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Research Article

Enhanced extraction of proteins using cholinium-based ionic liquids as phase-forming components of aqueous biphasic systems

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Aqueous biphasic systems (ABS) composed of ionic liquids (ILs) are promising platforms for the extraction and purification of proteins. In this work, a series of alternative and biocompatible ABS composed of cholinium-based ILs and polypropylene glycol were investigated. The respective ternary phase diagrams, tie-lines, tie-line lengths and critical points were determined at 25°C. The extraction performance of these systems for commercial bovine serum albumin (BSA) was then evaluated. The stability of BSA at the IL-rich phase was ascertained by size exclusion high-performance liquid chromatography and Fourier transform infrared spectroscopy. Appropriate ILs lead to the complete extraction of BSA for the IL-rich phase, in a single step, while maintaining the protein's native conformation. Furthermore, to evaluate the performance of these systems when applied to real matrices, the extraction of BSA from bovine serum was additionally carried out, revealing that the complete extraction of BSA was maintained and achieved in a single step. The remarkable extraction efficiencies obtained are far superior to those observed with typical polymer-based ABS. Therefore, the proposed ABS may be envisaged as a more effective and biocompatible approach for the separation and purification of other value-added proteins.

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1 Introduction

Proteins exhibit remarkable biological properties and are important biomolecules in different fields, from organismal biochemistry to the chemical, food and pharmaceutical industries. Their function and activity is related to their fragile and very sensitive three-dimensional structure that can be disrupted by changes in the medium composition, pH or temperature [1]. Consequently, many efforts have been made aiming at developing efficient extraction and purification procedures while attempting to maintain native proteins conformations [2–4].

The demand to develop not only biocompatible but also cheaper and sustainable extraction and purification processes for proteins has led to intensive efforts by academia and industries [5, 6]. Traditional purification methods for proteins include precipitation with salts, electrophoresis, and ion-exchange and affinity chromatographies [7–9]. These methods are not only costly and time consuming, but may be also responsible for the loss of biological activity of some proteins [10]. In addition, some of these methods (electrophoresis, ion exchange and affinity chromatography) use organic compounds which are volatile and hazardous to human health and to the environment.

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Abbreviations: ABS, aqueous biphasic systems; **BSA**, bovine serum albumin; **[Ch][Ac]**, cholinium acetate; **[Ch][Bit]**, cholinium bitartarate; **[Ch][But]**, cholinium butanoate; **[C_4mim]Cl**, 1-butyl-3-methylimidazolium chloride; **[C_8mim]Br**, 1-octyl-3-methylimidazolium bromide; **[Ch]Cl**, cholinium chloride; **[Ch][DHCit]**, cholinium dihydrogen citrate; **[Ch][DHP]**, cholinium dihydrogen phosphate; **[Ch][Gly]**, cholinium glycolate; **[Ch][Lac]**, cholinium lactate; **[Ch][OH]**, cholinium hydroxide; **[Ch][Pro]**, cholinium propanoate; EE_{BSA}%, percentage extraction efficiency of BSA; **ILs**, ionic liquids; **PPG 400**, polypropylene glycol with a molecular weight of 400 g mol^{-1}; **TL**, tie-line; **TLL**, tie-line length

Aqueous biphasic systems (ABS) are a liquid-liquid biocompatible extraction technique that was proposed in the 1950s by Albertsson [11]. Two aqueous-rich phases are formed upon mixing two structurally different components, such as two polymers, two salts or one polymer and one salt in aqueous media [12]. Therefore, ABS represent an appealing alternative to the current request for fast, economic, and easy-to-implement processes. The clarification, concentration and purification in a single operation unit is possible, and since they are liquid-liquid systems, ABS can be easily scaled-up [2, 13–15].

Taking into account their advantages, ABS have been replacing other extraction/purification techniques commonly employed to proteins [16, 17]. For long, the studied ABS were based in the coexistence of two immiscible aqueous-rich phases formed by polymer–polymer or polymer–salt mixtures [18]. Nevertheless, after the seminal work of Rogers and co-workers [19] on the possibility of creating ABS by mixing ionic liquids (ILs) and inorganic salts in aqueous solutions, novel ABS composed of ILs + water + organic/inorganic salts, amino acids, polymers or carbohydrates have become the focus of many ABS-related investigations [20]. The main advantages of these new ABS, when compared to polymer-polymer systems, are their lower viscosities and the possibility of tailoring the polarities of the coexisting phases which allow their design for the extraction of a target compound [21, 22]. Ionic liquids (ILs) are composed of a large organic cation and an inorganic/organic anion and belong to the molten salts group (with melting temperatures below 100°C). Due to their ionic character, most ILs present a negligible volatility at atmospheric conditions, non-flammability, high thermal and chemical stabilities, and an enhanced solvation capability for a large variety of compounds [22, 23].

The extraction of proteins using IL-based ABS was firstly demonstrated by Du et al. (2007) [24]. The authors employed an ABS constituted by 1-butyl-3-methylimidazolium chloride ($[C_4mim]Cl$) and K_2HPO_4 to extract and concentrate proteins from human body fluids. After this innovative work, other authors studied the application of IL-based ABS in the extraction of proteins and enzymes, such as bovine serum albumin, lysozyme, trypsin, myoglobin, horseradish peroxidase, cytochrome c, hemoglobin, peroxidase and ovalbumin [25–28]. Nevertheless, most of these studies were carried out with imidazolium-based ILs with anions with a strong alkaline or acidic character. Therefore, inorganic phosphate-based salts as buffered solutions were typically used to maintain the pH of the aqueous medium in order to avoid the proteins denaturation. However, phosphate ions can bind to metal ions, like calcium, zinc or magnesium – essential to maintain the integrity of some proteins/enzymes [29]. Recently, a novel class of ILs (with buffering characteristics) was proposed as phase-forming components of ABS, while their extraction performance for proteins was also highlighted [30]. The inorganic salts usually employed lead to several environmental and biocompatibility concerns. To minimize these issues they can be replaced by biodegradable and more biocompatible organic salts or polymers. These include polypropylene glycol (propylene glycol is a generally recognized as safe (GRAS) additive for food and medical-related products), polyethylene glycol, natural polysaccharides, among others [31]. On the other hand, we are also facing an era where a vast plethora of ILs are available, and apart from the commonly studied imidazolium-based fluids, many of them are toxic to organisms and poorly biodegradable, ILs derived from natural sources, such as amino acids, carbohydrates or cholinium-based were already synthesized [32]. Hence, ABS composed of cholinium-based ILs and polymers appear as potential alternatives for the extraction of biologically active and added-value molecules.

In this work, a series of biocompatible ABS composed of cholinium-based ILs and polypropylene glycol, with a molecular weight of 400 g mol^{-1} (PPG 400), has been investigated for the extraction of bovine serum albumin (BSA). The respective ternary phase diagrams, as well as the critical points, tie-lines and tie-line lengths, were determined at 25°C. The extractive performance of these systems for BSA was then ascertained, both with commercial BSA and using a real bovine serum sample. The effects of the chemical structure of ILs, composition of the biphasic mixtures and protein concentration were investigated aiming at maximizing extraction yields while avoiding the protein denaturation.

2 Materials and methods

2.1 Materials

The ABS studied in this work were determined using polypropylene glycol with an average molecular weight of 400 g mol^{-1} (PPG 400), supplied by Aldrich, and used as received. The ILs used in this work were: cholinium [(2-hydroxyethyl)trimethylammonium] chloride, [Ch]Cl (99 wt% pure from Sigma-Aldrich), cholinium dihydrogen citrate, [Ch][DHCit] (98 wt% pure from Sigma-Aldrich), cholinium bitartarate, [Ch][Bit] (98 wt% pure Sigma-Aldrich), cholinium acetate, [Ch][Ac] (98 wt% pure from Iolitec), cholinium dihydrogen phosphate, [Ch][DHP] (99 wt% pure from Iolitec), cholinium propanoate, [Ch][Pro] (99.5 wt% pure and synthesized by us), cholinium glycolate, [Ch][Gly] (97 wt% pure and synthesized by us), cholinium butanoate, [Ch][But] (99 wt% pure and synthesized by us), and cholinium lactate, [Ch][Lac] (99 wt% pure and synthesized by us). The ILs synthesized by us were prepared according to well-established protocols [33, 34]. The required precursors were commercially acquired, namely cholinium hydroxide [Ch][OH] (40 wt% in methanol) and glycolic acid (99 wt% pure) were from

Sigma-Aldrich, propanoic acid (≥99.5 wt% pure) was acquired from Merck, and butanoic acid (99 wt% pure) and lactic acid (88–92 wt% pure) were acquired from Riedel-de-Haën. After the synthesis and before use, all ILs were purified and dried for a minimum of 24 h at constant agitation, at a moderate temperature (~70°C) under vacuum (10 Pa). The water employed was ultra-pure water, double distilled and passed through a Milli-Q plus 185 water purification apparatus. Fetal bovine serum was purchased from Life Technologies (Gibco® sera). In addition to PPG 400, also PPG 600 and PPG 1200 were tested to prepare ABS with the studied cholinium-based ILs; yet, PPG 600 and PPG 1200 were not able to form ABS with none of the investigated ILs (mainly due to the low solubility of these polymers in water).

2.2 Synthesis and characterization of cholinium-based ionic liquids

The cholinium-based ILs were synthesized via neutralization of the base with the corresponding acid. The mixture was continuously stirred for at least 12 h at room temperature (~25°C). The anion source (1.1 equivalents of acid) was added to the [Ch][OH] solution and the mixture was stirred at room temperature to produce the IL and water as the by-product. The mixture was then subjected to evaporation at 25°C under reduced pressure, for at least 24 h, and which gives rise to a viscous liquid. The unreacted acid in the prepared IL was further eliminated with acetone or ethyl acetate. The molecular solvents were then evaporated under vacuum and the obtained IL was dried under vacuum at 25°C for at least 72 h. All the above procedure was done under an inert atmosphere, thereby preventing the choline hydroxide degradation induced by oxygen. The ILs synthetized in this work showed high purity with no sign of decomposition. The purity of each IL was confirmed by ^{1}H and ^{13}C NMR spectra (Supporting information, Table S1). The water content of the synthesized and dried ILs was determined by Karl Fischer titration (using a Mettler Toledo DL 39 and the hydranal Coulomat AG reagent from Riedel-de-Haën). The water content in all ILs is <0.1 wt%.

2.3 Determination of the phase diagrams, critical points, tie-lines and tie-line lengths

The ternary phase diagrams for the ABS composed of PPG 400 + IL + water were determined with the following ILs: [Ch]Cl, [Ch][DHP], [Ch][Bit], [Ch][DHCit], [Ch][Ac], [Ch][Prop], [Ch][But], [Ch][Gly] and [Ch][Lac]. Each ABS binodal curve was determined by the cloud point titration method at (25 ± 1)°C and atmospheric pressure. The experimental procedure adopted has been validated in a previous work [35]. Briefly, aqueous solutions of PPG 400 at ~90 wt% and aqueous solutions of the different ILs at variable concentrations (from 55 to 80 wt%) were prepared gravimetrically and used for the determination of the binodal/solubility curves. Mixture compositions were determined gravimetrically (±10^{-4} g).

The tie-lines (TLs) of each phase diagram, considering also the mixtures compositions for which the extractions of BSA were carried out, were determined at 25°C according to the method proposed by Merchuk et al. [36], and already applied to IL-based ABS [37, 38].

The experimental binodal curves were fitted using Eq. (1),

$$[PPG] = A_{exp}[(B[IL]^{0.5}) - (C[IL]^{3})] \quad (1)$$

where [PPG] and [IL] correspond to the weight fractions percentages of each compound, while A, B and C are fitting parameters acquired by least-squares regression.

The critical point of each ABS was determined according to the fitting provided by Eq. (2),

$$[PPG] = f[IL] + g \quad (2)$$

where [PPG] and [IL] are the weight fraction percentages of PPG 400 and IL, and f and g are parameters obtained from the fitting.

2.4 Partitioning studies and extraction efficiencies of BSA

The ternary mixtures compositions used in the partitioning experiments were chosen based on the phase diagrams determined in this work for each PPG 400 + IL + water system. A ternary mixture with a common composition, and within the biphasic region, was prepared with 30 wt% of PPG 400, 30 wt% of IL and 40 wt% of a phosphate buffered saline (PBS) aqueous solution (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride to achieve a pH = 7.4) containing BSA at concentrations of circa 0.5, 1.0, 5.0 and 10 g L^{-1}. Furthermore, the same compositions of the ternary mixtures were used in the extractions of BSA from fetal bovine serum (for the [Ch][Ac]-, [Ch][Bit]-, and [Ch][Prop]-based systems). To explore the influence of the composition of the phase-forming components through the BSA extraction, the following mixtures were also evaluated with model systems: 30 wt% of PPG 400 + 20 wt% of IL, 30 wt% of PPG 400 + 10 wt% of IL, 20 wt% of PPG 400 + 30 wt% of IL and 10 wt% of PPG 400 + 30 wt% of IL. Each mixture containing BSA was stirred, centrifuged and left to equilibrate for at least 10 min at (25 ± 1)°C to allow the BSA partitioning between the coexisting phases. After, the phases were separated and the quantification of BSA in each phase was accomplished by SE-HPLC (size exclusion high-performance liquid chromatography, using a Chromaster HPLC, Hitachi, coupled with an UV-Vis detector. Further details on the quantification method can be found elsewhere [30]. At least three independent

biphasic mixtures for each ABS were prepared and three samples of each phase were quantified. Blank control samples were also analysed to ascertain on possible interferences of the phase-forming components. The percentage extraction efficiency of BSA, EE_{BSA}%, corresponds to the ratio between the amount of protein in the IL-rich aqueous phase and that in the total/initial mixture as described elsewhere [30].

It should be highlighted that the influence of the PBS solution through the phase diagrams behavior was deeply analysed and it was found that at these concentrations of salts (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride) there are no changes on the phase diagrams.

3 Results and discussion

3.1 Ability of ILs to form ABS

Several ABS composed of water, PPG 400 and ILs were established in this work. The chemical structures of the ILs investigated and that have shown to be able to create ABS with PPG 400 are depicted in Fig. 1. The respective ternary phase diagrams are illustrated in Fig. 2. The experimental weight fraction data of each phase diagram are reported in the Supporting information, Tables S2–S4. In all studied ABS, the top phase corresponds to the aqueous PPG-rich phase while the bottom phase is mainly composed of IL and water.

The results depicted in Fig. 2 reflect the effect of the IL anion (since the cholinium-cation is common to all ILs) on the formation of ABS with PPG 400. The biphasic or two-phase regime is localized above the solubility curve; the larger this region, the higher is the ability of the IL to induce liquid-liquid demixing with aqueous solutions of PPG 400. The results are presented in weight fraction due to its convenience in the design of extraction processes.

In general, the ability of the investigated ILs, to form ABS in presence of a fixed amount of PPG, e.g. at 30 wt% of PPG, decreases in the following order: [Ch][DHP] ≈ [Ch][Gly] > [Ch][Ac] > [Ch]Cl ≈ [Ch][Lac] > [Ch][Prop] > [Ch][But] > [Ch][Bit] > [Ch][DHCit] (Fig. 2). Anions derived from carboxylic acids with shorter alkyl side chains have a higher affinity for water and more easily induce the formation of a second liquid phase enriched in a moderately hydrophobic polymer, such as PPG. This trend is confirmed with the ILs comprising the anions $[Ac]^-$, $[Prop]^-$ and $[But]^-$. Furthermore, the addition of hydroxyl groups, as in the lactate- and glycolate-based

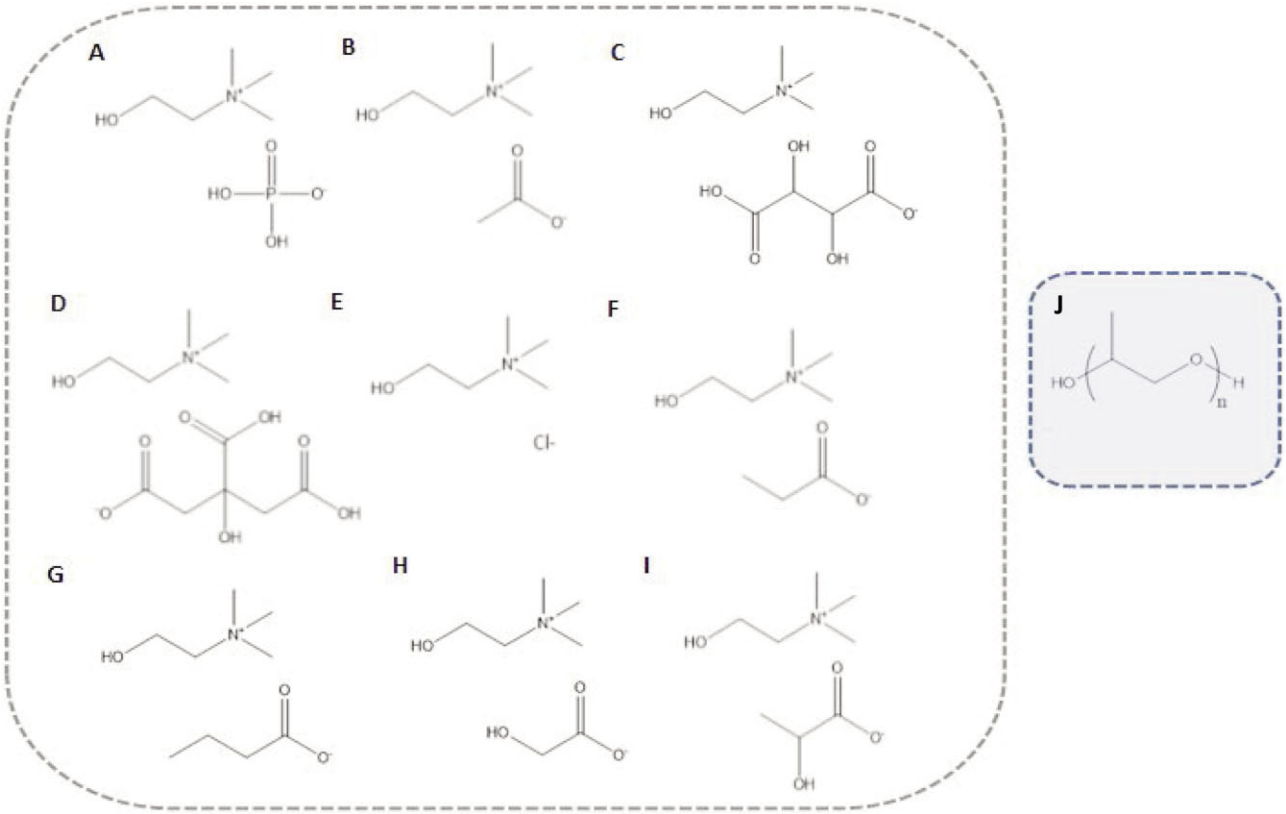

Figure 1. Chemical structure of the investigated ILs and PPG: (**A**) [Ch][DHP]; (**B**) [Ch][Ac]; (**C**) [Ch][Bit]; (**D**) [Ch][DHCit]; (**E**) [Ch]Cl; (**F**) [Ch][Prop]; (**G**) [Ch][But]; (**H**) [Ch][Gly]; (**I**) [Ch][Lac]; and (**J**) PPG.

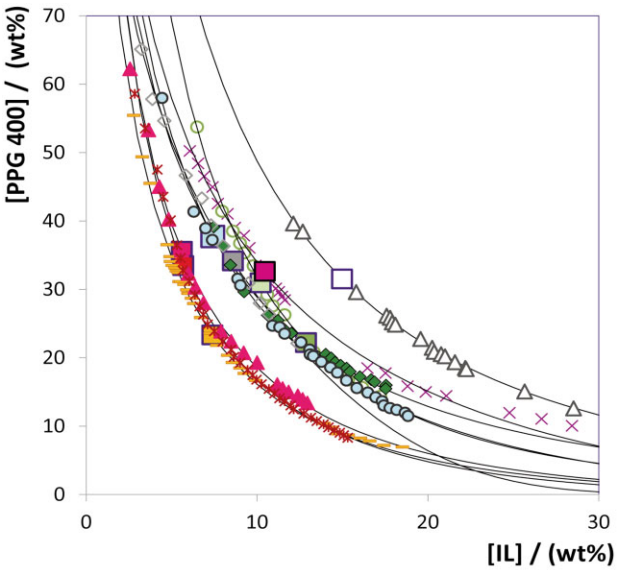

Figure 2. Phase diagrams for ABS composed of PPG 400 + IL + H_2O at 25°C: [Ch][DHP] (—); [Ch][Ac] (▲); [Ch][Bit] (×); [Ch][DHCit] (△); [Ch]Cl (♦); [Ch][Prop] (◇); [Ch][But] (○); [Ch][Gly] (✳); [Ch][Lac] (●), with the corresponding fitting of the experimental data using Eq. (1) (–) and critical points (■).

ILs, makes them more able to phase separate (when compared with the propanoate- and acetate-based fluids with the same number of carbon atoms). The addition of hydroxyl groups favors the hydrogen-bonding between the IL and water while providing a more hydrophilic character to the IL. [Ch][DHP] is amongst the ILs with a higher ability to promote ABS. This phenomenon complies with the idea that an increase in the anion polar surface improves the IL ability to induce ABS when combined with a more hydrophobic polymer. ILs with higher affinity for water are more capable to exclude PPG to a second liquid phase. The [Ch][DHCit] is the IL with the lowest ability to form ABS with PPG 400. $[DHCit]^-$ has a capability to act either as an H-bond donor or acceptor and, therefore, a strong salting-out effect from this IL was expected. However, as verified in a previous work comprising ABS constituted by polyethylene glycol [39], this IL shows a weaker ability to form ABS when compared with other anions derived from carboxylic acids. This may be due to the self-aggregation of the citrate anion as a result of the intermolecular hydrogen-bonding between the hydroxyl hydrogen atoms and one of the oxygens of the central carboxylic group [40].

For the studied systems, the experimental binodal data were further fitted by the empirical relationship described by Eq. (1) [36]. These correlations are depicted in Fig. 2 together with the experimental data. The regression parameters were estimated by least-squares regression, and their values and corresponding standard deviations are provided in the Supporting information, Table S5. In general, a good fitting of the experimental data was observed. The experimental TLs, along with their respective length (TLL), are also reported in the Supporting information, Table S6 and Fig. S1. In addition, the critical point of each system is also depicted in Fig. 2 (Supporting information with detailed data, Table S7). Amongst similar anions, close critical points were obtained. In general, the compositions of the coexisting phases become equal at higher amounts of PPG 400 when compared with the contents of IL. It is important to remark that some longer tie-lines were not used in the determination of the critical point because they are not parallel – a result of the high amount and almost saturation of the polymer-rich phase for mixture compositions continuously more rich in IL (Supporting information, Fig. S2).

3.2 Enhanced extraction of BSA

In the present work, the extraction efficiencies of commercial BSA in ABS composed of cholinium-based ILs + PPG 400 + water have been comprehensibly examined. The extraction efficiencies of BSA, at 25°C and at a common mixture composition (30 wt% of IL + 30 wt% of PPG 400), are shown in Fig. 3. The detailed extraction efficiencies and compositions of the coexisting phases of the mixtures used for the extraction of BSA are presented in Supporting information, Tables S8–S10. In all systems it is observed the preferential partitioning of BSA for the IL-rich aqueous phase. In general, the partitioning of proteins between the two phases of an ABS is a complex phenomenon, guided mainly by several competing interactions between the solute being partitioned and the phase-forming components. This surface-dependent phenomenon is very complex since a protein can interact with the surrounding molecules through hydrogen-bonding, electrostatic interactions and dispersive forces. Furthermore, steric effects can also dictate the protein preferential migration. In the studied systems, at pH 7.4, BSA is negatively charged (the isoelectric point of BSA is 4.8 [41]), and electrostatic interactions between the amino acid residues on the protein surface and the IL cation can occur. Nevertheless, all studied ILs share the same cation and therefore the differences observed amongst the extraction efficiencies are a result of other effects, such as preferential hydrogen bonding and dispersive interactions between the protein and the IL-rich phase forming constituents. Furthermore, in the ABS investigated, BSA preferential migrates to the more hydrophilic phase (IL-rich phase). In previous studies of extraction of BSA in ABS formed by ILs and salting-out salts, a preferential partitioning of BSA for the IL-rich phase was also observed [30, 42, 43]. Yet, in these salt-IL systems, the IL-rich layer corresponds to the most hydrophobic phase. Hence, these combined results suggest that the BSA partition is not dominated by the relative hydrophobicity of

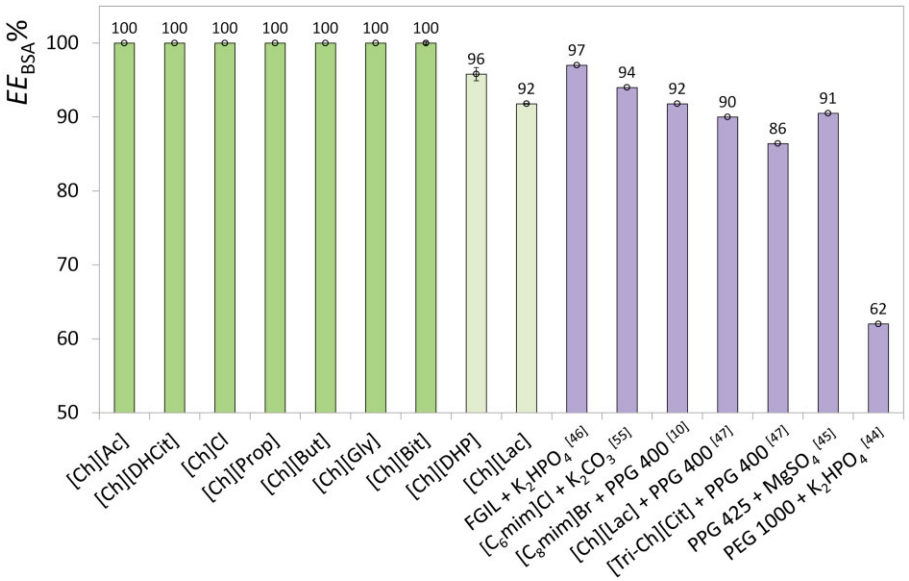

Figure 3. Average extraction efficiencies of BSA (EE_{BSA}%) in the systems composed of PPG 400 (30 wt%) + IL (30 wt%) + H_2O (at least three replicates for each system were accomplished). Experiments were carried out in samples with a total of 3 g at 25°C and pH 7.4. BSA at an initial concentration of 0.5 g L^{-1} was used. The comparison with literature results is also included.

the phases but actually by specific interactions established with the IL.

The extraction efficiencies of BSA for the IL-rich phase vary between 92 and 100%, and increase in the following order: [Ch][Lac] < [Ch][DHPh] < [Ch][Bit] ≈ [Ch][DHCit] ≈ [Ch][But] ≈ [Ch][Prop] ≈ [Ch][Gly] ≈ [Ch][Ac]. Remarkably, the complete extraction of BSA, in a single step, was achieved with the ILs [Ch][DHCit], [Ch][But], [Ch][Prop], [Ch][Ac] and [Ch][Gly]. It should be remarked, that for [Ch]Cl, despite allowing a complete extraction of BSA for the IL-rich phase, a visible precipitation of the protein at the interface was observed (around 70 w% of precipitated protein, determined by mass balance through the quantification of BSA in the coexisting phases by SE-HPLC). Based on these overall results, it seems that the extraction of BSA does not depend on the hydrophilicity of the IL-rich phase provided by the water content (see the TLs data in Supporting information, Table S8) or on the hydrophilicity of the IL anion (reflected by the phase diagrams trend). Although BSA does not preferentially migrate for the more hydrophobic PPG-rich phase it is still verified that it has a higher affinity for the more hydrophobic ILs investigated. Overall, these evidences suggest that there is a delicate balance among hydrogen bonding and dispersive interactions occurring between the protein and the IL ions and which dictate the protein partitioning.

Outstandingly, the biocompatible and biodegradable ABS formed by PPG 400 and [Ch][DHCit], [Ch][But], [Ch][Prop], [Ch][Ac] or [Ch][Gly] allow the complete extraction of BSA for the IL-rich phase in a single step. The high performance of these ABS is further supported by other studies comprising more conventional ABS already published in the literature, and where the reported extraction efficiencies are depicted in Fig. 3 for comparison purposes. For instance, an ABS formed by PEG and a phosphate salt led to a maximum BSA recovery of 62% [44]**.** Moreover, other ABS composed of PEG or PPG, combined with three inorganic salts, resulted in a maximum extraction of BSA of 90.4% [45]. Ding et al. [46] investigated the potential of different ABS composed of eight functionalized guanidinium-based ILs and phosphate-based salts. The authors of [46] reached a maximum extraction efficiency of BSA 97% (after an extensive study on the optimization of the system composition, pH, temperature and IL chemical structure). In addition, even with PPG-IL ABS, only extraction efficiencies of BSA up to 90% have been reported [47]. The ILs selected for such a purpose were cholinium lactate and tri-cholinium citrate, and amongst the ILs investigated in our work, the [Ch][Lac]-based ABS is the one which displays the lowest extraction efficiency. Li et al. [47] presented most of their results in partition coefficients. A more extensive comparison using the partition coefficient values of Li et al. [47] and those obtained in this work is provided in Supporting information, Table S11. Overall, the partition coefficients of BSA for the IL-rich phase achieved in this work are higher than those previously reported [47] with similar ABS. In summary, and as shown in this work, the combination of polymers and suitable ILs as phase-forming components of ABS, as well as their compositions, clearly leads to better and tailored extraction efficiencies of BSA.

Given the outstanding results afforded by [Ch][Ac], this IL was chosen to further study the ABS extraction ability at different compositions of IL and polymer. The IL and polymer amounts were decreased in order to minimize the content of phase-forming components able to form ABS while keeping the complete extraction of the protein. ABS containing varying concentrations of IL

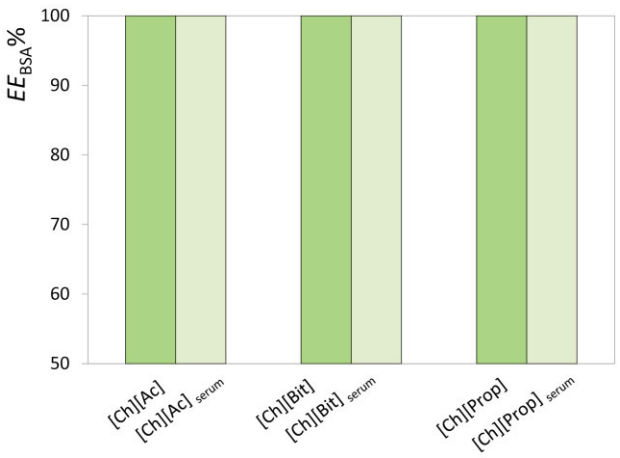

Figure 4. Average extraction efficiencies of BSA (EE_{BSA}%) in the systems composed of PPG 400 (30 wt%) + IL (30 wt%) + H_2O: Commercial BSA (dark bars) and BSA from a real matrix (fetal bovine serum) (lbright bars). At least three replicates for each system were accomplished. Experiments were carried out in samples with a total of 3 g at 25°C and pH 7.4. BSA at an initial concentration of 0.5 g L^{-1} was used, and for which the real bovine serum was previously diluted (the real sample contained 45 g L^{-1} as determined by us by SE-HPLC).

(from 10 to 30 wt%) and PPG (from 10 to 30 wt%) were evaluated and the results obtained are depicted in the Supporting information, Tables S9–S10. The compositions of the coexisting phases (or TLs data) for these mixture compositions are also presented in the Supporting information, Table S6. In general, a decrease in the IL or in the polymer concentration (down to 10 wt%) does not change the extractive performance of the investigated ABS. In all situations, the complete extraction of BSA for the IL-rich phase was achieved in a single step. Similar studies were carried out with higher concentrations of BSA aiming at detecting an upper limit of the BSA amount as a result of the saturation of the IL-rich phase. The study was carried out with 1.0, 5.0 and 10 g L^{-1} of BSA at the aqueous phase. The results showed that it is possible to maintain the complete extraction of BSA for the IL-rich phase (in a single step) even at BSA concentrations up to 10 g L^{-1}.

After fine-tuning the ILs and compositions of the phase-forming components with extractions carried out with model systems using commercial BSA, the extraction of BSA from fetal bovine serum was further attempted with three of the ABS that lead to complete extraction in a single step, namely those composed of 30 wt% of PPG 400 + 30 wt% of [Ch][Ac], [Ch][Bit] or [Ch][Prop], to evaluate the performance of these systems when applied to real matrices. In the three systems, BSA was completely extracted to the IL-rich phase (Fig. 4) with no denaturation and/or precipitation observed. Indeed, by the SE-HPLC chromatograms, no BSA is detected at the top phase (PPG-rich phase). Although no definite conclusions can be drawn on the purification factors achieved, since the peak of the IL overlaps the peaks of the major contaminant proteins present in the bovine serum, it seems that most of the contaminant proteins in the serum sample are also being extracted for the IL-rich phase, meaning that further work should be carried out aiming at the purification of BSA.

3.3 Improved stability of BSA in the IL-rich phase

The proteins stability can be strongly influenced by the type of ILs (or other phase-forming components) used to create a given ABS. However, the interactions between proteins and ILs are still not well understood. Several studies on protein interactions with ILs have been carried out by different authors [48, 49]. Furthermore, the effect of ILs on the proteins stability was suggested to be correlated with the Hofmeister series [50].

The stability of proteins during the extraction and purification procedures is an essential requirement for further applications. Changes in the protein environment, such as temperature, pH and solvents can alter the proteins native state. Most of the extraction studies based on the application of ILs focused on their extractive performance while few studies have inferred on the protein stability in ABS. Examples include the work of Dreyer and Kragl [51] who studied an ammonium-based IL and proved that it is not only effective in forming aqueous two-phase systems, but that this IL can also increase the specific activity of enzymes. Recently, Desai et al. [52] investigated the stability of different proteins (rubisco, BSA and IgG) in aqueous solutions of two ionic liquids: Iolilyte 221PG and Cyphos 108. The results showed the formation of high molecular weight aggregates when increasing the concentration of the IL [52]. Saadeh et al. [53] tested the interaction of tetrabutylammonium salts with BSA and catalase (CAT) and confirmed the enzyme denaturation by the influence of tetrabutylammonium salts. Some imidazolium-based ILs can also lead to the unfolding of polypeptides within BSA, resulting in the loss of the secondary and tertiary structure [54].

In addition to a high extraction performance it is of vital importance to guarantee the protein stability in the extractive phase. The SE-HPLC chromatograms, depicted in Fig. 5, reveal that the peak intensity and profile of BSA does not significantly change in presence of different concentrations of [Ch][Ac] (from 0 to 50 wt%). Furthermore, higher molecular weight aggregates were not detected indicating that BSA retains its native structure in the IL-rich phase. SE-HPLC chromatograms of BSA at different concentrations in aqueous solutions containing 30 wt% of [Ch][Ac] are reported in Supporting information, Fig. S3. No evidences of protein self-aggregation were observed. These results reveal the viability of cholinium-based ILs for the extraction and preservation of proteins. Although only some examples are provided in Fig. 4, it should be remarked that all the SE-HPLC chromatograms, used in the quantification of BSA, were

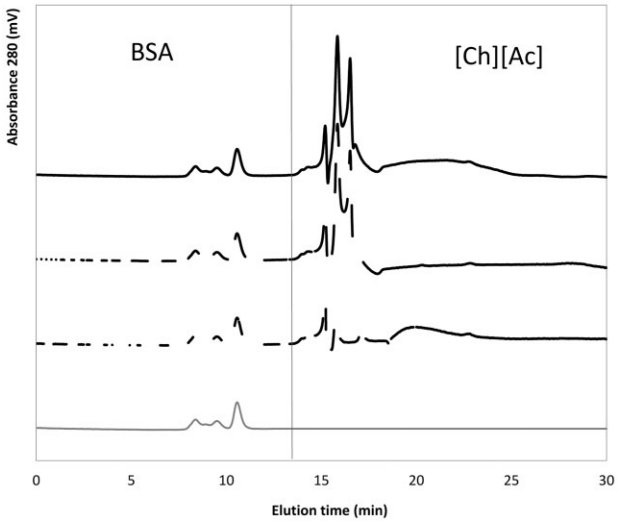

Figure 5. Size exclusion chromatograms of a BSA solution in PBS (0.5 g L^{-1}) (—), BSA solution with 12.5 wt% of [Ch][Ac] (-----), BSA solution with 25 wt% of [Ch][Ac] (·····) and BSA solution with 50 wt% of [Ch][Ac] (—). A Chromaster HPLC, Hitachi, coupled with an UV-Vis detector was employed. A 100 mM phosphate buffer in MiliQ water as a mobile phase was run isocratically with a flow rate of 1 mL min^{-1}. The column oven and autosampler were kept at a constant temperature of 25°C. The wavelength was set at 280 nm.

deeply analysed and allowed us to confirm that BSA maintained its native structure.

BSA in [Ch][Ac] aqueous solutions was also analyzed by FT-IR after being submitted to a range of different temperatures (from 4 to 50°C). The results obtained (Supporting information, Fig. S4) show that there are no significant changes in the protein secondary structure at lower temperatures (including 25°C used in the partitioning experiments). However, for 55°C it is evident the deviation of the amide I peak (after 20 min of equilibration), meaning that the protein may lose its native secondary structure at higher temperatures. Nevertheless, the phase diagrams and extraction routes proposed in this work are carried out at 25°C, where no loss of stability was observed.

In the present work, remarkable extractions efficiencies of BSA ranging between 92 and 100% for the IL-rich phase were obtained in a single step, without evidence of protein denaturation in concentrations at the aqueous media up to 10 g L^{-1}. In addition, the investigated biocompatible ABS are by far better systems for the extraction of BSA when compared with more traditional polymer-based or other IL-based ABS. The applicability of these systems, and the one-step complete extraction of BSA, was confirmed using a more complex and real matrix – fetal bovine serum sample. These new ABS may thus be envisaged as a new platform for the separation and purification of a wide variety of value-added proteins, such as biopharmaceuticals.

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4 References

[1] Patel, R., Kumari, M., Khan, A. B., Recent advances in the applications of ionic liquids in protein stability and activity: A review. *Appl. Biochem. Biotechnol.* 2014, *172*, 3701–3720.

[2] Peters, T. J., Partition of cell particles and macromolecules: Separation and purification of biomolecules, cell organelles, membranes and cells in aqueous polymer two phase systems and their use in biochemical analysis and biotechnology. in: P-A. Albertsson, (Ed.), *Cell Biochemistry and Function*, John Wiley and Sons, Chichester 1987, pp. 233–234.

[3] Vargas, M., Segura, A., Herrera, M., Villalta, M. et al., Purification of IgG and albumin from human plasma by aqueous two phase system fractionation. *Biotechnol. Progr.* 2012, *28*, 1005–1011.

[4] Scholz, M., Lüking, A., A protein-stabilizing technology for enhanced antibody stability and antibody-binding profiles in a microchip array. *Biotechnol. J.* 2012, *7*, 1002–1007.

[5] Andrews, B. A., Nielsen, S., Asenjo, J. A., Partitioning and purification of monoclonal antibodies in aqueous two-phase systems. *Bioseparation* 1996, *6*, 303–313.

[6] Zeng, Q., Wang, Y., Li, N., Huang, X. et al., Extraction of proteins with ionic liquid aqueous two-phase system based on guanidine ionic liquid. *Talanta* 2013, *116*, 409–416.

[7] Aguilar, O., Albiter, V., Serrano-Carre"n, L., Rito-Palomares, M., Direct comparison between ion-exchange chromatography and aqueous two-phase processes for the partial purification of penicillin acylase produced by E. coli. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* 2006, *835*, 77–83.

[8] Alm, T., Steen, J., Ottosson, J., Hober, S., High-throughput protein purification under denaturating conditions by the use of cation exchange chromatography. *Biotechnol. J.* 2007, *2*, 709–716.

[9] Nfor, B. K., Ripic, J., Van der Padt, A., Jacobs, M., Ottens, M., Model-based high-throughput process development for chromatographic whey proteins separation. *Biotechnol. J.* 2012, *7*, 1221–1232.

[10] Lin, X., Wang, Y., Zeng, Q., Ding, X., Chen, J., Extraction and separation of proteins by ionic liquid aqueous two-phase system. *Analyst* 2013, *138*, 6445–6453.

[11] Albertsson, P. A., Particle fractionation in liquid two-phase systems; the composition of some phase systems and the behaviour of some model particles in them; application to the isolation of cell walls from microorganisms. *Biochim. Biophys. Acta.* 1958, *27*, 378–395.

[12] P-A, A. (Eds), Partition of cell particles and macromolecules, *Acta Biotechnol.* John Wiley and Sons, New York 1986, pp. 146–146.

[13] Sutherland, I., Hewitson, P., Siebers, R., van den Heuvel, R., Arbenz, L., Kinkel, J., and Fisher, D., Scale-up of protein purifications using aqueous two-phase systems: Comparing multilayer toroidal coil

chromatography with centrifugal partition chromatography. *J. Chromatogr. A* 2011, 1218, 5527–5530.
[14] Rito-Palomares, M., Practical application of aqueous two-phase partition to process development for the recovery of biological products. *J. Chromatogr. B* 2004. 807, 3–11.
[15] Asenjo, J. A. and Andrews, B. A., Aqueous two-phase systems for protein separation: A perspective. *J. Chromatogr. A* 2011, 1218, 8826–8835.
[16] Rosa, P. A. J., Azevedo, A. M., Sommerfeld, S., Mutter, M. et al., Continuous purification of antibodies from cell culture supernatant with aqueous two-phase systems: From concept to process. *Biotechnol. J.* 2013, *8*, 352–362.
[17] Li, M., Kim, J.-W., Peeples, T. L., Amylase partitioning and extractive bioconversion of starch using thermoseparating aqueous two-phase systems. *J. Biotechnol.* 2002, *93*, 15–26.
[18] Platis, D., Labrou, N. E., Application of a PEG/salt aqueous two-phase partition system for the recovery of monoclonal antibodies from unclarified transgenic tobacco extract. *Biotechnol. J.* 2009, *4*, 1320–1327.
[19] Gutowski, K. E., Broker, G. A., Willauer, H. D., Huddleston, J. G. et al., 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.
[20] Freire, M. G., Pereira, J. F. B., Francisco, M., Rodríguez, H. et al., Insight into the interactions that control the phase behaviour of new aqueous biphasic systems composed of polyethylene glycol polymers and ionic liquids. *Chem. Eur. J.* 2012, *18*, 1831–1839.
[21] Pereira, J. F. B., Rebelo, L. P. N., Rogers, R. D., Coutinho, J. A. P., Freire, M. G., Combining ionic liquids and polyethylene glycols to boost the hydrophobic-hydrophilic range of aqueous biphasic systems. *Phys. Chem. Chem. Phys.* 2013, *15*, 19580–19583.
[22] Cláudio, A. F. M., Freire, M. G., Freire, C. S. R., Silvestre, A. J. D., Coutinho, J. A. P., Extraction of vanillin using ionic-liquid-based aqueous two-phase systems. *Sep. Purif. Technol.* 2010, *75*, 39–47.
[23] Ventura, S. P. M., Sousa, S. G., Freire, M. G., Serafim, L. S. et al., Design of ionic liquids for lipase purification. *J. Chromatogr. B* 2011, *879*, 2679–2687.
[24] Du, Z., Yu, Y.-L., Wang, J.-H., Extraction of proteins from biological fluids by use of an ionic liquid/aqueous two-phase system. *Chem. Eur. J.* 2007, *13*, 2130–2137.
[25] Freire, M. G., Cláudio, A. F. M., Araújo, J. M. M., Coutinho, J. A. P. et al., Aqueous biphasic systems: a boost brought about by using ionic liquids. *Chem. Soc. Rev.* 2012, *41*, 4966–4995.
[26] Pei, Y., Wang, J., Wu, K., Xuan, X., Lu, X., Ionic liquid-based aqueous two-phase extraction of selected proteins. *Sep. Purif. Technol.* 2009, *64*, 288–295.
[27] Selvakumar, P., Ling, T. C., Walker, S., Lyddiatt, A., Partitioning of haemoglobin and bovine serum albumin from whole bovine blood using aqueous two-phase systems. *Sep. Purif. Technol.* 2012, *90*, 182–188.
[28] Yan, J.-K., Ma, H.-L., Pei, J.-J., Wang, Z.-B., Wu, J.-Y., Facile and effective separation of polysaccharides and proteins from Cordyceps sinensis mycelia by ionic liquid aqueous two-phase system. *Sep. Purif. Technol.* 2014, *135*, 278–284.
[29] Good, N. E., Winget, G. D., Winter, W., Connolly, T. N. et al., Hydrogen ion buffers for biological research. *Biochemistry* 1966, *5*, 467–477.
[30] Taha, M., e Silva, F. A., Quental, M. V., Ventura, S. P. M. et al., Good's buffers as a basis for developing self-buffering and biocompatible ionic liquids for biological research. *Green Chem.* 2014, *16*, 3149–3159.
[31] Erickson, B., Nelson, Winters, P., Perspective on opportunities in industrial biotechnology in renewable chemicals. *Biotechnol. J.* 2012, *7*, 176–185.
[32] Wasserscheid, P., Welton, T. (Eds), *Ionic Liquids in Synthesis*, Wiley-VCH, Weinheim 2008.
[33] Pernak, J., Syguda, A., Mirska, I., Pernak, A. et al., Choline-derivative-based ionic liquids. *Chem. Eur. J.* 2007, *13*, 6817–6827.
[34] Muhammad, N., Hossain, M. I., Man, Z., El-Harbawi, M. et al., Synthesis and physical properties of choline carboxylate ionic liquids. *J. Chem. Eng. Data* 2012, *57*, 2191–2196.
[35] Mourão, T., Cláudio, A. F. M., Boal-Palheiros, I., Freire, M. G., Coutinho, J. A. P., Evaluation of the impact of phosphate salts on the formation of ionic-liquid-based aqueous biphasic systems. *J. Chem. Thermodyn.* 2012, *54*, 398–405.
[36] Merchuk, J. C., Andrews, B. A., Asenjo, J. A., Aqueous two-phase systems for protein separation. Studies on phase inversion. *J. Chromatogr. B* 1998, *711*, 285–293.
[37] Passos, H., Ferreira, A. R., Cláudio, A. F. M., Coutinho, J. A. P., and Freire, M. G., Characterization of aqueous biphasic systems composed of ionic liquids and a citrate-based biodegradable salt. *Bioch. Eng. J.* 2012, *67*, 68–76.
[38] Cla´udio, A. F. M., Ferreira, A. M., Shahriari, S., Freire, M. G., and Coutinho, J. o. A. P., Critical assessment of the formation of ionic-liquid-based aqueous two-phase systems in acidic media. *J. Phys. Chem. B* 2011, 115, 11145–11153.
[39] Pereira, J. F. B., Kurnia, K. A., Cojocaru, O. A., Gurau, G. et al., Molecular interactions in aqueous biphasic systems composed of polyethylene glycol and crystalline vs. liquid cholinium-based salts. *Phys. Chem. Chem. Phys.* 2014, *16*, 5723–5731.
[40] Glusker, J. P., Citrate conformation and chelation: Enzymic implications. *Acc. Chem. Res.* 1980, *13*, 345–352.
[41] Ge, S., Kojio, K., Takahara, A., Kajiyama, T., Bovine serum albumin adsorption onto immobilized organotrichlorosilane surface: Influence of the phase separation on protein adsorption patterns. *J. Biomater. Sci. Polym. Ed.* 1998, *9*, 131–150.
[42] Chen, J., Wang, Y., Zeng, Q., Ding, X., Huang, Y., Partition of proteins with extraction in aqueous two-phase system by hydroxyl ammonium-based ionic liquid. *Anal. Methods* 2014, *6*, 4067–4076.
[43] Kohno, Y., Saita, S., Murata, K., Nakamura, N., Ohno, H., Extraction of proteins with temperature sensitive and reversible phase change of ionic liquid/water mixture. *Polym. Chem.* 2011, *2*, 862–867.
[44] Rito-Palomares, M., Dale, C., Lyddiatt, A., Generic application of an aqueous two-phase process for protein recovery from animal blood. *Process Biochem.* 2000, *35*, 665–673.
[45] Salabat, A. R., Abnosi, M. H., Motahari, A., Application of aqueous mixtures of polypropylene glycol or polyethylene glycol with salts in proteomic analysis. *J. Iran. Chem. Soc.* 2010, *7*, 142–149.
[46] Ding, X., Wang, Y., Zeng, Q., Chen, J. et al., Design of functional guanidinium ionic liquid aqueous two-phase systems for the efficient purification of protein. *Anal. Chim. Acta* 2014, *815*, 22–32.
[47] Li, Z., Liu, X., Pei, Y., Wang, J., He, M., Design of environmentally friendly ionic liquid aqueous two-phase systems for the efficient and high activity extraction of proteins. *Green Chem.* 2012, *14*, 2941–2941.
[48] Rodrigues, J. V., Prosinecki, V., Marrucho, I., Rebelo, L. P. N., Gomes, C. M., Protein stability in an ionic liquid milieu: On the use of differential scanning fluorimetry. *Phys. Chem. Chem. Phys.* 2011, *13*, 13614–13616.
[49] Xue, L., Zhao, Y., Yu, L., Sun, Y. et al., Choline acetate enhanced the catalytic performance of Candida rogusa lipase in AOT reverse micelles. *J. Colloids Surf. B* 2013, *105*, 81–86.

[50] Kumar, A., Venkatesu, P., Does the stability of proteins in ionic liquids obey the Hofmeister series? *Int. J. Biol. Macromol.* 2014, *63*, 244–253.
[51] Dreyer, S., Salim, P., Kragl, U., Driving forces of protein partitioning in an ionic liquid-based aqueous two-phase system. *Biochem. Eng. J.* 2009, *46*, 176–185.
[52] Desai, R. K., Streefland, M., Wijffels, R. H., H. M. Eppink, M., Extraction and stability of selected proteins in ionic liquid based aqueous two phase systems. *Green Chem.* 2014, *16*, 2670–2670.
[53] Saadeh, S. M., Yasseen, Z., Sharif, F. A., Abu Shawish, H. M., New room temperature ionic liquids with interesting ecotoxicological and antimicrobial properties. *Ecotoxicol. Environ. Saf.* 2009, *72*, 1805–1809.
[54] Shu, Y., Liu, M., Chen, S., Chen, X., Wang, J., New insight into molecular interactions of imidazolium ionic liquids with bovine serum albumin. *J. Phys. Chem. B* 2011, *115*, 12306–12314.