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The Effect of Guanidine Hydrochloride on Phase Diagram of PEG- Phosphate Aqueous Two-Phase System

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***Abstract*—**This report focus on phase behavior of polyethylene glycol (PEG)4000/ phosphate/ guanidine hydrochloride/ water system at different guanidine hydrochloride concentrations and pH. The binodal of the systems was displaced toward higher concentrations of the components with increasing guanidine hydrochloride concentrations. The partition coefficient of guanidine hydrochloride was near unity and increased with decreasing pH and increasing PEG/salt (%w/w) ratio.

***Keywords*—**Aqueous two-phase system, guanidine hydrochloride, partition coefficient, phase diagram.

I. Introduction

MIXING two or more incompatible polymers or a polymer and a structuring salt in aqueous conditions generally forms an aqueous two-phase system (ATPS), which the percentage of water in both phases being 75-90% (v/v) [1]. These systems present a gentle, scalable and efficient procedure for separation of various biological materials such as recombinant proteins and enzymes [2]-[5].

Inclusion body refolding processes are poised to play a major role in the production of recombinant proteins. One step of general strategy used to recover active protein from inclusion bodies is solubilization of the aggregated protein with denaturant such as guanidine hydrochloride and urea [6]. Many articles have been written about applying guanidine hydrochloride in aqueous two-phase system for the initial recovery step, but little has been published about the complex problem of how the guanidine hydrochloride effects on the phase diagram behavior and determining partition coefficient the guanidine hydrochloride in these systems. Aqueous two-phase systems, based on polyethylene glycol and sodium sulfate, have earlier been successfully used in the presence of urea for the recovery of active insulin like growth factor (IGF-1) from inclusion bodies [7]. Recovery of active protein in phase systems containing PEG and a chaotropic salt such as guanidine hydrochloride has also been shown to be possible [8].

Novel bioseparation research based on aqueous two-phase systems needs to focus on determining phase diagrams, partition coefficients and other thermodynamic data for the design of industrial-scale process [1]. A comprehensive review of the early experimental liquid–liquid equilibria (LLE) of the aqueous two-phase systems containing two different kinds of polymers or a polymer and a salt have been reported by Albertsson [2] and Walter et al. [3], but the knowledge of phase systems containing chaotropic compounds is very limited [9], Rämsch et al. [9],[10], determined phase diagrams of poly(ethylene glycol)(PEG)/ sodium sulfate/ urea/ water and PEG/ dextran T-500(DEX)/ phosphate buffer/water at different concentration of urea and different PEG molecular weights. Guanidine hydrochloride is preferred due to the problem that urea solutions may contain and spontaneously produce cyanate [11], which can carbamylate the amino groups of the protein [12]. In addition, inclusion body solubilization by urea is pH dependent and optimum pH conditions must be determined for each protein [12].

At the present study, the effect the guanidine hydrochloride on phase behavior of PEG4000/ phosphate/ guanidine hydrochloride/ water at different guanidine hydrochloride concentrations and pH was investigated. Furthermore the partition coefficient of guanidine hydrochloride and parameters that affect it, e.g., pH and PEG/Salt (%w/w) ratio was studied. These data would be useful to increasing the knowledge of aqueous two-phase separation process and improving the yield of protein refolding.

II. Experimental Section

A. Materials

Polyethylene glycol, with a mass average 4000, di-potassium hydrogen phosphate and sodium di-hydrogen phosphate were of analytical grade (Merck) and were used without further purification. Guanidine hydrochloride was purchased from Sigma-Aldrich. Distilled water was used in all experiments.

B. Preparation of the Aqueous Two-Phase Systems

Biphasic systems were prepared by a mixture of PEG 4000 and phosphate salt solution at required pH. The pH of the salt solution was adjusted by mixing appropriate ratio of sodium di hydrogen phosphate and di potassium hydrogen phosphate. In

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this work for preparation of basic biphasic system the experimental data reported by Haghtalab et al. [13] were used. For each of the mentioned systems four samples including 2.5 %, 5%, 7.5% and 10 % (w/w) of guanidine hydrochloride were arranged.

All components were added into a graduated 15 ml test tube as a dry powder or as stock solution at constant pH and temperature (298.15k) , resulting in a 10 g system. The pH values of the solutions were measured precisely with a pH meter of JENWAY 3345 model.

In order to speed up phase separation the resulted solution was mixed by inverting the test tube upside down for 2 min and was centrifuges at 2400 rpm for 10 min. then the tubes were placed in a room temperature for 2 h; the solution reach to equilibrium and the samples of the top and bottom phase were carefully withdrawn, with care being taken to leave a layer of solution at least 0.2 cm thick above the interface.

C. Measurement of Salt Concentration

The analysis methods for salt concentrations (K_2HPO_4, NaH_2PO_4) were determined by using atomic absorption spectroscopy (AAS), shimatsu AA-6300 model.

D. Measurement of Guanidine Hydrochloride Concentration

The concentration of guanidine hydrochloride was determined by conduct meter at 298.15K using a JENWAY 4510 model. Since the conductivity of phase samples depends on both guanidine hydrochloride and salt concentration, but is independed on PEG concentration, calibration plots of conductivity versus guanidine hydrochloride concentration were prepared for deferent concentration of salt.

E. Measurement of PEG Concentration

The concentration of PEG was determined by refractive index measurements at 298.15K using an ATAGO-DTM1 model (Fig. 1). Since the refractive index of phase samples depends on PEG, guanidine hydrochloride, and salt concentration, calibration plots of refractive index versus polymer concentration were prepared for deferent concentrations of salt and guanidine hydrochloride. An example of calibration plot for PEG-K_2HPO_4-water system was shown in Fig. 2.

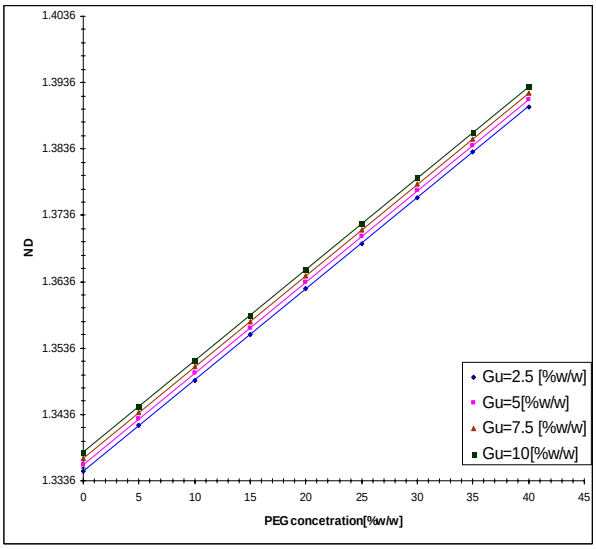

Fig. 1 Refractive index calibration curves for PEG (4000) - K_2HPO_4-water at 298.15K. (salt=2.5g)

III. Results and Discussion

In these systems opposing components was found, considering the lyotropic series H_2PO^{4-} and K^+ are so-called structure-making salts, while guanidine hydrochloride is described as structure breaking agent. The combination of these two competing components on phase separation is very interesting and cannot be predicted.

For measuring the concentration of components in bottom and top phases of aqueous two-phase systems standard calibration curves should be built.

The PEG concentration has no any effect on the calibration curve of guanidine hydrochloride concentration in PEG/ guanidine hydrochloride/ phosphate/ water system (Fig. 1). In the results, the calibration curves were related to the concentrations of salt and guanidine hydrochloride. A change of PEG concentration in the range of 10- 50% (w/w) results 0.05 ms (milli-Siemens) in conductivity of the solution which is negligible relative to 20 ms changes related to changes in GuHCl concentration.

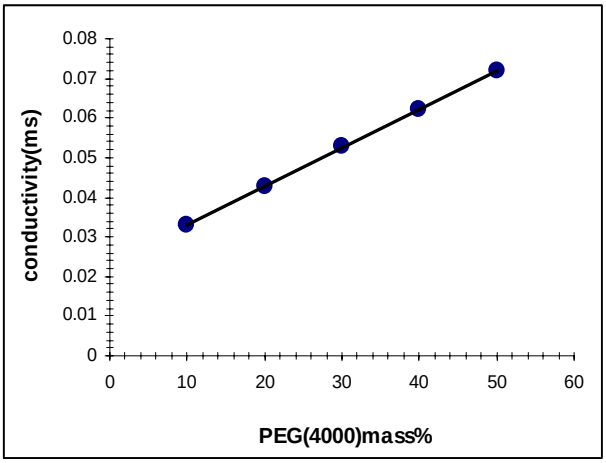

Fig. 2 Conductivity calibration curves for PEG (4000)-water at 298.15K

A typical calibration curve for guanidine hydrochloride/ K_2HPO_4/ water system was shown in Fig. 3. The guanidine concentrations varied between 0 and 50 (% w/w) and the concentration of phosphate were varied between 10 and 30 (%w/w).

To increase the knowledge of aqueous two-phase systems containing denaturants, the phase diagrams of a broad range of systems based on PEG4000/ phosphate/ water at 298.15K and different pH (7.2, 9.1, and 10.8) in the presence of guanidine hydrochloride were determined.

The experimental data of binodal for these systems at pH=7.2 are reported in table 1 and plotted in Figs. 4-6.

Figs. 4-6 show the influence of the guanidine hydrochloride concentrations on the binodal curve of PEG4000/ phosphate aqueous two-phase system at constant pH. The amount of phase components necessary to affect separation of the phases increases only slightly with increasing guanidine concentrations.

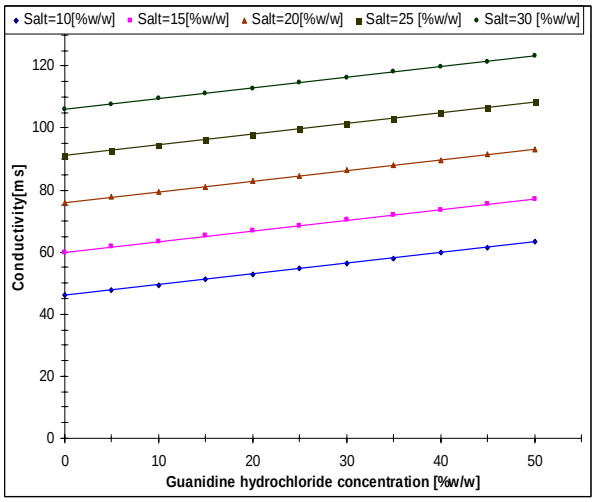

Fig. 3 Conductivity calibration curves for PEG (4000)-K_2HPO_4-water at 298.15K in different guanidine and salt concentrations

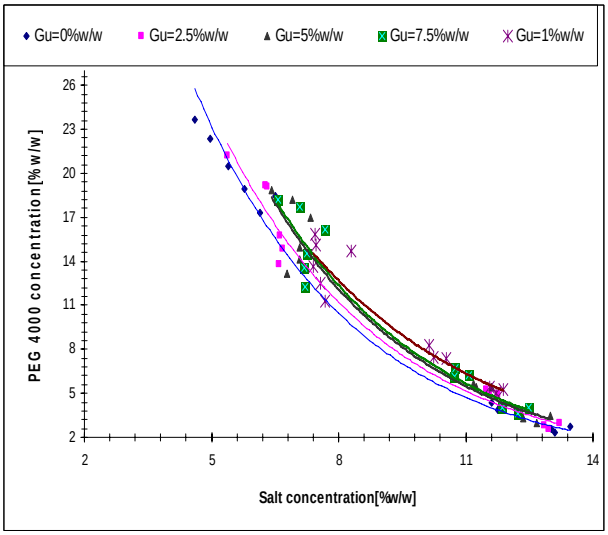

Fig. 4 Binodal of PEG4000/ phosphate aqueous two-phase systems at 298.15 and pH=7.2 at different guanidine hydrochloride concentrations

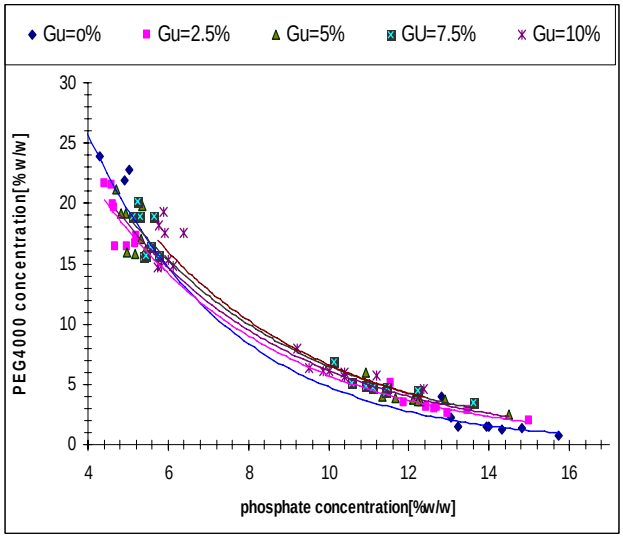

Fig. 5 Binodal of PEG4000/ phosphate aqueous two-phase systems at 298.15 and pH=9.1 at different guanidine hydrochloride concentrations

TABLE I
BINODAL CURVE DATA OF THE PEG4000/ PHOSPHATE/ GUANIDINE HYDROCHLORIDE/ WATER SYSTEM AT 298.15 AND PH=7.2

Salt TOP (%w/w)	PEG 4000 TOP (%w/w)	Salt BOT (%w/w)	PEG 4000 BOT (%w/w)	Salt FEED (%w/w)	PEG 4000 FEED (%w/w)	GuHCl FEED (%w/w)
4.61	23.64	13.47	2.72	10.85	8.87	0
4.97	22.36	13.02	2.6	10.32	9.54	0
6.51	18.42	11.82	3.85	9.47	9.57	0
5.39	20.51	13.1	2.3	9.42	10.9	0
6.14	17.27	11.61	4.35	8.97	10.9	0
5.78	18.92	11.75	3.85	8.49	12.27	0
5.38	21.23	13.2	3	8.38	8.93	2.5
6.26	19.17	12.85	2.78	10.25	11.31	2.5
6.6	13.78	11.75	4.95	10.29	5.67	2.5
6.31	19.11	12.96	2.54	9.53	11.21	2.5
6.67	14.86	11.48	5.28	8.58	9.42	2.5
6.62	15.75	11.57	4.93	8.13	8.56	2.5
6.92	18.2	12.99	3.45	10.9	8.75	5
6.41	18.84	12.35	3.32	10.19	9.34	5
6.78	13.17	11.6	5.46	10.81	9.74	5
7.34	17	12.68	3	9.36	10.86	5
7.09	14.17	11.18	5.66	10.02	11.1	5
7.08	14.91	11.24	5.42	9.09	12.54	5
7.08	17.68	12.48	4	10.75	8.57	7.5
6.57	18.19	11.86	4.06	10.11	9.3	7.5
7.21	12.3	11.08	6.26	10.6	9.78	7.5
7.69	16.16	12.23	3.65	9.34	10.82	7.5
7.19	13.55	10.75	6.7	9.88	11.15	7.5
7.25	14.55	10.73	6.15	8.94	12.63	7.5
7.45	15.87	11.89	5.24	10.72	8.65	10

7.47	15.15	11.56	5.42	10.04	9.41	10
7.68	11.26	10.55	7.39	10.48	9.74	10
8.3	14.7	11.71	4.99	9.31	10.84	10
7.58	12.47	10.14	8.29	9.75	11.11	10
7.41	13.64	10.24	7.45	8.44	12.65	10

From these figures it can be observed that the binodal was displaced toward higher concentrations with increasing guanidine hydrochloride concentrations and the compositions of the polymer-rich phase (and hence the tie- line length) behave in a slightly shorter manner. This effect was also seen for urea [14]. It could be related to structure breaking effect of guanidine hydrochloride on the water or the preferential interaction with aqueous interface [15]. But, it seems that further investigations are necessary to prove if guanidine hydrochloride really changes the water structure. PEG is a hydrophilic polymer, and due to the effects of guanidine on water structure, the depletion force between PEG- GuHCl-water increased. As a result, the ratio of PEG in two phases was changed and the two phase region shrinks.

Increasing the pH resulted in a slightly larger two-phase area. With increasing of pH, the charge of salt ions changed and for a constant PEG concentration lower concentration of salts is needed.

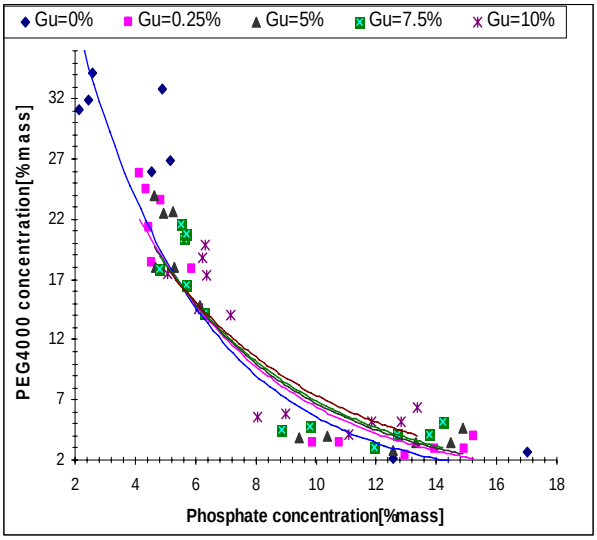

Fig. 6 Binodal of PEG4000/ phosphate aqueous two-phase systems at 298.15 and pH=10.8 at different guanidine hydrochloride concentrations

The partition coefficient guanidine hydrochloride (K) in two-phase system defined as:

$$K= [G]_{top}/ [G]_{bottom} \quad (1)$$

Where $[G]_{top}$ and $[G]_{bottom}$ are equilibrium concentrations of the guanidine hydrochloride in the top and bottom phases, respectively.

The tie lines length (TLL) is defined as:

$$TLL\ (\%) = \{[(PEG\%)_{top} - (PEG\%)_{bot}]^2 + [(salt\%)_{top} - (salt\%)_{bot}]^2\}^{0.5} \quad (2)$$

The tie line length, partition coefficient of guanidine hydrochloride and PEG/ Salt (%w/w) ratio for pH=7.2 are given in Table II.

Diagram of partitioning coefficient of guanidine hydrochloride vs. tie line length for different concentration of guanidine has shown in Fig. 7. It can be seen that for a constant TLL, partition coefficient of guanidine hydrochloride increased with increasing guanidine concentration. Also the partitioning coefficient increased with increasing TLL at constant guanidine hydrochloride concentration. The slope of portioning coefficient of guanidine vs. tie line length decreased with increasing GuHCl concentration. Also for a constant concentration of guanidine hydrochloride, the partitioning coefficient of guanidine decreased with increasing pH.

TABLE II
EXPERIMENTAL DATA FOR PEG4000/ PHOSPHATE/ WATER/ GUANIDINE HYDROCHLORIDE

[PEG/Salt]{%w/w]	TLL(%w/w)	K	GuHCl Feed(%w/w)
1.07	11.33	0.95	2.5
1.1	16.32	1	2.5
0.55	5.68	0.92	2.5
1.18	18.45	1.03	2.5
1.1	11.03	0.96	2.5
1.05	12.19	0.98	2.5
0.8	14.61	0.98	5
0.92	14.32	1.05	5
0.9	4.35	0.93	5
1.16	18.75	1.05	5
1.11	5.66	0.99	5
1.38	7.44	1.01	5
0.8	13.46	1.01	7.5
0.92	12.08	1.09	7.5
0.92	3.55	0.95	7.5
1.16	16	1.06	7.5
1.13	4.53	1.03	7.5
1.41	6.72	1.04	7.5
0.81	8.85	1.07	10
0.94	9.1	1.05	10
0.93	2.35	1.04	10
1.16	12.53	1.12	10
1.14	2.85	1.05	10
1.5	5.5	1.06	10

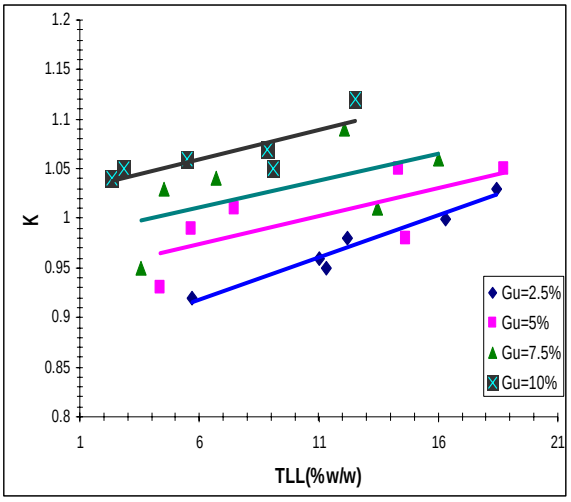

Fig. 7 Effect of tie line length and guanidine hydrochloride concentration on partition coefficient of guanidine hydrochloride at pH = 7.2

REFERENCES

[1] P. C. Singh and R. K. Singh, "Choosing an appropriate bioseparation technique", *Trend in Food science & Technology*, vol.7, 1996.

[2] P. Å. Albartsson, *Partition of cell particles and macromolecules*, 3rd Ed. New York: Willy, 1986.

[3] H. Walter, D. Brooks, and D. fisher, *Partitioning in aqueous two-phase systems*, New York: Academic Press, 1985.

[4] F. Rahimpour., F. Feyzi., S. Maghsodi and R. H. Kaul, "Purification of plasmid DNA with polymer-Salt aqueous two-phase system: optimization using response surface methodology", *Biotechnol. Bioeng.*, vol. 95, 2006, pp 627-637.

[5] F. Rahimpour., G. Mamo., F. Feyzi., S. Maghsoudi and R. H. Kaul, "Optimization refolding and recovery of active recombinant bacillus halodurans xylanase in polymer-salt aqueous two-phase system using surface response analysis", *J. Chromat. A*, vol. 1141, 2007, pp. 32-40.

[6] E. D. B. Clark, "Protein refolding for industrial processes", *Current Opinion in Biotechnol.*, 2001, vol.12, pp. 202-207.

[7] R.A. Hart, P.M. Lester, D.H. Reifsnyder, J.R. Ogez, S.E. Builder, "Large scale, In Situ isolation of periplasmic IGF-I from E. coli." *Bio./Technol.* 12 (1994) 1113.

[8] D. Forciniti, "Protein refolding using aqueous two-phase systems" *J. Chromatogr. A* 668 (1994) 95–99.

[9] C. Rämsch, L. B. Kleinelanghorst, E. A. Knieps, J. hommes and M.-R. Kula, "Aqueous two-phase system containing urea; influence on phase separation and stabilization of protein conformation by phase components", *Bitechnol. Prog.*, Vol. 15, 1999, pp. 493-499.

[10] C. Rämsch, L. B. Kleinelanghorst, E. A. Knieps., J. Thommes, and M. -R. Kula, "Aqueous two-phase systems containing urea: influence of protein Structure on Protein Partitioning", *Biotechnol. Bioeng.*, vol. 69, 2000, pp. 83-90.

[11] P. Hagel., J. J. T. Gerding., W. Fieggen and H. Bloemendal, "Cyanate formation in solutions of urea, I. Calculation of cyanate concentrations at different temperature and pH", *Biochim. Biophys. Acta*, vol. 243, 1971, pp. 366-373.

[12] J. Cejka, Z. Vodražka and J. Salgk, "Carbamylation of globin in electrophoresis and chromatography in the presence of urea", *Biochim. Biophys. Acta*, vol. 154, 1968, pp. 589-591.

[13] A. Haghtalab and Mokhtarani, B.; "The new experimental data and a new thermodynamic model based on group contribution for correlation liquid- liquid equilibria in aqueous two-phase systems of PEG and (K2HPO4 or Na2SO4)". *Fluid Phase Equilibria*, vol. 215, 2004, pp. 151-161.

[14] D. Estapé and U. Rinas, "Optimized procedures for purification and solubilization of basic fibroblast growth factor inclusion bodies", *Biotechnol. Tech.*, vol.10, 1996, pp. 481-484.

[15] O. Annuziata., N. Asherie., A. Lomakin.,J. Pande., O. Ogun and G. B. benedek, "Effect of polyethylene glycol on the liquid-liquid phase transition in aqueous protein solutions" *PNAS*, vol.99, no.22, 2002, pp. 14165-14170.