity always changed linearly with increasing peptide chain length, the type of amino acid being added affects the magnitude of the hydrophobic increment. In addition, the ionic composition of the aqueous media has a differential effect depending on the amino acid residues involved. Chirality of the residues in the peptide sequence is also shown to affect the peptide relative hydrophobicity.

21. PARTITIONING OF DRUGS IN OCTANOL-BUFFER AND AQUEOUS DEXTRAN-PEG TWO-PHASE SYSTEM

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Partitioning of a variety of organic compounds, the majority of which represent therapeutic drugs, was examined in octanol-buffer, pH 7.3, and in aqueous dextran-PEG two-phase system containing 0.15 M NaCl in 0.01 M sodium phosphate buffer at pH 7.3. The possibility of introducing the compounds in an organic solvent, and the effect of this solvent on the solute partitioning was explored. Relative hydrophobicity of the compounds was estimated and expressed in equivalent numbers of methylene groups. Analysis of quantitative structure-activity relationship (QSAR) for a subset of drugs examined clearly demonstrates the advantage of aqueous two-phase partitioning over that in octanol-water system.

22. MOLECULAR MODELING STUDIES ON PENICILLIN ACYLASE AFFINITY PARTITION

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The selectivity of aqueous two-phase systems can be increased by the presence in one of the phases of a specific ligand to the target compound (affinity partition). This is achieved by selecting the appropriate conditions or by binding the ligand to the polymer predominating in that phase. Therefore it is expected that the target compound will be mainly distributed in the phase containing the ligand. In a previous work¹ the partition of penicillin acylase in the presence of derivatized polymers with specific ligands was studied. However, no significant steer of the enzyme to the phase containing the ligand was observed under the several conditions tested. This was suggested to be due to the decrease of enzyme affinity for the ligand upon binding to the polymer.

This previous hypothesis was tested in this work by molecular modeling studies. Molecular modeling of the interaction of proteins with large molecules like polymers, although feasible, is not straightforward, and so a simpler approach was chosen. Ligands bound to molecules of n-ethyleneglycol were n ranges from 1 to 5, were docked in the enzyme active site. The binding modes and the interaction energies were compared. Results show that the docked structures for each ligand with different units of ethylene glycol almost overlap. The main differences were observed on the ethyleneglycol tails. The affinities computed by the interaction energy show a similar increasing trend until n = 3 and a decrease afterwards. These results suggest that the increase in polymer length, although it does not change the binding mode of the ligands, could decrease their affinity for the enzyme. In addition, it indicates that ligands bonded to tails of n-ethyleneglycol could be more effective in directing the partition of penicillin acylase to a poly(ethylene glycol)-containing phase than the previously used derivatized polymers.

1. Marcos, J. C.; Fonseca, L. P.; Ramalho, M. T.; Cabral, J. M. S.; Johansson, G. Affinity partition studies with penicillin acylase from *Escherichia coli*, 2nd European Symposium on Biochemical Engineering Science, 16-19 Setembro de 1998, Porto, Portugal.

23. PREDICTING THE PARTITION COEFFICIENTS OF PROTEINS IN AQUEOUS TWO-PHASE SYSTEMS: RULES, MODELS, AND PRACTICAL REALITY

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The ability to predict the partition coefficient of a protein in PEG/salt and PEG/dextran Aqueous Two-Phase Systems (ATPS) based on the molecular properties of the protein is clearly an important task. This paper will review the present state-of-the-art of the effect of fundamental protein properties on partition coefficient. Physicochemical properties such as hydrophobicity, charge, molecular weight and affinity as well as protein concentration in the ATPS will be considered. This analysis will include studies made with a large number of proteins and with chemically modified ones where a single property is changed.

The question of the evaluation and measurement of a protein's hydrophobicity and charge (charge vs. volumetric charge or charge density) will be analyzed as well as a range of ATPSs with application potential such as PEG/sulfate, PEG/citrate, PEG/phosphate and PEG/dextran in the absence and presence of NaCl. Finally, a critical evaluation will be made of the patterns followed, the existing models and their ability to predict the partition coefficient of individual proteins.

24. A NEW AQUEOUS TWO-PHASE SYSTEM BASED ON CASHEW-NUT TREE GUM AND POLYETHYLENE GLYCOL

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Recently, there has been much interest in the use of aqueous two-phase systems for the commercial purification and concentration of biological fermentation product. In some cases, inexpensive polyethylene glycol(PEG)/salt systems is limited by the high salt concentration necessary for the formation of the phases. Polymer/polymer phase systems are more generally useful, but commercial exploitation of these products has been restricted by the high cost of the fractionated dextran used in the dextran/PEG phase systems on which most of the literature is based. Therefore, there have been a number of attempts to develop other polymer pairs that combine to form phase systems having comparable properties to, but lower costs than dextran/PEG systems. A new aqueous two-phase system based on cashew-nut tree gum, the exsudate polysaccharide from *Anacardium occidentale* L. employed locally as a substitute for arabic gum in pharmaceutical uses, and polyethylene glycol is described. Crude gum was collected as natural exsudate from cultivated *A. occidentale* trees in Pernambuco state. Clear nodules free of bark were selected to be isolated via ethanol precipitation of an aqueous solution. Phase diagrams for the system cashew tree gum and PEG molecular weight of 4000 and 8000 at pH 7.0, 25°C, were described. Three different "tielines" for each phase-diagram were analyzed for Bovine serum albumin (BSA) partition coefficient determination. The partition coefficient (K) was defined as the ratio between BSA in the upper (PEG rich phase) and lower (cashew-nut tree gum rich phase) phases. BSA partition coefficient increased with decrease in polymer molecular mass. The influence of "tieline" length on protein partition was also investigated. It was observed a decrease in partition coefficient with increase in "tieline" length. Results indicate that this is a potentially useful aqueous two-phase system for protein extraction.

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25. PHASE BEHAVIOR AND PROTEIN PARTITIONING IN AQUEOUS TWO-PHASE SYSTEMS OF CATIONIC-ANIONIC SURFACTANT MIXTURES

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Cationic-anionic surfactant mixtures can spontaneously separate into two phases, one surfactant enriched phase and a surfactant depleted phase. Such aqueous surfactant two-phase systems (ASTP systems) can be used for the partitioning of biomolecules. We have studied phase behavior and protein partitioning of BSA and lysozyme in the ASTP system formed by mixtures of dodecyltriethylammonium bromide and sodium dodecylsulfate. This mixture separates into two phases in two regions in the phase diagram, either with excess of cat-ionic or an-ionic surfactant. BSA and lysozyme can be separated from each other, and for both proteins the partitioning can be strongly one-sided. The partitioning is mainly related to electrostatic effects. A separation based on different net-charge of proteins can be achieved. The phase behavior of cationic-anionic surfactant mixtures has some unique properties that can be utilized for increased selectivity and to remove phase components and recycle them into a new phase system. When the surfactant-rich phase is diluted the solution forms a new ASTP system. Based on this, a multi-step partitioning procedure is achieved that can increase the selectivity of the system. The further dilution of the surfactant-rich phase induces precipitation of mixed surfactants. This phase behavior allows an easy way to remove surfactants from target proteins after partitioning and allows an easy way of recycling phase components. We show that enzymes can maintain activity in ASTP systems. The extremely low CMC (critical micelle concentration) of cationic-anionic surfactant mixtures makes the concentration of surfactant monomers very low and thus prevents binding of surfactants to proteins.

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26. CHARACTERIZATION AND IMMOBILIZATION ON SOLID SUBSTRATES OF UCON/SALT AQUEOUS TWO-PHASE SYSTEMS

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We have developed a series of aqueous two-phase systems whose phase forming species are copolymers of ethylene oxide (EO) and propylene oxide (PO) (UCON) and sodium chloride. We have investigated the wetting and the wetting kinetics of agarose, hydrophobic agarose, and polystyrene by the upper and lower phases of these systems. These wetting studies show that on highly hydrophobic substrates, like polystyrene, the UCON-rich phase preferentially wet the substrate. We also observe that some adsorption of the polymers takes place. Therefore, dynamic light scattering was used to determine the thickness of the adsorbed layer of UCON on polystyrene beads from both the UCON-rich and the Salt-rich phases. Our studies demonstrate that the UCON-rich phase can be immobilized in hydrophobic supports. We expect to use these systems to develop liquid-liquid partition chromatography systems.

27. EFFECTIVE SEPARATION OF PROTEINS USING LIPOSOMES AS AQUEOUS TWO-PHASE SYSTEMS MODIFIED BY HEAT/SALT COMBINED STRESSES

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Recently, we have clarified that liposomes assist the refolding of cytoplasmic protein, carbonic anhydrase¹ and that the cytoplasmic β -galactosidase is translocated across the liposome membrane under heat stress conditions.² In both cases, the optimal stress conditions to induce the above functions was shown to be predominated by their hydrophobic properties on their surfaces.¹ From the practical viewpoints, the refolding process³ and selective separation⁴ of target protein have also been investigated by using the above phenomena on liposomes.

The liposome system, where two phases are composed of the inner and the outer aqueous phase separated by phospholipid bilayer membrane, can be defined as a variation of the aqueous two-phase system. By combining the different kinds of stresses (such as heat and/or salt), the distribution and translocation behaviors of various proteins were effectively controlled based on the variation of physicochemical properties of the liposome membranes and proteins.

1. Kuboi, R. *et al.*, *Biotechnol. Progress*, **1997**, *13*, 828-836.
2. Umakoshi, H. *et al.*, *Biotechnol. Progress*, **1998**, *14*, 218-226.
3. Yoshimoto, M. *et al.*, *J. Chromatography B*, **1998**, *712*, 59-71.
4. Umakoshi, H. *et al.*, *J. Chromatography B*, **1998**, *711*, 111-116.

28. IMMOBILIZED LIPOSOME CHROMATOGRAPHY FOR REFOLDING AND PURIFICATION OF PROTEIN

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Refolding and purification of recombinant proteins usually require multiple processes which cause dilution of target protein. In addition, in the refolding step, it is a serious problem that refolding intermediates of proteins tend to form inactive aggregates especially at high protein concentration. Therefore, a simple method is desired for obtaining purified recombinant protein at high concentration.

We have reported that liposomes, composed of aqueous two-phase systems separated by closed phospholipid bilayer membranes, dynamically interact with refolding intermediate of proteins as evaluated by immobilized liposomes chromatography (ILC).¹ Because liposomes have fluctuated surface, denatured proteins refold into its native state upon the interaction with liposomes including translocation across membranes.^{2,3} In addition, from ILC analysis, it is revealed that liposome-protein interactions (retention time on ILC) can be controlled based on local hydrophobicity of proteins determined with the aqueous two-phase partitioning method.¹ These results indicate that ILC can be utilized not only for chromatographic protein refolding but for selective separation of proteins.

In this study, a novel method was proposed for refolding and purification of protein utilizing dynamic liposome-protein interactions and simple ILC method.

1. Yoshimoto, M. *et al.*, *J. Chromatogr. B* **1998**, 712, 59-71.
2. Kuboi, R. *et al.*, *Biotechnol. Prog.* **1997**, 13, 828-836.
3. Umakoshi, H. *et al.*, *Biotechnol. Prog.* **1998**, 14, 218-226.

29. AMINO ACIDS AND PROTEIN PARTITIONING IN AQUEOUS TWO-PHASE SYSTEMS CONTAINING BLOCK COPOLYMERS AND ELECTROLYTES

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Recent work on bioseparation using aqueous two-phase systems revealed large partition coefficients when using block copolymers. In this work, we present new data on the phase behavior of three ATPS systems: Poly(ethylene glycol) PEG 3350/water/potassium phosphate, Poly(ethylene glycol) PEG 10000/water/potassium phosphate and the block copolymer PEO-PPO-PEO/water/potassium phosphate, as well as the application of such systems in the partitioning of some amino acids (tryptophane, phenylalanine and 1-tyrosine) and proteins (insulin and bovine serum albumin, BSA). Partition coefficients for each solute were determined as a function of temperature and solution pH. Data obtained for the amino acids considered revealed larger partition coefficients for tryptophane, than for phenylalanine and 1-tyrosine. This can be attributed to the greater hydrophobicity of the tryptophane molecule as compared to the other amino acids and points to the potential separation of amino acid mixtures using these ATPS. Partition coefficients observed for insulin in systems containing the PEO-PPO-PEO block copolymer were found to be almost an order of magnitude larger than those observed in systems containing polymer (PEG 3350 and 10000). Possible micelle formation is believed to be the reason for the large coefficients observed in block copolymer systems. BSA, on the other hand, was totally recovered in the potassium phosphate bottom phase..

The data obtained for the ATPS systems PEG/salt were correlated by a modified UNIQUAC equation. The inclusion of a Pitzer term accounts for the presence of electrolytes. Cross-association occurring between PEG and water molecules is treated as a chemical equilibrium between solvated and free PEG species and that a fixed number of water molecules is bonded to each solvated PEG molecule.

30. PURIFICATION OF RABBIT LACRIMAL GLAND PLASMA MEMBRANES BY AQUEOUS TWO-PHASE AFFINITY PARTITIONING

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We have previously reported on a technique to analyze membrane populations from lacrimal epithelial cells by combining sorbitol density gradient fractionation and two-phase partitioning. However, in order to study redistribution of proteins involved in the secretory response in these cells it would be of interest to develop a less time-consuming technique to purify the plasma membranes. The present study describes the purification of lacrimal gland plasma membranes by affinity partitioning using a two-phase system containing polyethylene glycol and dextran in which wheat germ agglutinin (WGA) conjugated to dextran is used as affinity ligand. Our results indicate that a fraction enriched in 5'-nucleotidase, a plasma membrane marker, can be obtained from a microsomal fraction by pulling the plasma membrane into the bottom phase in a single step.

This project is supported by an University of Kalmar Faculty Research Grant (JPG) and by the Swedish Natural Science Research Council (BJ).

31. CHARACTERIZATION OF MIXED CULTURES BY MULTI-STEP PARTITION IN AQUEOUS TWO-PHASE SYSTEM CARRIED OUT ON A THIN-LAYER COUNTER CURRENT DISTRIBUTION APPARATUS

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There is a need to characterize the complex mixture of bacteria involved in different degradation processes. This can be done by getting a fingerprint of the microbial population. One way to achieve this is by aqueous polymer-polymer two-phase partition carried out on a thin-layer counter current distribution apparatus. Different bacteria partition differently between two polymer phases due to differences in surface properties. If the partitioning is done in several steps there will be a separation of the bacterial groups. After separation the fractions are analyzed by measuring *e.g.*, cell density and/or fluorescence and a pattern of the distribution will be achieved.

In this investigation, anaerobic cultures are studied and characterized by partition in aqueous two-phase systems. Phase systems with dextran and polyethylene glycol are used with salt and phosphate buffer. The partition depends on the concentration and molecular weight of the polymers used, and on type and concentration of the salt, pH and temperature. These factors were investigated to find the suitable phase system. The partition was carried out in 60 steps on a thin-layer counter current distribution apparatus. The distribution of cells in the different fractions were analyzed by measuring cell density and fluorescence, and the microbial population was studied by microscopy. This separation method was used to study anaerobic cultures in different operational conditions, *e.g.*, in resting or stressed states, to investigate if different distribution patterns will reflect the metabolic status of the culture.

This method is promising for characterizing the composition in all fields where mixed cultures are involved in the process.

This research is supported by Swedish National Energy Administration.

32. SOLUBILIZATION IN SURFACTANT AGGREGATES ON SILICA SURFACES: IMPACT OF SURFACE AND OF AGGREGATE STRUCTURE

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It is well known that charged micelles bind counterions. Much less well known is that surfactant aggregates adsorbed on charged surfaces can also bind counterions. Since the structure of such aggregates is not well established the location of the counterions is less certain still. From the influence of ionic strength on surfactant adsorption it can be inferred that co-adsorption of counterions can affect the structure and adsolubilization properties of the surfactant aggregates. Recent work by Treiner and co-workers [P. Favoriti and C. Treiner, *Langmuir* **1998**, *14*, 7493-7502] has shown how co-adsorption onto silica of salicylate ions with cetylpridinium can dramatically change the density of adsorption of the surfactant. We have found that the spin-probe peroxylamine disulfonate (PADS) is strongly bound to cationic micelles and is extremely sensitive to the presence of the salicylate counterions. Interpretation of the local viscosity and polarity as measured by PADS and other spin-probes has been used to give insight into the affect of the co-adsorbed salicylate.

33. CONFORMATIONAL TRANSITION AND MASS TRANSFER IN EXTRACTION OF PROTEINS BY AOT/ALCOHOL-ISOOCTANE REVERSE MICELLAR SYSTEMS

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The reverse micellar system (RVMS) has been widely used for protein extraction systems, because it is a variation of aqueous two-phase systems (ATPS) composed of the bulk aqueous and the micro aqueous phases surrounded by surfactants and dispersed in the organic phase. There is a key factor in the protein extraction processes to control the property of micellar interface. Recently, various alcohols have been often used for the improvement of the extraction processes (particularly back-extraction processes) because of their marked effect on the control of the formation and destruction of the reverse micelles. The alcohols are also known to have destructive effects on native proteins. In order to clarify the merit and demerit of the addition of alcohols, we examined quantitatively the effect of alcohols on protein and reverse micellar structures. We used circular dichroism (CD) to compare the effects of various alcohols on proteins, and percolation phenomena to evaluate the effects of various alcohols on reverse micellar structure.

Upon the addition of alcohols in bulk aqueous phase, the proteins were denatured notably, depending on the alcohol species and concentration. While upon the addition of a small amount of alcohol in RVMS, the reverse micellar structures were influenced considerably, suggesting a possibility to improve the protein back-extraction. Practically in the back-extraction of proteins, the addition of alcohols in RVMS, which suppress the cluster formation of reverse micelles, remarkably improved the back-extraction processes.

34. PARTITION OF XYLANOLITIC COMPLEX FROM *PENICILLIUM JANTHINELLUM* BY AQUEOUS TWO-PHASE SYSTEM (ATPS)

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This work evaluates the influences of five parameters (pH, PEG molecular weight, PEG concentration, concentration of buffer K_2HPO_4/KH_2PO_4 and NaCl concentration) on total proteins partitioning produced by *P. janthinellum* fungus in aqueous two-phase systems (ATPS), using a 2^5 factorial experimental design. A mathematical model to quantify the influence of these parameters, individually and/or jointly, on proteins partitioning in ATPS was attained. This model predicted significant effects of PEG molecular weight (MM PEG), % PEG and % NaCl on their partition behavior, and insignificant effects of pH and potassium phosphate concentration. The model allowed us to observe that the pH, % PEG and % NaCl had significant effects. However, the effects MW PEG and potassium phosphate were not significant. This model was statistically tested. The optimum point for protein extraction was obtained under the following conditions: pH 7.0, PEG 10000, 3.67% PEG, 10% potassium phosphate and 12.4% NaCl. The partition coefficient (k) value experimentally obtained was 5.25 and the one predicted by model was 5.89. This result indicates that the model is suitable for the process studied. Electrophoretic analysis of the proteins extracted under optimum conditions showed that higher molecular weight proteins preferentially migrate to upper phase of the system.

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35. AQUEOUS BIPHASIC SYSTEMS: INFLUENCES OF POLYMER MOLECULAR WEIGHT ON SYSTEM BEHAVIOR

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Aqueous biphasic systems (ABS) are suitable for carrying out liquid/liquid separation of metal ions and organic molecules. These two-phase systems are formed when certain water soluble polymers are combined with certain inorganic salts in specific concentrations. The long range goal of our project is to reduce and/or eliminate the need for volatile organic compounds (VOCs) in many separation technologies by developing a fundamental understanding of the factors governing solute partitioning in ABS.

In this presentation, we will compare/contrast the phase-forming characteristics of different molecular weights of polyethylene glycol (PEG) and random polyethylene glycol/polypropylene glycol (PEG/PPG) copolymers. Also, we will present the partitioning behavior of several organic and inorganic molecules in the biphasic systems. Finally, the distributions of molecules will be graphically represented against tie-line length for direct comparison of several very different polymer/salt two-phase systems.

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36. IMPACT OF WASHING PROCEDURES UPON THE PARTITION BEHAVIOR OF NANOPARTICULATE INCLUSION BODIES

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Optimized *E. coli* fermentation conditions (37°C, pH 8, on M9 minimal medium, and an inducer (*i.e.*, IPTG) concentration of 5 mM) enabled the controlled production of cytoplasmic α -glucosidase inclusion bodies. Cell disruption by multiple passes through the APV-Gaulin homogenizer achieved total release of the inclusion bodies from the cell cytoplasm and micronized cellular debris, yielding a particulate suspension characterized by a mean particle diameter of ~150 nm. Fractionation of the target protein nanoparticles from this complex suspension was achieved by direct fractionation in PEG 8000/potassium phosphate aqueous two phase systems (ATPS).

SDS PAGE analyses of recovered inclusion bodies indicated the presence of other proteins variously physically associated, or coincidentally co-purifying, with these nanoparticles. The common protein banding pattern consisted of α -glucosidase (P, 67 kDa) and co-purifying proteins, C1, C2 and C3 with molecular masses corresponding to 45, 40 and 37 kDa respectively. Washing procedures performed on inclusion body pellets with a variety of chaotropes and detergents indicated that the contaminants C2 and C3 (identified by N-terminal sequence determination as *E. coli* outer membrane proteins *omp F* and *omp A*, respectively) strongly influenced the subsequent partition behavior of inclusion body particles in ATPS.

Partitioning studies with PEG 8000/phosphate demonstrated that the elimination of these associated membrane proteins caused a relocation of inclusion bodies from the interphase to a sediment within the bottom phase. Partitioning studies performed on the solubilized C2 and C3 components showed that they had a strong propensity to accumulate as an interphase. These studies invite analogies to be drawn with the ATPS-based fractionation of enveloped retroviruses (*i.e.*, unwashed inclusion bodies) and adenoviruses (*i.e.*, washed-membrane free inclusion body particles). These analogies will be discussed in the context of manufacturing processes customized for gene therapeutics.

37. PHASE FORMATION BEHAVIOR AND KINETICS OF THE SEPARATION OF PHASES AND PROTEINS IN AQUEOUS TWO-PHASE SYSTEMS

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Aqueous Two-Phase Systems (ATPS) have started to be used industrially for the isolation and separation of recombinant and enzyme proteins. The use of liquid-liquid extraction makes easier, issues such as scale-up, but also continuous steady-state operation. Two phase separation can reduce substantially the number of initial downstream steps integrating in one unit operation clarification, concentration and partial purification. Until now most studies have concentrated on investigations of factors affecting the partition coefficient of molecules but there are virtually no studies on the variables affecting phase formation and separation behavior and kinetics which is the critical issue for scale-up and design for practical application, in particular in the presence of "real" cell culture supernatants which will include proteins, cell debris and whole cells.

At present we are investigating fundamental aspects of phase formation and separation behavior including droplet formation and droplet size studies as a function of system physical properties (size of PEG, composition, density, viscosity, surface tension) and operational variables. (agitation, Re) and droplet coalescence studies as a function of system properties. First, we are carrying out the initial investigations in pure PEG/salt systems in both batch and continuous separators. In addition, we are using systems in the presence of proteins, proteins and cell debris and with whole cells.

All the data collected (and the related modeling studies) will be essential for design and scale-up of separators using ATPS. In this paper we will present and discuss our results in all the areas presented above.

38. AQUEOUS POLYMER TWO-PHASE SYSTEMS AS CARRIERS IN SPRAY-DRYING OF BIOLOGICAL MATERIAL

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Aqueous polymer-polymer two-phase systems provide a new concept in formulation of carrier systems for spray-drying of biological material. One of the two phases is easily dispersed as small droplets in the other phase, thus facilitating encapsulation of the former phase. By varying the composition of the system, the effective structure can be varied from being continuous in polymer A to continuous in polymer B. The advantage of such a two-phase system over conventional one-phase systems in spray-drying is the improved encapsulation of biological material being partitioned to the encapsulated phase. A two-phase system composed of poly (vinylpyrrolidone) (PVP) and dextran was applied to spray-drying of live probiotic bacteria. Electron spectroscopy for chemical analysis (ESCA) was used for analysis of the surface composition of the powders, providing information on the distribution of the polymers and the encapsulated material in the powder and thus also in the spray-droplets. The bacteria partitioned virtually exclusively to the dextran-rich phase and were well encapsulated in the dried powders. The surface composition of these powders was similar to that of the corresponding powders without bacteria. The survival rate and storage stability of the bacteria depended on the composition of the two-phase system. These results indicate that this type of formulation holds promise for future applications for encapsulation of sensitive biological material.

39. ELECTROSTATIC AND HYDROPHOBIC EFFECTS OF OLIGOPEPTIDE INSERTIONS ON PROTEIN ADSORPTION AT SOLID SURFACES

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Protein adsorption is a process of importance not only in numerous biological systems but also in a range of biomedical applications. Despite this, and despite a tremendous amount of work devoted to improve our understanding of protein adsorption, much is still unknown about its mechanism, at least to that of polymers and polyelectrolytes. One of the reasons for this has been the lack of well-defined proteins. The adsorption is driven by an interplay between surface properties, protein surface properties (exposed amino acid residues) and solvent properties.

In situ ellipsometry was used for adsorption studies at different surfaces of genetically engineered derivatives of Z and its dimer ZZ, where Z is an IgG binding domain derived from staphylococcal protein A. In particular, studies of the interplay between hydrophobic and electrostatic interactions as driving force for adsorption was investigated by studying the effects of oligopeptide insertions of the type Tn ((AlaTrpTrpPro)n), In ((AlaIleIlePro)n), Nn ((AlaTrpTrpAspPro)n) and Pn ((AlaTrpTrpLysPro)n) on the adsorption at poly(ethylene glycol) (PEG), silica, methylated silica and diaminocyclohexane plasma polymer surfaces. For comparison, the adsorption of the inserted oligopeptide stretches was investigated.

For the Tn and In modified proteins qualitatively the adsorption could be modeled with a Flory-Huggins mean-field lattice approach where the protein was regarded as a block copolymer with a hydrophilic and a hydrophobic part. The properties of exposed amino acid residues (hydrophobic or charged) were found to have a major influence on protein interfacial behavior. The relative importance of hydrophobic and electrostatic interactions as driving force for the adsorption is discussed. PEG-HIC column interaction data is compared with the interactions at solid flat surfaces.

40. ENTROPIC INTERACTION CHROMATOGRAPHY: PROTEIN PURIFICATION USING END-GRAFTED POLYMER BRUSHES

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Separation of macromolecules on the basis of their molecular weight is traditionally done using size-exclusion chromatography (SEC), where separation is achieved by the geometry dependent partition of macromolecules between a continuous phase and the porous interior of a gel or cross-linked bead. The volume of a pore accessible to a solute is limited by its size, so larger molecules will have access to a smaller volume and will remain in a bead for a shorter period of time than smaller solutes. Distances of intraparticle diffusion in SEC resins are typically on the micrometer scale, resulting long elution times and relatively broad peaks. In this work, we demonstrate that size-based separations can also be achieved by contacting the sample with an end-grafted brush of either poly(ethylene glycol) or poly(acrylamide), where the brush height is on the order nm, leading to significantly shorter diffusion lengths and elution volumes. The concept is based on cylindrical self-consistent field calculations which predict partition coefficients from a thermodynamic model for the free energy of mixing of the solute with the polymer phase. Size-dependent exclusion is predicted due to the unfavorable entropy of mixing associated with partition, modified by enthalpic interactions between the solute and the gel phase. This concept is used to design a highly efficient separation technology, which we call Entropic Interaction Chromatography, based on the interaction of solutes with a layer of terminally attached polymer chains.

41. REVIEW AND UPDATE ON SURFACTANT-MEDIATED CLOUD POINT EXTRACTIONS

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Aqueous solutions of some surfactant micelles exhibit phase separations behavior upon temperature alteration. This phenomenon can be exploited in separation science for the development of extraction, purification and preconcentration schemes for desired target analytes. This presentation will review the most recent developments concerning this alternative extraction approach, termed cloud point extraction (CPE) or micelle-mediated extraction (ME), including the utilization of zwitterionic surfactants in CPE and employment of CPE in conjunction with gas chromatographic analyses. In addition, the advantages, limitations and anticipated future directions of this methodology will be discussed.

42. AFFINITY ISOLATION OF INTEGRAL MEMBRANE PROTEINS WITH DETERGENT/POLYMER AQUEOUS TWO-PHASE SYSTEMS CONTAINING METAL CHELATING POLYMER

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Phase behavior and protein partitioning have been examined in novel detergent/polymer aqueous two-phase systems formed between PEG or dextran mixed with non-ionic detergents such as Triton, Tween, alkylglucosides and alkylpolyethyleneoxides. Detergent/polymer aqueous two-phase systems are formed where a polymer/water phase is in equilibrium with a micelle/water phase. Both membrane-bound and water-soluble proteins have been studied. Membrane proteins such as bacteriorhodopsin and cholesterol oxidase partition strongly ($K=5-20$) to the micelle phase, while water-soluble proteins such as BSA and lysozyme prefer the polymer phase. These results suggest the use of detergent/polymer mixtures for extraction of membrane proteins. The main driving forces for the partitioning of both water soluble and membrane proteins in these systems will be discussed.

For the first time it has been possible to achieve a biospecific affinity partitioning of an integral membrane protein, cytochrome bo₃ ubiquinol oxidase from *E. coli*. A metal chelating polymer was used in the detergent/polymer phase system for purification of the histidine-tagged protein. Thus, a fast and mild isolation method with high selectivity for integral membrane proteins has been created. Detergent/polymer aqueous two-phase systems are especially suitable for purification of labile membrane proteins, such as receptors, due to the rapid separation at low temperatures with many different mild non-ionic detergents.

43. AQUEOUS TWO-PHASE SYSTEMS FORMED BY POLYMER AND CATIONIC-ANIONIC SURFACTANT MIXTURES

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A novel aqueous two-phase system (ATPS) can be formed by polymer and cationic-anionic surfactant mixtures. In this work we investigated the aqueous two-phase systems formed by mixtures of a cationic-anionic surfactants (dodecyltriethylammonium bromide/sodium dodecylsulfate) and a polymer (Dextran, polyethylene glycol, ethylene oxide (EO)-propylene oxide (PO) random copolymers, differently). The phase behaviors of ATPS, including phase diagrams, phase separation time, effects of temperature, salts (buffers), molecular weight of polymers, were investigated. The composition, viscosity, density and interfacial tension of the phases were determined. The partitioning of proteins and the effects of salts (buffers) and pH values on partitioning were investigated. Affinity ligands were attached to polymers and affinity partitioning of proteins was investigated. It was shown that, in such an aqueous two-phase system, one phase is rich in surfactants and another phase is rich in polymers. Proteins can be distributed into different phases because of the marked differences between the phases. The partitioning behaviors of proteins could be optimized and controlled by tuning micelle properties, especially the charges on micellar surfaces, which could be achieved by changing the molar ratio of cationic to anionic surfactants. The affinity ligands attached to polymers gave high selectivity of protein partitioning. When the surfactant-rich phase were diluted, a new ATPS was formed, by which a multi-step partitioning procedure were developed. When EO-PO copolymers were used in ATPS, the multi-step partitioning could be achieved by raising the temperature of polymer-rich phase. When the phases were further diluted, cationic-anionic surfactants were precipitated out, so surfactants could be separated after partitioning has been completed. EO-PO copolymers could be separated after partitioning by raising the temperature above the copolymer cloud point. The recycle of cationic-anionic surfactants and EO-PO copolymers were discussed.

44. SOLUBILIZATION IN SURFACTANT AGGREGATES ON SILICA SURFACES: IMPACT OF SURFACE AND OF AGGREGATE STRUCTURE

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Surfactant micelles are well known for increasing the solubility of many hydrophobic molecules in aqueous phases. When surfactants adsorb on silica and other oxide surfaces, surfactant aggregates are formed which can also solubilize hydrophobic molecules. Partitioning between the adsorbed surfactant phase and the aqueous and/or micellar phases forms the basis for micellar and admicellar chromatographies, and impacts a range of environmental issues. The structure of adsorbed surfactant aggregates is a matter of dispute and a wide range of structures which are expected to have different solvation properties have been postulated. We will present the results of spectroscopic studies on how silica surfaces influence surfactant aggregate structure and how this affects the solubilization properties of the aggregates. For neutral solutes the solubilization can be well described using Linear Solvation Energy Relationships (LSER).

Aggregates of ionic surfactants can also bind oppositely charged counterions, both small inorganic counterions and large organic ions. Preliminary results on the use of spectroscopic probes to study counterion binding will be presented.

45. INITIAL ISOLATION OF RECOMBINANT PROTEINS FROM WHOLE FERMENTATION LYSATES USING AQUEOUS TWO PHASE SYSTEMS

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As the demand for therapeutic recombinant proteins, antibodies and antibody fragments grow, there is an ever-increasing search for a universally applicable initial extraction/isolation step for products in whole fermentation broth. And since 'bottom-line' economics are still at the core of the industry, the initial extraction/isolation step should not negatively impact the overall cost of goods sold. In light of these market pressures and previous successes using Aqueous Two Phase Separation (ATPS), it is clear that this technology has tremendous potential to play a critical role in the future of industrial protein purification.

There is a broad body of data covering the use of ATPS on model proteins, or proteins that have been semi-purified from clarified fermentation broth. Recent experiments in our laboratories show that ATPS can be successfully applied on fermentation broth lysates containing up to 40% solids by volume, and we have identified 'generic' conditions that can be applied to a variety of products. The work presented in this presentation investigates the effects of pH, ionic strength and polymer concentrations on the partitioning of several target proteins, cell fragments and host proteins in Ucon/Reppal aqueous two phase systems.

46. TWO-PHASE AQUEOUS EXTRACTION AS A PROCESS DEVELOPMENT TOOL

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Two-phase aqueous extraction provides the process development scientist with a very useful development tool. In our work at Genencor Intl., two-phase liquid extraction was developed from the bench scale to the industrial scale for the production of chymosin. The success with two-phase extraction came by coupling the extraction with ion exchange chromatography. These orthogonal purification techniques gave a two step process from the fermenter to customer. This process proved very useful in chymosin purification from both natural and recombinant sources. Kilogram quantities of active enzyme were produced for the customer. Later work has used two phase extraction as an intermediate process to purify several other enzymes from natural sources. For example, endoglucanase was purified from a multi-enzyme fermentation broth at the pilot scale. Two-phase extraction provided the means to separate the various components to produce samples for biochemical characterization and customer evaluation. In summary, two-phase liquid extraction has proved to be a versatile, robust and scaleable process in the industrial setting.

47. SEPARATION OF GENETICALLY MODIFIED CELLULASES USING DETERGENT BASED AQUEOUS TWO-PHASE EXTRACTION

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Extraction of proteins in aqueous two-phase systems are especially suited for large scale due to the ease of operation and scale up.¹ The partition coefficient is a property of the individual protein. To adapt the technology better to changing products in the EU-project "Integrated Process Design for Large Scale Production and Isolation of Recombinant Proteins" genetic engineering is used as a tool to fuse hydrophobic tags to target proteins to enable these proteins to be well separable by ATPS.² Different ATPS were investigated for two enzymes. Very successful in this investigation proved to be detergent-based systems. As an example the separation of a tagged cellulase, the Endoglucanase 1 will be reported. Based on former work³ the separation was investigated for different tags and different detergents, most of them nonionic Polyoxyethylene Alkyl Ethers. For an example, with EG1-fusion and 2% of the detergent C12-18EO5 we obtained yields of 89 % and partition coefficients of 52. Using wild type EG1 in comparison we obtained yields of 2-3% and partition coefficients of 0.3. The yield can be further improved applying additional optimization techniques. It can be concluded that directed genetic manipulations of proteins and the use of detergent based systems is a very suitable method to increase partition coefficients and consequently yield in a predetermined way.

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48. RECOMBINANT PROTEIN PARTITIONING IN THERMOSEPARATING AQUEOUS TWO-PHASE SYSTEMS BY GENETIC ENGINEERING OF PEPTIDE TAGS TO TARGET PROTEINS

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Aqueous two-phase systems containing thermoseparating ethylene oxide (EO)-propylene oxide(PO) copolymers in mixtures with dextran or hydroxypropyl starch have been used for protein separations. The target proteins are partitioned in a primary step to the phase containing the thermoseparating polymer. Genetic engineering was used to fuse peptide tags to target proteins to enhance the partitioning to the more hydrophobic thermoseparating phase. Tryptophan has previously been shown to partition to EO-PO copolymer phases and was therefore chosen as the main component in the peptide tags.

The partitioning was investigated for tryptophan containing peptides (Trp)_n and (Trp-Pro)_n, as well as for peptides also with charged residues, (Trp-Pro-Asp)_n and (Trp-Pro-Arg)_n. The recombinant proteins cutinase, a lipase, and ZZ-cutinase were studied as target proteins. Dextran T500 was used as bottom phase polymer and EO30PO70, a random copolymer of ethylene oxide and propylene oxide, as top phase polymer. The effects of the different amino acids were evaluated in a reference system with potassium sulfate, where the interfacial potential was close to zero. The effects of sodium perchlorate and triethylammonium phosphate were also studied.

For the peptide partitioning a linear relationship between log K and the number of tryptophan residues in the peptide was found for (Trp)_n. The prolines included in the (Trp-Pro)_n peptides did not affect the partitioning significantly. For the partitioning of protein-peptide fusions, all fusion tags containing tryptophan resulted in an increased partitioning to the top phase compared to the wild type protein. However, the contribution from the fusion tag did not in all cases correspond completely to the expected value from peptide partitioning data. A possible explanation could be that the tag is not fully exposed.

The effect of the non-ionic surfactants Triton X-100 and C₁₂EO₉ on the partitioning of wild type cutinase and cutinase-(Trp-Pro)₄ was studied in EO-PO/Reppal PES 200 systems. For the cutinase-(Trp-Pro)₄ construction an almost linear increase of the K-value vs. the concentration of detergent was found. This shows the possibility to use a system with detergent and thermoseparating polymer for purification of protein-peptide constructions.

Sponsor: EU project "Integrated bioprocess design for large scale production and isolation of recombinant proteins" (B104-CT96-0435).

49. PURIFICATION OF A RECOMBINANT PROTEIN FROM MAMMALIAN CELL CULTURE: AN INDUSTRIAL APPLICATION

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Purification of a recombinant protein from a mammalian cell culture fermentation is described. This protein is a good candidate for two-phase partitioning for several reasons. It has a very high cost per unit, is intrinsically unstable and current process yields are low. The emphasis was to develop an initial capture step for the rProtein from the cell culture broth which would result in a high recovery, some increase in purity, and a significant reduction in volume. It was also important that the resulting extract could be applied directly onto an ion exchange column for the next purification step, with little or no additional manipulation. Several different purification strategies were tried, including Peg/Dextran, Peg/Salt and Peg/Antibody systems. The system yielding the best purity and recovery was a Peg/Pluronic F68/Ammonium Sulfate System. Overall recovery of the target protein was >90%, with an increase in purity of over 100. A proposed purification strategy for direct capture of the rProtein from fermentation broth is described.

50. INDUSTRIAL PROSPECTS OF AQUEOUS TWO-PHASE PROCESSES

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Liquid-liquid extraction and separation of proteins represent a paradigm of process integration in biochemical engineering. It allows the integration of the separation of whole cells or cell homogenate debris and nucleic acids from the target product protein as well as initial protein purification in one step. In addition, it allows the continuous steady-state operation which is beneficial both for large scale and for quality control, an essential feature of modern process biotechnology. The prediction of partition coefficients of individual proteins is a crucial aim for the design of separation and purification processes based on liquid-liquid two phase systems. The most important properties of such a system that can be manipulated in the phases, without chemically modifying the polymers used as is usually done for biological affinity, are hydrophobicity and charge. This requires the development of appropriate models and correlations to predict their behavior. Similarly it is essential to build appropriate steady state and dynamic mathematical models to simulate and predict phase behavior in continuous two-phase extraction and back-extraction systems.

This presentation will focus on a critical analysis of the present state of the art of Aqueous-Two Phase Processes in terms of present industrial application mainly in the USA, Japan and Germany (*e.g.*, separation of recombinant chymosin from *Aspergillus awamori*, separation of α -amylase from *Bacillus* supernatant, separation of recombinant IGF-1 in *E. coli* inclusion bodies and transgenic sheep proteins) and of potential for future application. It will also identify **key** areas of process research and development needed in order to ensure widespread application of this technology. If one draws a parallel with the extraction of antibiotics in the biotechnology industry, the use of liquid-liquid extraction only became widespread with the development of very large scale processes. Hence, in the case of protein separation we can foresee that the use of liquid-liquid extraction will become commonplace when the need for mass production of pure proteins either for therapeutic applications, for analytical purposes or as novel industrial catalysts (next generation of recombinant biotechnological proteins) becomes urgent.

In order for liquid-liquid extraction technology for proteins using aqueous two-phases to become fully mature there is an urgent need to develop appropriate **correlations to predict partitioning** of proteins in practical systems (*e.g.*, PEG/phosphate and PEG/sulfate with and without the addition of salts) based on the physicochemical properties of the protein (hydrophobicity, charge and molecular weight) and also to develop process models to predict phase behavior and phase separation in batch and continuous extraction systems. These can be used to investigate **system stability** and thus develop **robust** processes not susceptible to small changes in process conditions. Dynamic models will also have use in the development of appropriate control strategies. The state of the art and bottlenecks for applications and modeling for prediction of partitioning and phase separation in "real" situations will be presented and discussed in this presentation.

51. RECOVERY OF NANOPARTICULATE BIOPRODUCTS FROM SUSPENSION FEEDSTOCKS: RELEVANCE TO THE MANUFACTURE OF GENE THERAPEUTICS

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There is an intense interest in the creation of viral and non-viral gene delivery vehicles designed to operate in the burgeoning field of gene therapy. The rush to assemble material appropriate for clinical trials has necessarily limited the amount of basic research to establish selective and scaleable recovery technologies which will (i) integrate efficiently with upstream cultures and (ii) yield the product in a form suited to validated operations of polishing, formulation and delivery. This position is exacerbated by the relative scarcity and high value of representative vectors in quantities suitable for appropriate experimentation. The nanoparticulate nature of extant gene delivery systems (particle size range 20-150 nm) and the associated property of low diffusivity, together with low molalities in culture feedstock and the requirement to maintain infective integrity, pose unique process engineering problems for the design and implementation of selective recovery and formulation operations.

Presentation will be made of the role that polymer-polymer and polymer-salt aqueous two-phase systems (ATPS) can play in circumventing the process bottlenecks currently posed by a dependency upon scale-limited, high-performance centrifugation for the manufacture of clinical lots of recombinant adenovirus gene therapy vectors. The value of exploiting nanoparticulate protein inclusion bodies (available in gram quantities) as surrogate mimics for adenovirus in method scouting experiments to establish candidate ATPS will be discussed. The evaluation of these systems with respect to the partition behavior of adenovirus from semi-purified states and crude cell lysates will be presented. The importance of studies with nanoparticulate mimics will be emphasized in a discussion of the difficulties posed by limitations in the availability of *bona fide* gene therapy vectors in quantities and concentrations representative of projected clinical doses.

52. PARTITION ACCOMPANIED BY ADSORPTION AT THE INTERFACE IN AQUEOUS TWO-PHASE SYSTEM: ACCUMULATION OF LOW MOLECULAR MASS AT THE INTERFACE SIMULTANEOUSLY WITH ADSORPTION OF HIGH MOLECULAR MASS

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On the partitioning of particles in aqueous two-phase systems, they are often accumulated at the interface. The accumulation can be caused not only by the adsorption at the interface, which corresponds to the selective partitioning to the interface, but also by the casual location to the interface as the result of precipitation. The accumulation along with partition is selective and expected to be utilized for separation or concentration but has not been clarified in detail. We previously showed the phenomenon of the former case where the high molecular mass RNA was adsorbed at the interface in the salt/PEG two-phase system. Its partition behavior was investigated quantitatively and showed to be reproducible, furthermore, unique and interesting with respect to the partitioning of the soluble macromolecule not the particle. In this presentation the partitioning of low molecular mass RNA along with adsorption of coexisting high molecular mass RNA at the interface will be shown. Low molecular mass RNA is inherently soluble and partitions between the top and bottom phases if partitioned alone in the potassium phosphate/PEG-1500 system. However, low molecular mass RNA was caught into the interface to a considerable extent if partitioned together with the high molecule. On the partition of various RNA preparations with different ratios of high and low molecular masses, the accumulation of low molecular mass RNA increased with an increase in percentage of the high molecular mass coexisting in the preparation, while the high molecular mass was mostly adsorbed at the interface. Characteristics of partitioning accompanied by accumulation at the interface will be discussed with other results.

53. PHAGE PARTITIONING: FUNCTIONAL SELECTION OF SURFACE DISPLAYED PEPTIDES

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This project involves genetically altering surface exposed regions of the primary coat protein of a phage particle so as to (a) investigate the effects of such alteration on particle partition in aqueous polymer phase systems, and (b) identify oligopeptides with enhanced affinity for the phases in such systems. The latter can find use in various applications, such as controlled phase wetting, or controlled phase affinity and purification of genetically altered proteins.

Various researchers have investigated the relationship between protein structure (1^o, 2^o, 3^o) and the affinity of proteins for the phases in aqueous polymer phase systems. Much of this work has been driven by an interest in improving the ability of phase partition to serve as a large scale commercial bioseparations method. Many interesting observations have been made such as the relatively high affinity of tryptophan residues, or proteins naturally or genetically enriched in tryptophan (*e.g.*, B-galactosidase), for poly(ethylene glycol) containing phases. However there is relatively little understanding about (I) molecular factors influencing oligopeptide or protein partition, as well as (II) how this influences the interaction of the phases with particles or surfaces covered in oligopeptides or proteins.

Viral particles whose surfaces are primarily composed on a single coat protein, whose terminal oligopeptide sequence can be genetically altered in a controlled manner,¹ provide an avenue to investigate I and II, above. Via "surface display technology" large libraries of up to 1010 different peptide or protein variants, expressed on bacterial phage can be screened via partitioning, for terminal oligopeptides with enhanced affinity for different phases, in phase systems of commercial bioprocessing interest. The beauty of the method lies in the genetic information of the displayed protein being contained inside the corresponding phage particle. This serves as a direct route to amplification of the information (via bacterial infection by target phage particles) and identification of structures through DNA sequencing. The library sizes are so large it should be possible to identify oligopeptide domains that exhibit desirable secondary traits (biocompatibility, high affinity, stability, alteration of phase affinity with pH or temperature) in addition to basic phase affinity.

During the past year initial studies, involving *E. coli* M13K07 phages from a library of altered pVIII coat proteins, and partitioning in both PEG-dextran or PEG-salt two phase systems of commercial interest, have shown that:

- a. Partition of M13K07 phage between the phases can be enumerated.
 - b. Phase systems exist which partition native M13K07 into the phase not enriched in PEG - so that the systems can be used to select on the basis of enhanced PEG-enriched phase affinity.
 - c. The phage survive phase system exposure and are viable - that is able to infect bacterial cells and multiply the genetic material coding for the surface modification (amino acid sequence).
 - d. Many oligopeptide sequences which appear to have enhanced PEG-rich phase affinity contain tryptophan residues.
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54. FRACTIONATIONS BASED ON UREA COMPLEX AND TWO-PHASE SYSTEM FORMATION

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In some solutions urea molecules form crystalline complexes with "linearly shaped" long chain, saturated fatty acids or fatty acid esters, but not unsaturated alkyl analogues. So too, in some solutions urea molecules form complexes with polymers such as poly-L-(lactic acid)s (PLLs) but not analogous polymers containing poly-D regions. Material that does not form complexes can be recovered from the liquid phase, while solid phase (complexed) material is subjected to further processing. For example, urea-fatty acid ester complexes dissolve in hot water to form liquid two-phase systems consisting of an organic phase plus an aqueous phase (enriched in urea). So too, urea-PLL complexes dissolve in hot water to form liquid-solid, two-phase systems consisting of an aqueous phase and precipitated PLL phase. In both examples, phase system formation may provide for efficient separations and reagent recycling. Urea complex formation has been studied for some time.^{1,2} We are currently exploring its use for large scale, primary fractionation, of seed oil preparations,³ and polymer preparations.⁴

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55. AQUEOUS BIPHASIC SYSTEMS FOR THE SEPARATION OF LIGNINS FROM CELLULOSE IN THE PAPER PULPING PROCESS

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The high efficiency of chemical use and recovery is the reason the chemical pulping of wood produces the majority of the United State's paper products. Further increases in the demand for paper products and environmental regulations have led to the development of technologies that are environmentally benign and more efficient in the separation of lignins from cellulose. The "Organosolv" Process employs the use of organic solvents to efficiently solubilize lignins from the cellulosic fraction of wood. Although these types of processes have shown to increase pulp yield and reduce environmental stress, they have not been adopted by the paper industry because of the difficulties in engineering high temperature, high pressure processes involving organic solvents. Polymer-based aqueous biphasic systems are suitable for the extraction and separation of organic compounds, and could eliminate the use of organic solvents. Our efforts to develop an aqueous polymeric extraction process for paper pulping will be presented.

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56. TWO-PHASE PARTITIONING OF RECOMBINANT CUTINASE

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Partitioning of recombinant cutinase, expressed in the culture supernatant of *Saccharomyces cerevisiae* (obtained from Unilever Research Laboratories, The Netherlands) was investigated as a result of modifications in the two-phase system and the protein, respectively. A 2-3 fold increase in partition coefficient (K) of the wild type enzyme was achieved by inclusion of 0.05% (w/v) detergent like Triton X-100 or Tween 20 in 20% polyethylene glycol (PEG) 1500-15% phosphate system at pH 7. The fatty acid, butyrate (0.5% w/v) increased the partitioning by more than 8 fold, resulting in K of 135. Among the fatty acids, the butyrate seemed to have a specific effect on cutinase partition as revealed by increase in K also in the presence of butyrate analogs, butyryl amide and isobutyrate but a very minor change by other fatty acids. Quantitative yields of the enzyme were obtained with a purification factor of 26 in the two-phase system with butyrate. Cutinase modified genetically with (Trp-Pro)_n were also partitioned preferentially to the top phase in polymer-salt systems, including PEG-phosphate and polyvinylpyrrolidone-phosphate. Fusion of (Trp-Pro)₄ was more effective than (Trp-Pro)₂ in altering the partition. Higher K of cutinase, both wild type and mutants, was obtained during partitioning of the whole broth. Interaction of the wild type enzyme with butyrate and that of enzyme mutants with polymers was studied by fluorescence spectroscopy.

The research is supported by a grant from European Commission (BIO4-CT96-0435).

57. POLYMER-POLYMER ORGANIC TWO-PHASE SYSTEMS AS MEDIA FOR BIOCATALYSIS. ENZYME-POLYMER INTERACTIONS AS STABILIZING MEANS WITH SPECIAL REFERENCE TO BIOCATALYSIS IN WATER-POOR MEDIA

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Mixtures of polystyrene and ethyl cellulose form polymer two-phase systems in organic solvents like toluene or chloroform. Ethyl cellulose also has the property that one can dissolve/suspend it in aqueous solution. An enzyme can be added to the aqueous solution/suspension and after drying a powder is obtained that is soluble in organic solvent. This means that the enzyme by complexing with the ethyl cellulose molecule is getting the property that it can be dissolved in very apolar organic solvents like toluene and chloroform. When adding a small amount of water to such systems, it is possible to use the dissolved enzyme to carry out biocatalytic reactions. The complexes are broken upon addition of excess of water, since then a two-phase system is formed with organic solvent in one phase together with most of the polymers and the aqueous phase containing the enzyme. The enzyme-polymer complexes showed an increased stability to elevated temperature as compared to native enzyme.

58. STRATEGIES TO MAKE AQUEOUS TWO-PHASE SYSTEMS MORE ROBUST AND EASILY HANDLED IN DOWNSTREAM PROCESSING SITUATIONS

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A limiting factor when presenting aqueous two-phase systems as reliable alternatives in DSP has been that the behavior of the systems have been regarded as unpredictable and that a lot of optimization work is needed before a suitable system is obtained. There are different strategies to address this issue:

- polymer-salt systems are more easily predictable than polymer-polymer systems
- variation of polymer content and salts in the system influences the partition behavior in a predictable way
- use of affinity ligands has made partitioning more predictable
- use of separator particles can control the partition behavior
- introduction of smart polymers in aqueous two-phase systems has added one more way to manipulate the system

Strategies for developing robust 2-phase systems to be applied in DSP will be discussed.

59. DEVELOPMENT OF A METHOD FOR EFFICIENT OPTIMIZATION OF AQUEOUS TWO-PHASE EXTRACTION

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The common way to investigate the feasibility and the optimization of an Aqueous Two-Phase System is to optimize empirically one parameter after the other. Using this approach, the true optimum might not be found (*e.g.*, if a local optimum exists or if a ridge is built in the response area). This can be the case as found in the present investigation. For the same reason, a factorial analyses with a single regression could be suboptimal.

Three different algorithms were applied to optimize extraction in PEG/salt systems: the method of steepest ascent,¹ a modified simplex-procedure² and a genetic algorithm.³ These algorithms were adapted and applied for the use of optimization in ATPS.

Separation of three different enzymes from whole broth was investigated: a recombinant protease and leucin dehydrogenase. Only the method of steepest ascent and the genetic algorithm could be applied successfully. The simplex procedure is too cumbersome. Optimizing the yield for the example of leucin dehydrogenase from E-coli a yield of 70% (steepest ascent) and of 80% (genetic algorithm) could be obtained using four and five system parameters, respectively. The two methods differ, *e.g.*, in the amount of experimental points, the needed number of experimental series, the number of process variables etc. A fusion of the steepest ascent and the genetic algorithm seem to be the fastest way to find an optimum since it fuses the fast approach of steepest ascent and the painstaking procedure of the genetic algorithm. This procedure is not limited in the amount of parameters and addition of auxiliary chemicals like NaCl and chaotropes can be investigated simultaneously.

The investigation provides further proof that mathematical tools are very helpful for the effective and fast optimization of protein extraction in ATPS.

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60. A MODEL FOR THE PARTITION OF METAL IONS IN AQUEOUS TWO-PHASE SYSTEMS

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Aqueous Two-Phase Systems (ATPS) composed of polyethylene glycol (PEG) as the polymer and inorganic salts have a great potential to separate metallic ions from aqueous solutions. Most published work focuses on the experimental determination of partition coefficients for metal ions. Generally, in these systems other components are often added as extractants, acids, bases or other inorganic ions.

A model is proposed for the partition coefficient of the metallic ion D_M as a function of operational variables, including characteristics of the metallic ion, characteristics of the ATPS and the addition of inorganic salts.

The model can be summarized by the following expression:

$$D_M = D_0 + \frac{a_1 \cdot (X)^{a_2} \cdot (r^*)^{a_3}}{1 + b_1 \cdot \exp(-b_2 \cdot Y) \cdot \exp(-b_3 \cdot Z)}$$

X is a characteristic variable of the ion that considers the electric charge and electronegativity. The variable Y is a characteristic of the ATPS and it considers the type of inorganic salt, the solubility and concentration of this salt and the concentration of PEG. Z is a variable that considers the concentration and solubility of additional salts in the original ATPS. The variable r^* it is the dimensionless ion size.

The model was tested for complex anions of BiX_4^- (BiCl_4^- , BiBr_4^- , and BiI_4^-) and cations from groups I and II (Na^+ , Cs^+ , Ca^{+2} , Sr^{+2} , and Ba^{+2}) giving a good adjustment for both systems.

It was found that for both systems there is an increase in partitioning coefficient with ion size and the variables Y and Z. On the other hand the ions partitioning coefficient BiX_4^- increases with the variable X. Meanwhile the cations from groups I and II have an inverse behavior which is attributable to the ion charge.

61. DROWNING-OUT CRYSTALLIZATION OF SODIUM SULFATE USING AQUEOUS TWO-PHASE SYSTEMS

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This presentation will describe a novel application of aqueous two-phase systems : drowning-out crystallization. This method can be used to obtain crystals of pure, anhydrous salt. A concentrated salt solution is mixed with Polyethylene glycol (PEG), upon which three phases are formed: salt crystals, a PEG rich liquid and a salt rich liquid. After removal of the solid salt, a two-phase system is obtained. Both phases are recycled within the process, allowing the design of a continuous process, which could be exploited industrially.

We have used the phase diagram of the system H₂O-Na₂SO₄-PEG-3350 at 28°C. Several process alternatives are proposed and their economic potential has been broadly analyzed. The novel process steps to produce sodium sulfate crystals include mixing, crystallization, settling and, optionally, evaporation of water. All of these issues will be presented and discussed in this paper.

62. COORDINATION VS. HYDRATION OF METAL IONS WITH PEG POLYMERS AS A FUNCTION OF POLYMER LENGTH

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Our interest in polyethylene glycol (PEG)-based aqueous biphasic systems (ABS) for the separation of metal ions from industrial processes or environmental waste led us to study a variety of heavy metal ion separations. We have previously demonstrated that heavy metal ions partition to the PEG-rich phase in ABS if a sufficient quantity of a coordinating hydrophobic anion is present. In order to more fully understand the inorganic coordination chemistry responsible for the observed extraction behavior of these metal ions, we have investigated the structural chemistry of heavy metal PEG complexes. The heavy metal PEG interactions were modeled by crystallization of low molecular weight PEGs, triethylene glycol (EO3) to hexaethylene glycol (EO6), in order to monitor the influence of PEG on the heavy metal coordination sphere as a function of chain length. This presentation will discuss the coordination of metal ions vs. their hydration as a function of PEG chain length.

63. POLYETHYLENE-GLYCOL BASED AQUEOUS BIPHASIC SYSTEMS: PHASE EQUILIBRIA AND PARTITIONING OF 1,10-PHENANTHROLINE COPPER(II) SULFATE COMPLEX

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Solvent extraction is a proved technology for the selective removal and recovery of metal ions from aqueous solutions: the use of aqueous biphasic systems can be attractive for many separation processes. These systems have usually been used previously at temperatures around 25°C; however it is possible that better separations may be achieved at other temperatures: phase diagrams have been determined for polyethylene glycol (AMW 3350)-ammonium sulfate-water system over the temperature range of 269-343 K and the effect of temperature on partitioning has been determined for 1,10-phenanthroline Cu(II) sulfate complex.

64. SUMMER UNDERGRADUATE RESEARCH PROGRAM (SURP) AT THE UNIVERSITY OF ALABAMA: SYNTHESIS AND CHARACTERIZATION OF ROOM TEMPERATURE IONIC LIQUIDS AND PHASE DIAGRAMS FOR POLYETHYLENE GLYCOL/SALT MIXTURES

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The Summer Undergraduate Research Program (SURP) provides undergraduate students in chemistry with an enticing opportunity to work as research assistants in functional research laboratories. The students' appointments provide them with the opportunity to conduct research and interact with others which introduces them to the highly demanding, yet rewarding, field of modern chemical science research.

In this presentation, three such undergraduate students present a portion of their ongoing summer projects. Ms. Derrica Boochee presents her work in the characterization of phase diagrams of polyethylene glycol (PEG) and salt mixtures (*e.g.*, NaOH, Na₂S, and Na₂CO₃) with respect to research ongoing in the paper/pulping industry in our laboratory. Ms. Leslie Dunklin and Mr. Richard Lee present their work on the synthesis and characterization of room temperature ionic liquids (RTIL) with interest toward separation science.

This work is supported by the National Science Foundation (Grant No. CTS-9522159), the UA Center for Green Manufacturing, and the UA Summer Undergraduate Research Program (SURP).

65. PARTITIONING OF WHEY PROTEINS, BOVINE SERUM ALBUMIN, AND INSULIN IN AQUEOUS TWO-PHASE SYSTEMS

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Biomaterial extraction using two-phase systems made from aqueous solutions of two water-soluble polymers and one polymer and a salt, is a powerful technique for the separation of biological molecules. The development of such extraction processes requires experimental results for the partitioning of model substances, as well as methods for correlating and predicting such phase equilibria. In this work, the partitioning of small amounts of porcine and human insulin, bovine serum albumin (BSA), α -lactalbumin, α -lactoglobulin and whey protein was investigated in polymer/salt systems, such as poly(ethylene glycol) and dipotassium hydrogen phosphate, sodium citrate, sodium sulfate and polymer/polymer systems, such as PEG and maltodextrin at 298.15 K. The systems were studied in equilibrium cells and the protein concentrations were determined by Ion Exchange HPLC, Bradford Method and absorbance at 280 nm. The phase compositions of the systems were obtained using a gravimetric method.

Porcine and human insulin are partitioned in an aqueous two-phase system composed by PEG (molecular weight of 600, 1450 and 3350) and sodium citrate or sodium sulfate at pH = 4.5, 7.0 and 9.5 and 298.15. The insulin concentration was determined by absorbance at 280 nm. In general, insulin concentrates in PEG-rich phase and very high partition coefficients were obtained. The partition behavior of bovine serum albumin (BSA), α -lactalbumin and α -lactoglobulin in PEG/maltodextrin(MD) systems was studied under varying conditions of MD/PEG concentration ratios and PEG (1450, 8000, 10000) and MD (1000, 4000) molecular weights. The partition coefficient of BSA is 0.30 and exhibit only a small dependence of PEG and MD molecular weights. Thus, BSA concentrates in the MD-rich phase. Such results are similar to those obtained for other PEG/Polysaccharide systems.

Isolated whey proteins were partitioned in an aqueous two-phase system composed by PEG 1500 and potassium phosphate at pH = 7.0 and 298.15K. The quantification of proteins in both phases was done by Ion Exchange HPLC analysis with a MONO-Q HR 5/5 column. The α -La and the α -Lg concentrate in the top and in the bottom phase respectively, as reported in the literature.

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66. APPLICATION OF AQUEOUS TWO-PHASE PARTITION TO THE PRODUCTION OF HOMOGENEOUS PREPARATIONS OF FLUORESCENTLY LABELLED HUMAN SERUM ALBUMIN

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The selective covalent coupling of a fluorophore to a protein was investigated in order to assemble a readily traceable macromolecular probe for the monitoring and quantitation of the partition of biomolecules in aqueous two phase systems (ATPS). The development of such a probe was based upon the selective labeling of the single free thiol group of human serum albumin (*i.e.*, cysteine 34 in the amino acid sequence of HSA) with N-(iodoacetyl aminoethyl)-5-naphthylamine-1-sulphonic acid (*i.e.*, 1,5-IAEDANS). The products of the labeling reaction included unreacted and reacted albumin (both specifically and non-specifically labeled) as well as free fluorophore. Thus rigorous fractionation of target product from the other components was essential in the manufacture of a homogeneous macromolecular probe having a defined and consistent fluorescent signal per unit mass as required for the performance studies in ATPS.

Since the labeling reaction did not involve major modification of the properties of the albumin molecule, the structural distinction between reacted and unreacted protein was small. Conventional methods of purification such as hydrophobic interaction chromatography (HIC) were found to be insufficiently selective for product fractionation and recovery. However, a combination of size exclusion chromatography and multi-step ATPS could be fine tuned to exploit such small molecular variation.

Presentation will be made of the design and operation of the purification sequence comprising two-size exclusion chromatography steps and a series of three ATPS operations. The latter comprised PEG 1450 and potassium phosphate assembled with appropriate tie-line lengths. The generic approach of exploiting ATPS for similar manufacturing operations will be discussed.

67. NADH OXIDASE ACTIVITY OF BRINE SHRIMP OSCILLATES WITH A PERIOD OF 25 MINUTES AND IS ENTRAINED BY LIGHT

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Plants have a surface NADH oxidase that measures time by oscillating with a 24 min period.¹ The plant clock is set by light.² With plants, a new maximum is observed exactly 12 min after the beginning of the light exposure. These experiments were to determine if animals exhibited a similar periodicity and to answer the question, does the periodicity in animals respond to light? Using brine shrimp as a model, the findings show that plants and animals exhibit the same oscillating NADH oxidase periodicity clock and that the clock in both plants and animals can be set by light. Brine shrimp were grown for two to three days and transferred to darkness for 45 min. After return to light for one min, NADH was added and measurements were taken to 50 min with a spectrophotometer. The brine shrimp exhibited a cell surface NADH oxidase that oscillated with a period of 25 min. After being subjected to light, the brine shrimp showed a new NADH oxidation maximum at 12 min after the beginning of the light exposure thus demonstrating that the internal oscillations in NADH oxidation of brine shrimp respond to light.

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68. A LIGHT ENTRAINABLE AND PERIODIC NADH OXIDASE ACTIVITY OF *TETRAHYMENA*

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Oxidation of external NADH (NADH is an impermeant substrate) by cells of *Tetrahymena pyriformis* oscillated with a temperature compensated period of 24 to 26 min. The period length in darkness (25.6 min) appeared to be slightly longer than the period in light (ca 24 min). In plants and in brine shrimp (see accompanying poster), the period was synchronized (entrained) by light. Twelve min after a light exposure, a new maximum was observed. A similar phenomenon was observed with *Tetrahymena*. When *Tetrahymena* were placed in darkness for 30 to 50 min and then returned to light, a new maximum in the rate of NADH oxidation was observed 12 to 13 min after the beginning of the light treatment. The response was given primarily by red light. Blue light was relatively ineffective. The nature of the light sensing chromophore is under investigation.

69. HEXOKINASE AND GLUCOSE 6-PHOSPHATE DEHYDROGENASE EXTRACTION FROM A *SACCHAROMYCES CEREVISIAE* CRUDE HOMOGENATE BY AQUEOUS TWO-PHASE SYSTEM

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Aqueous two-phase systems (ATPS) have found large applications in biotechnology for protein purification. In this work, the effect of polyethylene glycol (PEG) molecular weight on partition coefficient (K) of hexokinase (HK) and glucose 6-phosphate dehydrogenase (G6PDH) in ATPS was studied. Several ATPS were prepared from PEG and phosphate. The crude cell homogenate was prepared through disruption of 45g of baker's yeast cells. Crude cell homogenate was mixed (vortex) with PEG and phosphate and then centrifuged (2,500 g x 10 min). Top and bottom phases were assayed for enzymatic activity and protein concentration. The results attained for partition coefficients are shown on the following table:

PEG Molecular Weight	K _e *		K _p **	MB*** (%)	
	HK	G6PDH	Total Protein	HK	G6PDH
300	57.5	10.3	1.9	87	77
400	207.8	18.5	6.2	110	71
600	43.2	41.8	3.2	110	77
1000	33.9	5.5	1.9	58	-
1500	11.4	0.1	0.7	64	-
4000	0.2	0.1	0.3	47	54

*K_e = Enzyme partition coefficient = A_t/A_b (A_t and A_b are enzyme activities in the top and bottom phase, respectively);

**K_p = Total protein partition coefficient = P_t/P_b (P_t and P_b are total protein concentration in the top and bottom phase, respectively);

***MB = [(A_t.V_t+A_b.V_b).100]/[(A_i.V_i)] (A_i is the enzyme activity in the initial medium; V_i is the sample volume added to the system; V_t and V_b are top and bottom volume, respectively).

The mass balance did not achieve 100% for several experiments, since part of the enzyme activities was retained at the interface. Two possibilities for purification of HK and G6PDH can be observed. In the first one, employing PEG with molecular weight below 600 it is possible to recovery both enzymes in the top phase with high yield (MB ≅ 100%) and enrichment, since cellular debris are retained at the interface and part of total protein goes to the bottom phase. Extraction at PEG 1500 gives another possibility of partition, with a higher enrichment of both enzymes but with a lower yield than the first one. With ATPS in PEG 1500 a significant difference in K value between HK (K = 11.4) and G6PDH (K = 0.1) allows a separation of both enzymes (HK goes preferentially to the top phase while G6PDH goes to the bottom phase), and a separation of the cellular debris and total protein from them.

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70. DNA-POLYMER COVALENTLY CONJUGATED SYSTEMS FOR AFFINITY SEPARATION

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We have developed a couple of methods to prepare hybrid materials between DNA and thermoseparating polymer, poly(N-isopropylacrylamide) (polyNIPAAM). One of the methods relies on a vinyl monomer having a psoralen moiety which can be photochemically conjugated to double-stranded (ds) DNA. Near UV irradiation to the mixture of DNA and the monomer gives a vinyl derivative of DNA (DNA macro-monomer) which can be copolymerized with NIPAAM. The conjugate between polyNIPAAM and dsDNA was water soluble, while it was found to form precipitate over 31°C and was easily collected by centrifugation. In this process, DNA-binding proteins present in the system were found to accompany the conjugate to be separated from the solution.

On the other hand, single-stranded DNA was conjugated with PolyNIPAAM using an oligonucleotide, 5' terminus of which was modified with a vinyl group. Upon the temperature-dependent precipitation, this conjugate was revealed to be accompanied by the complementary DNA.

In addition to these DNA conjugates having the thermoseparating functions, we have prepared a DNA-PEG conjugate by the photochemical reaction between dsDNA and psoralen-terminated PEG. Interestingly, the DNA-PEG conjugate was soluble in thick PEG solution in which native DNA is insoluble to give precipitates. The DNA conjugate was found to distribute perfectly to PEG phase in aqueous biphasic system from PEG and dextran. This polymer system comprising dsDNA in PEG phase would be useful for ATPS-based affinity separation of DNA-binding proteins in mild conditions.

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71. LIQUID-LIQUID EXTRACTION OF ALKALINE XYLANASE IN AQUEOUS TWO-PHASE SYSTEMS

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Xylan, a group of heteropolysaccharides, is an abundant biopolymer found in plant tissues as major component of cell wall which is hydrolyzed by xylanases. Most of the xylanases commercially available produced by fungi are active at neutral or acidic pH and their optimum temperature is below 45°C. Various applications for xylanases in bioconversion and food industries have been suggested and one of the major potential applications of xylanases involves the pulp and paper industry. This way, extremophilic enzymes which are active at alkaline conditions have great potential in bleaching process without any need for changes in pH and with the advantage in lowering the release of polluting organic chlorine compounds.

The aim of this work is to extract and to purify the enzyme from the crude fermentation broth, produced by *Bacillus pumillus*, achieving high purification factors and enzyme yields using aqueous two-phase systems (ATPS).

The enzyme from crude fermentation broth was extracted by partitioning in ATPS composed of phosphate and polyethyleneglycol (PEG). The effect of tie-line length, PEG molecular weight and NaCl concentrations upon the purification factors and yields of xylanase were investigated by statistical design. The xylanase was mostly extracted at the top phase (PEG rich phase). The best system studied was the one containing 22% PEG6000, 10% K₂HPO₄ and 12% NaCl with a purification factor of 33 and 98% yield of enzyme activity.

The same composition of the best system was used to carry out a continuous extraction in a perforated blade column. Material collected from the top phase (PEG rich phase) was further applied to a SP-Sepharose column (cationic matrix).

72. BOVINE SERUM ALBUMIN PARTITIONING IN AN AQUEOUS TWO-PHASE SYSTEM: EFFECT OF pH AND SODIUM CHLORIDE CONCENTRATION

Ufuk Gündüz and Konca Korkmaz

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The partitioning of Bovine serum albumin (BSA) in a polyethylene glycol 3350 (8%)-dextran 45,000 (6%)-0.05 M phosphate aqueous two-phase system was investigated at different pHs, at varying concentrations of sodium chloride at 20 °C. The effect of NaCl concentration on the partition coefficient of BSA was studied for the PEG-dx systems with initial pH values of 4.2, 5.0, 7.0, 9.0, and 9.8. The NaCl concentrations in the phase systems with constant pH value were 0.06 M, 0.1 M, 0.2 M, 0.3 M, and 0.34 M. It was observed that the BSA partition coefficient decreased at concentrations smaller than 0.2 M NaCl and increased at concentrations greater than 0.2 M NaCl for systems with initial pHs of 4.2, 5.0, 7.0, and 9.0.

73. VISCOSITY PREDICTION OF PEG-DX-WATER SOLUTIONS USED IN AQUEOUS TWO-PHASE SYSTEMS

Ufuk Gündüz and Aysel Ergen

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In an earlier work we proposed the equation:

$$\ln \eta^{\text{mixture}} = [(c_1/(c_1 + c_2))\ln \eta_1] + [(c_2/(c_1 + c_2))\ln \eta_2] + a(c_1/(c_1 + c_2))(c_2/(c_1 + c_2))$$

to evaluate PEG-dextran-water mixture viscosities, where η_1 and η_2 , respectively, are the dynamic viscosities of the PEG-water and dextran-water solutions, a is a disposable parameter, and c_1 and c_2 are respectively, the weight percentages of PEG and dextran in solution. The validity of this model had been observed for PEG-dextran-water systems having equal concentrations at 10°C. In this present work, we report new viscosity data for the same polymers (PEG 8000 and Dextran 500,000) with different compositions in water representing PEG and dextran compositions of homogeneous systems at temperatures of 30, 50, and 70°C. The disposable parameter a is calculated as 2.36 for this temperature range, giving relative errors between 0.02 and 7.86 in absolute value. We have seen from our results that the proposed model works constantly well at different temperatures with comparable values for the disposable parameter a .

74. DETERMINING VALINE-ACETOHYDROXY ACID SYNTHASE (AHAS) DISSOCIATION CONSTANT K_d , BY USE OF AQUEOUS TWO PHASE SYSTEM (ATPS)

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AHAS is the enzyme that catalyzes the first common step in the biosynthesis of the branched chain amino acids in plant, yeast and bacteria. Isoenzyme III in *E. coli* is composed of a large catalytic subunit, I, and a small regulatory subunit, H. It is reversibly inhibited by one of the pathway end products-valine. The enzymatic activity of the catalytic subunit alone is resistant to valine, but valine sensitivity is restored upon the reconstitution of the holoenzyme. The binding site of valine is in the regulatory H subunit.

ATPS was used to estimate the K_d of valine with H. Valine interaction with H should influence its partition coefficient in the system. ATPS conditions were chosen when H is located solely in the salt phase, whereas valine is distributed almost equally between the phases. The amount of the bound and free radioactive valine in the system was determined for different concentrations of valine and the protein. A Scatchard plot of the data revealed a 1:1 valine-protein-binding ratio and K_d of 150 μM . The same K_d value was obtained previously using the equilibrium dialysis method. H protein isolated from a mutant resistant to valine, did not bind valine at all. The ability of H from other valine resistant mutants to bind valine is being examined.

Our new method to determine valine interaction with AHAS regulatory subunit is very sensitive, rapid, and requires very little protein compared to the presently used method.

75. PURIFICATION OF ACETOHYDROXY ACID SYNTHASE (AHAS) IN AQUEOUS TWO PHASE SYSTEM (ATPS)

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AHAS is an enzyme that catalyzes the first common step in biosynthesis of the branched chain amino acids in plants, yeast and bacteria. AHAS is of considerable biotechnological interest, because it is responsible for production of essential amino acids, and is the target of major herbicides. The purification of the enzyme is elaborate and prolonged, including use of an expensive hydrophobic column. Pretreatment of a crude *E. coli* extract containing the recombinant enzyme with PEG-salt ATPS was used in order to facilitate the purification. Partition experiments were carried out with pure enzyme and *E. coli* background proteins separately, in an attempt to find conditions for their maximal extraction into the PEG and phosphate phase, respectively. The partition coefficients of both target product and background proteins were compared for different stability ratios, pH, KCl concentrations and PEG molecular weights.

On a basis of these results two conditions which gave maximal separation of target enzyme from background proteins were chosen. Then the chosen systems were tested on a crude extract containing the target protein. The partition was optimized in terms of the protein concentration and the volumetric phase ratio. The specific enzyme activity after this one-step treatment was approximately 70% of that of conventionally purified enzyme. The PEG phase containing most of the target enzyme was passed through an ion exchange chromatography column for further removing of contaminants and separation from PEG. The overall yield of this two-step process reached 90% of that of the conventional purification and the specific activity was as that of the pure enzyme. The process was performed on the catalytic subunit of the wild type isozyme AHAS III. The possibility of using the conditions found, for purification of mutant enzymes as well as other AHAS isozyme, is being examined.

76. BIOAFFINITY EXTRACTION OF GLUCOAMYLASE IN AQUEOUS TWO-PHASE SYSTEM USING STARCH AS FREE BIOLIGAND

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Aqueous biphasic systems (ABS) with bioligands bound to the polymer, usually polyethylene glycol (PEG), have high selectivity. However, bounding the bioligand in PEG is costly, making these systems unviable for bioaffinity extraction of low cost proteins. The use of free bioligands is an alternative for decreasing the high cost of these systems.

This work describes the effect of starch addition on the partition of glucoamylase in 15.55% (w/w) PEG 300/18.75% (w/w) phosphate pH 7 system. The results were separated in two parts. In the first part, the starch distribution between the phases were analyzed in three concentrations of starch in the above described system: 0.1%, 0.07% and 0.04% in w/w. The starch showed one-sided distribution to the bottom phase for all three concentrations.

In the second part, filtered broth from submerged cultivation of *Aspergillus awamory*, containing glucoamylase and contaminants, was added in the system. The partition coefficient of glucoamylase showed a increase in system decreasing the starch concentration. The recovery of glucoamylase activity in bottom phase was 53%, 50% and 36% relative to systems with 0.1%, 0.07% and 0.04% of starch, respectively. The small decrease in recovery of glucoamylase activity relative to the decrease in starch concentration from 0.1% to 0.07%, indicate that the maximum effective starch concentration in system is close to 0.1%.

The starch addition of 0.1% into the system improved the recovery of glucoamylase in bottom phase of 14% to 53% without changing the partitioning of contaminants, which were concentrated in the top phase. Therefore, the addition of starch in the system made possible the separation of glucoamylase from the contaminants.

77. AQUEOUS TWO-PHASE PARTITION APPLIED TO ISOLATION OF MEMBRANES FROM CULTURED MAMMALIAN CELLS – AN OVERVIEW

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Partitioning in dextran-polyethyleneglycol (PEG) aqueous-aqueous phase systems represents a mature technology with many applications to separation of cells and to the preparation of membranes from mammalian cells.¹ Most applications to membrane isolation and purification have focused on plasma membranes, plasma membrane domains and separation of right side-out and inside-out plasma membrane vesicles. The method exploits a combination of membrane properties, including charge and hydrophobicity. Purification is based upon differential distributions of the constituents in a sample between the two principal compartments of the two phases (upper and lower) and at the interface. The order of affinity of animal cell membranes for the upper phase is: endoplasmic reticulum < mitochondria < lysosomes < Golgi < plasma membranes. Salt concentration and temperature affect partitioning behavior and must be precisely standardized.

Examples of the application of two phase partitioning to liver Golgi apparatus have been described.² Mitochondria and synaptosomes from adult rat brain can be separated, with the mitochondria recovered in the dextran-rich lower phase and the synaptosomes in the upper phase.³

To improve the resolution, apparatus have been devised that allow multiple extractions and transfers.¹ Their application to rat liver homogenate fractionation will be discussed along with speculation as to why the utility of the method for mammalian systems has been limited to separation of whole cells and plasma membranes.

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78. AQUEOUS TWO-PHASE PARTITION APPLIED TO FRACTIONATION OF PLANT CELLS AND RECENT DEVELOPMENTS

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Phase separations involve mixing of membranes with a mixture of polymers that themselves will separate into different phases. The procedure was first developed by Albertsson¹⁻³ and takes advantage of the different surface properties of membranes. Since different membranes and perhaps even the same membranes from different species may possess different surface charges, it was necessary initially to evaluate a series of polymer and salt solutions. Currently standardized mixtures of polyethylene glycol, dextran and potassium phosphate are utilized almost exclusively.

To effect the separation, the phase system containing the membranes is inverted and returned upright 20-40 times and then centrifuged at 4°C at low speed for short times in a swinging bucket rotor to resolve the polymers into two phases. The phases can be re-partitioned or washed to yield further purification or collected directly.

The principal applications of aqueous two-phase partition in plants – purification of plasma membranes and preparation of chloroplast subfractions -- will be reviewed briefly. Uses to remove plasma membrane contaminants from gradient purified Golgi apparatus, endoplasmic reticulum and tonoplast⁴ and to resolve preparations of right side-out and inside-out plasma membrane vesicles (*e.g.*, 5) will be illustrated. Discussion will address some practical limitations to advancing aqueous two-phase technology for isolation and purification of plant membranes.

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79. CONTINUOUS FLOW TWO-PHASE ELECTROPHORESIS FOR PROTEIN RECOVERY FROM CELL LYSATE

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The cell debris-removal step in the recovery of proteins from fermentation broth is problematic. Commonly used centrifugation and filtration methods are hampered by the solution properties of fine particles and gum-like polysaccharides as well as processing constraints of time consuming and costly cleaning and validation procedures. Partitioning in aqueous two-phase systems provides an alternative cell debris-removal method since cell debris generally partitions strongly to the lower phase in these systems. If the target protein partitioned strongly to the upper phase, efficient protein recovery from cell debris could be achieved. Since this is seldom the case, a large upper-phase to lower-phase ratio is generally required to provide acceptable protein yield.

We have developed a two-phase electrophoresis method that applies an electric field perpendicular to the phase interface to dramatically improve the effective partitioning of a positively-charged target protein into the upper phase while directing negatively-charged DNA, endotoxin, contaminating protein and cell debris into the lower phase. In this talk we will review our results on the recovery of beta-lactamase from *E. coli* fermentation broth in a batch two-phase electrophoresis device and present new results on the development of a continuous flow two-phase electrophoresis method.

80. PRELIMINARY DEVELOPMENT OF A NEW METHOD FOR LIQUID-LIQUID EXTRACTION IN AQUEOUS TWO-PHASE PARTITIONING SYSTEMS

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Successful proof-of-principle testing of a single apparatus for transferring chemical components between two immiscible aqueous phases and subsequent separation of the phases has been performed. The key feature of the mass transfer method developed is the ability to achieve relatively high continuous solution throughputs and efficient phase separation in a single device.

The apparatus used in the process is a centrifugal solvent extraction contactor similar in concept to a device used for the purification of metals by selective transfer between immiscible aqueous and organic solutions. A significant difference between conventional organic/aqueous extraction applications and aqueous two-phase partitioning systems is that phase densities in the latter are very nearly equal as both phases consist primarily of water.

A preliminary evaluation of phase separation performance using a conventional (unmodified) contactor was performed for the ATPP system water-polyethylene glycol-dextran. Results from the test indicated that acceptable phase separation could be achieved only at very low solution throughputs over a very narrow range of contactor speeds.

The mass transfer efficiency of the unmodified apparatus was evaluated in the unmodified contactor using the same ATPP system with bromophenol blue added as the solute. Concentration results obtained were compared against equilibrium distribution results which were obtained in the laboratory. Mass transfer efficiencies obtained in the contactor were 80 to 85% of theoretical.

Analysis of phase separation results led to the conclusion that separation performance had been severely limited because of the near-equal-density of the immiscible phases. A computational model was used to predict the effect of contactor modifications that could reasonably be expected to counter the near-equal-density condition. Certain aspects of the contactor were modified accordingly and a second phase separation test was performed. A substantial improvement in phase separation performance was realized using the modified device.

*Managed by Lockheed Martin Energy Research Corp., for the U. S. Department of Energy.

81. ROLE OF SURFACE EXPOSED AMINO ACID RESIDUES IN PROTEIN PARTITIONING: POINT MUTATIONS AND COMPUTER ANALYSIS

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The partitioning of a protein in an aqueous two phase system depends on protein surface properties, such as hydrophobicity and charge, which are determined by the exposed amino acid residues. The partitioning of di- to hexa-peptides of one type of amino acid residue has been studied to determine the contribution to the partition coefficient of Phe, Tyr, Trp, Ala, Ile, Asp and Lys. Genetic engineering offers possibilities to make planned alterations in amino acid composition of a protein.¹ A series of 15 point mutated variants of the protein cutinase have been partitioned to analyze effects on partitioning from mutations in surface exposed residues. The exposure of amino acid residues on the protein surface was analyzed with the computer program GRASP. The results from the partitioning of peptides have been compared to the partitioning of the point mutated cutinases have been compared to one and other. Effects of salts on partitioning was also studied for the cutinase variants differing in net charge.

The two-phase system was composed of dextran and a random copolymer of ethylene oxide (EO) and propylene oxide (PO). The advantage of using EO-PO copolymers is their ability to phase separate in water solution by an increase in temperature, *i.e.*, temperature induced phase separation. A two-phase system with one concentrated polymer phase and one water phase almost free from polymer is then formed. So far, proteins have been found to partition exclusively to the water phase. The aim is to partition a target protein to the EO-PO phase in a primary dextran/EO-PO two-phase system, and subsequently separate the protein from the EO-PO copolymer by removing the EO-PO phase and raising the temperature above the copolymer cloud point.

Peptide partitioning showed that Ile, Tyr, Phe, and Trp directed the partitioning of peptides to the EO-PO copolymer phase while Lys and Asp directed the partitioning to the dextran phase. Peptides of Ala partitioned equal between the two phases. The same trends were obtained for the point mutated cutinases and the partition coefficient could for several of them be approximately predicted from the peptide partitioning and GRASP analysis of the exposure of the residues on the protein surface. Salt effects on partitioning were directly proportional to the net charge of the protein.

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82. GENETIC ENGINEERING OF THE *FUSARIUM SOLANI PISI* LIPASE CUTINASE FOR ENHANCED PARTITIONING IN PEG-SODIUM PHOSPHATE AQUEOUS TWO PHASE SYSTEMS

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The *Fusarium solani pisi* lipase cutinase has been genetically engineered to investigate the influence of C-terminal peptide extensions on the partitioning of the enzyme in PEG-phosphate based aqueous two-phase systems. Peptide tags containing one or several tryptophans have previously been shown to have a preference for PEG-rich phases. Therefore, a set of lipase variants were constructed containing various C-terminal peptide extensions including tryptophan rich peptide tags [-(WP)2, -(WP)4]. Furthermore, in order to investigate the role of charge in PEG-phosphate systems positively [-(RP)4] and negatively [-(DP)4] charged tags without and with tryptophan residues [-(WPR)4, -(WPD)4] were constructed. The modified cutinase variants were produced in *Escherichia coli* as secreted to the periplasm from which they were efficiently purified by IgG-affinity chromatography employing an introduced N-terminal IgG-binding ZZ affinity fusion partner present in all variants. Partitioning experiments performed in a PEG/sodium phosphate aqueous two-phase system showed that for variants containing either (WP)2 or (WP)4 peptide extensions, 10- to 70-fold increases in the partitioning to the PEG rich top-phase was obtained, when compared to the wild type enzyme. An increased partitioning, although not that dramatic, was also seen for cutinase variants furnished with tags containing both tryptophan- and charged amino acid residues. In this case the (WPD)4- compared to the (WPR)4-extension performed better. On the other hand, the effects of solely charged peptide extensions were rather insignificant. In conclusion, this work points out that the use of genetic engineering for making minor changes in protein primary structure should be of general interest for the design of production processes for industrially important proteins including bulk enzymes.

83. AFFINITY EXTRACTIONS USING A CELLULOSE-BINDING-DOMAIN TAG AND THERMOSEPARATING HYDROXYETHYL CELLULOSE-BASED POLYMERS

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Cellulose binding domains (CBDs) provide generic affinity tags for purifying fusion protein constructs through specific binding to cellulose-based polymers. CBDN1, a 12-kDa domain from the endoglucanase CenC of *Cellulomonas fimi*, binds a wide range of derivatized celluloses, including the thermoseparating polymers hydroxyethyl cellulose (HEC) and ethyl(hydroxyethyl cellulose) (EHEC). Affinity constants are in the 10⁵ M⁻¹ range, forcing proteins containing a CBDN1-tag to partition quantitatively to a phase enriched with either polymer. We describe a novel affinity extraction technology which first exploits the tendency of CBDN1 to partition quantitatively to the PEG-rich phase of a PEG/salt two-phase system due to the large number of solvent-exposed aromatic residues (Tyr and Trp) on the CBD. Affinity back-extraction is then achieved by contacting the fusion-protein loaded PEG-rich phase with a second phosphate-rich phase containing either HEC or EHEC. Finally, target protein and affinity polymer are recovered by thermoseparating the polymer, which breaks the protein/polymer complex, and yields a solution-phase containing the purified fusion protein and a precipitate-phase containing pure polymer which can be recycled back into the process.

Previously, we have described a set of proprietary vectors which allow production in recombinant *E. coli* of g L-1 quantities of soluble fusion proteins containing a CBD tag. This, when combined with the affinity extraction technology described above, therefore provides a new and powerful generic method for large-scale affinity extraction of recombinant proteins and peptides.

84. PARTITIONING OF NATIVE AND UNFOLDED ENZYMES IN AQUEOUS TWO-PHASE SYSTEMS

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During recombinant *E. coli* fermentation with high expression levels, inclusion bodies are often formed. Aqueous two-phase systems have been used in the presence of urea for the initial recovery steps. In order to investigate phase behavior of such systems we determined phase diagrams of PEG/Sodium Sulfate/Urea/Water and PEG/DEX T-500/Urea/Phosphate-Buffer/Water at different concentrations of urea and different molecular weight of PEG.

PEG/Na₂SO₄ aqueous two-phase systems could be obtained including up to 30% w/w urea at 25°C and PEG/DEX T-500 up to 35% w/w urea. The binodal was displaced towards higher concentrations with increasing urea concentrations. The partition coefficient of urea was near unity. Mutants of T4-Lysozyme were used to analyze the effect of phase components on the conformation of the enzyme. We showed that partitioning of tryptophan peptides is not dependent on the concentration of urea in the phase system but only a function of the number of amino acids. The influence of phase components on the protein charge was modeled with TITRA at the University of Aalborg. The T4-Lysozyme partitioning was modeled with the VERS Model developed at the University of Kaiserslautern. (R. Tintinger, J. Zhu, C. Grossmann, G. Maurer, Partitioning of some Amino Acids and Low Molecular Mass Peptides in Aqueous Two-Phase Systems of PEG and Dextran in the presence of small amounts of K₂HPO₄/KH₂PO₄-Buffer at 293 K: Experimental Results and correlation, *J. Chem. Eng. Data* **1997**, 42, 975-984). Calculated and experimental results are discussed.

85. SEPARATION PROCESS USING THERMO-SEPARATING POLYMER TWO-PHASE SYSTEMS BASED ON UNFOLDING AND REFOLDING OF PROTEINS

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The possibility of a separation process based on unfolding and refolding of proteins was studied by investigating the partitioning behavior of proteins in thermoseparating polymer two-phase systems with varying solution conditions (*i.e.*, temperature).

The partitioning of chymotrypsin inhibitor 2 (CI2), for which a simple two-state model can be applied, was performed in a system composed of random copolymer of ethylene oxide and propylene oxide (Breox: cloud point 323 K and dextran T-500). The partition coefficients of CI2 in Breox/dextran T-500 systems were increased because of the increase of the surface hydrophobicity of CI2 at given solution conditions. The partitioning behavior of CI2 was also found to be partitioned to the hydrophobic polymer phase at specific solution conditions. These results on the CI2 partitioning in both systems were in good agreement with protein conformational change between folded and unfolded state. It is considered that the partitioning behavior of other proteins can also be controlled on the basis of the change of the surface properties of proteins, followed by their conformational change under the given solution conditions.

A bioseparation process is finally presented based on the partitioning behavior of unfolded and refolded proteins by controlling the solution conditions in thermo-separating polymer two-phase systems, where the target protein can be recovered through i) the selective separation in Breox/dextran system, ii) the protein refolding in the top phase, and iii) the recovery from the water phase in the Breox/water system. The present process can be extended for the separation of the other proteins considering both the conformational change of proteins and their surface properties under the given solution conditions.

86. AQUEOUS POLYMERIC SOLUTIONS AS ENVIRONMENTALLY-BENIGN SOLVENT EXTRACTION MEDIA

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Several liquid-phase extraction technologies employing environmentally-benign phase- or micelle-forming polymers in aqueous solution have the potential to replace volatile organic compounds (VOCs) in classical solvent extraction technologies (*e.g.*, aqueous biphasic systems, cloud point extraction, micellar extraction, and thermoseparating polymer systems). The apparent similarities of these systems, their phase separating properties, and their ability to solubilize a wide variety of solutes ranging from metal ions, organic compounds, and biologicals will be discussed. Some comparative data from the literature whereby the solvating power of these systems may be compared to traditional solvents will be presented along with new data on the polarity of the phases in typical aqueous biphasic systems. The need for additional comparative data in this area and the need to demonstrate the validity of the approach in operational processes will also be emphasized. A 'toolbox' approach to implementing environmentally-benign polymers in clean separations science and technology could lead to new and better solvent extraction systems.

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87. SEPARATION, CONCENTRATION AND IMMOBILIZATION OF TECHNETIUM FROM ALKALINE SUPERNATE WASTE WITH EICHROM'S ABEC[®] RESIN

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Development of remediation technologies for the characterization, retrieval, treatment, concentration and final disposal of radioactive and chemical tank waste stored within the Department of Energy complex represents an enormous challenge. For example, more than 90 million gallons of waste are stored in 335 underground storage tanks at four different DOE sites. The majority of this waste is alkaline in nature, contains a high concentration of nitrates, nitrites and other salts, and is contaminated with radioactive components. The principal radioactive species are predominantly cesium, strontium, and technetium. Eichrom with its research partners at Argonne National Laboratory, Northern Illinois University, and The University of Alabama has commercialized and demonstrated a new separation material, Aqueous Biphasic Extraction Chromatography Resin (ABEC[®]), to address the separation and concentration requirements for the technetium component in these wastes.

The new ABEC material is distinct in its ability to function in the presence of high salt concentration without loss of selectivity for technetium, as the pertechnetate anion. Unlike anion exchange resin which are sometimes proposed for the separation of pertechnetate, no other anions are absorbed from the alkaline waste, and regeneration of the resin and stripping of the pertechnetate is accomplished simply by rinsing the column material with water.

This report gives an overview of the proposed separation and immobilization techniques and presents data received by the investigation of simulant and actual waste streams (*e.g.*, actual Hanford tank waste). Moreover, the results of leaching studies and dose calculation of the final waste form are shown.

88. PARTITIONING OF IODIDE IN AQUEOUS BIPHASIC SYSTEMS AND UPTAKE ON ABEC[®] RESINS

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The possibilities of using polyethylene glycol (PEG)-ABS to separate and recover ions from environmental and nuclear wastes has led us to study a variety of ion separations in ABS including iodide. We have previously demonstrated that iodide will partition to the PEG-rich phase in an ABS. The importance of radioactive iodide removal in environmental applications provided the impetus to initiate a detailed study of the partitioning behavior of iodide in ABS and onto ABEC resins. This presentation will discuss our recent investigations with iodide, iodate, and iodine in PEG-ABS and onto ABEC resins.

This research is supported by the PG Research Foundation.

89. A NEW RHENIUM-188 GENERATOR TECHNOLOGY USING POLYETHYLENE GLYCOL-BASED AQUEOUS BIPHASIC EXTRACTION CHROMATOGRAPHIC RESINS

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Rhenium-188 is an isotope useful as a radiopharmaceutical tracer because it produces short-lived energetic gamma rays. ^{188}Re -labelled radiopharmaceuticals have found use predominantly in cancer treatment. For most medical applications, the chemically stable +7 oxidation state of ReO_4^- and its chemical congener, TcO_4^- , are most commonly used as is or as precursors for radiopharmaceuticals. Rhenium-188 is the daughter of beta decay of the tungsten-188 parent. Currently, tungsten-188 is produced by neutron capture of tungsten-186. Methods for the separation of rhenium-188 from tungsten-188 require the conversion of ^{188}W to tungstate, WO_4^{2-} , in base and adsorption onto an alumina column with elution of perrhenate.

Polyethylene glycol (PEG)-based aqueous biphasic systems (ABS) have been developed to provide separation of perrhenate ($^{188}\text{ReO}_4^-$)/tungstate ($^{188}\text{WO}_4^{2-}$) in alkaline, high tungstate media. Partitioning properties of $^{99}\text{TcO}_4^-$ and $^{188}\text{ReO}_4^-$ in PEG-ABS and ABEC are similar for each. We have shown that ReO_4^- can be concentrated from a load solution of high WO_4^{2-} in NaOH using aqueous biphasic extraction chromatography (ABEC). The chromatographic progress of the column was monitored by a $^{99}\text{TcO}_4^-$ spike. The retained $\text{ReO}_4^-/^{99}\text{TcO}_4^-$ is eluted with water off the column with recorded capacities of 160 mg NaReO_4/g dry resin.

This work is supported by the U.S. National Science Foundation (Grant No. CTS-9522159).

90. NAPHTHOL AND RESORCINOL-BASED AZO DYES AS METAL ION COMPLEXANTS IN AQUEOUS BIPHASIC SYSTEMS

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Aqueous biphasic systems (ABS) have demonstrated applications as environmentally-friendly methods to separate anionic species, such as pertechnetate, from high ionic strength solutions. In contrast, partitioning of hydrated metal ions such as Fe^{3+} to the PEG-rich phase is negligible without the addition of a metal ion complexant to the system. As the nature of the extracting phase is aqueous, new classes of water soluble molecules are available that were not amenable for use with organic solvents. Enhancing the metal ion partitioning to the PEG-rich phase in ABS is challenging as both phases are 80% water on a molal basis. To effectively enhance the partitioning, the extractant should quantitatively prefer the PEG-phase. Naphthol and resorcinol-based azo dyes including 1-(2-pyridylazo)-naphthol (PAN), 1-(2-thiazolyazo)-naphthol (TAN), 4-(2-pyridylazo)-resorcinol (PAR), and 4-(2-thiazolyazo)-resorcinol (TAR) are known metal complexants in traditional solvent extraction systems. This presentation will examine the affinity of these azo dyes for the PEG phase as a function of ligand speciation to determine the partitioning behavior of the ionized forms in ABS. Metal ion partitioning studies will demonstrate the influence of these complexants on the distribution of metals in ABS.

This work is supported by the U.S. National Science Foundation (Grant No. CTS-9522159).

91. AROMA COMPOUNDS RECOVERY FROM MICELLIAL CULTURES IN AQUEOUS TWO-PHASE PROCESSES

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Flavor and aroma chemicals used in food, cosmetic and detergent industries are products of great commercial significance. Existing technologies of flavor and aroma production by micro-organisms have opened up an alternative source in comparison with the chemical synthesis. In this context, the production of 6-pentyl-alpha-pyrone (6PP) represents a very interesting case because the concentration of the product of interest (aroma) in the fermentation broth causes inhibition of the growth of the microorganism and, as a result the production of the aroma is inhibited. Extractive fermentation or in situ removal of products represents an alternative technology to remove the product from the fermentation broth as it is formed. Therefore, the productivity of the processes can be increased. In the case of the production of 6PP, several attempts to remove the molecule have been published (*e.g.*, using adsorbent, pervaporation and immobilization). However, these processes have disadvantages as complications and negative effect upon the performance of the micro-organism. Aqueous two-phase systems (ATPS) have been suggested as an alternative for extractive fermentation of biological products. In the present research, a representative model (production of 6PP by *Trichoderma harzianum*) has been chosen to evaluate the recovery of aromas using ATPS. A practical approach which exploits the known effect of systems parameters upon protein partition was used to examine the partition behavior of 6PP from *Trichoderma harzianum* in polyethylene glycol (PEG)-dextran and salt two-phase systems. The partition behavior of this type of flavor compound in aqueous two-phase systems (ATPS) is for the first time reported here.

This research is supported by CONACyT México (Grant 4046P-B).

92. PARTITIONING OF BOVINE SERUM ALBUMIN IN AN AQUEOUS TWO-PHASE SYSTEM: OPTIMIZATION OF PARTITION COEFFICIENT

Ufuk Gündüz

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Partitioning of proteins in aqueous two-phase systems has been shown to provide a powerful method for separating and purifying mixtures of biomolecules by extraction. These systems are composed of aqueous solutions of either two water-soluble polymers, usually Polyethylene Glycol (PEG) and dextran (Dx), or a polymer and a salt, usually PEG and phosphate or sulfate. There are many factors which influence the partition coefficient, *K* (ratio of biomolecule concentration in top phase to that in the bottom phase), in aqueous two-phase systems. The value of the partition coefficient relies on the physico-chemical properties of the target protein and the contaminant and their interactions with those of the chosen system. In this work, the partition behavior of pure Bovine Serum Albumin in aqueous two-phase systems was examined in order to investigate the effects of changes in phase properties on the partition coefficient, *K*. pH and concentration of NaCl were found to be the factors having influence on *K*. Optimal conditions of these factors were obtained using the Box-Wilson experimental design. The optimum value of *K* was found as 0.02 when NaCl concentration, and pH were 0.2 M and 8.9, respectively, for a phase system composed of 8% PEG (3,350) – 6% Dx (45,000) – 0.05M Phosphate at 20°C.

93. EXTRACTION AND PURIFICATION OF PEROXIDASE OF SOYBEAN (*GLYCINE MAX*) BY METAL AFFINITY PARTITIONING

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Affinity partition employing metal as ligand is an effective method to purify proteins. The most commonly metal complex used is IDA-Cu²⁺, which, according to the literature, is stable under high saline concentrations, interacts specifically with the target biomolecule, is easily recycled and not expensive. Proteins with histidine, cysteine and triptophane residues on the surface are favored for this technique

Initially, the model protein lysozyme (from chicken egg white), was partitioned in the metal-affinity PEG-salt system. PEG 4,000 was activated with thionyl chloride, attached to iminoacetic acid (IDA) and then, the PEG-IDA was complexed with copper ions. The ATPS without ligands was composed of 13% (w/w) PEG 4,000 and 9% (w/w) phosphate. PEG-IDA-copper concentrations used in the metal-affinity systems were 1, 5 and 10%. The partition coefficient of lysozyme increased 9-fold when 5% PEG 4000-IDA-Cu²⁺ system was employed to extract the enzyme.

Then, the extraction of peroxidase from a crude extract of defatted soybean (*Glycine max*) by metal-affinity in ATPS was studied. A liquid-liquid extraction process using metal-ligand was developed in two steps aiming to purify the peroxidase. In the first purification step, the system was composed of 14% (w/w) PEG 4000-IDA-Cu²⁺ and 8% (w/w) Na₂SO₄ and the peroxidase partitioned mainly to the top phase (K = 24). In the second step, a system formed by 14% PEG 4000 and 10% phosphate was used to revert the value of the partition coefficient of peroxidase to the bottom salt-rich phase (K = 0.05), providing the purification and recovery of 60% of the enzyme. The top PEG 4000-IDA-Cu²⁺-rich phase was washed by ultrafiltration in order to remove the sulfate salt and reused in another cycle of peroxidase purification.

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94. PARTITIONING OF ERYTHROMYCIN BY TEMPERATURE-INDUCED AQUEOUS TWO-PHASE SYSTEM

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The partition coefficients of erythromycin were measured at 25°C in ethylene oxide-propylene oxide random copolymer (EOPO)/hydroxypropyl starch (HPS) and polyethylene glycol (PEG)/HPS aqueous two-phase polymer systems. It was not suitable to use these systems to extract the erythromycin. The partition behavior of erythromycin in PEG/K₂HPO₄ and EOPO/K₂HPO₄ systems was investigated in great detail. The effects of a large number of factors on the partitioning behavior, including the molecular weight of PEG, the tie line length, the concentration of EOPO and K₂HPO₄, the addition of salt, solution pH, the heteroprotein and the reduced sugar in fermentation broth, were studied. Erythromycin partitioned unevenly in the EOPO/K₂HPO₄ systems. It could thus be extracted and purified by this system.

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95. BIOSPECIFIC AFFINITY PARTITIONING OF LIPOSOMES

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Biospecific affinity partitioning is a method that can be used for the purification of membranes and membranous structures such as organelles, cells, and viruses. Affinity partitioning is carried out in aqueous polymer two-phase systems in an environment compatible with membrane structures. In order to explore basic requirements for the method to work model experiments have been performed in which biotinylated liposomes were partitioned in a polyethylene glycol/dextran two-phase system, using avidin-dextran as affinity species.

In model experiments under strictly defined conditions avidin-dextran efficiently pulled biotinylated PC liposomes from top to bottom phase. One biotin per liposome was sufficient to redistribute them. Further studies using mixed biotinylated liposomes, for instance PC/PS, showed that these were not attracted by avidin-dextran to the bottom phase even at low percentage of PS. A closer study suggested that the negative charge introduced through PS to these liposomes affect the otherwise very strong avidin-biotin interaction thereby precluding affinity partitioning. Various ways to overcome this problem, including the use of a long spacer arm for the biotin ligand to decrease the effect of adjacent liposomal negative charges, will be discussed.

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96. METAL ION BOUND PARTICLES IN AQUEOUS TWO-PHASE SYSTEM FOR PROTEIN PURIFICATION

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Integration of different separation techniques to form a new unit operation has the potential to reduce the number of single steps during downstream processing of proteins and hence reduce product losses and process costs. The feasibility of an integrated affinity adsorption-two phase extraction process was investigated using Ni(II)-bound Sepharose in an aqueous PEG/sulfate two-phase system for the purification of recombinant *Bacillus stearothermophilus* lactate dehydrogenase (LDH) carrying genetically attached polyhistidine (His₆) tail, expressed in *Escherichia coli*. The recombinant product was bound to immobilized nickel very strongly compared to native LDH. When high concentration of NaCl was used in binding buffer, most of the engineered LDH from the crude feedstock was bound to Ni(II)-Sepharose particles which were quantitatively partitioned to the interface of the system. The particles were separated from the two phases and the bound enzyme was completely recovered by treatment with imidazole. The results show that tailored recombinant proteins could be isolated efficiently from crude mixture in a single step by this separation process.

97. METAL SEPARATION CAPABILITIES USING AQUEOUS BIPHASIC SYSTEMS ON MAGNETIC RESINS

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Waste minimization and recycling practices can often constitute a significant fraction of chemical processing and operating costs. The combination of Aqueous Biphasic Systems (ABS) and magnetic microparticle supports is a simple technology that utilizes micrometer-sized magnetic composite materials containing a bonded layer of polyethylene glycol (PEG) as an effective manner to separate metals. This paper presents PEG used to form ABS on magnetic microparticles for recovering metals (*e.g.*, pertechnetate, iron) from high ionic strength solutions. Furthermore, we evaluate the use of water activity to further understand the thermodynamics of ABS.

98. QUANTITATION OF THE EFFECTIVE TIE-LINE LENGTH OF WORKING AQUEOUS TWO-PHASE SYSTEMS: GENERIC APPLICATION TO THE OPTIMIZATION OF PRACTICAL PROTEIN RECOVERIES FROM PARTICULATE FEEDSTOCKS

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The nature and amount of biomass loaded into an aqueous two phase system (ATPS) is empirically recognized to strongly influence phase separation. For example, in PEG-phosphate systems the presence of complex mixtures of biomolecules and particulates disrupts the equilibrium between PEG, salt and water by modifying the thermodynamic properties of the system. The incompatibility between phase chemical components increases in the presence of biomass which promotes phase separation at lower PEG and salt concentrations. This results in an effective shift in the position of the binodal curve toward the x- and y-axis with concomitant modification of the tie-line length for all original biphasic systems.

A method has been described in an accompanying abstract, whereby the distribution at partition equilibrium of a selection of tritiated amino acids can be exploited to redefine PEG-phosphate ATPS loaded with complex biological feedstocks in terms of an equivalent tie-line length (TLL_e). Presentation will be made of the application of this method to the redefinition of PEG-salt ATPS variously loaded with disrupted microbial cells or whole blood. Recombinant *E. coli* expressing yeast α -glucosidase as nanoparticulate inclusions bodies was subjected to five passes through a Manton-Gaulin homogenizer, whilst *S cerevisiae* (out-dated bakers' yeast) was passed once through a continuously operated bead-mill.

PEG-phosphate ATPS were loaded with various challenges of biomass (5-70% w/w) and characterized in respect of equivalent TLL and the distribution of bulk and individual protein products. It was found that different sources of biomass had a characteristic impact upon phase separation, the location of the binodal curve, and the TLL_e of the original biphasic systems. This observation will be discussed in the context of the predictive design of generic ATPS for the recovery of protein products from particulate feedstocks in intensified processes. Of particular interest are situations where biomass contributes significantly to the imparting of phase forming characteristics upon monophasic mixtures of PEG and potassium phosphate.