Good's buffers as novel phase-forming components of ionic-liquid-based aqueous biphasic systems

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ABSTRACT

Aiming at the development of self-buffering and benign extraction/separation processes, this work reports a novel class of aqueous biphasic systems (ABS) composed of ionic

liquids (ILs) and organic biological buffers (Good's buffers, GBs). A large array of ILs and GBs was investigated, revealing that only the more hydrophobic and fluorinated ILs are able to form ABS. For these systems, the phase diagrams, tie-lines, tie-line lengths, and critical points were determined at 25 °C. The ABS were then evaluated as alternative liquid-liquid extraction strategies for two amino acids (L-phenylalanine and L-tryptophan). The single-step extraction efficiencies for the GB-rich phase range between 22.4 and 100.0 % (complete extraction). Contrarily to the most conventional IL-salt ABS, in most of the systems investigated, the amino acids preferentially migrate for the most biocompatible and hydrophilic GB-rich phase. Remarkably, in two of the studied ABS, L-phenylalanine completely partitions to the GB-rich phase while L-tryptophan shows a preferential affinity for the opposite phase. These results show that the extraction efficiencies of similar amino acids can be tailored by the design of the chemical structures of the phase-forming components, creating thus new possibilities for the use of IL-based ABS in biotechnological separations.

Keywords: Liquid-liquid extraction, Bioseparation, Aqueous biphasic system, Ionic liquid, Good's buffers, Amino acid.

1. Introduction

The development of benign separation techniques has been a hot topic of research envisaging the extraction and purification of added-value compounds from biological media [1, 2]. Biocompatibility is a crucial feature in the design of these platforms, particularly when dealing with biologically active products [3]. Typical organic solvents employed in liquid-liquid extractions from aqueous media are usually highly volatile and toxic. In this context, aqueous biphasic systems (ABS) - liquid-liquid systems mostly composed of water - are potential alternatives for extraction and purification

processes. These systems consist in two aqueous-rich phases and can be created by the mixture of two polymers, a polymer with a salt or two salts. Liquid-liquid extractions by ABS have been intensively explored and used to separate and purify several biological products [4-6] and also to recover metal ions, radiochemicals, drugs molecules, dyes, among others, from complex mixtures [7, 8].

In the last decade, it was demonstrated that ionic liquids (ILs) can be also employed as phase-forming components of ABS [9]. In general, ILs are organic salts with a melting temperature below 100 °C. Due to their ionic nature, ILs display exceptional properties, such as a negligible vapour pressure, a wide liquid temperature range, a high solvating ability for a wide variety of compounds or materials, and high thermal and chemical stabilities [10]. ILs are also good extraction solvents, both neat and in aqueous solutions, and are able to increase the stability of added-value biomolecules, namely proteins, enzymes and DNA [11-15]. Taking into account these features, IL-based ABS have been extensively investigated as alternative liquid-liquid extraction processes, allowing enhanced and selective extractions [16]. These ternary systems consist of two immiscible aqueous-rich phases, and could be designed by IL + salt, IL + carbohydrate, IL + amino acid or IL + polymer pairs dissolved in aqueous solution [12]. However, most of the investigations carried out with IL-based ABS are focused on combinations of ILs and high-charge density salts [16]. The major reason behind such a selection is related to the large ability of high-charge density salts, and thus to their salting-out nature, to create IL-based ABS. The salting-out effect is mostly a result of the formation of water-ion complexes that results on the “dehydration” of the IL [17-19]. Nevertheless, these systems suffer from major drawbacks resulting from the use of inorganic salts, namely their high charge-density and the creation of solutions of high ionic strength and extreme pH values that may damage the biological products, such as

proteins, if buffers combinations or mixtures of salts are not employed. Moreover, the high concentration of these salts may be deleterious when discharged into aqueous effluents [20]. Even so, most studies regarding IL-based ABS comprised inorganic salts based on phosphate, carbonate and sulphate anions [14, 21-23]. Most of the systems investigated lead to the formation of alkaline aqueous salt solutions (*e.g.*, with the use of K_2HPO_4 , K_3PO_4 , K_2CO_3 , KOH , Na_2HPO_4 and NaOH) [9, 21, 23, 24]. On the other hand, the use of salt mixtures and buffered solutions with pH close to biological values has been less explored [25, 26]. In this context, the phase-forming ability of IL-based ABS by the addition of non-electrolytes, self-buffering and biocompatible salting-out agents (Good's buffers, GBs) was here investigated for the first time.

GBs, recognized as inert biological buffers for biochemical and biological studies, were developed by Good and co-workers [27-29]. GBs are zwitterionic compounds consisting of *N*-substituted taurine and glycine derivatives. Most of these buffers display pK_a values between 6 and 8, have a good solubility in water, do not readily permeate through cell membranes, do not chelate with metal-ions, are chemically stable and resist to enzymatic degradation, and are easily prepared and purified from low-cost materials [27, 29, 30]. Taking into account the collective benefits of GBs and ILs, we propose here their combined use as phase-forming components of ABS. Among the available GBs, *N*-tris(hydroxymethyl)methylglycine (Tricine), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES), and 2-(*N*-morpholino)ethanesulfonic acid (MES) were selected because they are

suitable buffers for pH control in the physiological pH region (although an alkaline species needs to be added for this purpose) [29,30]. Novel ternary phase diagrams for ABS composed of GBs and ILs were determined at 25 °C, and their ability as novel liquid-liquid systems for extraction/purification purposes was evaluated by partitioning studies carried out with standard aqueous solutions of two amino acids that can be produced in fermentative medium (L-tryptophan and L-phenylalanine) [31, 32].

2. Material and Methods

2.1. Materials

The GBs investigated were Tricine (purity > 99 wt %), HEPES (purity > 99.5 wt %), TES (purity > 99 wt %), CHES (purity > 99 wt %), and MES (purity > 99 wt %), all purchased from Sigma-Aldrich. The ILs studied for ABS formation were 1-butyl-3-methylimidazolium trifluoromethanesulfonate ([C₄mim][CF₃SO₃], purity ≥ 99 wt %), 1-butyl-3-methylimidazolium tetrafluoroborate ([C₄mim][BF₄], purity ≥ 99 wt %), 1-butyl-3-methylimidazolium dicyanamide ([C₄mim][N(CN)₂], purity > 98 wt %), 1-butyl-3-methylimidazolium thiocyanate ([C₄mim][SCN], purity > 98 wt %), 1-butyl-3-methylimidazolium tosylate ([C₄mim][TOS], purity 99 wt %), 1-ethyl-3-methylimidazolium trifluoromethanesulfonate ([C₂mim][CF₃SO₃], purity 99 wt %), 1-ethyl-1-methylpyrrolidinium trifluoromethanesulfonate ([C₂mpyr][CF₃SO₃], purity 99 wt %), all purchased from Iolitec. Tetrabutylphosphonium bromide ([P₄₄₄₄][Br], purity > 95 wt %), kindly supplied by Cytec Industries Inc., was also investigated. To reduce the volatile impurities to negligible values, IL samples were purified under constant agitation, under vacuum, and at moderate temperature (60 °C), for a minimum of 24 h. After this procedure, the purity of each IL was further checked by ¹H, ¹³C and ¹⁹F NMR spectra (whenever applicable) and found to be in accordance with the stated purity level

provided by the suppliers. L-tryptophan (purity > 99.0 wt %) and L-phenylalanine (purity 99.0 wt %) were acquired from Sigma-Aldrich and Merck, respectively. Although several combinations of ILs and GBs were tested to create novel ABS, only aqueous mixtures composed of the more hydrophobic ILs ([C₄mim][CF₃SO₃] and [C₄mim][BF₄]) and the GBs Tricine, HEPES and TES were able to form ABS. The chemical structures of the GBs and ILs able to undergo liquid-liquid demixing towards the formation of ABS, and of the amino acids used in the extraction experiments, are shown in Fig. 1. The water used was double distilled, passed through a reverse osmosis system, and additionally treated with a Milli-Q plus 185 purification apparatus.

2.2. Methods

2.2.1. Phase diagrams, tie-lines, tie-line lengths and critical points

The solubility (saturation) curves were determined through the cloud point titration method [33] at (25 ± 1) °C and atmospheric pressure. Aqueous solutions of ILs at 80-90 wt % and aqueous solutions of the different GBs at concentrations below, yet close, to their saturation values in water (Tricine at 20 wt %, HEPES at 50 wt % and TES at 55 wt %) were prepared gravimetrically and used for the determination of the binodal curves. Repetitive drop wise addition of the aqueous IL solution to the aqueous solution of each GB was carried out until the detection of a cloudy (biphasic) mixture, followed by the drop wise addition of ultrapure water until the observation of the monophasic region (clear and limpid solution). The ternary system compositions were determined by weight quantification within $\pm 10^{-4}$ g. The experimental binodal curves were fitted to Eq. (1) [34]:

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

where $[IL]$ and $[GB]$ are the IL and GB weight fraction percentages, respectively, and the coefficients A , B , and C are the fitting parameters.

The tie-lines (TLs) were determined by a gravimetric method originally proposed by Merchuk et al. [34] for polymer-based ABS, applied later by Rogers and co-workers [7] and by us to IL-based ABS [33]. A ternary mixture composed of IL + GB + water at the biphasic region was gravimetrically prepared within $\pm 10^{-4}$ g, vigorously agitated, and left to equilibrate for at least 12 h and at (25 ± 1) °C, aiming a complete separation of the coexisting phases. After this period, both phases were carefully separated and individually weighed. Each TL was determined by the lever-arm rule through the relationship between the IL-rich phase composition and the overall system composition, for which the following system of four equations (Eqs. (2) to (5)) and four unknown values ($[IL]_{IL}$, $[IL]_{GB}$, $[GB]_{IL}$, and $[GB]_{GB}$) was solved [34]:

$$[IL]_{IL} = A \exp[(B \times [GB]_{IL}^{0.5}) - (C \times [GB]_{IL}^3)] \quad (2)$$

$$[IL]_{GB} = A \exp[(B \times [GB]_{GB}^{0.5}) - (C \times [GB]_{GB}^3)] \quad (3)$$

$$[IL]_{IL} = \frac{[IL]_M}{\alpha} - \frac{1-\alpha}{\alpha} [IL]_{GB} \quad (4)$$

$$[GB]_{IL} = \frac{[GB]_M}{\alpha} - \frac{1-\alpha}{\alpha} [GB]_{GB} \quad (5)$$

where subscripts “IL”, “GB” and “M” designate the IL-rich phase, the GB-rich phase and the mixture composition, respectively; α is the ratio between the mass of the IL-rich phase and the total mass of the mixture. The system solution results in the composition

(wt %) of the GB and IL in the top and bottom phases. The identification of the IL- and GB-rich aqueous phases was carried out through the IL quantification in each phase at 212 nm by UV-spectroscopy using a BioTeck Synergy HT microplate reader.

For the calculation of each tie-line length (TLL) the following equation was used:

$$TLL = \sqrt{([GB]_{IL} - [GB]_{GB})^2 + ([IL]_{IL} - [IL]_{GB})^2} \quad (6)$$

The critical point of each system (the mixture composition at which the composition of the two aqueous phases becomes equal) was also estimated. The critical point was determined by extrapolating the TLs' slopes of distinct systems by the fitting provided by Eq. (7) [34].

$$[IL] = f[GB] + g \quad (7)$$

where f and g are fitting parameters.

The pH values (± 0.02) were measured at (25 ± 1) °C using a Mettler Toledo SevenMultiTMdual pH meter.

2.2.2. Partitioning of amino acids

Aqueous solutions of amino acids were prepared at the concentration of $0.73 \text{ g}\cdot\text{dm}^{-3}$ ($3.6 \times 10^{-3} \text{ mol}\cdot\text{dm}^{-3}$) for L-tryptophan and $0.60 \text{ g}\cdot\text{dm}^{-3}$ ($3.6 \times 10^{-3} \text{ mol}\cdot\text{dm}^{-3}$) for L-phenylalanine. At these concentrations, the amino acids can be considered at infinite dilution and completely solvated in aqueous media, avoiding thus specific interactions between the biomolecules. The ternary mixtures compositions were chosen based on the phase diagrams determined before for each IL-GB ABS. To avoid discrepancies in the results which could arise from the different compositions of the phases, all the

partitioning studies were performed at a constant TLL (*ca.* 50) and weight ratio ($\alpha \sim 0.5$). Each mixture was vigorously stirred and left to equilibrate for at least 12 h (a time established in previous optimizing experiments), to achieve the complete partitioning of each amino acid between the coexisting phases. After a careful separation of both phases, the quantification of each amino acid in each phase was carried by UV-spectroscopy, using a BioTeck Synergy HT microplate reader, at a wavelength of 279 nm for L-tryptophan and 257 nm for L-phenylalanine using calibration curves previously established. At least three individual biphasic systems were prepared in order to determine the average in the amino acids partition coefficients and extraction efficiencies, as well as the respective standard deviations. Possible interferences of the GBs and ILs with the analytical method were taking into account, and control samples were always prepared at the same weight fraction composition, using pure water instead of the amino acid aqueous solution. The partition coefficients of L-tryptophan (K_{Try}) and L-phenylalanine (K_{Phe}) are defined as the ratio of the concentration of the each amino acid in the GB-rich phase to that in the IL-rich phase Eq. (8),

$$K_{\text{AA}} = \frac{[\text{AA}]_{\text{GB}}}{[\text{AA}]_{\text{IL}}} \quad (8)$$

where $[\text{AA}]_{\text{GB}}$ and $[\text{AA}]_{\text{IL}}$ are the concentration of each amino acid (AA) in the GB- and in the IL-rich aqueous phases, respectively.

The percentage extraction efficiency of L-tryptophan ($EE_{\text{Try}} \%$) and phenylalanine ($EE_{\text{Phe}} \%$) are defined as the percentage ratio between the amount of amino acid in the GB-rich aqueous phase and that in the total mixture, according to Eq. (9).

$$EE_{AA} \% = \frac{w_{AA}^{GB}}{w_{AA}^{GB} + w_{AA}^{IL}} \quad (9)$$

where w_{AA}^{GB} and w_{AA}^{IL} are the amount of amino acid (AA) in the GB-rich and in the IL-rich aqueous phase, respectively.

3. Results and Discussion

3.1. Phase diagrams, tie-lines, tie-line lengths and critical points

Novel ternary phase diagrams were determined in this work, and the respective solubility curves are illustrated in Fig. 2. The detailed experimental weight fraction data are given in the Supplementary Data. In the studied ABS, the bottom phase corresponds to the IL-rich phase, while the top phase is mainly composed of GB and water. The only exception was observed with the ABS composed of $[C_4mim][BF_4] + TES + H_2O$. The identification of the IL-rich phase in each system was carried out by UV-spectroscopy.

Although a large range of ILs was investigated, only those composed of $[CF_3SO_3]^-$ and $[BF_4]^-$ anions coupled to the imidazolium cation with a longer alkyl side chain (at least butyl) were able to form ABS with GBs. This is a result of the weak salting-out ability of the GBs for instance when compared with high-charge density salts [16], and agrees with previous results on the formation of IL-based ABS with weaker salting-out species, such as carbohydrates [35] or amino acids [36]. The GBs seem to be preferentially hydrated and lead to the “dehydration” of the IL and to the formation of a second liquid phase taking into account that only the most hydrophobic ILs are able to form ABS. In Fig. 2, the solubility curves are presented in weight fraction, whereas their representation in molality units is provided in the Supplementary Data. However, it should be highlighted that the same trend on the ILs and GBs ability to form ABS is observed in both units. In all phase diagrams, the biphasic region is localized above the solubility curve whereas the monophasic region corresponds to compositions described

below the solubility curve. The larger the biphasic region, the higher the ability of the IL or GB to undergo liquid–liquid demixing.

For the studied systems, the experimental binodal data were further fitted by the empirical relationship described by Eq. (1). The regression parameters A , B and C were estimated by the least-squares regression method, and their values and corresponding standard deviations (σ) are provided in Table 1. In general, good correlation coefficients were obtained for all systems, indicating that these fittings can be used to estimate the phase diagram in a region where no experimental results are available. The representation of the fitting by Eq. (1) is also depicted in Fig. 2.

Additionally, the critical point of each system was determined and is represented in Fig. 2 - detailed data are given in the Supplementary Data. The compositions of the coexisting phases become equal at concentrations of GB ranging between 3.87 and 22.35 wt % and at concentrations of IL ranging from 41.12 to 51.07 wt %. The critical point is more dependent on the concentration of the GB than on the IL composition and, in general, the concentration of IL at the critical point is higher for systems with less ability to form ABS.

The experimental TLs, along with their respective length (TLL), as well as the pH values of both phases in each ABS, and for the compositions for which the TLs were determined, are given in Table 2. An example of the TLs obtained is depicted in Fig. 3, while the TLs for the remaining systems are depicted in the Supplementary Data.

The pH values of these systems are in the acidic region due to the acidic nature of the pure GBs (aqueous solutions of 30 wt % of HEPES, TES, and Tricine present pH values from 3 to 5 as determined by us). The pH values determined for the coexisting phases of the IL-based ABS range between 2 and 6, with an average pH value of circa 4.9. Although not attempted in this work, it should be stressed that the pH of these systems

can be adjusted to the physiological pH range by the addition of an alkaline species [26]. However, acidic ABS are particularly valuable for the extraction of high-value compounds with low acidic dissociation constants to guarantee their neutral form [37]. The ABS studied in this work are also a particular class of acidic systems, since most works in the literature on IL-based ABS display alkaline coexisting phases that may be deleterious to a number of pH sensitive biomolecules [21, 38].

Fig. 2 depicts the effect of different GBs in the formation of ABS. For instance, at 40 wt % of $[C_4mim][BF_4]$, the ability of the GBs to undergo liquid-liquid demixing follows the trend: Tricine $\gg$ HEPES $>$ TES. Overall, a decrease on the hydrophobicity of the GB was expected to facilitate the creation of ABS. The $\log(K_{ow})$ values of each buffer are, respectively, -3.11 for HEPES, -4.48 for TES, and -5.25 for Tricine [39]. Although all the GBs investigated are quite hydrophilic, the trend observed for the ABS formation does not follow the K_{ow} values. Moreover, if Tricine is the GB with the highest ability to form ABS with $[C_4mim][BF_4]$, the same should be observed with $[C_4mim][CF_3SO_3]$. Instead, with $[C_4mim][CF_3SO_3]$, only the most hydrophobic GB (HEPES) revealed to be able to induce liquid-liquid demixing. It should be highlighted that more hydrophobic GBs, namely MES ($\log(K_{ow}) = -2.49$) and CHES ($\log(K_{ow}) = -0.59$) [39], were also tested with both ILs, although no phase separation was observed. Even so, and since only highly hydrophobic and fluorinated ILs were able to form ABS, the overall results seem to indicate that GBs display a higher affinity for water compared to the ILs, and thus, there is the preferential exclusion of the IL from the aqueous solution leading to the formation of ABS. However, and in general, it seems that more complex interactions between the GBs and the ILs, in addition to preferential hydration of the GBs, are taking place.

The formation of ABS depends also on the type of IL. In the studied phase diagrams it is possible to observe that the IL $[\text{C}_4\text{mim}][\text{BF}_4]$ is more able to form ABS than $[\text{C}_4\text{mim}][\text{CF}_3\text{SO}_3]$. $[\text{C}_4\text{mim}][\text{CF}_3\text{SO}_3]$ was only able to form ABS with HEPES, while solid-liquid equilibrium was observed when this IL is mixed with aqueous solutions of Tricine or TES in the whole composition range. It should be remarked that although $[\text{C}_4\text{mim}][\text{BF}_4]$ is not the best candidate to form ABS, since it may suffer hydrolysis when in aqueous media [40], the pH values presented in Table 2 support that if hydrolysis occurs, it is very limited – at least within the 12 h of equilibrium used for the determination of the TLs. In fact, the pH values of the coexisting phases corresponding to the system formed by $[\text{C}_4\text{mim}][\text{BF}_4]$ + HEPES are very similar to those displayed by the water-stable IL $[\text{C}_4\text{mim}][\text{CF}_3\text{SO}_3]$. Even though other ILs should be more adequate to form ABS, and as attempted and explored in this work with $[\text{C}_4\text{mim}][\text{N}(\text{CN})_2]$, $[\text{C}_4\text{mim}][\text{SCN}]$, $[\text{P}_{4444}]\text{Br}$, $[\text{C}_4\text{mim}][\text{TOS}]$, $[\text{C}_2\text{mim}][\text{CF}_3\text{SO}_3]$ and $[\text{C}_2\text{mpyr}][\text{CF}_3\text{SO}_3]$, these were not able to form ABS with GBs, and thus IL-GBs ABS are confined to highly hydrophobic and fluorinated ILs.

It was already demonstrated that $[\text{C}_4\text{mim}][\text{CF}_3\text{SO}_3]$ could form IL-based ABS with others salting-out agents, such as salts, *e.g.* NaCl [41], carbohydrates [35], amino acids (L-lysine, D,L-lysine HCl and L-proline) [36] and polymers [42]. Fig. 4 depicts the comparison on the capacity of GBs to induce liquid-liquid demixing of $[\text{C}_4\text{mim}][\text{CF}_3\text{SO}_3]$ aqueous solutions against the previously reported salting-out agents [35, 36, 41, 42]. The results are shown in molality units to avoid distortions on the ability of the phase-forming components to create ABS as a result of their different molecular weights. The ability of HEPES to induce ABS is quite similar to that afforded by a weak salting-out salt such as NaCl (the solubility curves almost overlap). On the other hand, the polymer PEG 2000, L-lysine or D-(+)-glucose display a higher ability to

form ABS (larger biphasic regions above the solubility curves), whereas L-proline and D-(+)-xylose are weaker salting-out species than HEPES (smaller biphasic regions).

Although a few number of GBs and ILs presented the capacity of create ABS, it should be highlighted that their characteristics, such as the possibility of adjusting the pH of the systems to the physiological pH range, make of these systems a potential route for the recovery and purification of value-added biomolecules.

3.2. Extraction of amino acids

Fig. 5 depicts the percentage extraction efficiencies of L-tryptophan and L-phenylalanine in several IL-based ABS for the GB-rich phase. The results obtained for the partition coefficients of L-tryptophan and L-phenylalanine are illustrated in the Supplementary Data. The composition of the coexisting phases corresponding to the initial mixtures of [C₄mim][CF₃SO₃] + HEPES (36 wt% of HEPES, 30 wt% of IL, and 34 wt% of water), [C₄mim][BF₄] + HEPES (13.5 wt% of HEPES, 49 wt% of IL, and 37.5 wt% of water), [C₄mim][BF₄] + TES (19 wt% of TES, 43 wt% of IL, and 38 wt% of water) and for [C₄mim][BF₄] + Tricine (9 wt% of Tricine, 38 wt% of IL, and 53 wt% of water) used in the partitioning experiments are provided in Table 2. All the experiments were performed at a constant TLL (*ca.* 50) and weight ratio ($\alpha \sim 0.5$). The extraction efficiencies of L-tryptophan ($EE_{\text{Try}}\%$) and phenylalanine ($EE_{\text{Phe}}\%$) for the GB-rich phase range between 22.4 and 100.0 %. These results reveal that the partitioning of the two amino acids depends either on the IL or on the GB that compose a given ABS.

The octanol-water partition coefficients (K_{ow}) of both amino acids are very similar and their values translate their hydrophilic character ($\log(K_{\text{ow}})_{\text{Try}} = -1.06$ and $\log(K_{\text{ow}})_{\text{Phe}} = -1.38$) [43]. In most situations, there is a preferential partition of both amino acids for the

GB-rich phase – the most hydrophilic phase as can be ascertained by the water content in the coexisting phases given in Table 2. The extraction efficiency of L-tryptophan for the GB-rich phase follows the trend: $[\text{C}_4\text{mim}][\text{BF}_4]\text{-Tricine} > [\text{C}_4\text{mim}][\text{CF}_3\text{SO}_3]\text{-HEPES} > [\text{C}_4\text{mim}][\text{BF}_4]\text{-HEPES} > [\text{C}_4\text{mim}][\text{BF}_4]\text{-TES}$. On the other hand, the trend observed for L-phenylalanine is: $[\text{C}_4\text{mim}][\text{BF}_4]\text{-HEPES} \approx [\text{C}_4\text{mim}][\text{BF}_4]\text{-TES} > [\text{C}_4\text{mim}][\text{BF}_4]\text{-Tricine} > [\text{C}_4\text{mim}][\text{CF}_3\text{SO}_3]\text{-HEPES}$.

At the pH of the coexisting phases of the ABS studied, both amino acids are predominately on their zwitterionic form (with no net charge) - for L-tryptophan: $\text{p}K_{\text{a}1} = 2.38$ and $\text{p}K_{\text{a}2} = 9.39$; and for phenylalanine: $\text{p}K_{\text{a}1} = 2.13$ and $\text{p}K_{\text{a}2} = 8.62$ [43]. Consequently, the partitioning results obtained in this work are related with the affinity of each amino acid for the coexisting phases ruled by H-bonding and dispersive forces. Aqueous biphasic systems composed of polymer + salt, polymer + IL and salt + IL were already evaluated for the extraction of amino acids. For instance, partition coefficients for tryptophan of ≈ 1 in conventional polymer-polysaccharide [44], between 1 and 7 in polymer-salt [22] and between 10 and 120 in IL-salt [45] ABS were already reported.

Zafarani-Moattar and Hamzehzadeh [46] also determined the partitioning coefficients of several amino acids in ABS composed of 1-butyl-3-methylimidazolium bromide ($[\text{C}_4\text{mim}]\text{Br}$) and potassium citrate at 25 °C as a function of the pH of the aqueous media. The authors [46] concluded that dispersive interactions were the main driving force for the amino acids partitioning, although salting-out effects and electrostatic interactions also play a role. Nevertheless, in ABS composed of IL and salts, *e.g.* potassium citrate or potassium phosphate, aromatic amino acids always preferentially migrate for the IL-rich phase [33, 38, 45]. It was previously suggested that hydrophilic imidazolium-based ILs are good at extracting aromatic amino acids due to preferential $\pi \cdots \pi$ and H-bonding interactions [33, 38, 45]. In this work, and though in presence of

imidazolium-based ILs, both phenylalanine and tryptophan do not always preferentially partition towards the IL-rich phase. Therefore, in previous works [33, 38, 45], the salting-out effect afforded by the high-charge density salt must play a major role whereas, in this work, there is a more delicate balance between the favourable $\pi\cdots\pi$ interactions with the IL-rich phase and H-bonding interactions that can occur between the amino acids and all the phases components. Even though the chemical structure of carbohydrates was found to be negligible in the extraction of L-tryptophan with ABS formed by $[\text{C}_4\text{mim}][\text{CF}_3\text{SO}_3]$ and a wide range of monosaccharides, disaccharides and polyols [35], in IL-GB-based ABS the chemical structure of the GB seems to be an important factor.

Outstanding results were achieved with ABS composed of $[\text{C}_4\text{mim}][\text{BF}_4]$ and HEPES or TES, where the total recovery of L-phenylalanine to the GB-rich phase was observed in a single-step ($EE_{\text{Phe}}\% = 100$). These two systems have the additional interest of L-tryptophan showing a preferential affinity for the IL-rich phase. Based on these results, it can be envisaged that these systems can be optimized to reach a complete selective extraction, in a single-step, by the optimization of the mixture composition and pH of the coexisting phases. Furthermore, successive extractions can be applied to improve the separation of both amino acids, as well as by the use of more complex technologies, such as Centrifugal Partition Chromatography (CPC).[47] In summary, these systems open the possibility to deal with the selective separation of complex mixtures of amino acids, for instance from a fermentation broth or from hydrolysed peptide mixture.

Although the preferential migration of aromatic amino acids was not observed for the IL-rich phase, as observed before with IL + salt [33, 38, 45, 48] and IL + carbohydrate [35] ABS, it should be remarked that partition coefficients of L-tryptophan and L-phenylalanine similar to those observed in polymer-salt-based ABS [22] have been

obtained, and up to complete extraction, in a single-step. Moreover, the IL-GB ABS studied here allow a tailoring on the extraction of similar amino acids by an adequate manipulation of the chemical structure of either the IL or GB. Finally, the amino acids can be enriched in a benign and biocompatible GB-rich phase instead of the usual IL-rich phase.

The scalability of ABS is a well-known feature. For instance, Kroner et al. [49] reported the application of polymer-based ABS on the purification of formate dehydrogenase from *Candida boidinii* while Selber et al. [50] showed the application of detergent-based ABS on the purification of a fusion protein. The easiness in scaling-up and the almost non-existence of instrumental complexity supports the high potential of ABS as integrated extraction and purification strategies.

4. Conclusions

The extraction and purification of (bio)molecules by means of ABS is a promising approach due their water-rich environment and inherent biocompatibility. In the past decade, IL-based ABS have been studied as novel extraction routes while being able to demonstrate an outstanding performance on the extraction and purification of a large variety of compounds; yet, most of the IL-based ABS investigated are formed with high-charge density inorganic salts. Aiming at developing self-buffering and more biocompatible liquid-liquid extraction systems, in this work, we proposed the use of GBs as novel phase-forming components of IL-based ABS. A large array of ILs and GBs was investigated and, for the systems able to form ABS, the respective ternary phase diagrams, TLs, TLLs and critical points were determined. In general, only highly hydrophobic and fluorinated ILs are able to undergo liquid-liquid demixing with GBs in aqueous media.

The applicability of these ABS was also evaluated through the determination of the extraction efficiencies of two amino acids (L-tryptophan and L-phenylalanine) usually produced by fermentation processes. Contrarily to the most previously investigated IL-salt ABS, in most situations, the amino acids preferentially partition to the more hydrophilic GB-rich phase. The extraction efficiencies obtained range between 22 % and 100 %, and are highly dependent on the IL and GB chemical structures. Outstanding results were obtained with the systems formed by [C₄mim][BF₄] and HEPES or TES, where the complete extraction of L-phenylalanine for the GB-rich phase was observed in a single-step, while L-tryptophan preferentially migrates for the opposite phase. These results confirm a tailoring ability of the novel IL-GB ABS which can be useful in the selective extraction of mixtures of amino acids in biotechnological separations.

Acknowledgments

This work was developed in the scope of the project CICECO-Aveiro Institute of Materials (Ref. FCT UID/CTM /50011/2013), financed by national funds through the FCT/MEC and when applicable co-financed by FEDER under the PT2020 Partnership Agreement. The authors are also grateful for financial support from FCT - *Fundação para a Ciência e a Tecnologia* for the project PTDC/QUI-QUI/121520/2010 and post-doctoral and doctoral grants, SFRH/BPD/78441/2011 and SFRH/BD/85248/2012, from M. Taha and H. Passos, respectively. M. G. Freire acknowledges the European Research Council (ERC) for the Starting Grant ERC-2013-StG-337753.

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Figures Caption

Fig. 1. Chemical structure of the GBs, ILs and amino acids studied: (i) Tricine; (ii) HEPES; (iii) TES; (iv) $[\text{C}_4\text{mim}][\text{CF}_3\text{SO}_3]$; (v) $[\text{C}_4\text{mim}][\text{BF}_4]$; (vi) L-tryptophan; (vii) L-phenylalanine.

Fig. 2. Phase diagrams at 25 °C for the ABS composed of [C₄mim][BF₄] + Tricine + H₂O (◆); [C₄mim][BF₄] + HEPES + H₂O (■); [C₄mim][BF₄] + TES + H₂O (●); and [C₄mim][CF₃SO₃] + HEPES + H₂O (▲). The lines represent the fitting of the experimental data by Eq. (1) (—) and the yellow symbols represent the critical point of each system.

Fig. 3. Phase diagram for the ternary system composed of [C₄mim][CF₃SO₃] + HEPES + H₂O: binodal data (▲); TL data (●); critical point (▲); adjusted binodal data through Eq. (1) (—).

Fig. 4. Phase diagrams for ABS composed of [C₄mim][CF₃SO₃] and different salting-out agents: PEG 2000 (■); L-lysine (●); D-(+)-glucose (—); NaCl (◆); HEPES (▲); D-(+)-xylose (✕); and L-proline (*).

Fig. 5. Percentage extraction efficiencies of amino acids between the GB- and IL-rich aqueous phases at 25 °C: L-phenylalanine (green bars) and L-tryptophan (blue bars).

Figures

Fig. 1. Chemical structure of the GBs, ILs and amino acids studied: (i) Tricine; (ii) HEPES; (iii) TES; (iv) [C₄mim][CF₃SO₃]; (v) [C₄mim][BF₄]; (vi) L-tryptophan; (vii) L-phenylalanine.

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Fig. 5. Percentage extraction efficiencies of amino acids between the IL- and GB-rich aqueous phases at 25 °C: L-phenylalanine (green bars) and L-tryptophan (blue bars).

Tables

Table 1. Correlation parameters used to describe the experimental binodal data by Eq. (1) and respective standard deviation (σ).

GB	$A \pm \sigma$	$B \pm \sigma$	$10^5(C \pm \sigma)$	R^2
$[C_4mim][BF_4] + GB$				
Tricine	125.6 ± 4.8	-0.517 ± 0.027	18.48 ± 9.28	0.9922
HEPES	159.3 ± 8.0	-0.351 ± 0.019	1.60 ± 0.68	0.9941
TES	108.6 ± 40.7	-0.224 ± 0.107	1.00 ± 0.70	0.9535
$[C_4mim][CF_3SO_3] + GB$				
HEPES	158.0 ± 8.1	-0.256 ± 0.013	5.50 ± 0.59	0.9992

Table 2. Experimental data for TLs, TLLs and pH of the coexisting phases of the investigated ABS.

GB	Weight fraction composition / wt %								TLL
	[<i>IL</i>] _{IL}	[<i>GB</i>] _{IL}	pH _{IL}	[<i>IL</i>] _M	[<i>GB</i>] _M	[<i>IL</i>] _{GB}	[<i>GB</i>] _{GB}	pH _{GB}	
[C ₄ mim][BF ₄] + GB									
Tricine	52.70	2.79	5.09	39.73	5.98	15.11	12.04	4.82	38.71
	56.15	2.41	3.72	38.19	7.54	6.62	16.56	3.62	51.52
	60.47	1.99	3.77	38.21	9.05	2.56	20.36	3.65	60.76
HEPES	71.60	5.16	5.00	49.07	13.39	26.38	21.68	5.21	48.14
	85.67	3.12	5.56	38.06	19.94	19.31	26.57	5.13	70.38
	88.89	2.76	5.53	37.87	21.93	14.18	30.82	5.48	79.80
TES	58.80	7.42	2.05	43.51	17.04	28.66	26.38	2.00	35.61
	65.50	5.08	4.37	43.67	18.56	22.48	31.65	3.94	50.56
	72.26	3.31	2.34	43.44	19.97	21.12	32.88	1.95	59.08
[C ₄ mim][CF ₃ SO ₃] + GB									
HEPES	69.49	10.19	5.21	51.14	20.96	28.18	34.44	5.15	47.90
	82.58	6.42	6.08	49.72	24.88	20.85	41.21	5.95	70.86
	90.64	4.72	5.87	43.88	30.26	17.60	44.61	5.81	83.22