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New approaches to aqueous polymer systems: Theory, thermodynamics and applications to biomolecular separations

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Abstract – A summary is presented of the main qualitative features of aqueous two-phase systems. Some aspects of the application of such systems to biomolecular separation are discussed. A brief description of a recent theoretical approach to aqueous two-phase systems based on statistical geometrical concepts is considered and some links are made to measurable thermodynamic properties.

Some polymer-polymer pairs or polymer-inorganic salt pairs, when in a "good" solvent, can form two immiscible homogeneous liquid phases.[1,2] If the solvent is water, we call the systems formed "aqueous two-phase systems." Fig. 1 illustrates these two types of aqueous two-phase systems with typical compositions.

Examples of other pairs of polymers which form aqueous two-phase systems include: Dextran + Ficoll, polyvinylalcohol or hydroxypropylstarch, and polyethylene glycol + Ficoll, polyvinylalcohol.* Other examples of polymer + salt aqueous two-phase systems are (see also ref. 2 and 3): methoxypolyethylene glycol + potassium phosphate, polyvinylpyrrolidone + potassium phosphate, polyethylene glycol+ sodium sulphate, polyethylene glycol + sodium carbonate and polyethylene glycol + sodium chloride.

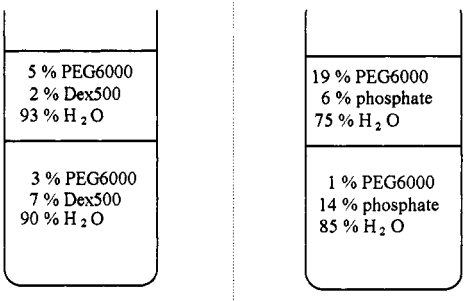

Figure 1 An illustration of the typical phase compositions of PEG 6000+Dex500 and PEG 6000+phosphate aqueous two-phase systems. The concentrations shown are based on weight.

Special types of aqueous two-phase systems are those containing polyoxylene detergents.[4] The formation of these systems arises from the inverse temperature/solubility behaviour of non-ionic detergents carrying polyoxyethylene groups as the hydrophilic moiety. On raising the temperature above room temperature phase separation occurs. In the two resulting phases, one is a detergent rich coacervated phase and the other is the water rich depleted phase. Terstappen *et al.*[5] have systematically and quantitatively studied protein partitioning in

* Usually polyethylene glycol (PEG) is a linear polymer and has the structural unit – (CH_2CH_2O) –. Methoxypolyethylene glycol has one end of the PEG molecule methoxylised. Dextran is composed predominantly by poly(α-1,6-glucose), Ficoll is a nonionic synthetic polymer of sucrose, polyvinyl alcohol has the structural unit – (CH_2CHOH) –.

such detergent-based aqueous two-phase systems, employing a series of similar polyoxyethylene detergents and proteins of varying hydrophobicity.

Some other similar aqueous two-phase systems[6] composed of aliphatic alcohols, salts and water (*e.g.*, ethanol-phosphate-water, ethanol-sulphate-water, ethanol-citrate-water, 1-butanol-phosphate-water, 1-propanol-phosphate-water, 2-propanol-phosphate-water, 2-methyl-2-propanol-phosphate-water) have also been reported.

These aqueous two-phase systems can provide benign and non-disruptive environments for the separation and purification of biopolymers. They are potentially attractive since, as has been shown in some examples, they can give a means of separation which is easy to manipulate, reliable in scale-up and is simple and effective for process operation.[2] A procedure for the purification of β-interferon from tissue cultures of human fibroblasts[7,8] has been successfully used in the pharmaceutical industry for some years and this work has been recently extended to a recombinant human *E. coli* interferon.[9] We have recently developed a novel bioseparation process[10,11] in which the role of aqueous two-phase extraction is essential and which is shown in Fig. 2.

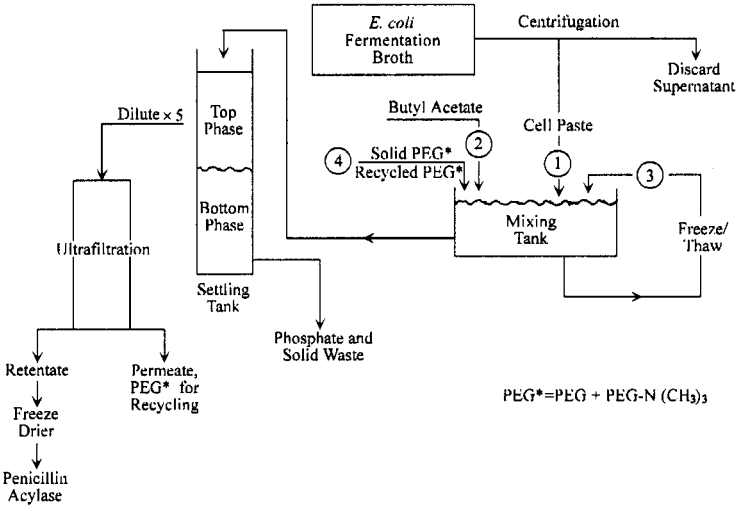

Figure 2 The extraction process for penicillin acylase recovery from *E. coli*.[10]

The understanding of the phase diagram of a particular aqueous two-phase system is necessary for the scientific application of this system to biological, biochemical, biotechnological or even metallurgical applications. The phase diagrams shown in Fig. 3 represent two types of commonly encountered aqueous two-phase systems.

FROM PHASE DIAGRAM TO SOLUTE PARTITIONING

As indicated earlier, one of the important features of aqueous two-phase systems is that in the two equilibrated phases water is the dominant component. If one adds biomolecules, especially biomacromolecules or cell organelles, into an aqueous two-phase system, uneven partitioning of the molecules between the two phases will usually result and this is illustrated in Fig. 4. Conventionally, this phenomenon is described by the partition coefficient of the added species and this is given as:

$$K_P = C_P^T / C_P^B \qquad (1a)$$

where C_P^T and C_P^B are the concentrations of the added solute in the top and bottom phases, respectively. The reason for the uneven distribution can be attributed to several factors such as the size of the solute, electrostatic interactions, hydrophobic (hydrophilic) interactions, biospecific affinity interactions and conformational effects in the different chemical and physical environments of the two phases. Generally, this partition coefficient is related to the factors above by[2]

$$\ln K_P = \ln K_P^\circ + \ln K_{el} + \ln K_{hfob} + \ln K_{biosp} + \ln K_{size} + \ln K_{conf} + \cdots \quad (2)$$

where K_p° is the "intrinsic" partition coefficient and el, hfob, biosp, size, conf represent the contributions to the partition coefficient from electrostatic, hydrophobic, biospecific, size and conformational effects.

The partition coefficients of biomacromolecules show a wide variation and it is this variation which is the foundation for using two phase systems for the separation of biologically active molecules or cell organelles.

If small amounts of proteins or enzymes are added to an aqueous two-phase system, usually the partition coefficient is independent on the total concentration of this component. The yield, Y_T, of the target product, if it is enriched in the top phase, is given by

$$Y_T = \frac{C_T V_T}{C_T V_T + C_B V_B} \times 100\% = \frac{K_P R}{K_P R + 1} \times 100\% \quad (3)$$

where C_T and C_B are the enzyme activities or protein concentrations in the top and in the bottom phases respectively, R is the volume ratio of the top and bottom phases.

In practical situations, the systems are complex and biomass is usually present in aqueous two-phase systems. We observed for many cases that cell mass (cell debris) is collected at the interface between the phases. The nature of this aggregation is similar to precipitation rather than the formation of another liquid phase. Therefore, aqueous two-phase systems characteristically both separate the target protein from impurities through equilibrium partitioning and coagulate cell and cell debris at the interface of the two liquid phases. Interestingly, there are two extreme cases: (i) the top phase is so small that the interface appears to be the top phase, (ii) the bottom phase is so small that the interface appears to be the bottom phase.

To illustrate these effects we consider some experiments on PEG + phosphate aqueous two-phase systems containing *E. coli* cell mass. Two broad types of observation are seen: firstly, the top phase looks clear but the bottom phase is turbid (referred to as I), and secondly, both top and bottom phases look transparent but cell mass is coagulated at the interface (referred to as II). Table 1 shows the influence of PEG concentration on the transition between these two phenomenon. High concentrations of PEG and/or high of cell mass loadings always tend to give the latter situation (case II). Although we have categorised the observations as being distinct and different, the main difference between the above two phenomenon is that in the former the bottom phase volume is so small that it becomes negligible.

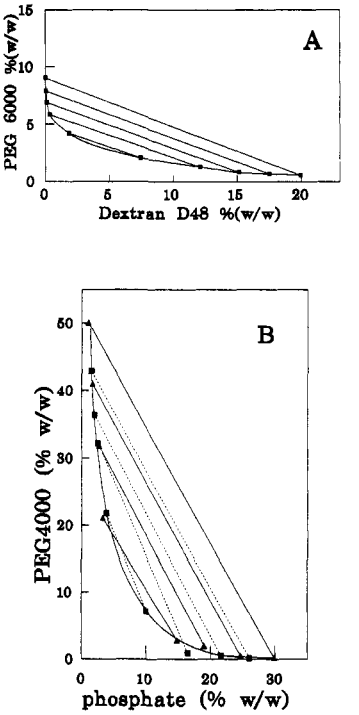

Figure 3 Phase diagrams of aqueous two-phase systems: (A) PEG 6000-Dextran D48 at 20°C (adapted from ref. 2); (B) PEG 4000 + tripotassium phosphate in the absence and in the presence of *E. coli* cell mass: ■ ··· without cell mass, ▲ ··· with cell mass (adapted from ref. 10).

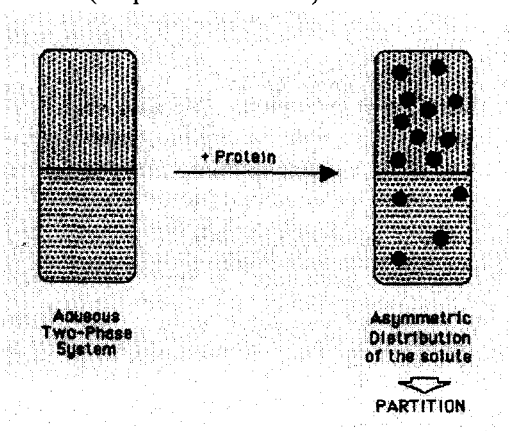

Figure 4 Distribution of a homogeneous protein species in an aqueous two-phase system

For systems where cell mass is present, the loss of some of enzyme activity by enclosure in the cell or cell organelles is unavoidable. The enzyme yield in, for example, the top phase (*cf.* eqn. 2) changes to:

$$Y_T = \frac{C_T V_T}{M_T} \times 100\% \quad (4)$$

where M_T is the total amount of enzyme added to the entire partitioning system.

Table 1 The influence of PEG concentration and cell mass load on PEG + potassium phosphate aqueous two-phase systems†

	Cell mass (%)				
PEG 4000 (% w/w)	5	10	15	20	25
10	I	I	I	I	I
12	I	I	I	I	II
14	I	I	I	II	II
16	I	I	II	II	II
22	I	II	II	II	II

†Potassium phosphate concentration was kept at 20% (w/w) and pH at 7.1. See text for the meaning of categories I and II.

The industrial applications of these systems need a fundamental understanding of the factors involved in the formation of two-phase systems and of the features which determine the partitioning behaviour of biomolecules. It has been indicated (eqn. 2) that the partition of solutes, or indeed cells, between the two aqueous phases is affected by many factors such as hydrophobicity and charge, and to date, optimisation in separation processes has largely been obtained by empirical manipulation of such properties. This is generally unsatisfactory and inefficient and the object of our work has been to study, from a theoretical viewpoint, the behaviour of aqueous two-phase systems. The ultimate objective is to obtain methods for the rational design of commercial purification and separation processes which use such systems.

SOME NEW APPROACHES IN DEALING WITH AQUEOUS TWO-PHASE SYSTEMS

If one is to have a predictive method which allows the rational design of commercial purification and separation processes there are several problems which must be addressed and these include the following. (i) The establishment of the molecular criteria necessary to describe the coexistence curves (binodals) of the two phases in the absence of biomaterials. (ii) The establishment of the tie-line relationships in the systems. (iii) The prediction of how biomolecules partition when present at concentrations such that no significant perturbation of the phase behaviour occurs. (iv) The prediction of the behaviour of systems when the target species and/or "impurities" are present at high concentrations. Superimposed upon these are problems on issues associated with polymer modification, scale-up and multi-stage partitioning. Some of the outstanding aspects are as follows:

Coexistence curves

There have been several attempts to use the Flory-Huggins theory of polymer solutions to describe both coexistence curves and tie-line relationships. Such approaches are doomed at the outset, at least if one is aiming for any real molecular understanding of the phenomena involved, since some of the fundamental assumptions made in the formulation by Flory and Huggins[12] are transparently not applicable to systems containing water. Consequently, we have developed a new approach, which we have termed the "Binodal Model", to polymer-containing systems which is based on the concepts of statistical geometry. Its wide adaptability has also been illustrated.[13] In many cases, the Binodal Model[14] takes the form

$$\ln\left(\langle V\rangle_{210}\frac{\rho N_A w_2}{\langle M\rangle_2}\right)+\langle V\rangle_{210}\frac{\rho N_A w_1}{\langle M\rangle_1}=0 \qquad (5)$$

where w_1 and w_2 are the weight concentrations of two phase-forming species along the binodal respectively, $\langle V\rangle_{210}$ is the effective excluded volume (EEV) of species 2 in the species 1 aqueous solution, ρ is the solution density, N_A is Avogadro's number, for polymer molecules $\langle M\rangle_1$ and $\langle M\rangle_2$ are mean molecular weights for species 1 and 2, and usually the root-mean-square average molecular weights for polydisperse components are taken.

This novel theory is an advance towards the solution of phase separation problems and allows the prediction of the coexistence behaviour of two-phase systems using only one parameter, the EEV, and the physical meaning of this parameter has been demonstrated. A historical outline of efforts in describing binodals has been given elsewhere.[13]

It is appropriate to mention here that this theory also has potential applications to many other problems in polymer and colloid science and we are currently extending this treatment to the important and outstanding problems of the partitioning of soluble particulates and the precipitation of macromolecules by polymers or salts. In practice, we are aware that a correct understanding of phase separation and partition in partly aqueous systems is important to those who wish to avoid phase separation. This situation often occurs in food technology, surfactant and oil recovery industries. The theory is therefore expected to find more applications to many other problems in polymer and colloid science.

Tie-line relationships

The Binodal Model, although able to predict coexistence curves, cannot be applied to tie-line relationships, *i.e.* to linking the compositions of the phases in equilibrium. Most of the approaches which have been used to obtain relationships between phase compositions have invoked, in one form or another, virial expansions in solute concentrations. Although such expansions have been frequently used, we have recently shown[15] that there are some fundamental problems in their use to describe aqueous two-phase systems. Briefly, the situation is that there is a conflict between the virial expansion formulation and molecular events in that, at the solute concentrations necessary to give two phases, third and higher order virial coefficients are required to represent system properties. However, when these coefficients are given their proper physical meaning, there is always a fundamental inconsistency. A means of circumventing this difficulty by, *e.g.*, the introduction of what we have termed "effective" second virial coefficients has been suggested.[15]

Protein partitioning at low concentrations

Strictly speaking, the protein partitioning coefficient in eqn. 1a will be a constant only at very low protein concentration in the system, *i.e.*

$$K_P = \lim_{C_P\to 0} C_P^T/C_P^B = \text{constant} \qquad (1b)$$

In recent years this has been one of the most popular subjects in this area and some progress has been made using both nonelectrostatic and electrostatic approaches.[16,17,18] This work is summarised in a recent review article.[19]

The combination of these two types of molecular interactions were firstly realised by Albertsson[2] and he gave the following expression

$$\ln K_P = \ln K_P^\circ + FZ_P\Delta\psi/RT \qquad (6)$$

where K_P and K_P^o are the protein partition coefficients at a given pH and at the protein isoelectric point, respectively, $\Delta\psi$ is the electrostatic potential difference between the two phases and Z_P is the charge on the protein.

Equation 6 shows that protein partitioning can be represented as two factors, one from electrostatic interactions and the other from short range interactions. In other words, it is possible to deal with short range (van der Waals) and long range (electrostatic) molecular interactions separately. We will also develop a protein partitioning model which is consistent with the concepts used in developing our Binodal Model.[20]

Protein partitioning at high solute and/or high cell mass concentrations

If the experimental conditions are such that the phase system is perturbed significantly by the added materials, the problems will be more complicated. There are three possible scenarios:

(i) the soluble target protein could be present at very high concentrations.
(ii) the target protein could be present at low concentrations but there is also present a high concentration of soluble impurities.
(iii) a large amount of insoluble cell and/or cell debris could be present.

In the first and second situations, rather than having a system with two phase-forming components, in effect, such systems now consist of at least three phase-forming components. As a consequence, the binodal will shift from its original position, but the tie-line slope will be little changed. In the third situation, most of the cell mass will be precipitated or will coagulated at the interface of the two phases and the binodal will be less influenced, but the total phase composition will alter due to the removal of some bulk water by the cell mass. The common feature in all three cases will be the alteration of the initial tie-line length. There is little coherent information available on any of the above aspects, but such information is necessary to complement the theoretical studies. Comprehensive studies of all three areas need to be undertaken.

Affinity Partitioning

Some useful results on affinity partitioning were obtained from studies of metal affinity extraction in both polymer + polymer[21] and polymer + salt aqueous two-phase systems.[22]

Theories of affinity partitioning stemmed from the original work of Flanagan and Barondes.[23] For proteins with n independent binding sites and very high ligand concentration, they derived

$$K = K_o K_L^n (K_{aT}/K_{aB})^n \tag{7}$$

where K and K_o are the protein partition coefficient in the presence and absence of polymer ligand, K_L is the partition coefficient of the polymer-bound ligand, K_{aT} and K_{aB} are the binding constants for the protein-ligand complex in the top and bottom phases respectively.

Later Brooks *et al.*[24] obtained the more general expression where the limitation to the ligand concentration is removed and this is:

$$K = K_o\left(\frac{1+K_{aT}L_T}{1+K_{aB}L_B}\right)^n = K_o\left(\frac{1+K_{aT}L_T}{1+K_{aB}L_T/K_L}\right)^n \tag{8}$$

Equation 8 was also obtained by Cordes *et al.*[25] Interestingly, as was observed by Flanagan and Barondes[23] a linear relationship exists between $\ln(K_o/K)$ and L_T^{-1}. The following expression

$$\left(\log\frac{K}{K_o}\right)^{-1} = \left(\log\frac{K_{E,\max}}{K_o}\right)^{-1} + \left(\log\frac{K_{E,\max}}{K_o}\right)^{-1} K_D(L_T)^{-1} \tag{9}$$

derived by Johansson but shown by Cordes *et al.*[25] fits the experimental results very well. We also found, in our study of the recognition of affinity interactions,[11] that this relationship holds.

Equation 8 has been made more general[26] and applied to the situation where one has a protein with m binding sites for metal ion association or hydrogen ion association, the association constants being K_b and

$K_{H,b}$ respectively, and n sites with corresponding constants K_a and $K_{H,a}$. The following expression was derived:

$$\ln\left(\frac{K}{K_o}\right) = n\ln\left[\frac{1+K_{aT}[M]_T+K_{H,a}[H]}{1+K_{aB}[M]_B+K_{H,a}[H]}\right] + m\ln\left[\frac{1+K_{bT}[M]_T+K_{H,b}[H]}{1+K_{bB}[M]_B+K_{H,a}[H]}\right] \quad (10)$$

where the subscripts T and B refer to top and bottom phases respectively.

This model takes into account inhibition of ligand binding by hydrogen ion and the possibility that more than one type of ligand site contributes to the affinity partitioning.

When one comes to consider the incorporation of chemical functionality on phase-forming polymers (*i.e.* affinity partitioning), eqns. 7-10 show that the influence of affinity interactions can be superimposed on the partition coefficient in a simple way. This amalgamation could be very useful in practical situations.

Thermodynamic measurements

We are currently investigating thermodynamically based measurements of the pertinent molecular interactions to provide independently measurable model parameters. Since the EEV is related to thermodynamic variables by:[14]

$$V^*_{ji0} = \frac{\rho N_A}{\upsilon_i} \cdot \frac{\Delta A_{ji0}}{k_B T} \quad (11)$$

where ΔA_{ji0} is the difference between the Helmholtz free energy necessary to create a cavity with volume V_{ji0} and that of the mean value.

The determination of EEV's and their variation with composition, temperature and with other measurable thermodynamic variables is an important part of our studies. We are determining the solubility of a range of proteins under a variety of conditions (pH and solution concentrations) in both binary (water + one polymer or salt) and ternary (water + two polymers and/or salt) in one phase regions. This will give some measures of the interplay between the tendencies proteins have to self-interact and their propensities to be solvated by the solvent components. In essence, the problems we are addressing experimentally and how these are related to our predictive models are illustrated in Fig. 5. An aqueous two-phase system for protein separation in a biotechnological process contains at least four components, but molecular information on interactions between components can be obtained by studying some three-component and two-component systems. Links could be investigated (once a reasonable body of information is obtained) between the volumetric properties of the solvent systems investigated and the Effective Excluded Volume. Some link must exist since the EEV is a function of the spacing among the molecular centres of the neighbouring species in the system, but we do not know what this is at the moment. However, it would be very useful in practice to establish the link

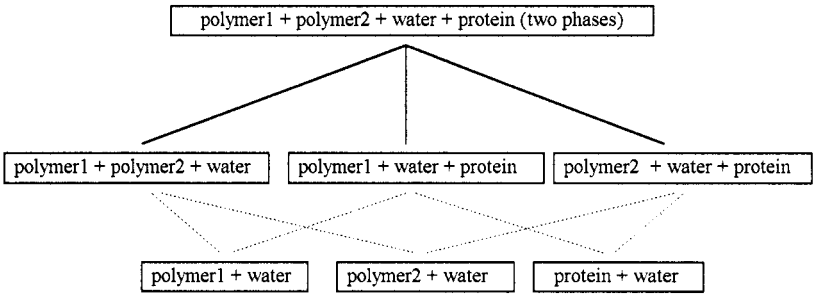

Figure 5 A schematic experimental strategy for the prediction of protein extraction in aqueous two-phase systems, showing that pertinent experimental information for aqueous two-phase systems is obtained from simpler systems containing only some of the components.

between the EEV and solute volumetric properties since the latter are easily measured and the former determines the coexistence curve in two-phase systems.

We are also studying the volumetric properties of mixed solutions containing proteins and exploiting links between changes in volumetric properties and preferential solvation.

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