Analytical Methods

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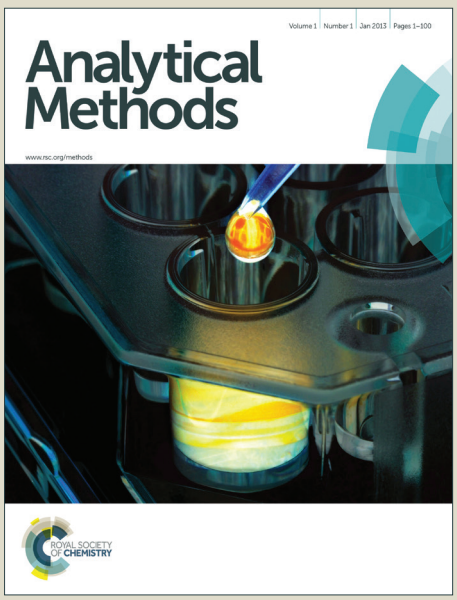

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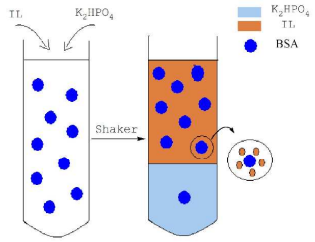

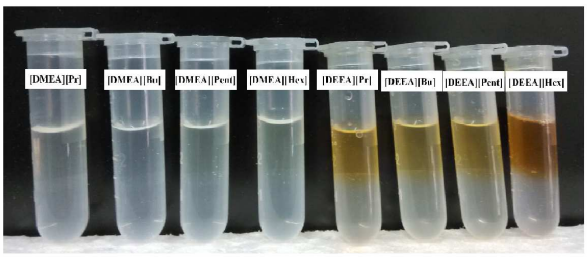

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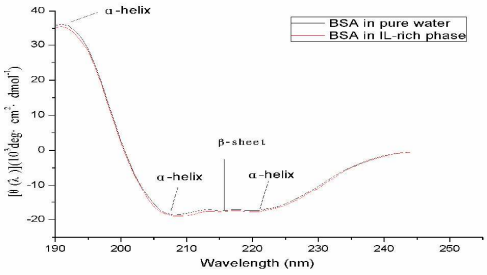

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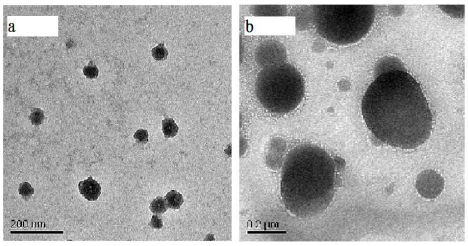

the TEM of before ILs-ATPS (a) and after (b)

Partition of proteins with extraction in aqueous two-phase system by hydroxyl ammonium-based ionic liquid

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Abstract

A series of hydroxyl ammonium ionic liquids (ILs) have been designed and synthesized. An ionic liquid aqueous two-phase system (IL-ATPS) based on N, N-dimethylethanolamine propionate ([DMEA] [Pr]) ionic liquid was studied firstly for the extraction of proteins. Based on the single-factor experiment, an initial serial investigative test was used to identify the optimal conditions. Five factors and four levels orthogonal experiments were used to verify the optimum extraction conditions. The results showed that under the optimum conditions, the extraction efficiency could reach up to 99.50%. The RSD of extraction efficiencies in precision experiment, repeatability experiment and stability experiment were 0.3% (n=5), 1.1% (n=5) and 1.5% (n=5), respectively. The conformation of the proteins was not affected after extraction into the IL-rich phase in terms of the determination by UV–vis and FT-IR spectra. According to the determination of conductivity, dynamic light scattering (DLS), and transmission electron microscope (TEM) images, the microstructure of the IL-rich phase and the possible mechanism for the extraction were investigated. Hydrogen bonding interaction, salt out effect and the aggregation phenomenon played important roles in the extraction. The circular dichroism (CD) spectral experiment analysis indicated that the secondary structures of the protein were unchanged after extract. Based on these findings, it is suggested that the method of hydroxyl ammonium-based ILs- ATPs have the potential to offer new possibility in the extraction of bio-analysis.

Key Words: Hydroxyl ammonium-based ionic liquids, Aqueous two-phase system, Protein, Extraction, Circular dichroism.

1. Introduction

Proteins are components of living organism that play critical roles in phenomena like metabolism, gene expression, signal transduction, cellular and extracellular structures, and the like [1]. So, it is particularly necessary to prepare the pure proteins. Proteins always exist within complex mixtures, so centrifugation has been needed to remove bacterial cells in the initial separation step before further purification can be carried out [2,3]. In the past years, the traditional protein purification methods include ion exchange gel filtration chromatography, affinity chromatography [4], membrane separation, ammonium sulfate precipitation, salting out and electrophoresis [5], but they are expensive and not suitable for mass production [6]. Furthermore, due to the poor stability, proteins in the conditions of acids, alkali or heating are easily denatured [7].

Walden was the first one who described the compound ethylammonium nitrate as ionic liquids (ILs) in 1941 [8]. ILs have many fascinating properties including wide liquid ranges, low volatilities (negligible vapor pressure), good thermal stabilities, electrolytic conductivity, wide range of viscosities, adjustable miscibility, reusability, nonflammability and so on [9]. ILs have been widely applied in liquid–liquid extraction of various compounds, such as metal ions [10-13], small organic molecules [14], acids [15], phenols and amines [16,17] and biological compounds, such as proteins [18-23] and DNA [9] .Therefore, ILs have received attention in recent years as “greener solvents” and “designer solvents” [24].

With the beginning of the new century, a new generation of water stable ILs with

anions like [BF_4], [PF_6], [Tf_2N] or halides were synthesized [25]. Because the strong associative ability of anions within ILs can dissolve proteins, it can lead to the conformational change that can cause destruction [26, 27]. To solve this problem, some water added into the ILs can maintain the activity of the enzyme [28, 29].

The ionic liquid aqueous two-phase system (IL-ATPS) was reported by Rogers et al [30] for the first time in 2003. IL-ATPS is widely used in the biological separation and purification fields. The aqueous two-phase system formed by inorganic salts usually include poly (ethylene glycol) (PEG) + salt and IL + salt ATPSs. In IL-ATPS, the two phases contain a certain amount of water, and this meets the requirement of the active protein in an ionic liquid solution, which provided a biocompatible environment for the moderate extraction and purification of biological substances. In the previous works, some investigators had studied the extraction of medicinal compounds or proteins by imidazolium [31] or guanidine [7] ionic liquids, but no one had learned the partition of proteins by hydroxyl ammonium ionic liquids.

Hydroxyl ammonium compounds have attracted widespread concern of the chemist and pharmacologist for the identity of cheap, various form, chemical stability, high catalytic activity and strong physiological activity. Moreover, the hydroxyl ammonium ionic liquid can be synthesized simply by one step with high yields which easy to realize industrial scale production. In recent years, some scientists had studied the applications of hydroxyl ammonium ionic liquids [32] in SO_2 absorption [33], organic synthesis [34] and CO_2 absorption [35].

The aim of this work is to investigate the partition of proteins in those systems

based on hydroxyl ammonium IL-ATPS. Such ionic liquids have the similar structure with choline-base ionic liquids which are easy degradable. The concentrations of proteins in top phases were determined by measuring the absorbance at 278 nm for bovine serum albumin (BSA) and ovalbumin (OVA), and at 404 nm for bovine hemoglobin (BHb). BSA was chosen as a model protein to investigate the effect of system parameters and extraction mechanism. Because the synthetic simplicity and low cost of the proposed eight ionic liquids, it is easy to achieve the industrial production scale.

2. Experimental

2.1 Apparatus

Ionic liquids were dried by a 101-0E Ventilated drying oven (Beijing, China) and DZF-6051vacuum drying oven (Shanghai, China). A Thermostats cultivating shaker (Shanghai, China) was used to provided a certain temperature and rotation speed in the experiment. A UV-2450 UV-Vis Spectrophotometer (SHIMADZU, Japan) was used to determine the absorbance of the sample. ^{1}H NMR spectra were measured with aVarianInova-400 NMR spectrometer (Varian, USA) at room temperature by using TMS as internal standard. FTIR spectra were recorded on a Spectrum One FTIR Spectrometer (Perkin Elmer, USA). A JEM-3010 transmission electron microscope (JEOL, Japan) was used to examine the microstructures of samples before and after extraction. A DDS-2A conductivity (Shanghai, China) and A ZS-90 dynamic light scattering (Malvem, Britain) were used for the measurement of the mass of ILs and

sample. Circular Dichroism (CD) spectra were recorded in a MOS-500 spectropolarimeter (France).

2.2 Materials and reagents

All chemicals used were of analytical grade. Diethyl ethylene diamine (DEEA) was obtained from Aladdin Chemical Reagent Co., Ltd. N, N-dimethylethanolamine (DMEA) were purchased from Tianjin Kermel Fine Chemical Research Institute. Propionic acid (Pr), butanoic acid (Bu), pentanoic acid (Pent), and hexanoic acid (Hex), were all AR grade and purchased from Sinopharm Chemical Reagent Co., Ltd. Bovine serum albumin (BSA), Bovine hemoglobin (BHb) and Ovalbumin (OVA) were all from Sinopharm Chemical Reagent Co., Ltd. Potassium phosphate dibasic anhydrous (K_2HPO_4) were purchased from Aladdin Reagents Company (Shanghai, China) and were of 98% purity. Double distilled deionized water was used throughout the experiments.

2.3 Synthesis and characterization of ILs

As we all know, hydroxyl ammonium ionic liquids were synthesized simply by neutralization of the base with the appropriate acid. According to the literature, eight kinds of ILs (as shown in Table 1). By way of example, N,N-dimethylaminoethanol (0.1 mol) dissolved in 10 ml methanol was firstly added to a dried100ml flask. The flask was equipped with a magnetic stirrer and the temperature was controlled at 298.15 K. Propionic acid (0.11mol) was then dropwise added into the flask. After addition was completed within two hours, the solution was stirred at room temperature for 24 h to complete the reaction. Finally, the product was vacuum

distilled at 50□ to remove unreacted reactants and the methanol. All the synthetic ILs structures were confirmed by ^{1}HNMR and FT-IR, which were shown in Supplementary Information Table S1 and Supplementary Information Fig S1 respectively.

2.4 Preparation of phase diagram for IL/salt aqueous two-phase systems

The phase diagram for IL/salt aqueous two-phase systems were determined by the cloud-point method [36]. A few grams of pure ionic liquid were taken into a 10 ml test tube which contained a number of water. A salt solution of known mass fraction was added dropwise to the test tube and shaken. After a certain amount of the salt solution was added, the mixture became turbid or cloudy, and the IL/salt aqueous two-phase system was formed. Record the volume of the salt solution added. Then the water was added into the system drop by drop until one further drop made the mixture clear again, and one phase occurred. The volume of the water added was recorded and the composition of this mixture was calculated. The above procedure was repeated to obtain sufficient data to construct the phase diagram. There the X axis was the mass percentage of the salt solution and the Y axis was the mass percentage of the ILs solution.

2.5 Protein distribution in Ionic liquid-based aqueous two-phase system

A certain amount of the ionic liquids, K_2HPO_4 and the solution of proteins were taken into a 10mL graduated centrifuge tube. Then the mixture was shaken vigorously for 20 min to attain equilibrium, and the temperature of the systems was controlled at 278K with a Thermostats cultivating shaker (Shanghai, China). Wait a few minutes in

order to ensure the formation of two-phase system, then record down the volumes of top phase and bottom phase. After extraction, the top phase solution was taken to determine the concentration of protein after being diluted suitable times. At the same time, the blank experiment was provided to eliminate the other influencing factors in the same condition. Determine the concentration of protein with the external standard method which was measuring the absorbance at 278nm for bovine serum albumin (BSA) and ovalbumin (OVA), and at 404 nm for bovine hemoglobin (BHb) using a UV2450 UV–vis spectrophotometer. The linearity for analyzing BSA, BHb and OVA were in the concentration ranges of 0.05–1.00 mg ml^{-1}, 0.025–0.200 $mgml^{-1}$ and 0.05–1.00 mg ml^{-1}, respectively, with correlation coefficients between 0.9998 and 0.9999. Partition coefficients（K）of the proteins between the two phases were calculated by the formula :

$$K = C_t / C_b \qquad (1)$$

Phase volume ratio (R) is defined as volume ratio of the top phase to the bottom phase:

$$R = V_t / V_b \qquad (2)$$

The extraction efficiency (E) was calculated by the following equation:

$$E = C_t V_t / (C_t V_t + C_b V_b) = KR/(1+KR) \qquad (3)$$

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

2.6 Measurement of extraction mechanism

The conformations of proteins were investigated by UV–vis spectra. BSA in pure water and in IL-rich top phase after extraction were recorded from 200 to 400 nm.

FI-IR measurements were carried out at room temperature on Perkin-Elmer FT-IR spectrometer (America). FT-IR spectra of pure BSA, pure IL, and BSA in IL-rich top phase were recorded from 4000 to 400 cm^{-1}.

Conductivity measurements were performed at 298.15 K by A DDS-2A conductivity (Shanghai, China). The conductance cell was equipped with a water circulating jacketed glass vessel and different concentrations of IL were detected. The uncertainty in conductance measurements was about ± 0.02%.

The DLS measurements were carried out using a A ZS-90 dynamic light scattering (Malvem, Britain). All measurements were performed at 298.15 K and at 90° scattering angle.

The microscopic structure of the IL-rich top phase is detected by A JEM-3010 transmission electron microscope (JEOL, Japan). A carbon Formvar-coated copper grid was laid on a filter paper. One drop of sample solution was dripped on the copper grid as the staining agent and the excess liquid was removed by filter paper. After being dried, the samples were imaged under Vacuum conditions.

2.7 Determination of the Secondary Structure of protein

The circular dichroism of the pure protein and protein in IL-rich top phase after extraction were determined with MOS-500 Circular Dichroism Spectrometer. The concentration of the protein sample was 0.1mg·ml^{-1}, cell path was 1 mm, a bandwidth of 2 nm, the range of scan was 190-250 nm and scan speed was 1 nm·s^{-1}.

The sample was scanned for three times and detected at room temperature.

3. Results and discussion

3.1 Phase diagrams of ILs

As a promising class of new solvents, phase diagram data are required for the design of aqueous two-phase extraction process and for the development of models that predict partitioning of proteins. The binodal curves data determined at 298.15 K and atmospheric pressure for the IL/K_2HPO_4 systems were shown in Fig. 1. These binodal curves can illustrate the information about the concentrations of ILs and salts required to form a two phase systems. As can be seen from these figures, the top phase is IL-rich while the bottom phase is salt-rich. As we all know, for a given salt, the closer the binodal curve is to the origin, the lower the IL concentration required for the formation of two phases. Therefore, it can be seen from Fig. 1 that the ability of the ILs for phase separation follows the order: [DMEA][Pr]<[DMEA][Bu]<[DMEA][Pent]<[DMEA][Hex]<[DEEA][Pr]<[DEEA][Bu]<[DEEA][Pent]<[DEEA][Hex]. Similar trends have been reported in Li et al which reported that hydrophobicity of the ILs could conduce to the higher phase separation capacity [37]. This indicated that [DMEA] [Pr] has the strongest hydrophilic ability above all the ILs in this paper. Considering the fact that [DMEA][Pr],[DMEA][Bu],[DMEA][Pent],[DMEA][Hex],have the same cation but different anions, phase-forming ability of these ILs was determined by the nature of anions. It is easy to see that the ability to form aqueous two-phase is proportional to the anion alkyl chain length. The possible reason is that the more alkyl chain length

the stronger hydrophobicity, thus decreasing the amount of water available to hydrate ILs. The same principle can be used why the salting-out ability is [DEEA][Pr] > [DMEA][Pr]. By the same token, cation has more impact on the ability to form aqueous two-phase than anion which can be seen from the picture.

However, K_2HPO_4 was chosen for further study in this work because it not only can configurate ATPS with ILs, but also provide a suitable pH value for the protein extraction in the following study.

3.2 Single factor experiments

3.2.1 Selection of extraction ILs and Protein

In order to determine the extraction performance, eight kinds of ionic liquids used in the ATPS have been investigated for the extraction of three proteins namely BSA, BHb and OVA. The extraction efficiencies obtained are given in Table 2 and Fig. 2. It can be seen that the different ionic liquids have different abilities to extract various proteins. As an example, the values of the extraction efficiencies for BSA change from 58.69% to 99.47%. This demonstrates that the hydroxyl ammonium-based ILs ATPSs reported here may be a novel option for the purification and separation of biomolecules. Since the [DMEA][Pr] has the highest BSA extraction rate, so the [DMEA][Pr] + K_2HPO_4 ATPSs was chosen in the following experiment.

3.2.2 Effect of the mass of ILs

The effect of the amount of added ILs in the systems in which the [DMEA][Pr]/K_2HPO_4 ATPS were used as the extraction system with 2ml (15mg /ml)

BSA and K_2HPO_4(1.5g) has been investigated on the extraction efficiency of proteins, which was illustrated in Supplementary Information Table S2 and Fig. 3a. When the IL content varied from 0.9 to 1.0 g, the extraction yield increased rapidly and the highest efficiency was 94.59%. It means that the more the ILs, the more aggregates was formed which facilitated protein extraction. Even if the IL amount increased to 2.0 g, the increase of the extraction yield is not obvious. So considering the economic reason 1.0g of ILs for the ATPS was chosen in next experiment.

3.2.3 Effect of the mass of K_2HPO_4

As an example, the effect on BSA distribution of the mass of K_2HPO_4 was studied, and the results were illustrated in Fig. 3b. As indicated in this figure, the extraction efficiency changes with the various concentrations of K_2HPO_4 over the range of 1.4-3 g. With the increase of the amount of salt, the extraction efficiency of BSA was up to 98.97%.So the phenomenon indicated that the salt out effect was the driving force for the protein extraction. However, the extraction efficiency decreases rapidly when the salt mass is greater than 2.0g. The cause maybe that with the salt concentration increasing, the more water transferred from top phase to bottle phase then leads to reduce water content of IL.As the bottle phase is highly hydrophilic than the top phase, the hydrogen bonding interaction between the surface water of protein and amino acid residue is more easier for protein to transfer into salt- rich phase. It is obvious that salt out effect and hydrogen bonding interaction act as a combined effect for protein extraction. Therefore, the salt mass was determined as 2.0 g for the next experiments.

3.2.4 Effect of the mass of BSA

In order to find the optimum content of BSA studied on the influence of extraction efficiency, different concentrations of protein was adopted and the results were illustrated in Fig. 3c. It is clear that the extraction efficiency was increased with the increasing of the mass of BSA between 5mg-10mg and when the mass of BSA was higher than 10mg, the extraction efficiency had fallen rapidly. This may be because the extraction system has a limited ability of extraction with a certain amount of ionic liquid. So with the protein increases, the extraction efficiency were continue to reduce. The equilibrium concentrations of the partitioned protein in the IL-rich top phase and the phosphate-rich bottom phase were constantly changed, so the K was also changed. According to the above result, 10 mg BSA was selected for further experiment.

3.2.5 Effect of the separation time

The effect of the extraction time in IL-based ATPS was investigated by changing the shaking time of the IL-based ATPS. 1g [DMEA][Pr] and 2g K_2HPO_4 and 2ml of 5mg/ml BSA were added into a series of the test tubes which were shaken at 200 rpm for different shaking time periods of 2, 4, 8, 10, 15, 20, 25, 30 min and the time dependence of the extraction efficiency was illustrated in Fig. 3d. It can be seen that with the increase of the shaking time the extraction yield of BSA obviously increased from 68.29% to 99.24% in the range of 2-20 min. And when separation time was increased to30 min, the extraction efficiency was keeping an extraction efficiency of almost 99.50% and reached the equilibrium. Therefore, 20 min was chosen as the

shaking time for the extraction.

3.2.6 Effect of the temperature

To further confirm the extraction temperature studied on the influence of extraction efficiency of BSA in IL-based aqueous two-phase systems, the BSA content on its distribution behavior was also studied. In light of Fig. 3e, as the temperature increased from 15 to 25 □, the extraction efficiency of the protein increased from 86.59% to 99.24%.When the temperature kept at 30 □ or higher, the extraction yield decreased correspondingly. The possible reason for this phenomenon was that the increased extraction temperature could reduce the viscosity of the ionic liquid, enhance the solvent solubility and diffusion capacity. But when the temperature continues to rise, the extraction rate was reduced. On the one hand, it means that the temperature was high to destroy the hydrogen bonding interaction between the surface water of protein and amino acid residue. On the other hand, as the temperature rises, the extraction rate is reduced resulting in a tendency for the liquid homogeneous. Through further study found that when the temperature exceeds 60 □, BSA was denaturated. So the extraction was carried out at 25 □ because of the relatively high extraction yield.

3.3 Orthogonal experiment

As a promising class of new solvents, the optimize conditions were determined through an orthogonal array experimental design L_{16}（4^5） by mass of IL (factor A), mass of K_2HPO_4 (factor B), concentration of BSA (factor C), temperature (factor D) and separation time (factor E). An L_{16}（4^5）matrix, which is an orthogonal array of five

factors and four levels was employed to assign the considered factors and levels (as shown in Table 3). K1, K2, K3 and K4 mean the average extraction efficiencies of each factor in each of the level. The bigger the difference of R is, the more influence the factor has on the extraction efficiency which R was determined by the difference value between the maximum and minimum values of K. The results show that the impact of various factors on the importance of the extraction efficiency were: A>B>E>C>D, and the optimize conditions are $A_4B_4C_2D_3E_3$ by 99.62% of the extraction efficiency.

3.4 Method validation

At the optimal conditions, the ILs-ATPS were composed by1.0 g of IL, 2.0g of K_2HPO_4 and 2 ml of 5.0 mg/ml BSA which were validated by the precision experiment, repeatability experiment and stability experiment (as shown in Supplementary Information Table S3). Apparatus precision was investigated by the analysis of the top phase of the solution of BSA for five times by UV detection. The RSD obtained was 0.3%.The result indicates that the precision of the UV–vis spectra is great. Five copies of the same sample measured respectively under the same conditions. The calculation of RSD was 1.1% (n=5) which indicate that this method has excellent repeatability. Taking a sample detected continuously in five days under the same conditions to verify the stability experiment. The result of the relative standard deviation (RSD) of extraction efficiency was1.5% (n=5), which explain that the sample is recoverable within five days.

3.5 Extraction mechanism

3.5.1 UV-Vis spectroscopy

UV-vis spectra were investigated for BSA in order to examine the conformation of proteins before and after extraction. The UV–vis spectra of BSA in pure water and in IL-rich top phase after extraction were shown in Fig. 4. It is obvious that the UV–vis spectra of BSA are similar and its maximum absorption is at the same position (at 278 nm) in pure water and in the [DMEA][Pr]-rich upper phase after extraction. This explains that there are no direct chemical bonds interactions between BSA molecules and the ILs in the extraction process which further confirmed that the spatial structure of the protein was not destroyed.

3.5.2 FT-IR spectroscopy

FT-IR spectroscopy is one of the common experimental methods recognized as useful in providing information on structure features of compounds or chemical bonds in a molecule or an interaction system, attributable to the unique energy absorption bands for specific bonding environments or interactions [38]. As is known, proteins are irregular polymers made up essentially of 20 amino acids with four levels of spatial structure. Amide is the basic unit of the peptide bond: amide□is assigned to both C=O stretching vibration and ring stretching vibrations, while amide II is assigned to C–N stretching vibrations [7]. The absorption bands most widely used as structure probes in protein FT-IR spectroscopy have been the amide I vibrations (1690 -1600cm^{-1}) and amide II stretching vibrations (1600-1500) [39]. Fig. 5 showed the FT-IR spectra of pure BSA, pure IL, and BSA in IL. It was found that the amide□ bond (at1655 cm^{-1}) and amide II band (at 1550 cm^{-1}) shown the characteristics of the

BSA. From Fig. 5B, the two characteristics of the BSA absorption bands have remained in the similar region after extraction in the IL-BSA complexity. It can be seen that the conformation of the protein was not changed in the IL phase and no new chemical bonds generated which was further confirmed the conclusion with UV–vis detection. By the way, the 3237 cm^{-1} was assigned to both N–H and O–H stretching vibration of hydroxyl ammonium ionic liquid which illustrated that the hydroxyl ammonium ionic liquids could exist in a large number of hydroxyl groups with proteins to form intermolecular hydrogen bonds. Therefore hydroxyl ammonium IL-based aqueous two-phase extraction systems have great potential applications in purification of biological macromolecules.

3.5.3 Conductivity and DLS detection.

The conductivity was measured at 298.15 K with different concentrations of IL solution. 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)[7].Fig. 6a shows the CAC value of the [DMEA][Pr] was 0.13 g/ ml which was lower than the top phase of the ATPS investigated in the present work. It is illustrated that the IL aggregates were formed with BSA in the top phase.

Fig. 6b-d shows the DLS results of the aqueous protein solution and aqueous IL+ protein solution. A new and intensity aggregation emerged in the range of 100–1000 nm, which was larger than the aggregation of the pure protein. This is because the interaction may occur between the ionic liquid and the protein which

illustrates that the formation of aggregates is one of the main driving forces in the extraction of protein by IL-ATPS. As shown in Fig.6d, another intensity aggregation emerged in the range of 1000–10000 nm in the top phase of the ATPS with BSA added which may be the formation of aggregates by the excess ionic liquids and the intensity of the aggregation is less than that shown in Fig. 6b.

3.5.4 Detection of the microscopic structure of IL-rich top phase by TEM

The microscopic structure of the IL-rich top phase is detected by transmission electron microscopy for a further understanding of the separation process. Fig. 7a shows the conformation of IL-rich top phase without protein and the spot may be the ionic liquid without too much aggregation. Fig. 7b shows the appearance of pure protein and Fig. 7c-d show the distribution of IL-rich top phase after extract protein. From the TEM images it can be seen that the IL-aggregate-protein complex was taken shape after the BSA was extracted in the top phase. The results test by TEM consistent with DLS. That is to say, the aggregation was the main driving force of protein partitioning in an ionic liquid-based aqueous two-phase system.

3.6 Analysis of the Secondary Structure of protein

Far-ultraviolet circular dichroism (CD) can reflect the secondary structure of a protein from 190 to 250 nm. The characteristic of the α-helix structure of protein shows a positive band centered at192 nm and two negative bands centered at 208 and 222 nm. The β-sheet structure of protein shows a negative band centered at 216 nm. Fig. 8 shows the circular dichroism spectra of the pure BSA in water and BSA in IL-rich phase. The main observed spectral characteristic of BSA CD curve almost kept the same. The result demonstrated that the secondary structure of the protein was

unchanged after extraction by ionic liquid. In other words, hydroxyl ammonium ionic liquid aqueous two-phase extraction systems provide a gentle environment for protein extraction.

4. Conclusions

This paper systematically investigated the extraction efficiency of protein in hydroxyl ammonium ionic liquid/K_2HPO_4 of aqueous two-phase system. In comparison with the references reported method, the greatest benefit of the present method is that the adapted extraction solvent is timesaving (just 20 min in this study), cost saving (the synthetic materials of hydroxyl ammonium-based ionic liquid are abundant-sourced and low-cost), and also easy to achieve industrial scale production. Moreover, such ionic liquids are green and environmentally friendly because they have similarities structural with choline-base ionic liquids which are easy degradable. The high extraction rate (99.50%) illustrate that the proposed method of hydroxyl ammonium ILs-based ATPs for the selective separation of protein would have potential applications in bio-analysis and bio-separation. UV-vis and FT-IR spectra were investigated for BSA in order to examine the conformation of proteins before and after extraction. The determination of conductivity, DLS and TEM determinations proved that the hydrogen bonding interaction, salt out effect and the aggregation phenomenon played important roles in the extraction. The result of CD provides useful information for analyzing the advanced structure of the protein after extraction by ionic liquid.

Acknowledgements

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529 **Table 1The structures of eight ILs**

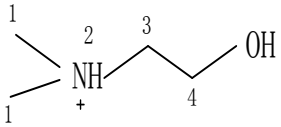
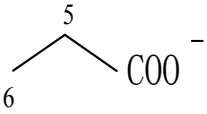
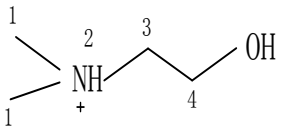
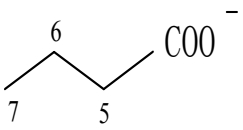
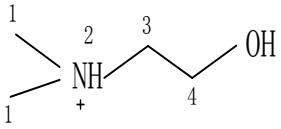
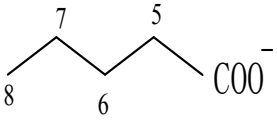
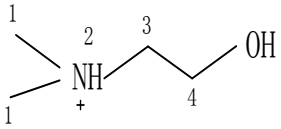
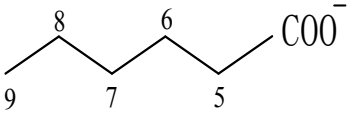
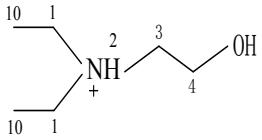
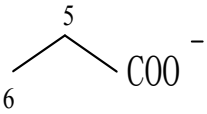
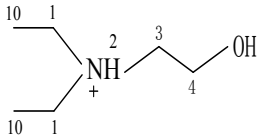
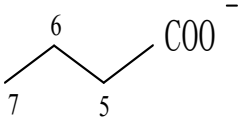
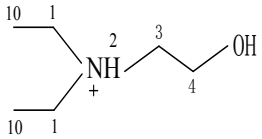
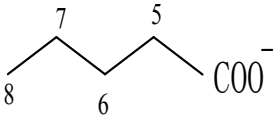
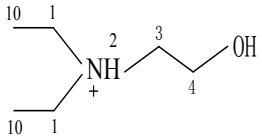
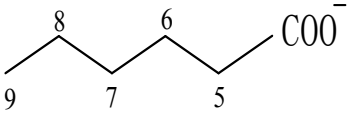
ILs	Cation	Anion
[DMEA][Pr]		
[DMEA][Bu]		
[DMEA][Pent]		
[DMEA][Hex]		
[DEEA][Pr]		
[DEEA][Bu]		
[DEEA][Pent]		
[DEEA][Hex]		

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Table 2.The extraction efficiencies of proteins by ATPSs based on ionic liquids (5.0mmol), and K_2HPO_4 (1.5g) solutions and Protein (15mg/ml, 2.0mL). (n=3)

Ionic liquid	Efficiency (%)		
	Bovine serum albumin	Bovine hemoglobin	Ovalbumin
[DMEA][Pr]	99.47	18.11	68.02
[DMEA][Bu]	95.87	20.09	63.69
[DMEA][Pent]	93.09	52.54	51.07
[DMEA][Hex]	64.80	60.64	51.69
[DEEA][Pr]	89.43	32.53	36.37
[DEEA][Bu]	88.50	47.32	38.80
[DEEA][Pent]	61.44	54.91	43.48
[DEEA][Hex]	58.70	59.01	39.17

Note: 1. E% was represented the extraction efficiency, which was calculated from equation (3).

558 **Table 3. Results of orthogonal experiment L_{16}（4^5）**

Experiment	A IL(g)	B salt(g)	C BSA(mg)	D T(□)	E t(min)	E(%)
1	1(0.8)	1(1.6)	1(5)	1(15)	1(15)	79.51
2	1	2(1.8)	2(10)	2(20)	2(20)	90.21
3	1	3(2)	3(15)	3(25)	3(25)	91.05
4	1	4(2.2)	4(20)	4(30)	4(30)	90.27
5	2(1)	1	2	3	4	94.54
6	2	2	1	4	3	97.10
7	2	3	4	1	2	97.35
8	2	4	3	2	1	95.74
9	3(1.8)	1	3	4	2	88.83
10	3	2	4	3	1	91.37
11	3	3	1	2	4	92.16
12	3	4	2	1	3	99.71
13	4(1.6)	1	4	2	3	94.91
14	4	2	3	1	4	93.05
15	4	3	2	4	1	98.57
16	4	4	1	3	2	99.86
K1	87.76	89.45	92.16	92.40	91.30	major and minor order: A>B>E>C>D optimize conditions:$A_4B_4C_2D_3E_3$
K2	91.52	92.93	95.76	93.25	93.17	
K3	93.24	94.78	92.17	94.20	95.69	
K4	94.82	96.39	93.48	93.69	92.50	
R	7.06	6.94	3.60	1.80	4.40	
Optimal level	A_4	B_4	C_2	D_3	E_3	

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Figure captions

Fig. 1. Phase diagram of IL/salt aqueous two-phase systems

Fig. 2. Effect of kinds of IL for extraction different protein

Fig. 3. Single factor effect of protein extraction: mass of IL (a), mass of K_2HPO_4 (b), mass of BSA (c), separation time (d), temperature (e).

Fig. 4.UV-vis spectra of BSA in pure water and in IL-rich top phase after extraction.

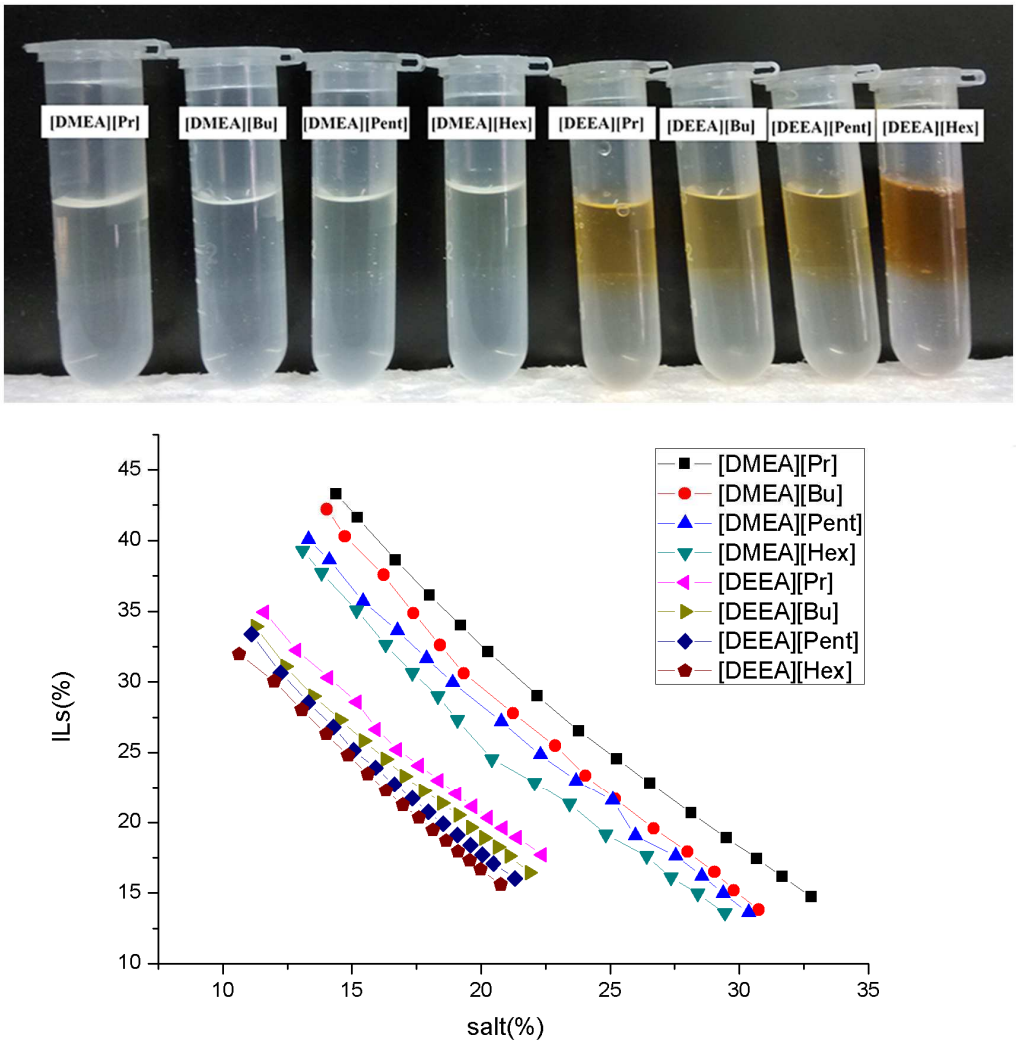
Fig. 5.FT-IR spectra of pure [DMEA][Pr], pure BSA and BSA in[DMEA][Pr]. (a) pure [DMEA][Pr]; (b) pure BSA; (c) BSA in IL-rich top phase.

Fig. 6. The aggregates size distribution:(a)Concentration dependence of the conductivity for [DMEA][Pr] in aqueous solutions at 25□. (b) IL in pure water. (c) BSA in pure water. (d) Size distribution of the top phase of the ATPS with BSA added.

Fig. 7. TEM images of the aggregates: (a) ILs-ATPS without BSA. (b) Pure BSA. (c, d) Image of the top phase of the ATPS with BSA added.

Fig. 8. Circular dichroism spectra of BSA in pure water and in IL-rich top phase after extraction.

Fig. S1 Infrared spectroscopy of ILs: a. [DMEA][Pr], b. [DMEA][Bu], c. [DMEA][Pent], d. [DMEA][Hex], e. [DEEA][Pr], f. [DEEA][Bu], g. [DEEA][Pent], h. [DEEA][Hex].



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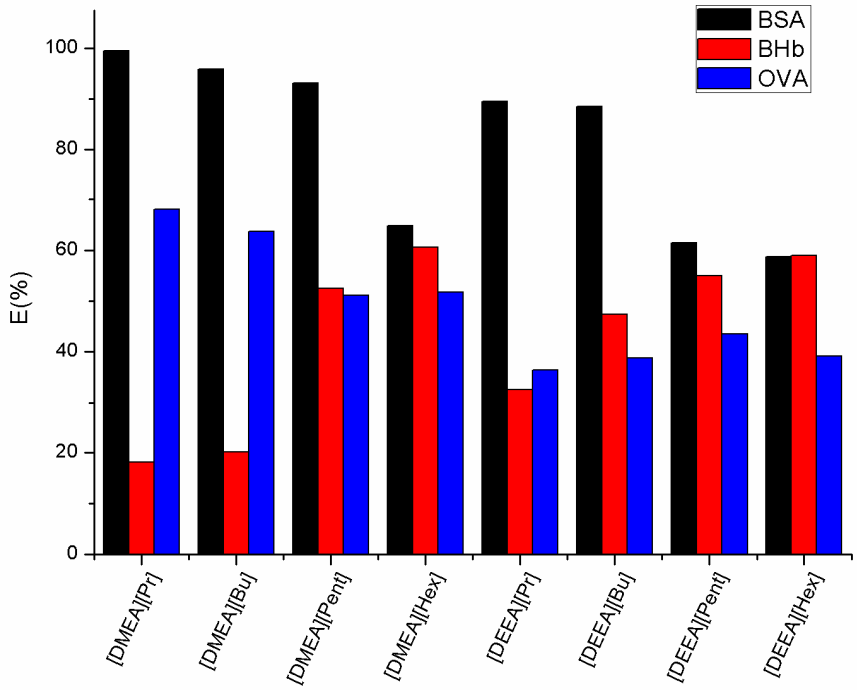

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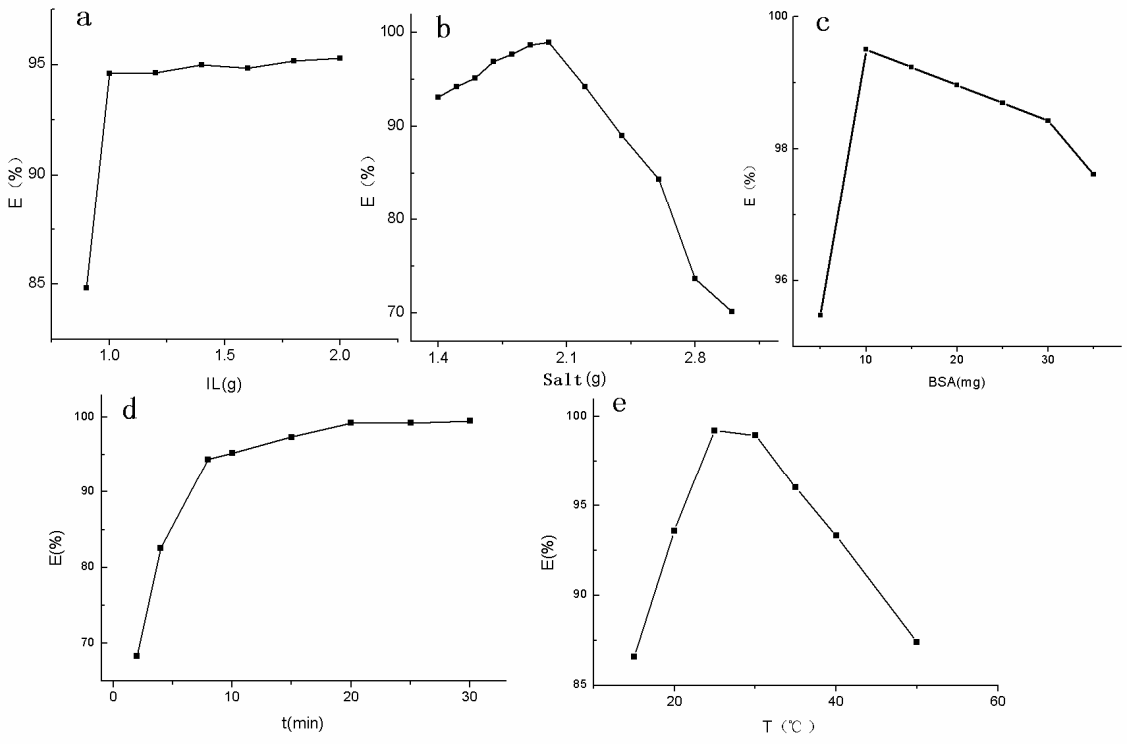

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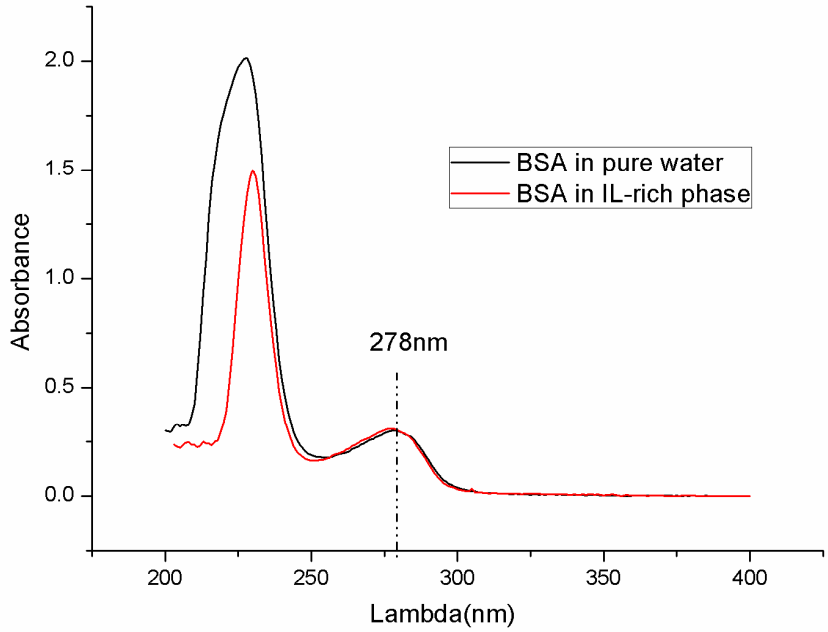

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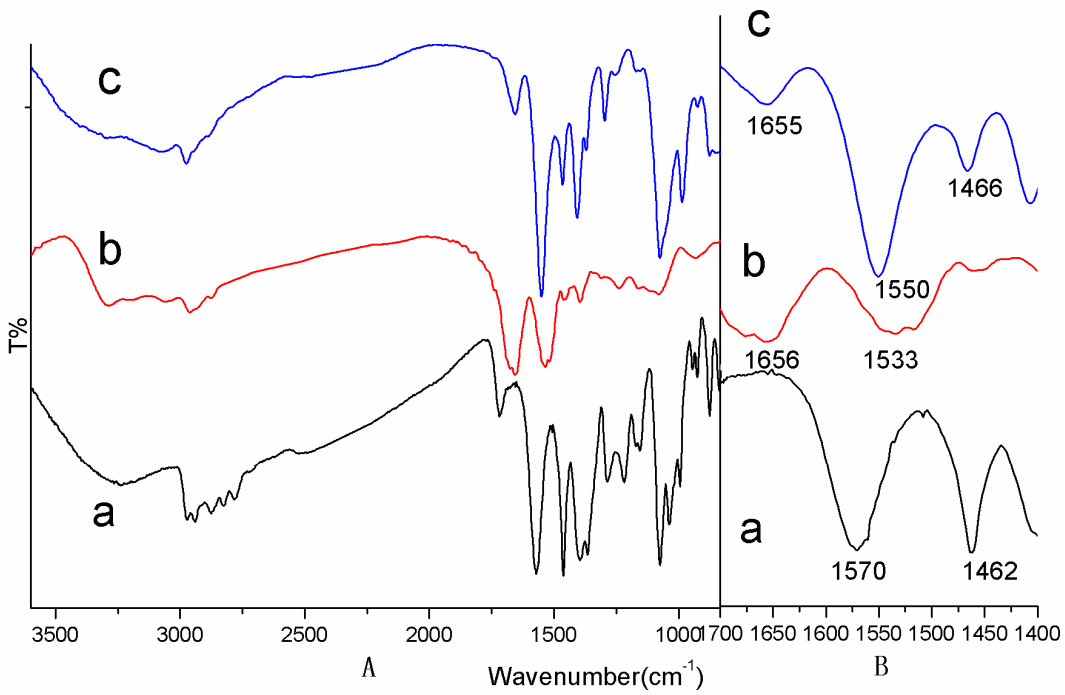

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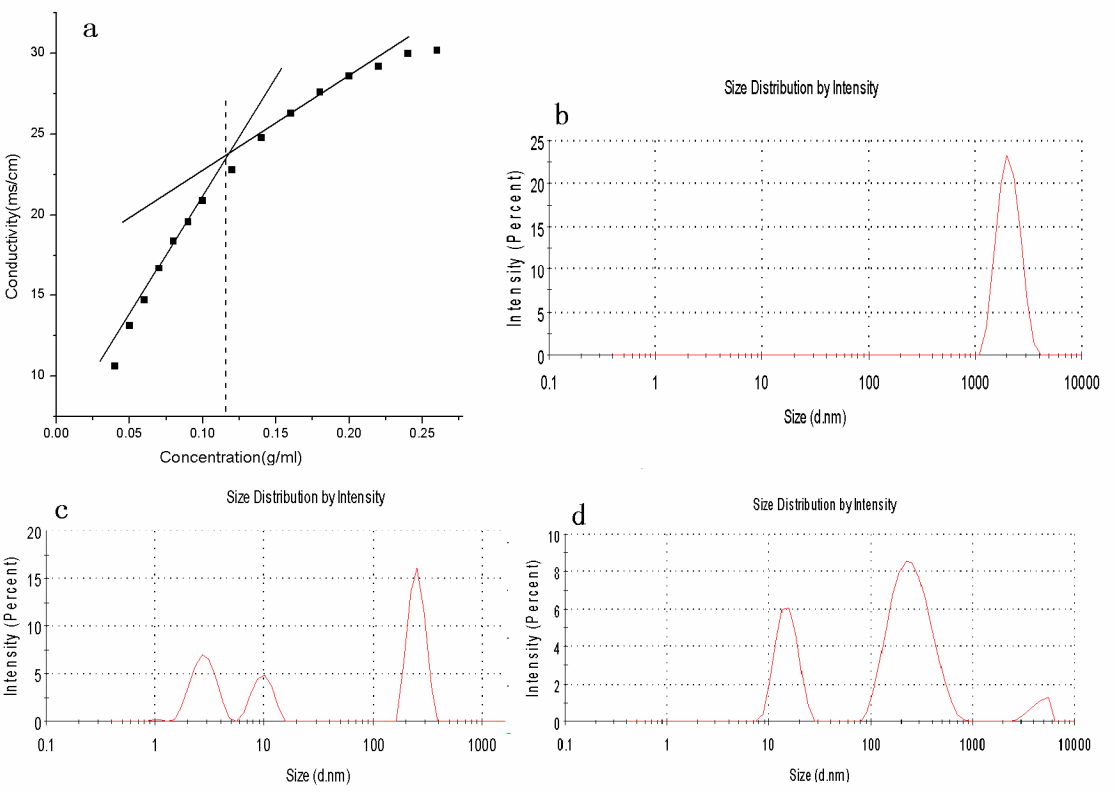

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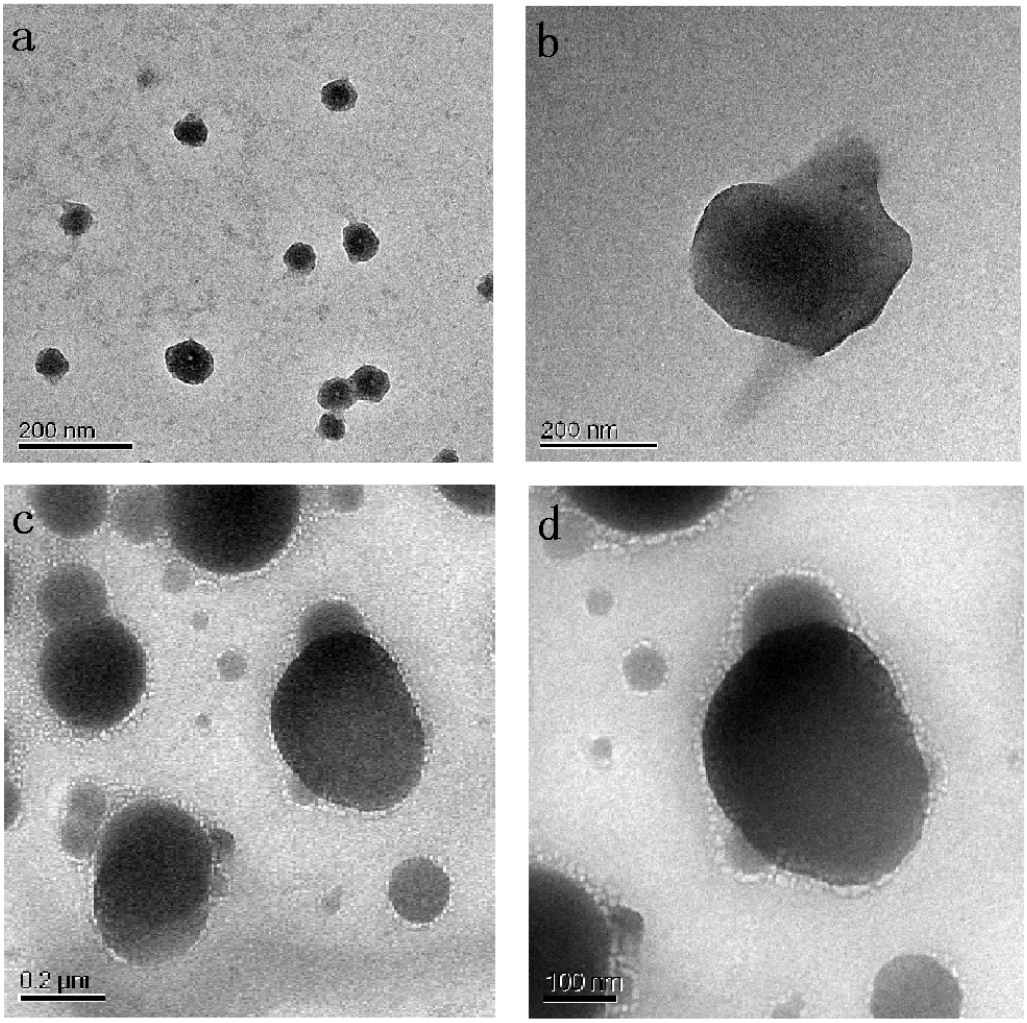

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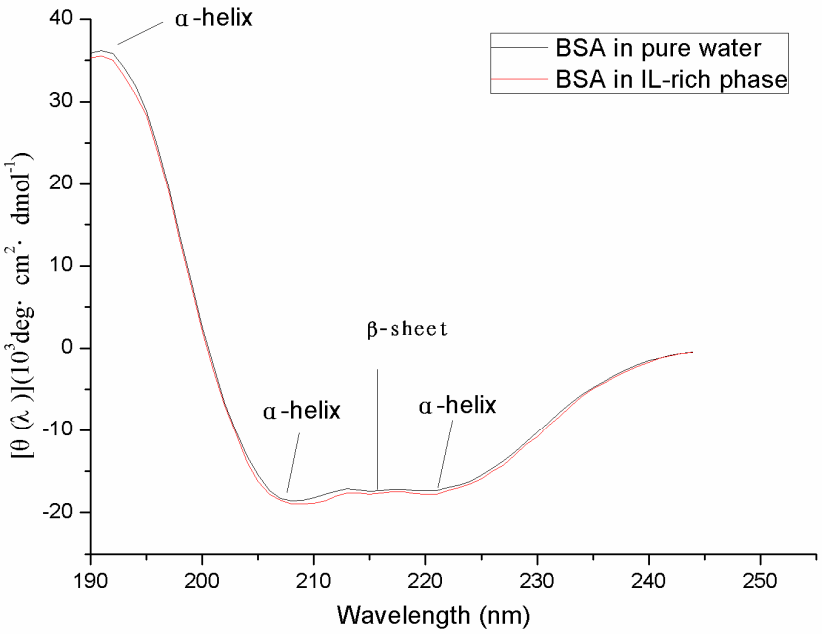

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731 Fig. 8. Circular dichroism spectra of BSA in pure water and in IL-rich top phase after

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