SCIENCE CHINA
Chemistry

• ARTICLES •

July 2010 Vol.53 No.7: 1554–1560
doi: 10.1007/s11426-010-4025-9

Selective separation of protein and saccharides by ionic liquids aqueous two-phase systems

PEI YuanChao, LI ZhiYong, LIU Li, WANG JianJi* & WANG HuiYong

Key Laboratory of Green Chemical Media and Reactions, Ministry of Education; School of Chemistry and Environmental Science, Henan Normal University, Xinxiang 453007, China

Received March 17, 2010; accepted May 31, 2010

In the present work, it was found that aqueous solution of a hydrophilic ionic liquid (IL), 1-butyl-3-methylimidazolium dicyanamide ($[C_4mim][N(CN)_2]$), could be separated into an aqueous two-phase system (ATPS) by inorganic salts such as K_2HPO_4 and K_3PO_4. The top phase is IL-rich, while the bottom phase is phosphate-rich. It was shown that 82.7%–100% bovine serum albumin (BSA) could be enriched into the top phase and almost quantitative saccharides (arabinose, glucose, sucrose, raffinose or dextran) were preferentially extracted into the bottom phase in a single-step extraction by $[C_4mim][N(CN)_2] + K_2HPO_4$ ATPS. The extraction efficiency of BSA from the aqueous saccharide solutions was influenced by the molecular structure of saccharides. The conductivity, dynamic light scattering (DLS) and transmission electron microscopy (TEM) were combined to investigate the microstructure of the IL-rich top phase and the possible mechanism for the selective separation. It is suggested that the formation of the IL aggregate and the IL aggregate-BSA complex plays a significant role in the separation of BSA from aqueous saccharide solutions. This is the first example for the selective separation by ILs-based ATPSs. It is expected that these findings would have potential applications in bio-analysis, separation, and IL recycle.

selective separation, bovine serum albumin, saccharide, ionic liquid, aqueous two-phase system

1 Introduction

As a new kind of green solvents, ILs have a variety of unique properties, such as negligible vapor pressure, non-flammability, thermal and chemical stability, tunable chemical structures and physical properties, and strong solubilization for organic and inorganic compounds [1–3]. This makes ILs attractive as novel extractants for various metal ions and organic compounds including amino acids, proteins and other biomaterials [4–6]. However, in the extraction process, hydrophobic ILs have to be used in order to create the IL/water biphasic systems. Compared with hydrophilic ILs, the hydrophobic ILs are more expensive, and their number is limited. Possible denaturation would take place during the extraction/separation process of biomacromolecules, such as proteins and DNAs, by such simple IL/water biphasic systems.

ATPSs were usually formed as a result of mutual incompatibility of two polymers or one polymer and one salt above a certain concentration. Because the bulk of their both phases consists of water and no organic solvents are used [7], ATPSs offer many advantages such as the short processing time, low energy consumption, relative reliability in scale-up and a biocompatible environment [8], and have been recognized as an economical and efficient downstream processing method. Thus they have been widely used for recovery and purification of various biomolecules such as proteins [9] and nucleic acids [10].

In 2003, Rogers *et al*. [11] reported for the first time that ATPSs could also be formed by hydrophilic ILs with a certain concentration of inorganic salts, and might have sig-

*Corresponding author (email: jwang@henannu.edu.cn)

nificant applications in separation. Since then, IL-based ATPSs have been used to separate/enrich amino acids, proteins and other biochemicals from aqueous solutions [12–19]. However, these studies were almost limited to the simple extraction process of the individual compounds from one phase to another phase. To the best of our knowledge, no selective separation of protein from its mixture with saccharides by IL-based ATPSs was reported previously, although such information is of prime significance for the separation of biochemicals. Therefore, the development of IL-based ATPSs for selective separation is highly desired.

In this paper, we report our results for the selective separation of protein from aqueous saccharides by using $[C_4mim][N(CN)_2] + K_2HPO_4$ ATPSs. For this purpose, BSA was used as a model protein, and arabinose, glucose, sucrose, raffinose and dextran were selected to include monosaccharide, oligosaccharide and polysaccharide. It was found that BSA was transferred into the IL-rich top phase and the saccharides were preferentially extracted into the phosphate-rich bottom phase. Factors affecting the selective separation process were investigated. Furthermore, conductivity, DLS and TEM were used to study the microstructure of the IL-rich top phase and the possible mechanism for the selective separation. It is suggested that aggregation of the IL in the IL-rich top phase plays a significant role in the separation of BSA from aqueous saccharide solutions, and the formation of the IL aggregate-protein complex is the driving force for the selective separation. Our findings suggest that $[C_4mim][N(CN)_2] + K_2HPO_4$ ATPSs have the potential to offer new possibilities for the selective separation of proteins and saccharides.

2 Experimental

2.1 Materials

1-Methylimidazole (C.P.) and 1-chlorobutane (98%) were purchased from Linhai Kaile Chem. Co., and Alfa Aesar, respectively. They were distilled twice before use. $NaN(CN)_2$, K_3PO_4, K_2HPO_4, K_2CO_3, H_2SO_4 and phenol from Shanghai Chem. Co., bovine serum albumin and raffinose pentahydrate from Beijing Jingkehongda Biotechnol. Co., Ltd., and arabinose, glucose, sucrose and dextran (Mr~1500) from Sigma were used without further purification. These chemicals were all of analytical grade unless otherwise stated.

2.2 Synthesis of ILs

$[C_4mim]Cl$. This IL was synthesized according to the procedure described previously [20], and will be used for the preparation of $[C_4mim][N(CN)_2]$. The final product was obtained as a white wax-like solid at room temperature. Yield: 87.4%; ^{1}H NMR: δ = 9.446 (s, 1H), 7.840 (d, 1H), 7.765 (d, 1H), 4.177 (t, 2H), 3.856 (s, 3H), 1.747 (m, 2H), 1.233 (m, 2H), and 0.873 (m, 3H) ppm.

$[C_4mim][N(CN)_2]$. For the synthesis of $[C_4mim][N(CN)_2]$, the procedure reported by Liu *et al*. [21] was closely followed. The final product was obtained as a colorless liquid. Yield: 89.4%; ^{1}H NMR: δ = 9.099 (s, 1H), 7.758 (d, 1H), 7.694 (d, 1H), 4.162 (t, 2H), 3.849 (s, 3H), 1.769 (m, 2H), 1.265 (m, 2H), and 0.902 (m, 3H) ppm; ^{13}C NMR: δ = 135.79, 122.77, 121.50, 118.37, 48.38, 35.08, 30.90, 18.35, and 12.30 ppm.

2.3 Preparation of binodal curves and tie lines for ATPSs

The phase diagram was prepared by turbid titration method [11]. A few grams of concentrated IL solutions were weighed into a test tube. A salt solution of known mass fraction was then added dropwise to the test tube until the mixture became turbid or cloudy. The mass of the salt solution added was recorded and the composition of this mixture was calculated. Addition of a few drops of water made the mixture clear again, and the above procedure was repeated to obtain sufficient data to construct a liquid-liquid equilibrium binodal curve. The concentration of the phase components was determined by weight quantification of all the components added within an uncertainty of $\pm 10^{-4}$ g. The tie lines, which describe the concentrations of the IL and salt in the two phases, were measured with the procedure outlined in our previous work [22].

2.4 Separation of the protein from sugars

Separation systems were prepared by mixing a given amount of the IL, K_2HPO_4, and the aqueous solutions of BSA and saccharides into a graduated glass tube. Another mixture with the same phase components but without BSA and saccharides was prepared as a blank to avoid interference. The procedure was similar to that described in one of our previous papers [18].

The concentrations of BSA in both phases were determined by measuring the absorbance at 280 nm using a Shanghai 752N UV-vis spectrophotometer. A mass balance check was carried out between the initial mass of protein and the amounts in the top and bottom phases on the basis of equilibrium composition. The error in the mass balance was within 3%. The extraction efficiency of BSA into the IL-rich phase was calculated by using eq. (1):

$$E_p = C_t V_t / \left(C_t V_t + C_b V_b \right) \quad (1)$$

where C_t and C_b are equilibrium concentrations of the partitioned BSA in the IL-rich top phase and the phosphate-rich bottom phase, V_t and V_b stand for the volume of the top phase and the bottom phase, respectively.

The concentrations of saccharides in the salt-rich bottom

phase were determined by a colorimetric method [23]. The procedure was as follows: 0.2 mL of aqueous saccharide solution was pipetted into a colorimetric tube and 1 mL of 5% phenol was added. Then 5 mL of concentrated sulfuric acid was added rapidly, with the stream of acid directed against the liquid surface rather than against the side of the test tube in order to obtain good mixing. The tubes were allowed to stand for 10 min, and then they were shaken and placed in a water bath at 25–30 °C for 20 min before readings were taken. The absorbance of the characteristic yellow-orange color was measured at 490 nm using a Shanghai 752N UV-vis spectrophotometer. A mass balance check was carried out between the initial mass of saccharides and the amounts in the top and bottom phases on the basis of equilibrium composition. The error in the mass balance was within 5%. The extraction efficiency of saccharides into the phosphate-rich phase was calculated by using eq. (2):

$$E_s = C_b V_b / C_0 V_0 \quad (2)$$

where C_b and V_b are equilibrium concentrations of the partitioned saccharides in the salt-rich bottom phase and the volume of this phase, respectively, C_0 and V_0 stand for the concentrations and volume of aqueous saccharides added into the test tube before the separation process.

2.5 Measurement of conductivity of the aqueous [C_4mim][$N(CN)_2$] solution

Conductivity measurements were performed at 298.15 K by a Wayne-Kerr 6430B Auto Balance Bridge with a resolution of 1×10^{-5} μS cm^{-1}. A Shanghai DJS-1 electrode was used for the conductivity measurements. The conductance cell was equipped with a water circulating jacketed glass vessel, and the temperature was controlled within ± 0.01 K with a HAAKE V26 thermostat (Thermo Electron, Germany). The cell was calibrated with aqueous KCl solutions at different concentrations, and a cell constant of 1.0474 cm^{-1} was determined. The uncertainty in conductance measurements was about ± 0.02%.

2.6 Determination of size distribution by DLS

The DLS measurements were carried out using a Nano-ZS 90 laser light scattering photometer (Malvern, U.K.). Light of $\lambda = 633$ nm from a solid-state He-Ne laser (4.0 mW) was used as the incident beam. All sample solutions were filtered through a 0.22 μm hydrophilic PVDF membrane filter, and then stayed at room temperature overnight. All measurements were performed at 298.15 K and at 90° scattering angle.

2.7 The morphology of IL aggregates and IL-protein complexes

The morphology of the IL aggregates and the IL aggregate-protein complexes in the IL-rich upper phase was investigated using TEM. A carbon Formvar-coated copper grid (200 mesh) was laid on one drop of the sample solution for 5 min, and the excess liquid was removed by filter paper. One drop of uranyl acetate solution (1%) was dripped on the copper grid as the staining agent. The excess liquid was removed by filter paper. After being dried, the samples were imaged under a Hitachi H-800 microscope operating at 200 keV.

3 Results and discussion

3.1 Phase diagram of ATPSs

Phase diagram data are required for the design of aqueous two-phase extraction process. The phase diagrams determined at 298.15 K for the [C_4mim][$N(CN)_2$]/salt systems are shown in Figures 1 and 2. Figure 1 shows the binodal curves for the ATPSs of [C_4mim][$N(CN)_2$] with K_2HPO_4 and K_3PO_4, and Figure 2 illustrates the tie-line data determined for the [C_4mim][$N(CN)_2$] + K_2HPO_4 ATPSs. Figure 1 shows the binodal curves are more close to the origin for the salts from K_2HPO_4 to K_3PO_4, thus requiring lower concentrations for phase separation. Therefore, the ability of these salts for phase separation follows the order: K_3PO_4 > K_2HPO_4. Similar trends have been reported for the ATPSs of [C_4mim]Cl, [C_4mim]Br, [C_6mim]Br or [C_8mim]Br ILs [22, 24]. Considering the fact that these salts have the same cation but different anions, the phase-forming ability of them was determined by the nature of anions. The above result implies that the higher the valence of the anions, the lower the concentration required for the formation of a two-phase system. This may be interpreted by their different salting-out effects: the anions with higher valence have stronger salting-out effect than those with lower valence because the former anions hydrate more water molecules

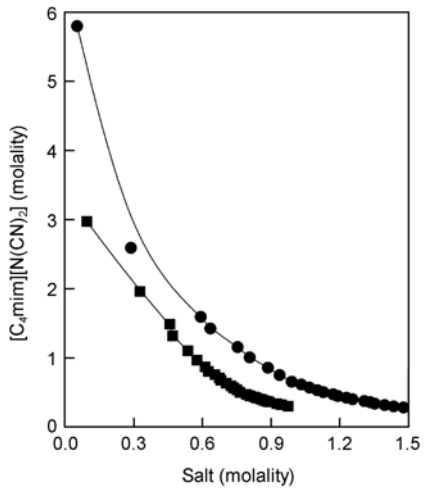

Figure 1 Binodal curves of [C_4mim][$N(CN)_2$] + salts ATPSs at 298.15 K. ■, K_3PO_4; ●, K_2HPO_4.

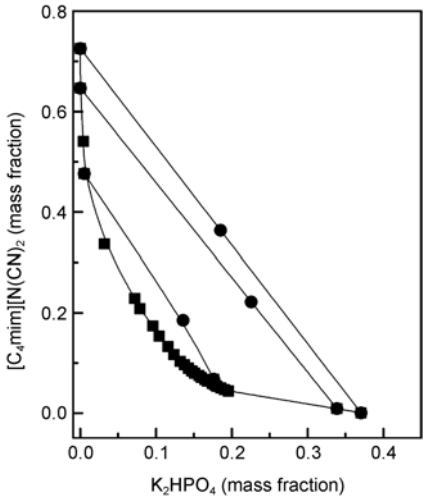

Figure 2 Phase diagram of $[C_4mim][N(CN)_2]$ + K_2HPO_4 ATPSs at 298.15 K. ■, binodal; ●, total composition.

than the latter ones, thus decreasing the amount of water available to hydrate ILs. This trend is in agreement with the order of Gibbs free energy of hydration of the anions [25, 26]: $PO_4^{3-}(\Delta G_{hyd} = -2765\ kJ\ mol^{-1}) > HPO_4^{2-}$ ($\Delta G_{hyd} = -1125$ kJ mol^{-1}). This order follows the Hofmeister series for the strength of the kosmotropic salts. Because K_2HPO_4 could result in an appropriate pH range for the gentle extraction of BSA, this salt was chosen for further study.

3.2 Partition of BSA and saccharides in the $[C_4mim][N(CN)_2] + K_2HPO_4$ ATPSs

The extraction efficiencies for BSA and saccharides are shown in Figure 3. It shows that about 82.7%–100.7% BSA was enriched into the top phase, while almost all the saccharides were preferentially extracted into the bottom phase

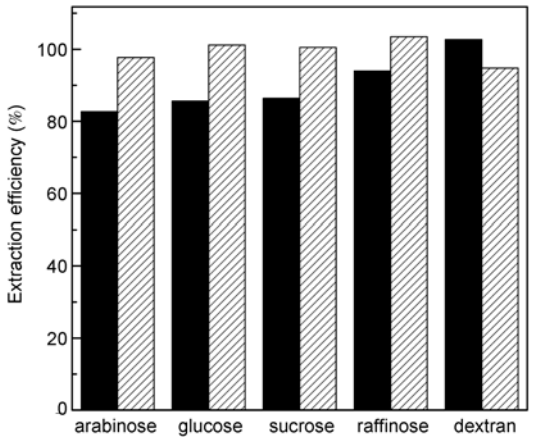

Figure 3 The extraction efficiencies of BSA and saccharides in $[C_4mim][N(CN)_2] + K_2HPO_4$ ATPSs at 298.15 K. ■, BSA; ▨, saccharides. The test concentrations of BSA and saccharides were all at 4 mg mL^{-1}; 0.6 g IL, 0.6 g K_2HPO_4 and 1.5 mL H_2O were added for the formation of ATPSs.

by the IL-based ATPSs. To the best of our knowledge, this is the first example for the selective separation of protein from saccharides by ATPSs.

The presence of high concentrations of K_2HPO_4 increased the hydrophobicity of the bottom phase [27], in which the solubility of protein was significantly decreased as a result of competition for water molecules between a large number of salt ions and the protein. Therefore, introduction of a salt into the aqueous protein solution promotes the transfer of the protein from the bottom phase to the top phase [18]. The extraction efficiency of BSA from the aqueous saccharide solutions was influenced by the saccharide structures. As indicated in Figure 3, the extraction efficiency of BSA from the aqueous saccharide solutions increases from arabinose, glucose, sucrose, raffinose to dextran. The reason for this phenomenon will be explained in the following sections.

Bridges *et al*. classified $[C_4mim]Cl$ as a chaotropic IL [24]. The anion $N(CN)_2^-$ is more weakly hydrated than Cl^-, indicating that $N(CN)_2^-$ is more chaotropic than Cl^- [28, 29]. When the kosmotropic ions were added in aqueous IL solutions, the water molecules close to these ions would be in electro-constriction state because of their water-structuring nature. Therefore, the hydrogen bond network of water was enhanced in this phase, which required more energy for cavity formation around the bulky organic $[C_4mim]^+$ cation [11]. At a certain concentration of kosmotropic salt, an aqueous solution of IL with the more hydrophobic cation and the less water-structuring anion was separated. Because the solutions of sugars are kosmotropic [30], the presence of sugar in the $[C_4mim][N(CN)_2] + K_2HPO_4$ ATPSs will assist the kosmotropic HPO_4^{2-} anion to separate from the phase, although only adding saccharides into the aqueous $[C_4mim][N(CN)_2]$ solution cannot form the ATPSs.

The OH groups on sugar molecules could destroy the natural hydrogen bond network of water by the formation of new hydrogen bond networks with water molecules. Thus, sugars could be called structure breakers [31, 32]. The more hydroxyl groups on sugar molecules fit into water structure, the more they break the water network, and the more kosmotropic the saccharides are. The form of hydrogen bond between water and sugar molecules will reduce the number of "free" water in the bottom phase of the ATPSs, and force more BSA or chaotropic IL to transfer in the top phase than the systems without sugars. Therefore, the number of hydroxyl groups on sugar molecules is another factor affecting the distribution behavior of BSA in the ATPS. The number of hydroxyl groups increases in the order of arabinose < glucose < sucrose < raffinose < dextran. On the basis of this, it is not difficult to understand the different extraction efficiencies of BSA in the presence of different sugars. After the separation, almost all the saccharides were extracted into the bottom phase. As mentioned above, HPO_4^{2-} and saccharides are all kosmotropic. They could destroy the natural hydrogen bond network of water, and form new hy-

drogen bond networks with water. The interactions between water and sugar are strong enough to enrich the sugars in the bottom phase.

3.3 The microscopic structure of the IL-rich top phase and the possible mechanism for the selective separation

Examination of the microscopic structure of the IL-based ATPSs is essential for a better understanding of the separation process. In the $[C_4mim][N(CN)_2] + K_2HPO_4$ ATPSs, the top phase was composed of the IL, water and a small amount of salt, and the bottom phase was composed of the salt, water and a small amount of IL. The molar fraction of water was always greater than 0.8 in the two phases. The IL concentration was about 3.0 mol L^{-1} in the IL-rich top phase, and the salt concentration was approximately 2.7 mol L^{-1} in the salt-rich bottom phase. In the top phase, such a high IL concentration would lead to a significant aggregation between cations of the IL because of their amphiphilic characteristics [33]. In order to determine the existing state of $[C_4mim][N(CN)_2]$ in the top phase of the ATPSs, aqueous solutions of $[C_4mim][N(CN)_2]$ were used to simulate the top phase, and their conductivities were measured at 298.15 K. Figure 4 shows the concentration dependence of the conductivity for aqueous $[C_4mim][N(CN)_2]$ solutions. The conductivities exhibit typical behavior with two linear fragments, and the concentration at which the two linear fragments intersect is assigned to the critical aggregation concentration (CAC). The CAC value of $[C_4mim][N(CN)_2]$ was found to be about 1.0 mol kg^{-1}. As the IL concentration is greater than the CAC, the IL aggregates would be formed. Therefore, it is appropriate to state that aggregates of $[C_4mim][N(CN)_2]$ were formed in the top phase of the ATPS investigated in the present work.

The size distribution of the IL aggregates was determined by DLS at different concentrations. As shown in Figure 5, the aqueous $[C_4mim][N(CN)_2]$ solution is not microscopically homogeneous, but polydisperse. The aggregate size increases with increasing concentration of the IL. The possible reason is that with increasing IL concentration, more IL monomers in aqueous solutions entered into the aggregates, resulting in the formation of the large-sized aggregates. Figure 6 shows the DLS results of the aqueous BSA, aqueous IL and aqueous IL+BSA solutions. It is noteworthy that after the addition of BSA into the aqueous IL solution, a new aggregate appears at about 550 nm. The size of this aggregate is greater than that of the BSA aggregate, and its intensity is greater than the IL aggregate. This suggests that an IL aggregate-protein complex was formed at 550 nm. Similar phenomena have been reported for surfactant and protein systems [34, 35]. We examined the morphology of the IL aggregate and the IL aggregate-protein complex by TEM, as shown in Figure 7. The result shows that the IL aggregate and the IL aggregate-protein complex are all spherical. Therefore, it is appropriate to state that the formation of the IL aggregate and the IL aggregate-protein complex

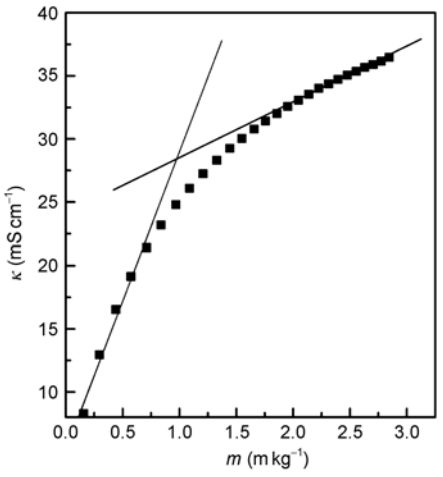

Figure 4 Concentration dependence of the conductivity for $[C_4mim][N(CN)_2]$ in aqueous solutions at 298.15 K.

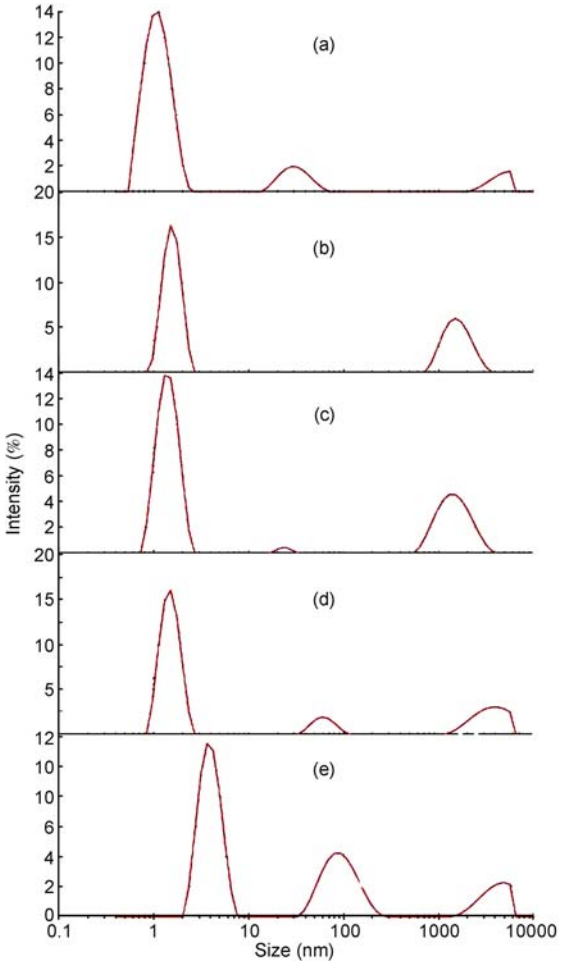

Figure 5 The size distribution of the $[C_4mim][N(CN)_2]$ aggregates in aqueous solution at different IL concentrations. (a) 1.5; (b) 2.0; (c) 2.5; (d) 3.0; (e) 3.5 mol L^{-1}.

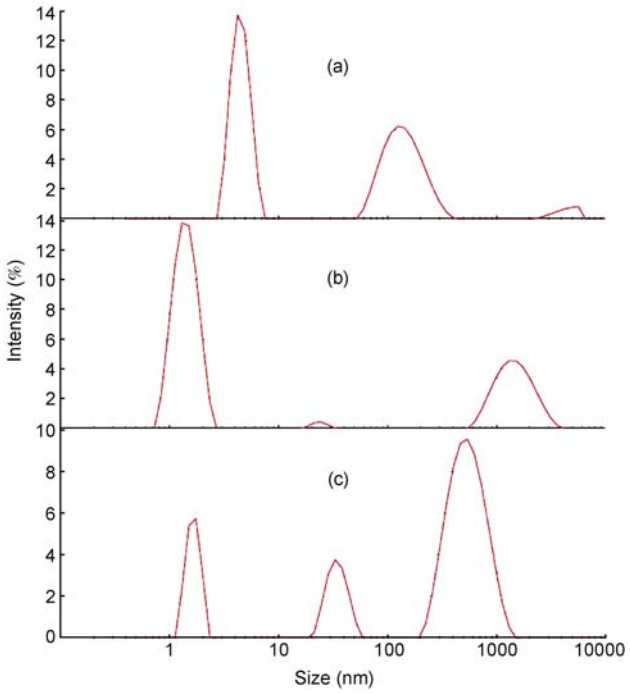

Figure 6 The size distribution of the aggregates for BSA (1.23 g L^{-1}), the IL (2.5 mol L^{-1}) and the IL (2.5 mol L^{-1})+ BSA (1.23 g L^{-1}). (a) BSA; (b) IL; (c) IL+BSA.

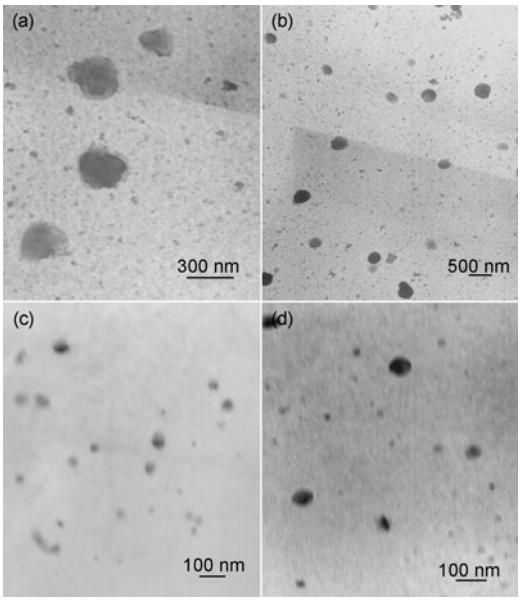

Figure 7 TEM images of the aggregates of the IL (2.5 mol L^{-1}) with and without BSA (1.23 g L^{-1}). (a,b) without BSA; (c,d) with BSA.

is responsible for the effectively selective separation.

4 Conclusions

In this article, $[C_4mim][N(CN)_2]$ + salt aqueous two-phase system was demonstrated to be effective for the selective separation of BSA from aqueous saccharides. It is found that about 82.7%–100% BSA was enriched into the top phase, while almost quantitative saccharides were preferentially extracted into the bottom phase of the ATPSs. The extraction efficiency of BSA from the aqueous saccharide solutions increases with increasing number of hydroxyl groups of the saccharides.

The conductivity, DLS and TEM studies suggest that the IL aggregates and IL aggregate-protein complexes were formed in the IL-rich top phase. The aggregates are spherical, and their size increases with increasing IL concentrations. The aggregation of the IL plays a significant role in the separation of BSA from aqueous saccharide solutions. The formation of the IL aggregate-protein complexes is the driving force for the selective separation. These results highlight new possibilities of IL-based ATPSs for the selective separation of biomolecules.

This work was supported by the National Natural Science Foundation of China (20873036) and the Innovation Scientists and Technicians Troop Construction Projects of Henan Province (084200510015).

1 Welton T. Room-temperature ionic liquids. Solvents for synthesis and catalysis. *Chem Rev*, 1999, 99: 2071–2084
2 Wasserscheid P, Keim W. Ionic liquids-new “solutions” for transition metal catalysis. *Angew Chem Int Ed*, 2000, 39: 3772–3789
3 Dupont J, de Souza RF, Suarez PAZ. Ionic liquid (molten salt) phase organometallic catalysis. *Chem Rev*, 2002, 102: 3667–3692
4 Pandey S. Analytical applications of room-temperature ionic liquids: A review of recent efforts. *Anal Chim Acta*, 2006, 556: 38–45
5 Han XX, Armstrong DW. Ionic liquids in separations. *Acc Chem Res*, 2007, 40: 1079–1086
6 Shimojo KNK, Kamiya N, Goto M. Crown ether-mediated extraction and functional conversion of cytochrome c in ionic liquids. *Biomacromolecules*, 2006, 7: 2–5
7 Baskir JN, Hatton TA, Suter UW. Protein partitioning in two-phase aqueous polymer systems. *Biotechnol Bioeng*, 1989, 34: 541–558
8 Hatti-Kaul R. *Aqueous Two-Phase Systems: A General Overview in Aqueous Two-Phase Systems: Methods and Protocols*. New Jersey: Human Press, 2000. 1–10
9 Andrews BA, Schmidt AS, Asenjo JA. Correlation for the partition behavior of proteins in aqueous two-phase systems: Effect of surface hydrophobicity and charge. *Biotechno Bioeng*, 2005, 90: 380–390
10 Cole KD. Purification of plasmid and high molecular mass DNA using PEG-salt two phase extraction. *Biotechniques*, 1991, 11: 18–24
11 Gutowski KE, Broker GA, Willauer HD, Huddleston JG, Swatloski RP, Holbrey JD, Rogers RD. Controlling the aqueous miscibility of ionic liquids: Aqueous biphasic systems of water-miscible ionic liquids and water-structuring salts for recycle, metathesis, and separations. *J Am Chem Soc*, 2003, 125: 6632–6633
12 He CY, Li SH, Liu HW, Li K, Liu F. Extraction of testosterone and epitestosterone in human urine using aqueous two-phase systems of ionic liquid and salt. *J Chromatogr A*, 2005, 1082: 143–149
13 Neves CMSS, Ventura SPM, Freire MG, Marrucho IM, Coutinho JAP. Evaluation of cation influence on the formation and extraction capability of ionic-liquid-based aqueous biphasic systems. *J Phys Chem B*, 2009, 113: 5194–5199
14 Li SH, He CY, Liu HW, Li K, Liu F. Ionic liquid-based aqueous two-phase system, a sample pretreatment procedure prior to high-performance liquid chromatography of opium alkaloids. *J Chromatogr B*, 2005, 826: 58–62
15 Ventura SPM, Neves CMSS, Freire MG, Marrucho IM, Oliveira J,

Coutinho JAP. Evaluation of anion influence on the formation and extraction capability of ionic-liquid-based aqueous biphasic systems. *J Phys Chem B*, 2009, 113: 9304–9310
16 Jiang YY, Xia HS, Guo C, Mahmood I, Liu HZ. Phenomena and mechanism for separation and recovery of penicillin in ionic liquids aqueous solution. *Ind Eng Chem Res*, 2007, 46: 6303–6312
17 Du Z, Yu YL, Wang JH. Extraction of proteins from biological fluids by use of an ionic liquid/aqueous two-phase system. *Chem Eur J*, 2007, 13: 2130–2137
18 Pei YC, Wang JJ, Wu K, Xuan XP, Lu XJ. Ionic liquid-based aqueous two-phase extraction of selected proteins. *Sep Purif Tech*, 2009, 64: 288–295
19 Cao Q, Quan L, He CY, Li N, Li K, Liu F. Partition of horseradish peroxidase with maintained activity in aqueous biphasic system based on ionic liquid. *Talanta*, 2008, 77: 160–165
20 Huddleston JG, Visser AE, Reichert WM, Willauer HD, Broker GA, Rogers RD. Characterization and comparison of hydrophilic and hydrophobic room temperature ionic liquids incorporating the imidazolium cation. *Green Chem*, 2001, 3: 156–164
21 Liu QB, Janssen MHA, Rantwijk FV, Sheldon RA. Room-temperature ionic liquids that dissolve carbohydrates in high concentrations. *Green Chem*, 2005, 7: 39–42
22 Pei YC, Wang JJ, Liu L, Wu K, Zhao Y. Liquid-liquid equilibria of aqueous biphasic systems containing selected imidazolium ionic liquids and salts. *J Chem Eng Data*, 2007, 52: 2026–2031
23 Michel D, Gilles KA, Hamilton JK, Rebers PA, Fred S. Colorimetric method for determination of sugars and related substances. *Anal Chem*, 1956, 28: 350–356
24 Bridges NJ, Gutowski KE, Rogers RD. Investigation of aqueous biphasic systems formed from solutions of chaotropic salts with kosmotropic salts (salt-salt ABS). *Green Chem*, 2007, 9: 177–183
25 Zafarani-Moattar MT, Hamzehzadeh S. Liquid-liquid equilibria of aqueous two-phase systems containing 1-butyl-3-methylimidazolium bromide and potassium phosphate or dipotassium hydrogen phosphate at 298.15 K. *J Chem Eng Data*, 2007, 52: 1686–1692
26 Marcus Y. Thermodynamics of solvation of ions. Part 5.—Gibbs free energy of hydration at 298.15 K. *J Chem Soc Faraday Trans*, 1991, 87: 2995–2999
27 Zasllavsky BY, Miheeva LM, Mesteckina NM, Rogozhin SV. Physicochemical factors governing partition behaviour of solutes and particles in aqueous polymeric biphasic systems. II. Effect of ionic composition on the hydration properties of the phases. *J Chromatogr A*, 1982, 253: 149–158
28 Constantinescu D, Herrmann C, Weingärtner H. Protein denaturation by ionic liquids and the Hofmeister series: a case study of aqueous solutions of Ribonuclease A. *Angew Chem Int Ed*, 2007, 46: 1–4
29 Zhao H. Effect of ions and other compatible solutes on enzyme activity, and its implication for biocatalysis using ionic liquids. *J Mol Catal B*, 2005, 37: 16–25
30 Wu B, Zhang YM, Wang HP. Phase behavior for ternary systems composed of ionic liquid + saccharides + water. *J Phys Chem B*, 2008, 112: 6426–6429
31 Galema SA, Engberts JB, Blandamer MJ. Stereochemical aspects of the hydration of carbohydrates. Kinetic medium effects of monosaccharides on a water-catalyzed hydrolysis reaction. *J Am Chem Soc*, 1990, 112: 9665–9666
32 Chang SC, Weaver MJ. *In situ* infrared spectroscopy at single-crystal metal electrodes: An emerging link between electrochemical and ultrahigh-vacuum surface science. *J Phys Chem*, 1991, 95: 5391–5400
33 Wang HY, Wang JJ, Zhang SB, Xuan XP. Structural effects of anions and cations on the aggregation behavior of ionic liquids in aqueous solutions. *J Phys Chem B*, 2008, 112: 16682–16689
34 Michalis A, Panagiotis S, Elefteria N, Herve R, Apostolos KR, Georgios T. Molecular size determination of a membrane protein in surfactants by light scattering. *Biochimica et Biophysica Acta*, 2003, 1615: 69–76
35 Spelzini D, Peleteiro J, Pico G, Farruggia B. Polyethyleneglycol-pepsin interaction and its relationship with protein partitioning in aqueous two-phase systems. *Colloids and Surfaces B*, 2008, 67: 151–156