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Extraction of Proteins from Biological Fluids by Use of an Ionic Liquid/Aqueous Two-Phase System

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Abstract: An ionic liquid/aqueous two-phase system based on the hydrophilic ionic liquid 1-butyl-3-methylimidazolium chloride (BmimCl) and K_2HPO_4 has been employed for direct extraction of proteins from human body fluids for the first time. Proteins present at low levels were quantitatively extracted into the BmimCl-rich upper phase with a distribution ratio of about 10 between the upper and lower phase and an enrichment factor of 5. Addition of an appropriate amount of K_2HPO_4 to the separated upper phase results in a further phase separation, giving rise to an improved enrichment factor of 20. FTIR and UV spectroscopy demonstrated that no chemical (bonding) interactions between the ionic liquid and the protein functional groups were identifiable, while no alterations of the natural properties of the proteins were observed. The partitioning of proteins in the two-phase system was assumed to have been facilitated by the electrostatic potential difference between the coexisting phases, as well as by salting out effects. The system could be applied successfully for the quantification of proteins in human urine after on-line phase separation in a flow system. The use of an ionic liquid, as a green solvent, offers clear advantages over traditional liquid–liquid extractions, in which the use of toxic organic solvents is unavoidable.

Keywords: biomolecules · human urine · ionic liquids · liquid-phase separation · protein extraction

Introduction

Levels of protein excreted in urine have diagnostic and prognostic importance in kidney transplant recipients.[1] Moreover, proteinuria can be used to assess the effectiveness of some therapies and as a surrogate marker of chronic nephropathy and cardiovascular disease.[2] The daily excretion of proteins into urine by a healthy individual is usually less than 150 mg, but the urinary protein excretion of patients with renal diseases may even exceed a few grams per day.[3] Recently, the appearance of very low levels of albumin (albumin excretion 300 mg day^{-1}) in a urine sample (i.e., the so-called microalbuminuria; MAU) has received extensive attention[4] as a sign of pathological changes in blood capillaries and the earliest sign of diabetes mellitus.

At the very beginning of kidney damnification, the increase in total urine proteins is related to the increment of urine albumin, and total proteins may be determined by using bovine serum albumin (BSA) as standard.[5,6] Several colorimetric laboratory methods for protein quantification in urine are available,[7] among which the most commonly used are the benzethonium chloride, the Ponceau-S, and the Coomassie Brilliant Blue dye-binding (Bradford) methods. For the determination of microalbuminuria in diabetic children, more sensitive methods such as radioimmunoassay or enzyme-linked immunosorbent assay are required.[8] Practically, the complex nature of the matrix components in human body fluids frequently causes serious interference in protein quantification. At this juncture, it would be highly desirable to develop a suitable procedure for early diagnosis of proteinuria and MAU.

Room temperature ionic liquids (ILs) have found wide application in the field of chemistry.[9–14] The distinct features of ILs in relation to water and organic solvents have indicated great promise as attractive green alternatives to or replacements for conventional volatile organic solvents,[15–17] and extensive efforts have been made in the field of sample pretreatment by ILs: that is, extraction and separation/purification of trace metal species, organic compounds,[18–20] and amino acids[21] from complex matrices. This technique also offers potential applications in bioprocessing or analytical biochemistry,[22,23] in the form of purification and separation of macromolecules of life science interest from complex biological matrices, together with their quantification.

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Proteins are usually insoluble in ionic liquids,[24–26] so only a very few reports concerning this issue are to be found in the literature: low levels of monellin were found to be soluble in butylmethyl pyrrolidinium bis(trifluoromethanesulfonyl) amide,[27] while ether structures have been used to induce solubility of a protein in a hydrophobic IL.[28] So far no exploration has been directed to the extraction of proteins by use of ionic liquids.

Here we report for the first time the direct extraction of proteins from a biological sample matrix—human urine—with the aid of an ionic liquid/aqueous two-phase system based on the hydrophilic ionic liquid 1-butyl-3-methylimidazolium chloride (BmimCl) and suitable quantities of K_2HPO_4. After phase separation, the proteins had transferred into the BmimCl-rich upper phase, while the majority of the other concomitants remained in the lower phase. The concentrations of total proteins in both phases were determined by the Bradford method,[29] through coupling of the extraction system with on-line phase separation. The employment of an ionic liquid as a green solvent offers clear advantages over the conventional liquid–liquid extraction procedures involving toxic organic solvent. In addition, UV/Vis and FTIR spectroscopic investigations indicated that the natural properties of proteins remained unchanged during the extraction process.

Results and Discussion

Studies of ionic liquid/aqueous two-phase systems: Various salts, including K_2HPO_4, K_3PO_4, K_2CO_3, K_2SO_4, $(NH_4)_2SO_4$, KOH, Na_2HPO_4, and NaOH, have been investigated for their suitability for the formation of ionic liquid/aqueous two-phase systems.[30] K_2HPO_4 was finally selected because of its higher solubility in water (1600 g L^{-1}), as well as a better ability to promote phase separation: Figure 1 illustrates the phase diagram for the BmimCl/K_2HPO_4 system at 25 °C to 60 °C recorded by turbidometric titration. It is obvious that the two-phase system was readily producible in the temperature range studied here, but with increasing temperature higher concentrations of both BmimCl and K_2HPO_4 were required in order to maintain the two-phase system: in any situation the formation of an ionic liquid/aqueous two-phase system at a certain temperature requires the amounts of both BmimCl and K_2HPO_4 to exceed a particular threshold. In order to investigate its effect on the formation of the two-phase system and capability for phase separation, various quantities of K_2HPO_4 were added at room temperature to a solution containing water (3.0 mL) and BmimCl (300 μL). The experiments indicated that a two-phase system was formed and phase separation was observed when 2.7 g of K_2HPO_4 were employed. As illustrated in Figures 1 and 2, further increases in the added amount of

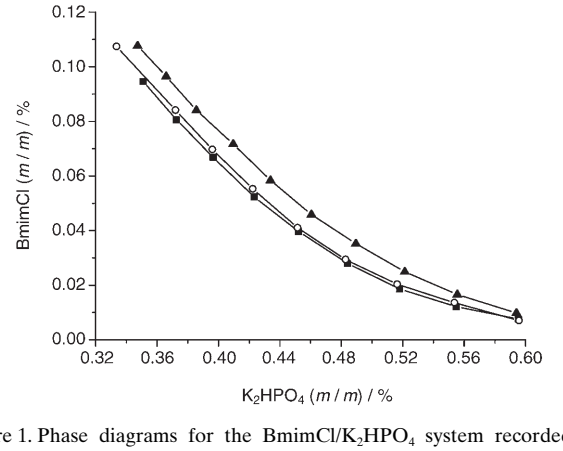

Figure 1. Phase diagrams for the BmimCl/K_2HPO_4 system recorded at various temperatures (25 °C: ■, 40 C: ○, 60 °C: ▲) and determined by turbidometric titration.

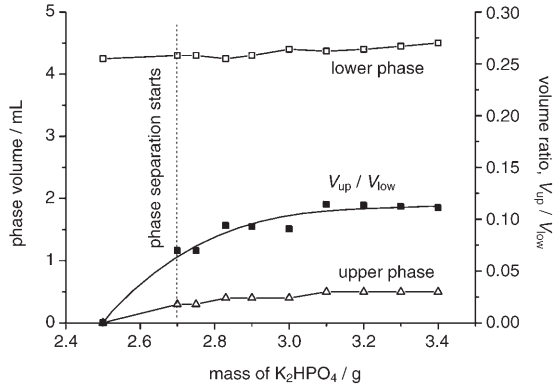

Figure 2. Effect of the amount of K_2HPO_4 used on the formation of the two-phase system and phase separation at room temperature. An aqueous solution (3.0 mL) and BmimCl (300 μL) were employed.

K_2HPO_4 ensured the production of the two-phase system and made phase separation easier, though at the same time giving rise to a slight increase in the upper phase volume, where the proteins were enriched, so that a minor decrease in the enrichment factor was recorded.

In a system consisting of K_2HPO_4 (3.0 g) and aqueous solution (3.0 mL), phase separation started when 0.3 mL of ionic liquid was introduced at room temperature (Figure 3). A further increase in the volume of BmimCl used resulted in a slight increment of the upper phase volume, which consequently caused a decrease on the enrichment factor for proteins. This result is also consistent with the observations in Figure 1.

The ensuing investigations of protein extraction with this two-phase system were carried out at room temperature, through the adoption of the same amount of aqueous solution (in volume) and K_2HPO_4 (in mass), along with an ionic liquid volume of 10% of that for the aqueous solution, as discussed below.

Protein distribution in the two-phase system

Effect on protein distribution of the amount of K_2HPO_4 used: As mentioned previously, proteins were transferred

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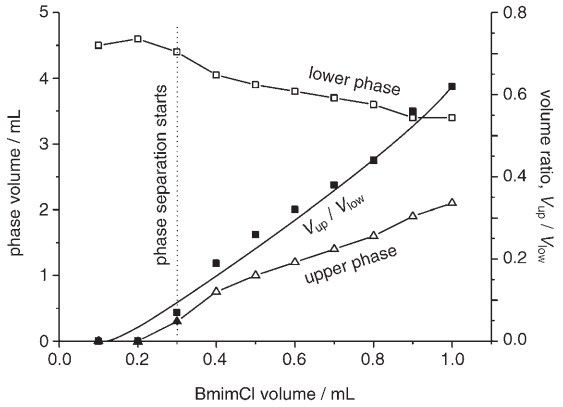

Figure 3. Effect of the volume of BmimCl used on the formation of the two-phase system and phase separation at room temperature. Aqueous solution (3.0 mL) and K_2HPO_4 (3.0 g) were employed.

into the upper phase with a certain level of enrichment. Figure 4 shows the extraction efficiency with BSA (0.5 mg mL^{-1}) and various amounts of K_2HPO_4 over the range of 0.45–0.75 g, in a system containing BSA solution

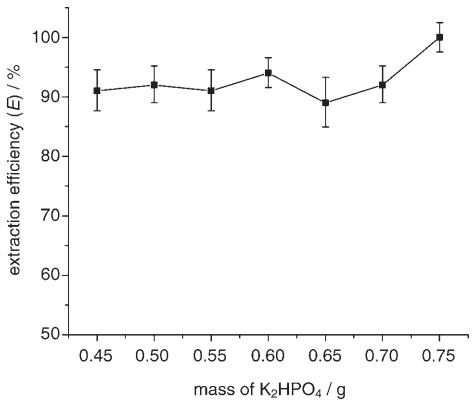

Figure 4. Amount of K_2HPO_4 employed on protein distribution/extraction efficiency, with use of BSA solution (0.5 mg mL^{-1}, 500 μL) and BmimCl (50 μL) for the extraction. The bars represent the standard errors of the means.

(0.5 mg mL^{-1}, 500 μL) and BmimCl (50 μL). It is clear that an extraction efficiency of about 90% was observed when the amount of K_2HPO_4 employed was less than 0.7 g, while an extraction efficiency of almost 100% was recorded as the K_2HPO_4 amount was increased up to 0.75 g or even more. In aqueous solution the concentration of K_2HPO_4 employed in this system seems to be high enough to induce protein precipitation, but no precipitation of proteins was observed during the extraction process with the dual-phase system over the concentration range studied here.

The mechanisms for the transfer of proteins into the BmimCl-rich upper phase and their enrichment there were investigated. It has been demonstrated that the solubility of BSA in the hydrophilic ionic liquid BmimCl is virtually negligible, so it appears conclusive that direct bonding interactions between the proteins and the BmimCl moieties are not included in the main driving forces behind the extraction of proteins in the two-phase system and their final enrichment in the upper phase (the ionic liquid-rich phase), a conclusion further demonstrated by FTIR and UV/Vis spectroscopy as described in the following sections.

Although identical acidities, of about pH 10, were maintained for the two-phase system, the compositions of the two phases are very different: the upper phase accounts for the majority of the BmimCl moieties, while the lower phase contains high concentrations of K_2HPO_4. Partitioning in aqueous two-phase systems is mainly a process in which the exposed groups of proteins come into contact with the phase components, and is therefore a surface-dependent phenomenon, depending strongly on the surface characteristics of the proteins. It has been demonstrated that electrostatic potential difference between coexisting phases is a common property at interfaces even though the phases are electrically neutral,[31] and in this particular case the uneven distribution of $Bmim^+$ and K_2HPO_4 generates a difference in electrical potential between the phases.[32–34] The dominant components of proteins found in human urine in this study include albumin and transferrin, as described below, with isoelectric points at pH 4.7–5.2, and hence negatively charged in basic media. Therefore, in the interfacial area, the positively charged $Bmim^+$ moieties attract the proteins into the upper phase, while the repulsion from the HPO_4^{2-} groups meanwhile further facilitates the transfer of proteins into the $Bmim^+$-rich phase.

On the other hand, the presence of high concentrations of K_2HPO_4 increased the hydrophobicity of the lower phase,[35] in which the solubility of proteins was significantly decreased as a result of competition from the large number of salt ions with proteins for water molecules: that is, the solvation spheres surrounding the proteins' ionized groups are removed[36] and salting out of proteins takes place. Consequently, the majority of the proteinic components were moved into the upper phase, with no obvious chemical or bonding interactions being involved during the whole process. In this extraction system, the transfer of protein was dominated by the salting out effect.

Effect of temperature on the extraction efficiency: The extraction of BSA was carried out over a temperature range of 25–70 °C, and the temperature dependence of the extraction efficiency is illustrated in Figure 5. It is obvious that at temperatures of <60 °C the overall extraction efficiency remained virtually unchanged, remaining at a level of more than 90%. This observation was consistent with reported observations in the literature that serum albumin goes through two structural stages when heat-treated. The first stage occurs at around 65 °C, and further heating above this temperature starts the second stage, in which denaturation of protein is encountered.[37–40] The first stage is reversible whilst the second stage is irreversible; the onset temperature of the conformation change as found by DSC was 58.1 °C,[41] while that for denaturation was 62 °C.[42] Obviously, the tem-

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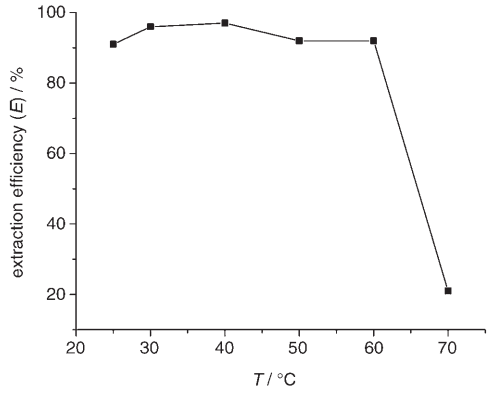

Figure 5. Temperature dependence on protein extraction efficiency into the upper phase, with BSA solution (0.5 mg mL^{-1}, 500 μL), K_2HPO_4 (0.5 g), and BmimCl (50 μL).

perature effect is negligible in this study, in which the temperature was controlled not to exceed 60 °C. At 70 °C, however, deposition of protein attributed to its denaturation was observed, and in addition, the phase separation was difficult under these circumstances. Further investigations indicated that the heat-tolerance of BSA was similar in both the upper and the lower phases.

Distributions of BmimCl, K_2HPO_4, and concomitants in the two-phase system: The distributions of BmimCl, K_2HPO_4, and water in the two-phase system were closely related to the partitioning of proteins in the two phases, so it would be highly desirable to quantify their individual contents in the two phases. Under the experimental conditions used in this study it has been shown that the majority of the employed BmimCl was to be found in the upper phase, only about 1.5% (*v*/*v*) being left in the lower phase, while about 95% (m m^{-1}) of the adopted K_2HPO_4 was found to remain in the lower phase. In the meantime, water contents in the upper and lower phases were 56% (m m^{-1}) and 58% (m m^{-1}), respectively.

As large amounts of salt might be encountered in the matrix of human urine samples, special attention should therefore be paid to the distributions of metal species in the two phases. In this system it was demonstrated that free cationic metal species—alkali, alkaline earth, and most of the transition metals—tend to remain in the lower phase, their extraction into the BmimCl-rich upper phase under the experimental conditions cited being practically unobserved, which facilitates the separation of proteins from the complex matrices very well. The extraction of metal species into the upper phase could only be facilitated in the presence of dedicated chelating reagents.[43–45]

The effect of BmimCl and K_2HPO_4 on BSA quantification by the Bradford method: After BSA extraction, the upper phase contains large amounts of BmimCl, while the lower phase is rich in K_2HPO_4. The effects of these species on the quantification of BSA were therefore investigated.

Various amounts of BmimCl (up to 100 μL) were mixed with CBBG solution (1.0 mL) for plotting of calibration graphs with BSA concentrations in the 0–50 μg mL^{-1} range. It was demonstrated that the slopes and intercepts of the calibration graphs remained unchanged when the volume of BmimCl was controlled so as not to exceed 10% of the volume of the added CBBG solution, and no detectable adverse effects on the quantification of BSA were encountered in the range studied. For the ensuing quantification of proteins after extraction, the volume ratio for the employed BmimCl-rich upper phase and the CBBG solution was kept at 1–10%.

On examination of the high concentration of K_2HPO_4 in the lower phase, its effect on the quantification of BSA was found to be very pronounced. Figure 6 illustrates the dependence of the pH value of the CBBG solution and the recorded increment of the blank absorbance on the amount of the lower phase added when the Bradford method was employed for the quantification of residual proteins in the lower phase after extraction. As the pH value of the lower phase was approximately 10, and also in view of the high concentration of K_2HPO_4 in it, a notable increase in the pH of the CBBG solution was observed after a small amount of the lower phase had been introduced. Consequently, the recorded absorbance (the blank signal) of the system was significantly enhanced with increasing volume of the lower phase, which resulted in a deterioration in the sensitivity through decreasing of the slopes of the calibration graphs. Therefore, whenever the residual proteins in the lower phase were quantified after extraction, as small an amount as possible of the lower phase (i.e., 10–15 μL) was employed.

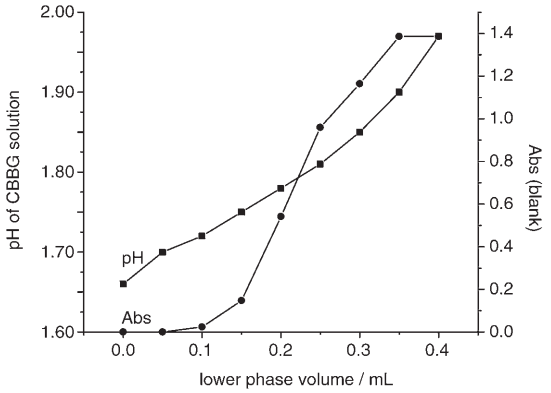

Figure 6. Dependence of the pH value of CBBG solution and the recorded blank absorbance on the amount of the lower phase added when the Bradford method was employed for the quantification of residual proteins in the lower phase after extraction. CBBG solution (1.0 mL) was employed.

Spectrometric studies after BSA has been extracted into the BmimCl-rich upper phase: The extraction of proteins through the use of this ionic liquid/aqueous two-phase

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system provides a novel approach for protein separation and preconcentration from complex matrices of biological fluids. In order to facilitate subsequent biological investigations, it is preferential that the natural properties of the proteins should remain unaltered during the extraction process, and so the properties of the BSA extracted into the BmimCl-rich upper phase were investigated by both UV/Vis and FTIR spectroscopy.

UV/Vis spectroscopy: The UV/Vis absorptions of proteins are mainly attributable to the lateral chains of aromatic series of amino acids, so in the ultraviolet region the chemical environment around the amino acids can be used to deduce the structure of a protein. In the case of BSA, some of the distinct features of its UV spectrum include a peak at approximately 280 nm, a trough at about 250 nm, and a rise between 245–240 nm. Figure 7 illustrates UV spectra recorded for BSA in various matrices, including in pure aqueous solution, in the BmimCl-rich upper phase extracted from artificial urine, and also aqueous solution. It is clear that the recorded UV spectra for BSA are almost identical regardless of the compositions of the matrices, and this observation clearly indicated that no alteration in the structure of BSA was encountered when it was extracted into the BmimCl-rich upper phase, which further confirmed the conclusion that no direct chemical (bonding) interactions were involved between the BSA molecules and the ionic liquid moieties.

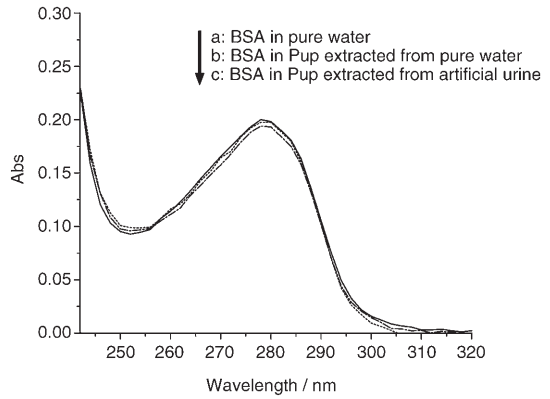

Figure 7. UV spectra of BSA recorded in various matrices (with final concentrations of 0.3 mg mL^{-1}). a) BSA in aqueous solution. b) BSA in the upper phase extracted from artificial urine. c) BSA in the upper phase extracted from aqueous solution. Pup = Protein extracted in the Upper Phase.

IR spectra: FTIR spectra provide useful information for identifying the presence of certain functional groups or chemical bonds in a molecule or an interaction system, attributable to the unique energy absorption bands for specific bonding environments or interactions.[46] Proteins are irregular polymers made up essentially of 20 amino acids with four levels of spatial structure. The primary structure in a polypeptide chain is its amino acid sequence, while linear segments of the polypeptide chain (i.e., α-helices, β-sheets, and β-turns) constitute the secondary structure, the conformation of which is stabilized by the main-chain hydrogen bonds.[47] Amide is the basic unit of the peptide bond: amide I is assigned to both C=O stretching vibration and ring stretching vibrations, while amide II is assigned to C–N stretching vibrations. The absorption bands most widely used as structure probes in protein FTIR spectroscopy have been the amide I vibrations, which fall between 1690 and 1600 cm^{-1}.[48] FTIR spectra for BSA, BmimCl, and BSA in BmimCl are illustrated in Figure 8, and it is obvious that the very broad absorption band around 3400 cm^{-1} provides no useful information in this particular case, while the absorption bands from 1700 to 1500 cm^{-1} are most informative. In Figure 8a (right), the two marker bands of the protein components (i.e., the amide I band at 1665 cm^{-1} and the amide II band at 1540 cm^{-1})[49] were readily identifiable in the FTIR spectra of BSA. It is thus obvious that after BSA has been extracted into the BmimCl-rich upper phase, as shown in Figure 8c (right), no new absorption bands are observable, while the two marker bands of BSA were clearly identified as shoulders of the two bands from BmimCl (i.e., 1635 and 1575 cm^{-1}) in the upper phase without any shift caused by the presence of a large amount of BmimCl. Meanwhile, the two absorption bands of BmimCl in this region (i.e., 1635 and 1575 cm^{-1}) had also remained unchanged after extraction. The observations here further demonstrated that there is no structural change in BSA when extracted into the BmimCl-rich upper phase of the ionic liquid/aqueous two-phase system, so no alterations in the natural properties of BSA were encountered after its separation and preconcentration.

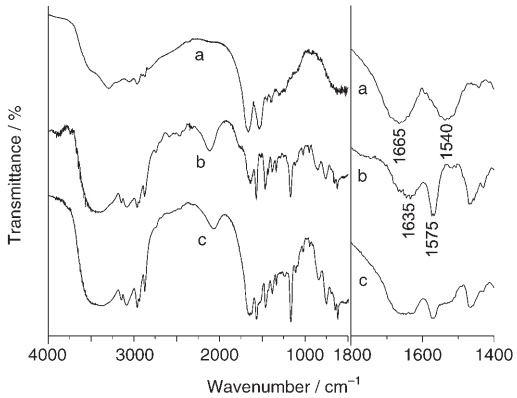

Figure 8. FTIR spectra of: a) pure BSA (powder), b) pure BmimCl, and c) BSA in BmimCl.

Analytical performance of the extraction system: On adoption of the experimental conditions described above [i.e., maintenance of a two-phase system by employment of the same amounts of aqueous BSA solution (in volume) and K_2HPO_4 (in mass), together with an ionic liquid volume of 10% of that for the aqueous solution] a distribution ratio of ca. 10 between the BmimCl-rich upper phase and the

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K_2HPO_4-rich lower phase was observed, along with an enrichment factor of 5, while a second phase separation of the previously separated upper phase results in an improved enrichment factor of 20.

SDS-PAGE for proteins in human urine after extraction: Human urine (3.0 mL) from a healthy volunteer was taken for the extraction process with the use of BmimCl (0.3 mL) and K_2HPO_4 (3.0 g), and after phase separation the proteins in the two phases were assayed by SDS-PAGE with respect to the SDS-PAGE marker; their electrophoretograms are illustrated in Figure 9. It is obvious that in the case of original

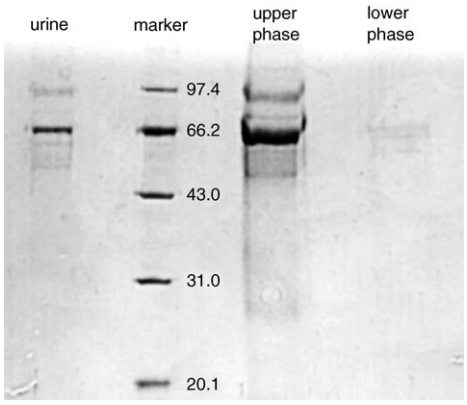

Figure 9. SDS-PAGE of proteins in: a) human urine, b) BmimCl-rich upper phase after extraction of urine, and c) K_2HPO_4-rich lower phase after extraction of urine.

human urine (Figure 9a), a clear albumin band at ca. 66 kDa was observable before extraction, together with a very blurry band in the 66–97 kDa range (ca. 88 kDa), which might be attributable to transferrin. After extraction, these two bands were significantly enhanced in the BmimCl-rich upper phase (Figure 9b), while nothing was detectable in the lower phase (Figure 9c), where the majority of the employed K_2HPO_4 remained. This observation clearly demonstrated that almost quantitative extraction of proteins could be achieved by use of this ionic liquid/aqueous two-phase system.

Protein quantification in human urine: Because human urine is a complex matrix the selection of an appropriate blank with similar matrix components is highly advantageous, so the artificial urine as described in the Experimental Section was employed.

The calibration graphs were made by spiking various quantities of BSA solution (5 mg mL^{-1}) in artificial urine (1.0 mL), followed by the same extraction procedure as described previously. A sample from the upper phase (10 μL) was then removed and mixed with CBBG solution (1.0 mL) in order to facilitate the chromogenic reaction, followed by absorbance measurement at 595 nm and protein quantification.

A detection limit of 0.8 μg mL^{-1} for total proteins, defined as three times the standard deviation of blank determinations, was determined for the procedure, along with precision of 1.9–7.8 % at the concentration levels found in human urine samples. The results are illustrated in Table 1. Spiking recoveries at various concentration levels were also performed for the three human urine samples, and recoveries in the range of 91.4–102.4 % were obtained.

Table 1. Proteins in human urine samples with spiking recoveries.

Sample	Found [μg mL^{-1}]	Spiked [μg mL^{-1}]	Recovery [%]	RSD [%]
1	84.5 ± 10.6	20	102.4	7.4
		50	91.4	7.8
2	48.2 ± 1.1	50	98.0	1.9
3	88.8 ± 6.4	60	100.8	3.1

Conclusion

The extraction of proteins in an ionic liquid (BmimCl)/aqueous two-phase system is reported for the first time. Quantitative extraction of proteins into the BmimCl-rich upper phase from the complex matrix that is human urine was facilitated in the presence of appropriate amounts of BmimCl and sufficient quantities of K_2HPO_4, while metal species and some other concomitants were separated, remaining in the K_2HPO_4-rich lower phase. No chemical (bonding) interactions between proteins and the BmimCl moieties were observed during the extraction process, and no alterations of the natural properties of proteins were therefore involved.

The employment of on-line extraction and phase separation in a flow system facilitates automation and fast operation.

This approach offers clear advantages over existing protein extraction procedures. Traditional liquid extraction procedures with organic solvents cause contamination of the obtained proteins, which might also pose critical problems for subsequent biological investigations, as such organic solvents might be toxic to bioprocesses.[50–53] In the present case, only water and hydrophilic ionic liquid are involved, the latter being a green solvent and therefore less harmful at this point, which is very appropriate for bioprocessing, in this particular case, for the purification/separation of proteins and their quantification in the ionic liquid-rich phase.

Experimental Section

Chemicals: All chemicals involved in this study were at least of analytical reagent grade, while doubly deionized water (18 MΩ cm^{-1}) was used throughout. Bovine serum albumin (BSA) (Sino-American Biotechnology Company, Beijing), Commassie Brilliant Blue G250 (CBBG, Fluka), K_2HPO_4 (Tianjin Kermel Chemicals, China), 85 % phosphoric acid (Liaohe Chemicals, Shenyang, China), and ethanol (Liaohe Chemicals, Shenyang, China) were used as purchased.

The Bradford reagent (CBBG working solution) contains CBBG (0.01 % w/v), ethanol (5 % v/v), and phosphoric acid (10 % v/v). A stock solution of BSA (10.0 mg mL^{-1}) was prepared by dissolving a suitable

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amount of BSA in deionized water, and standard solutions were obtained by stepwise dilution of this solution. A SDS-PAGE marker (Shanghai Institutes for Biological Sciences) contains: rabbit phosphorylase b (97.4 kDa), BSA (66.2 kDa), rabbit actin (43 kDa), bovine carbonic anhydrase (31 kDa), trypsin inhibitor (20.1 kDa), and hen egg white lysozyme (14.4 kDa).

Urine samples were collected from volunteers and were stored in a refrigerator at 5 °C. In order to match the matrix with urine an artificial urine was used for calibration; it was prepared by dissolving NaCl (Sinopharm Chemical Reagent China-SCRC, 55 mmol), KCl (SCRC, 67 mmol), calcium sulfate (2.6 mmol, SCRC), magnesium sulfate (3.2 mmol, SCRC), sodium sulfate (SCRC, 29.6 mmol), sodium dihydrogenphosphate (SCRC, 19.8 mmol), urea (SCRC, 310 mmol), and creatinine (Sigma, 9.8 mmol) in deionized water, with making up of the volume to 1.0 L.[54]

Apparatus: UV/Vis spectra were measured with a Lambda 950 UV/Vis spectrometer (Perkin–Elmer) or a T6 UV/Vis spectrophotometer (Purkinje General Instruments, Beijing). FTIR spectra were recorded on a Spectrum One FTIR spectrometer (Perkin–Elmer). Metal species in the extraction system were quantified with a WFX-130 A atomic absorption spectrometer (Rayleigh Analytical Instrument, Beijing) and an AFS-810 atomic fluorescence spectrometer (Titan Instruments, Beijing). A FIA-3110 flow injection system (Titan Instruments, Beijing) was used for on-line extraction. The pH values were measured with an Orion CHN060 pH-meter (ThermoElectron).

Preparation of ionic liquid BmimCl: 1-Butyl-3-methylimidazolium chloride was prepared by a documented route,[55] by treatment of 1-methylimidazole with chlorobutane (0.25 mol each) at 75 °C for 48–72 h with stirring and heating at reflux. The obtained viscous liquid was allowed to cool down to room temperature and washed three times with ethyl acetate (20 mL), and the product was finally dried under vacuum at 70–80 °C overnight. BmimCl is reported to be air- and water-stable at room temperature and to be miscible in any proportion with water.[56]

Extraction and determination of proteins in both phases: In a centrifugal tube (1.0 mL), the sample solution containing proteins (500 μL) was mixed with BmimCl (50 μL); that is, the aqueous/IL phase ratio (*v*/*v*) was fixed at 10. Afterwards, K_2HPO_4 (0.5 g) was added and the contents were shaken vigorously on an agitator for 1 min to facilitate the formation of the two-phase system and the extraction of proteins. The mixture was then allowed to stand for 2 min to facilitate the phase separation. For very low levels of proteins it is preferential to achieve a further improvement of the enrichment factor after extraction. In these circumstances, because the separated BmimCl-rich upper phase still contains a large amount of water, the introduction of a suitable quantity of K_2HPO_4 into this phase thus results in a second phase separation by elimination of the majority of the incorporated water, which gives rise to a significant enhancement of the protein concentration in the further separated upper phase.

A sample (10 μL) of the upper phase solution was taken and added to the CBBG working solution (1.0 mL). The mixture was then allowed to stand for 2 min to facilitate the colorimetric reaction, and was afterwards transferred by peristaltic pump into the flow cell of the UV/Vis spectrophotometer, where the absorbance of the system was measured at 595 nm.

Online extraction and phase separation: We have also investigated the protocol for on-line extraction, phase separation, and protein quantification by the Bradford method through the use of a two-phase system with a flow manifold as illustrated in Figure 10. The sample solution was first mixed with BmimCl at flow rates of 1.0 mL min^{-1} and 0.1 mL min^{-1} respectively, and the mixture was afterwards set to meet saturated K_2HPO_4 solution downstream at the same flow rate as that for the sample solution. The admixture was then directed so as to flow through a 300 mm mixing coil, in which the extraction of proteins was facilitated. After the extraction process, phase separation of the two-phase system was performed in a conical gravitational phase separator,[57] and the BmimCl-rich upper phase was collected for further quantification. It is worth mentioning that the use of solid K_2HPO_4 should be avoided when operated on-line, while a saturated solution was employed for the two-phase formation (i.e., an overall aqueous/IL phase ratio of 5 was adopted). In order to ensure the purity of the collected BmimCl-rich upper phase, a proportion of the upper phase (ca. 20%) was directed into the waste with the lower phase without separation.

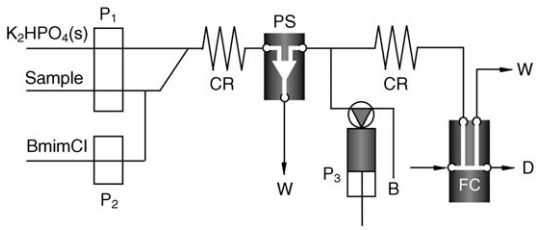

Figure 10. Flow manifold for on-line extraction and phase separation with the ionic liquid/aqueous two-phase system. Proteins were quantified by the Bradford method. K_2HPO_4(s): saturated K_2HPO_4 solution. B: CBBG solution. W: waste. P_1, P_2: peristaltic pumps. P_3: syringe pump. CR: PTFE knocked reactor as mixing coils. PS: gravitational phase separator. FC: flow through cell. D: flow through UV/Vis detector.

Distributions of BmimCl, K_2HPO_4, and water in the two-phase system: The distribution of BmimCl was determined by quantification of its contents in both the upper and lower phases after phase separation by spectrophotometry at 250 nm. For the quantification of K_2HPO_4 and water contents, as well as their distributions in the two-phase system, the upper and lower phases were dried at 70 °C until constant weights were attained, and from this the contents of K_2HPO_4 and water in both phases, as well as their distributions, were then calculated by weight difference.

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