ELSEVIER

Polymer 43 (2002) 3387–3390

polymer

www.elsevier.com/locate/polymer

Polymer Communication

Role of water structure on phase separation in polyelectrolyte–polyethyleneglycol based aqueous two-phase systems

Vandana Gupta, Sunil Nath, Subhash Chand*

Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology Delhi, New Delhi 110 016, India

Received 29 November 2001; received in revised form 4 February 2002; accepted 4 February 2002

Abstract

Partitioning of proteins in aqueous two-phase systems (ATPS) has emerged as one of the important downstream processing techniques in bioprocess technology. The phase separation behavior of polyelectrolyte–polyethyleneglycol (PEG) based ATPS have been studied to elucidate the mechanism controlling phase behavior. The effect of various inorganic salt additives revealed the importance of water structure as a major factor controlling phase separation in these systems. Nitrate and potassium (water structure breaking ions) elevated the binodial line while sulphate, phosphate and sodium (water structure making ions) depressed the binodial line in both polyacrylic acid–PEG as well as polyethylenimine–PEG based ATPS. The effect of increase in concentration of either of the constituent polymers in both systems (at constant salt concentration) always led to a greater propensity towards phase separation. These results point to a mechanism in which *salt-assisted polymer-modified water structure* interactions play a central role in phase separation in ATPS. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Polyelectrolyte; Phase separation; Aqueous two-phase system

1. Introduction

Partitioning and precipitation processes have been used successfully for the separation, purification and analysis of various biological materials [1–5]. The properties of the coexisting phases are important determinants of the partitioning of proteins in both precipitation and aqueous two-phase systems (ATPS). Several explanations for the phase separation behavior in aqueous mixtures of two non-ionic polymers or non-ionic polymer and a salt have been provided [2,6,7]. However, the role of water that constitutes the bulk component of the two phases in these systems has been neglected in almost all the explanations proposed. The state and/or structure of water is altered in an aqueous solution depending on the chemical nature and concentration of the solute(s) present [8] and they can play an important role in the design of biphasic aqueous polymeric systems for the partitioning of proteins. Zaslavsky et al. [9,10] extensively studied the phase separation process for non-ionic polymers and stressed on the role of 'water structure' as an important factor controlling two-phase formation. The phase separation in an aqueous mixture of two-polymers results from the effect of constituent polymers on the water structure and the phase separation is due to the incompatibility of these polymer-modified water structures.

Polyelectrolytes are water-soluble charged polymers that interact with oppositely-charged proteins by electrostatic interactions. They are inexpensive, non-toxic, recyclable and form two-phase systems at low polymer concentrations compared to non-ionic polymers [11,12]. However, very limited studies have been carried out on polyelectrolyte-based ATPS in terms of their phase separation behavior.

In the present investigation, the phase separation behavior of polyelectrolytes and polyethyleneglycol (PEG) based two-polymer systems have been studied. The studies focus on the phase behavior in the presence of various inorganic salt additives that affect the water structure. The role of polymer concentration on phase separation has also been studied to elucidate the mechanism controlling the phase behavior in polyelectrolyte–PEG based ATPS.

2. Experimental

2.1. Materials

Polyethylenimine (PEI) (number average molecular weight—60 000), polyacrylic acid (PAA) (average molecular

---

* Corresponding author. Tel.: +91-11-6459-1004; fax: +91-11-686-8521.

E-mail address: subhashc@dbeb.iitd.ernet.in (S. Chand).

PII: S0032-3861(02)00117-9

weight 250 000) were purchased from Sigma, St Louis, USA. PEG (average molecular weight 6000) was procured from SRL, India. All other chemicals were of reagent grade.

2.2. Construction of binodials

Stock solutions of polymers and salts were prepared in fresh buffer (phosphate buffer for PAA and tris-maleate buffer for PEI, 10 mM). The pH of these solutions was adjusted to pH 7.0, and stock solutions were mixed in the order—PEG, polyelectrolyte, buffer, and salt, vortexed and centrifuged at 900*g* for 15 min. The formation of two phases was monitored visually against a light source. Binodials were constructed in this way for two-phase systems of PEG–PAA and PEG–PEI.

3. Results and discussion

The local water structure around a given macromolecule usually consists of water molecules hydrogen-bonded to the polar hydrophilic site and the hydration water structure around the non-polar site. The interplay of these effects results in the specific structuring of water molecules around the macromolecule which overcomes the surface forces sufficiently to stabilize the macromolecules in aqueous solution. The size of the local water structures in polymer solutions is very large and it is possible to regard them as regions of water with particular structures [13]. Two solute species will generally attract or repel each other in water depending on the compatibility of the structures of water in their hydration shells [14]. Phase separation in an aqueous mixture of two polymers results from the effect of polymers on the water structure and could be due to the incompatibility of these polymer-modified water structures. To confirm the validity of this hypothesis the effect of water structure-perturbing factors such as inorganic salts, on phase separation in polyelectrolyte based ATPS has been studied in this work.

The effect of different inorganic salts on the phase behavior may be attributed to their role in affecting the structure of water in the system. The salts have been classified as 'structure breaking' and 'structure making' to describe the effects of different ions on the structure of water [15–18]. By these terms it is implied that the effect of a structure breaking ion on water is qualitatively similar to that of an increase in temperature, while a structure making ion produces an opposite effect like that of a decrease in temperature. The structure-affecting properties of the ions are displayed in such properties of their aqueous solutions as the viscosity (structure breaking ions reduce it), the rate of exchange of water molecules between the hydration shell and bulk water (structure breaking ions decrease its energy of activation), the longitudinal relaxation rate of the water molecule, measured by NMR (structure breaking ions increase it), etc. According to various measures of the effects of ions on the structure of water, the water structure making category of ions include cations, Li^+, Na^+, NH_4^+, Ca^{2+}, Mg^{2+}, etc., and anions, F^-, SO_4^{2-}, CO_3^{2-}, PO_4^{3-}, CH_3COO^-, etc. while the structure breaking ions are K^+, Rb^+, Cs^+, Cl^-, Br^-, I^-, SCN^-, NO_3^-, ClO_4^-, etc. [15–18].

3.1. Effect of inorganic salt additives on the phase separation in polyacrylic acid–PEG based ATPS

As discussed, salts have been classified into two categories: structure making and structure breaking to describe the effect of different ions on the structure of water. Therefore, depending on this classification, a number of ions were selected to study the effect on phase behavior. The effect of these ions on phase behavior is shown in Fig. 1. Here, different anions were selected with the cations being sodium and potassium in these cases. Nitrate is classified under structure breaking, and sulphate and phosphate under structure making. The water structure making salts depressed the binodial of the system to a greater extent than the water structure breaking salts as can be seen from the results in Fig. 1 which showed that sulphate and phosphate lowered the binodial to a greater extent than the nitrate ions and this trend was observed at all salt concentrations tried out.

The effect of cations was also observed on the phase behavior with sodium (structure making) and potassium (structure breaking) ions. As shown in Fig. 1, sodium lowered down the binodial in comparison with potassium irrespective of the anion. However, the effect of cations on the shifting of binodials was not as pronounced as that observed with anions. This fact is generally believed to be due to the orientation of the water molecules at anions radially through the O–H bond direction, leaving three other structure sensitive H-bonding vectors for interaction

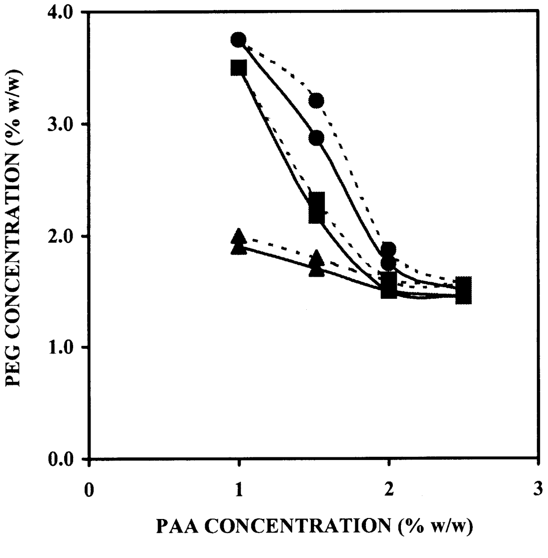

Fig. 1. Effect of salt ions on the binodial in PAA–PEG based ATPS at pH 7.0. The solid lines represent sodium ions while dashed lines represent potassium ions. (●) Nitrate; (■) sulphate; (▲) phosphate. The concentration of the salt additive was 0.5 M in all systems.

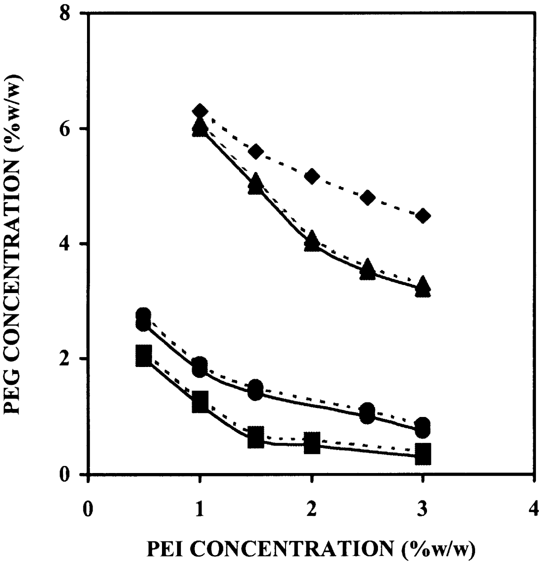

Fig. 2. Effect of salt ions on the binodial in PEI–PEG based ATPS at pH 7.0. The solid lines represent sodium ions while dashed lines represent potassium ions. (◆) Chloride; (▲) nitrate; (●) sulphate; (■) phosphate. The concentration of the salt additives was 1 M in all systems.

with water. Around cations, the water orientation is probably with the dipole axis colinear with the centre of the ion (less structure sensitive) [15]. The relationship between the effect of salt additives on the phase diagram of PAA–PEG system and the action of the salt on the structure of water clearly implied the importance of the water structure on the phase separation.

3.2. *Effect of inorganic salt additives on the phase separation in polyethylenimine–PEG based ATPS*

The studies were then extended to the phase separation behavior of the PEI–PEG system. PEI is a positively-charged polymer carrying primary, secondary, and tertiary amino groups. The effect of a number of structure making and breaking anions as well as cations was studied on the phase behavior in PEI–PEG based ATPS and the results are shown in Fig. 2. These results showed that the water structure making anions—phosphate and sulphate, considerably lowered the binodial in comparison with the water structure breaking anions such as chloride and nitrate. Similarly, a very slight depression in the binodial was observed in the presence of sodium (water structure making ion) in comparison with potassium (water structure breaking ion). These results are in accord with the results obtained on the effect of salt on phase behavior in PAA–PEG based ATPS.

Earlier workers have studied the effect of salt on phase behavior in PEI–hydroxyethylcellulose based ATPS. In the case of PEI–hydroxyethylcellulose based ATPS, the efficacy of anions to induce formation of two-phases also followed a similar trend as was observed with PAA–PEG and PEI–PEG based ATPS i.e. phosphate > sulphate > chloride = nitrate. This behavior was explained on the basis of polyvalent anions being efficient counterions to PEI, and thus promoting the formation of two-phase systems. The presence of cations showed no significant effect on the phase behavior [19].

3.3. *Effect of polymer concentration on phase separation in polyelectrolyte–polyethyleneglycol based ATPS*

The validity of the role of water structures in ATPS is also observed by the effect of polymer concentration on the phase separation behavior. The results in Figs. 1 and 2 show that increase in the concentration of one polymer lowers the concentration of the second polymer required for phase separation. In Fig. 1, as the concentration of PAA was increased, a lower concentration of PEG was required for phase splitting. A similar trend was also observed in the case of PEI–PEG based ATPS (Fig. 2).

In aqueous two-polymer mixtures, formation of two phases takes place above a critical concentration of the polymers, as clearly demonstrated by our experimental data (Figs. 1 and 2). Initially, the addition of polymers in the aqueous media results in the arrangement of water molecules around the polymer chains. The incompatibility of different water structures around different polymers results in separation of two different phases with each phase being enriched in one of the polymers. With increase in concentration of either polymer, the increased concentration of polymer-modified water structures leads to an increase in the degree of interactions between the two polymers. These incompatible interactions due to an increase in the polymer concentration result in a greater tendency for phase separation. Our studies also indicate the importance of water structures on the phase separation as seen by the effect of various inorganic salt additives. A number of possibilities may exist for the salt effect on phase separation behavior. It has not been established whether salt ions and their hydration shells form a separate entity from the polymer-modified water structures or salt ions also form a part of the polymer–water complex.

At low polymer concentrations, the degree of interactions among the polymers is low due to a high concentration of the bulk water. Significantly, our data also reveal a phase separation effect of salt above a critical concentration. The addition of an inorganic salt results in the arrangement of water molecules around salt ions. An ion in water is surrounded by the inner hydration layer of the oriented, immobilized, and closely packed water molecules, and the transition zone (or outer hydration sphere) in which the preferred arrangement of water molecules corresponds neither to that of the inner hydration sphere nor to that of the bulk water. The bulk water in an aqueous electrolyte solution is the part of water not included in both inner and outer hydration spheres of the ions. The addition of ions to the aqueous two-polymer mixture leads to an arrangement of water molecules around them and therefore, utilizes a part of the bulk water to form the hydration spheres. This leads to a decrease in the concentration of the bulk water in the system and therefore, enhances the interactions between the two different kinds of polymer-modified water structures. The addition of salt is necessary to facilitate the enhancement of these interactions. Thus, these enhanced incompatible interactions among the polymers due to salt-assisted polymer-modified water structures ultimately cause the splitting of the polymer solution into two phases. The addition of anions/cations showed the same effect on phase separation in both PAA as well as PEI based ATPS, i.e. the effect of added salt was found to be independent of the charge on the polyelectrolyte (positive or negative). Our results are in agreement with the mechanism of phase separation discussed earlier; they are not readily reconciled with any other mechanistic proposal, for instance phase separation due to a *direct* effect of added salt on the polymers. Our results are also in accord with the effect of these salt ions on phase separation in the neutral polymers-based ATPS (e.g. PEG-Dextran). Therefore, our studies strongly implicate the role of salt-assisted polymer-modified water structures as an important factor influencing phase separation in polyelectrolyte-based ATPS.

4. Conclusions

Phase behavior studies on the polyelectrolyte–PEG based ATPS indicated the role of water structures as a key factor controlling phase separation. The elevation of the binodial line in the presence of water structure breaking salts and its depression in the presence of water structure making salts indicated the water-structure driven phase separation in polyelectrolyte–PEG based ATPS. In particular, our experiments on phase separation behavior as a function of polymer concentration of either component of the mixture (at constant salt concentration) and the similar trends of the effects of salt on phase splitting (at constant polymer concentration) for both positively as well as negatively charged polyelectrolyte–PEG systems underscore a more subtle salt-assisted polymer-modified water structure mechanism of phase separation in ATPS.

Acknowledgements

The authors thank Dr B. Y. Zaslavsky (Analiza Inc., USA) for helpful discussions on the role of water structure in phase separation.

References

[1] Albertsson PA. Partition of cells and macromolecules. 3rd ed. New York: Wiley Interscience, 1986.
[2] Walter H, Brooks DE, Fisher D. Partitioning in aqueous two-phase systems. Theory, methods, uses, and applications to biotechnology. Orlando: Academic Press, 1985.
[3] Nath S. J Chem Technol Biotechnol 1995;62(3):295–300.
[4] Nath S, Patrickios CS, Hatton TA. Biotechnol Prog 1995;11(1):99–103.
[5] Gupta V, Nath S, Chand S. Biotechnol Lett 2000;22(11):927–9.
[6] Gustafsson A, Wennerström H, Tjerneld F. Polymer 1986;27(11):1768–80.
[7] Tompa H. Polymer solutions. London: Butterworths, 1951.
[8] Beall PT. Cryobiology 1983;20(3):324–34.
[9] Zaslavsky BY, Mestechkina NM, Rogozhin SV. J Chromatogr 1983;260(2):329–36.
[10] Zaslavsky BY, Bagirov TO, Borovskaya AA, Gulaeva ND, Miheeva LH, Mamhudov AU, Rodnikova MN. Polymer 1989;30(11):2104–11.
[11] Rastogi A, Chand S. Biotechnol Tech 1989;3(1):33–8.
[12] Hughes P, Lowe CR. Enzyme Microb Technol 1988;10(2):115–22.
[13] Gekko K. Ions and molecules in solution. Amsterdam: Elsevier, 1983.
[14] Molyneux P. Water, a comprehensive treatise. New York: Plenum Press, 1975.
[15] Conway BE. Ionic hydration in chemistry and biophysics. Amsterdam: Elsevier, 1981.
[16] Edsall JT, McKenzie HA. Adv Biophys 1983;16(1):53–183.
[17] Marcus Y. Ion solvation. Chichester: Wiley, 1985.
[18] Zaslavsky BY. Aqueous two-phase partitioning: physical chemistry and bioanalytical applications. New York: Marcel Dekker, 1995.
[19] Dissing U, Mattiasson B. Biotechnol Appl Biochem 1993;17(1):15–21.