

Ionic liquids as a novel class of electrolytes in polymeric aqueous biphasic systems

João H.P.M. Santos^a, Francisca A. e Silva^a, João A.P. Coutinho^a, Sónia P.M. Ventura^{a,*}, Adalberto Pessoa Jr.^b

^a Chemistry Department, CICECO, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

^b São Paulo University, Av. Prof. Lineu Prestes n 580 Bloco 16, 05508-000 São Paulo, SP, Brazil

ARTICLE INFO

Article history:

Received 19 November 2014
Received in revised form 17 January 2015
Accepted 1 February 2015
Available online 10 February 2015

Keywords:

Aqueous biphasic systems
Ionic liquids
Electrolytes
Polyethylene glycol
Sodium poly(acrylate)
Extraction

ABSTRACT

The use of ionic liquids (ILs) as electrolytes in aqueous biphasic systems (ABS) composed exclusively of polymers and their application to the extraction of biomolecules is here studied. The binodal curves of the quaternary systems formed by polyethylene glycol (PEG 8000), sodium poly(acrylate) (NaPA 8000), water and ILs or inorganic salts as electrolytes are established. These systems enable the assessment of the influence of the salt nature, chemical structure and concentration on the two-phase formation. These systems were characterized regarding the pH and the ILs' partition between both phases. Moreover the extractive potential of these ABS is evaluated using the protein cytochrome *c* (Cyt *c*) and the dye chloranilic acid (CA). The main results on the extraction efficiencies show that Cyt *c* is recovered in the NaPA 8000-rich phase ($EE_{\text{Cyt } c} > 96.13 \pm 3.22\%$), while CA preferentially migrates towards the PEG 8000-rich phase ($EE_{\text{CA}} > 80.13 \pm 1.45\%$). The optimal electrolyte concentration in the CA partition was found to be the lowest possible, *i.e.* 0.1 wt%, as it leads to a remarkable increase in the partition coefficient of CA (K_{CA}) towards the PEG 8000-rich phase. These results indicate that this class of polymeric ABS may be an advantageous tool in the development of novel extractive platforms.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

Polyethylene glycol (PEG) is a non-ionic polymer commonly used in the preparation of aqueous biphasic systems (ABS), since it presents advantageous characteristics in terms of biodegradability, toxicity and cost-effectiveness. In order to create polymeric ABS, blends of PEG with other polymers, such as maltodextrin [1,2] and specially dextran [3,4], are the most common options. The number of water soluble polymers and of their mixtures is very large; yet, some of the potential pairs of polymers are not able to form two-phase systems at operationally convenient amounts. This limitation triggered the creation of a novel type of ABS, capable of minimizing the polymers concentrations required, while increasing those of water [5–7]. This can be attained through the addition of small amounts of a salt (1–5 wt%) [6] to an aqueous solution of polymers, where they act as electrolytes and induce phase separation. When no salt is added the two-phase formation does not occur or is only observed at very high polymer concentrations, and

therefore, the presence of one electrolyte is essential for this type of ABS formation [5–7]. Molecular mechanisms ruling the phase separation phenomenon were proposed by Johansson et al. [6]. They showed that the phase separation was promoted by the presence of the electrolyte ions which decrease the entropy of compartmentalization of the counter ions in the co-existing phases. The adequate selection of the electrolyte allows the control of the width of the biphasic regions [6], in which it is possible to work at very low polymers' concentrations (*i.e.* lower than 10 wt% of each polymer), and the partition of the solutes between the phases. These systems have been applied to the extraction and purification of an extensive range of molecules of biotechnological importance from several sources namely, distinct proteins [8,9], enzymes [8,10–12], DNA [13,14] and antibiotics [15]. Since such systems were shown to maintain the native conformation and biological activity of various biomolecules including proteins, and allow high yields in the purification processes [8,9], they are believed to be biocompatible and effective extraction processes.

Ionic liquids (ILs) are salts, liquid at temperatures below 100 °C, composed of large organic cations with dispersed charge, and anions of either inorganic or organic nature [16]. They present unique characteristics [16] and an outstanding ability to solvate

* Corresponding author. Tel.: +351 234 370200; fax: +351 234 370084.
E-mail address: spventura@ua.pt (S.P.M. Ventura).

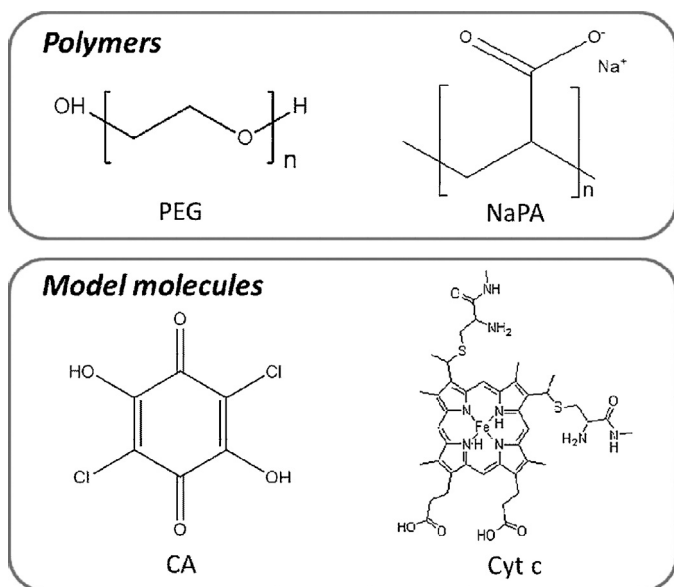


Fig. 1. Chemical structures of the polymers and the molecules under study.

compounds from a wide spectrum of polarities [17,18]. The tunability of the ILs' properties by anion–cation combinations [19] makes it possible to control the formation and design of ABS by the manipulation of the phases' polarities allowing the creation of tailored systems for a given purpose. Their applications are well beyond the ABS domain since these ionic compounds are widely used in several areas of expertise such as organic chemistry [20], electrochemistry [21] and nanotechnology [22].

This work aims at studying the use of ILs as electrolytes to prepare polymeric ABS. Firstly, binodal curves of quaternary systems composed of PEG + sodium polyacrylate (NaPA) (both with an average molecular weight of 8000 g mol^{-1}) + ILs or inorganic salts (as electrolytes) + water were established at $(298 \pm 1) \text{ K}$ and atmospheric pressure. Several ILs formed by distinct cations and anions and inorganic salts were applied as electrolytes, allowing the comparative assessment of their performance. The second part of this work evaluates the use of these systems in extraction processes, in which cytochrome *c* (Cyt *c*) and chloranilic acid (CA) were tested as model molecules. For this purpose only ABS containing imidazolium-based ILs and inorganic salts at either fixed or variable concentrations were considered. The ILs partition among the two phases, the pH and extraction parameters (extraction efficiencies for the two model compounds and partition coefficients for CA) were determined. Insights into the partitioning phenomenon are provided, allowing the development of heuristic rules for the design of these systems as powerful extraction platforms.

2. Materials and methods

2.1. Materials

PEG 8000 and the aqueous solution of NaPA 8000 (45 wt%) were used as received from Sigma–Aldrich®. Na_2SO_4 and NaCl were acquired from Synth® with 98% of purity. Chloranilic acid (purity >99 wt%) and cytochrome *c* from equine heart (purity = 95 wt%) were purchased from Merck® and Sigma–Aldrich®, respectively. The chemical structures of the polymers and the model molecules used are provided in Fig. 1. Eleven ILs sharing distinct cations and anions were investigated and their names, acronyms, chemical structures, purities and suppliers are listed in Table 1. The purity of all ILs was confirmed by ^1H and ^{13}C NMR and found to match the

suppliers' information. The water used was double distilled, passed by a reverse osmosis system and further treated with a Milli-Q plus 185 water purification apparatus.

2.2. Binodal curves determination

The experimental binodal curves were determined gravimetrically, within an uncertainty of $\pm 10^{-4} \text{ g}$, using the cloud point titration method [23] at $(298 \pm 1) \text{ K}$ and atmospheric pressure. Briefly, three stock aqueous solutions were prepared: 50.0 wt% of PEG 8000 + 5.0 wt% of IL or inorganic salt, 18.0 wt% of NaPA 8000 + 5.0 wt% of IL or inorganic salt and water + 5.0 wt% of IL or inorganic salt. Additionally, four distinct IL and inorganic salt concentrations (used as electrolytes) were tested, namely 5.0, 2.5, 1.0 and 0.1 wt%. Drop-wise addition of the PEG 8000-containing solution was carried out to the solution of NaPA 8000 (and/or *vice versa* when necessary) until the visual detection of two phases. Subsequently, drop-wise addition of the IL/inorganic salt-containing solution was conducted until the two phases disappear (monophasic region). This procedure was repeated several times in order to obtain the binodal curve, being performed under constant stirring. The experimental phase diagrams data were correlated using the Merchuk equation [24] (Eq. (1)):

$$[\text{PEG } 8000] = A \exp[(B \times [\text{NaPA } 8000]^{0.5}) - (C \times [\text{NaPA } 8000]^3)] \quad (1)$$

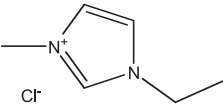
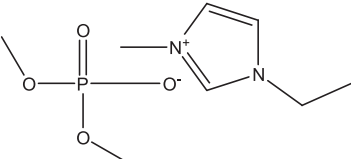
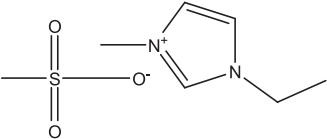
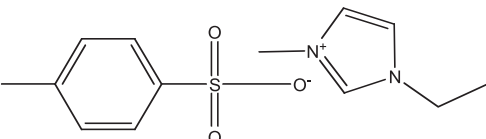
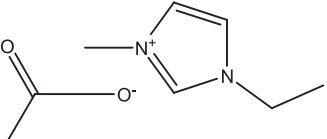
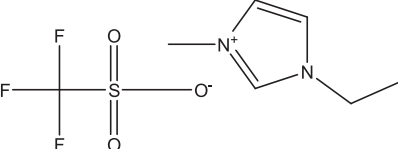
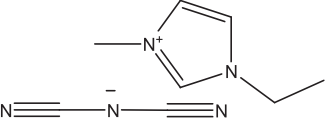
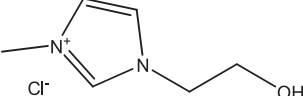
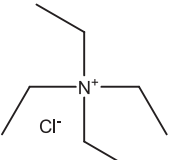
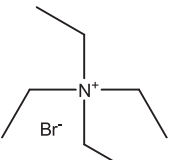
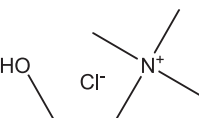
where [PEG 8000] and [NaPA 8000] represent the weight percentages of PEG 8000 and NaPA 8000, respectively. *A*, *B* and *C* are constants obtained by the regression of the experimental data. The Merchuk equation was chosen since it has a low number of adjustable parameters to correlate these data and it is the most commonly applied [25].

2.3. Partition of Cyt *c*, CA and imidazolium-based ILs

For the partition studies, only the imidazolium-based ILs and the inorganic salts were used. A mixture point within the biphasic region was selected and prepared by weighing the appropriate amounts of each compound: 15.0 wt% of PEG 8000 + 4.5 wt% of NaPA 8000 + 5.0 wt% of electrolyte + 0.1 g of a Cyt *c* stock aqueous solution (at circa 5.0 g L^{-1}) or 1 g of a CA stock aqueous solution (at circa 0.72 g L^{-1}) + water (fulfilling a total mass of 5 g). For further optimization, the effect of electrolyte concentration on the partition of CA was addressed by using systems containing either $[\text{C}_2\text{mim}][\text{N}(\text{CN})_2]$ or NaCl. In these cases, the same mixture compositions aforementioned were prepared by varying only the amount of electrolyte: 5.0; 2.5; 1.0 and 0.1 wt%. All the components were mixed together using a Vortex agitator (IKA, C-MAG HS7), and then equilibrated at $(298 \pm 1) \text{ K}$ and atmospheric pressure during 12 h, allowing the equilibrium to be reached. At the end, the final systems generated two clear phases, a PEG 8000-rich as the top and a NaPA 8000-rich as the bottom layer, with a well-defined interface in between. The coexisting phases were carefully separated and collected for the measurement of their volumes and pH values, as well as for the molecules quantification assays.

The target compounds and the imidazolium-based ILs content was determined using a Molecular Devices Spectramax 384 Plus | UV–Vis Microplate Reader, at the respective maximum wavelength of absorbance, namely 409 nm for Cyt *c*, 330 nm for CA, and 211 nm for the $[\text{C}_2\text{mim}]\text{X}$. At least three independent samples of each system were prepared, being the average values reported along with the respective standard deviations.

Table 1
List of names, acronyms, chemical structures, purities and suppliers of the ILs investigated.

Name Acronym	Chemical structure	Purity (wt%)	Supplier
<i>Imidazolium-based ILs</i>			
1-Ethyl-3-methylimidazolium chloride [C ₂ mim]Cl		>98	Iolitec® (Ionic Liquid Technologies, Germany)
1-Ethyl-3-methylimidazolium dimethylphosphate [C ₂ mim][DMP]		>98	
1-Ethyl-3-methylimidazolium methanesulfonate [C ₂ mim][CH ₃ SO ₃]		>99	
1-Ethyl-3-methylimidazolium tosylate [C ₂ mim][Tos]		99	
1-Ethyl-3-methylimidazolium acetate [C ₂ mim][CH ₃ CO ₂]		>95	
1-Ethyl-3-methylimidazolium triflate [C ₂ mim][CF ₃ SO ₃]		99	
1-Ethyl-3-methylimidazolium dicyanamide [C ₂ mim][N(CN) ₂]		>98	
1-(2-hydroxyethyl)-3-methylimidazolium chloride [OHC ₂ mim]Cl		99	
<i>Ammonium-based ILs</i>			
Tetraethylammonium chloride [N _{2,2,2,2}]Cl		>98	Sigma-Aldrich®
Tetraethylammonium bromide [N _{2,2,2,2}]Br		>98	Fluka AG – Chemische
Cholinium chloride [Ch]Cl		>98	Sigma-Aldrich®

The IL partition coefficient (K_{IL}) was calculated as the ratio between the amount of IL present in the PEG 8000-rich phase (top) and NaPA 8000-rich phase (bottom), as described by Eq. (2):

$$K_{IL} = \frac{Abs(IL_{PEG8000\text{-rich phase}})}{Abs(IL_{NaPA8000\text{-rich phase}})} \quad (2)$$

where $Abs(IL_{PEG8000\text{-rich phase}})$ and $Abs(IL_{NaPA8000\text{-rich phase}})$ are the IL absorbance output measured for the top and bottom phases, respectively.

The CA partition coefficient (K_{CA}) is defined by the ratio of the CA concentration in the top phase, $[CA]_{PEG8000\text{-rich phase}}$, to that in bottom the phase, $[CA]_{NaPA8000\text{-rich phase}}$ as indicated by Eq. (3).

$$K_{CA} = \frac{[CA]_{PEG8000\text{-rich phase}}}{[CA]_{NaPA8000\text{-rich phase}}} \quad (3)$$

Partition coefficients for Cyt *c* are not reported due to analytical limitations that prevented the determination of its concentration in the PEG 8000-rich phase (where its concentration is below the detection limit). Hereupon, the direct comparison between the partition of the two molecules under study will be made by the extraction efficiency (EE, %) parameter, calculated following Eq. (4):

$$EE = \frac{V_{\text{molecule-rich}} \times [M]_{\text{molecule-rich}}}{V_i \times [M]_i} \times 100 \quad (4)$$

V_i and $[M]_i$ are the initial volume and the initial concentration of molecule added in the extraction systems preparation, while $V_{\text{molecule-rich}}$ and $[M]_{\text{molecule-rich}}$ denote the volume and concentration of the molecule in the phase where it is more concentrated: top phase in the case of CA (EE_{CA}) and bottom phase for Cyt *c* ($EE_{Cyt\ c}$). It should be noted that blank controls, where the CA or the Cyt *c* solutions were replaced by water, were used to remove possible interferences from the phase components in the quantification methodologies. Finally, the pH values of both phases for all systems tested were measured at $(298 \pm 1)K$ using a Digimed DM-22 dual meter pH equipment with an uncertainty of ± 0.02 pH units.

3. Results and discussion

3.1. Preparation of polymeric ABS with ILs as electrolytes

Neither the use of ILs as electrolytes (they have been widely used in electrochemistry and in lithium batteries [26,27]) nor the use of electrolytes in polymeric ABS (Johansson et al. [6] have addressed the influence of inorganic salts as electrolytes) are a novelty. The application of ILs in the generation of novel ABS is vast [16]. In this field, they can be combined with a wide plethora of other solutes (inorganic [23,28] or organic [29,30] salts, polymers [31,32] and carbohydrates [33,34]) as one of the main two phase formation agents or even used as adjuvant [35] in polymer-salt-based systems (to tune the polarity of the aqueous phases). Yet, there is no previous report on the use of ILs as electrolytes in the domain of polymeric ABS, *i.e.* to induce the formation of ABS in aqueous solutions of two polymers at useful concentrations. To start, the experimental binodal curves were determined, being their graphical representations, along with the correlations by the Merchuk equation presented in Figs. 2 and 3. The detailed experimental weight fraction data and correlation parameters (A, B and C) are reported in Supporting Information (Tables A.1–A.4). The ILs used allow the investigation of several parameters related with the application of ILs as electrolytes, namely the nature of the anion moiety, the cation core and their mass concentration. Table 2 reports all trends gauged for the electrolytes' aptitude to promote the phase separation, organized according to each parameter evaluated.

From the results, it can be concluded that, as the inorganic salts, the ILs can also act as electrolytes inducing the formation of ABS as shown in Fig. 2. Despite the higher capability to induce phase separation demonstrated by the inorganic salts, probably due to their higher charge density, it seems that the phase split can also be created and manipulated by the addition of various ILs and by playing with the cation/anion combination (Fig. 2 and Table 2). However, it seems that on these systems they have, in general, a higher capacity to induce phase separation when compared with other IL-containing ABS (*e.g.* IL + polymer, polymer + inorganic salt + IL as adjuvant, IL + salt) [16] (*cf.* Figure A.1 in Supporting Information). This is of major importance, since there is a natural interest to find systems that can move the binodal curve towards the water-rich region in the phase diagram, due to the low component content

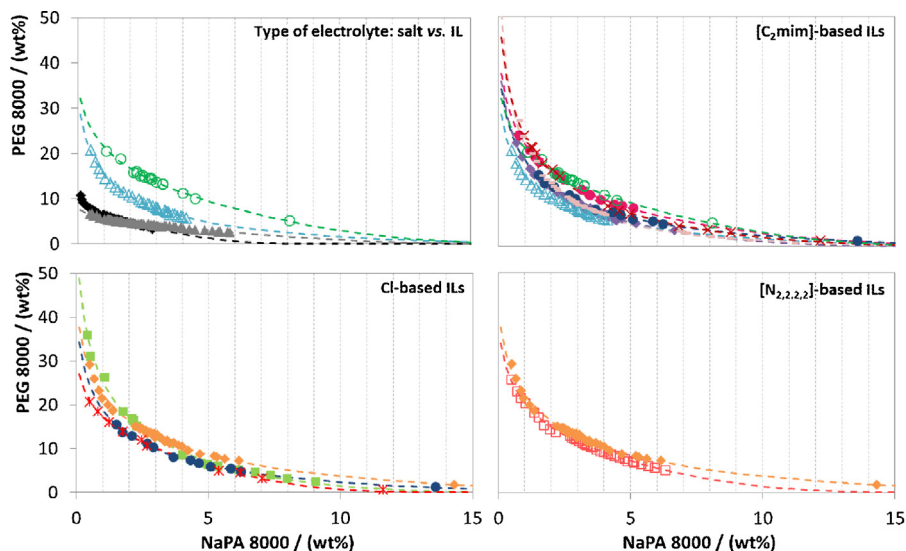


Fig. 2. Phase diagrams of the polymeric ABS containing 5.0 wt% of electrolyte (either inorganic salts or ILs) grouped according to the type of effect investigated (in order to simplify the analysis): NaCl (\blacktriangle), Na_2SO_4 (\blacklozenge), $[C_2mim][N(CN)_2]$ (\triangleleft), $[C_2mim][Tos]$ (---), $[C_2mim][CF_3SO_3]$ (\blacklozenge), $[C_2mim]Cl$ (\bullet), $[Ch]Cl$ (\ast), $[OHC_2mim]Cl$ (\blacksquare), $[C_2mim][CH_3CO_2]$ (\times), $[N_{2,2,2,2}]Br$ (\square), $[C_2mim][CH_3SO_3]$ (\bullet), $[N_{2,2,2,2}]Cl$ (\blacklozenge), $[C_2mim][DMP]$ (\circ). Binodal curves fitted using Eq. (1) are represented in dashed lines.

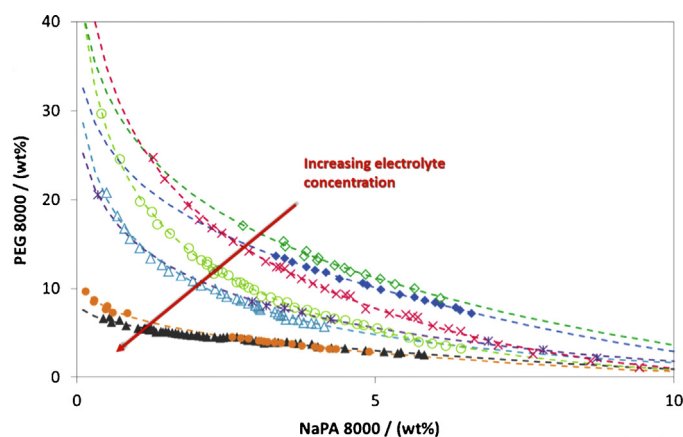


Fig. 3. Phase diagrams of polymeric ABS at different electrolytes' concentration: 5.0 wt% of NaCl (\blacktriangle), 2.5 wt% of NaCl (\bullet), 1.0 wt% of NaCl (\ast), 0.1 wt% of NaCl (\blacklozenge), 5.0 wt% of $[\text{C}_2\text{mim}][\text{N}(\text{CN})_2]$ (\triangle), 2.5 wt% of $[\text{C}_2\text{mim}][\text{N}(\text{CN})_2]$ (\circ), 1.0 wt% of $[\text{C}_2\text{mim}][\text{N}(\text{CN})_2]$ (\times), and 0.1 wt% $[\text{C}_2\text{mim}][\text{N}(\text{CN})_2]$ (\diamond). Binodal curves according to Eq. (1) are represented in dashed lines. The arrow is a guide to the eye.

(polymer/salt) required, allowing the reduction of the overall cost associated to the technology and the decrease in the viscosity, creating more biocompatible ABS. The impact of several anion moieties on the polymeric ABS formation was assessed by using either $[\text{C}_2\text{mim}]$ -based or $[\text{N}_{2,2,2,2}]$ -based ILs (binodal data depicted in Fig. 2). Eight distinct anions, namely Cl^- , Br^- , $[\text{DMP}]^-$, $[\text{CH}_3\text{SO}_3]^-$, $[\text{Tos}]^-$, $[\text{CH}_3\text{CO}_2]^-$, $[\text{CF}_3\text{SO}_3]^-$ and $[\text{N}(\text{CN})_2]^-$ were investigated, and their aptitude to promote the phase separation is detailed in Table 2 (the tendencies were retrieved at circa 4 wt% of NaPA 8000). Although the phase formation mechanism must be very different from that observed for IL + salt ABS, the trends here observed are surprisingly similar to those described in previous works for conventional imidazolium [23] or ammonium [36] + salt-based ABS. Since this follows the inverse trend of the salts hydrophobicity, i.e. the anions with a higher hydrogen bonding basicity are those with a lower ability to induce the phase separation, the mechanism seems to be related with the cation–anion interaction and their ability to dissociate in aqueous solution so that the anions induce a phase separation. The same type of effect was reported by Johansson et al. [6] for inorganic salts of different hydrophobic/hydrophilic character as electrolytes on polymeric ABS.

Fig. 2 also shows the effect of non-aromatic and non-cyclic ($[\text{N}_{2,2,2,2}]^+$ and $[\text{Ch}]^+$) and functionalized ($[\text{OHC}_2\text{mim}]^+$) cations regarding the ABS formation (details are reported in Table 2 for Cl-based ILs). It seems that two different driving forces influence the ABS formation. For the imidazolium-based ILs, the increasing hydrophobicity from $[\text{OHC}_2\text{mim}]^+$ to $[\text{C}_2\text{mim}]^+$ moves the binodal curve towards the water-rich region, as reported in literature

Table 2
Ability of electrolytes to promote the formation of polymeric ABS.

Parameters	Rank of the electrolytes' performance (at circa 4 wt% of NaPA 8000)
Salts vs. ILs	$\text{NaCl} \approx \text{Na}_2\text{SO}_4 > \text{ILs}$
IL anion	$[\text{C}_2\text{mim}]$ -based ILs: $[\text{N}(\text{CN})_2]^- > [\text{Tos}]^- \approx [\text{CF}_3\text{SO}_3]^- > \text{Cl}^- > [\text{CH}_3\text{CO}_2]^- > [\text{CH}_3\text{SO}_3]^- > [\text{DMP}]^-$ $[\text{N}_{2,2,2,2}]$ -based ILs: $\text{Br}^- > \text{Cl}^-$
IL cation	Cl-based ILs: $[\text{C}_2\text{mim}]^+ \approx [\text{Ch}]^+ > [\text{OHC}_2\text{mim}]^+ > [\text{N}_{2,2,2,2}]^+$
Electrolyte concentration	NaCl: $5.0 \text{ wt}\% \approx 2.5 \text{ wt}\% > 1.0 \text{ wt}\% > 0.1 \text{ wt}\%$ $[\text{C}_2\text{mim}][\text{N}(\text{CN})_2]$: $5.0 \text{ wt}\% > 2.5 \text{ wt}\% > 1.0 \text{ wt}\% > 0.1 \text{ wt}\%$

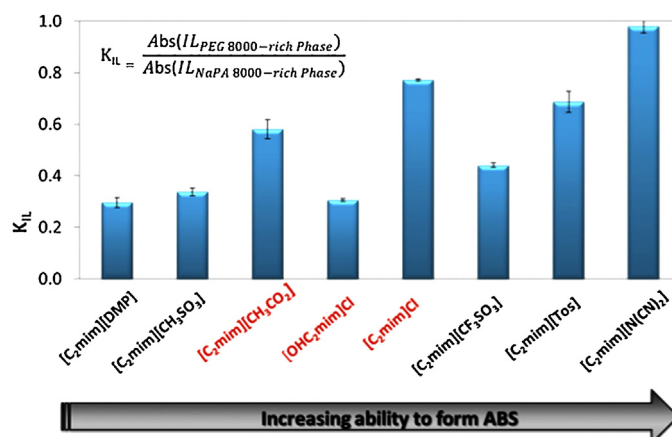


Fig. 4. Graphical representation of K_{IL} values determined for the $[\text{C}_2\text{mim}]$ -based ILs and their relationship with the ability for ABS formation. The ILs marked in red represent the outsiders. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

[16,18], while for the non-cyclic ammonium-based compounds ($[\text{N}_{2,2,2,2}]\text{Cl}$ and $[\text{Ch}]\text{Cl}$), a reverse effect is observed. In this case, $[\text{N}_{2,2,2,2}]^+$ represents the most hydrophobic core being however, contrarily to what was observed for the anions and the imidazolium cations, the less able to promote the separation of the phases. This suggests that both the cation–anion interaction and the hydrophobic nature of the cation are influencing the ABS formation in opposite ways.

The $[\text{C}_2\text{mim}]$ -based ILs' capacity to act as electrolyte in polymeric ABS (Table 2) seems to be correlated with the K_{IL} (the ratio between the amount of IL present in the PEG 8000-rich and NaPA 8000-rich phases, respectively, at the mixture point 15.0 wt% of PEG 8000 + 4.5 wt% of NaPA 8000 + 5.0 wt% of $[\text{C}_2\text{mim}]$ -based ILs). This partition coefficient varies with the IL under study in the order $[\text{C}_2\text{mim}][\text{DMP}] \approx [\text{OHC}_2\text{mim}]\text{Cl} < [\text{C}_2\text{mim}][\text{CH}_3\text{SO}_3] < [\text{C}_2\text{mim}][\text{CF}_3\text{SO}_3] < [\text{C}_2\text{mim}][\text{CH}_3\text{CO}_2] < [\text{C}_2\text{mim}][\text{Tos}] < [\text{C}_2\text{mim}]\text{Cl} < [\text{C}_2\text{mim}][\text{N}(\text{CN})_2]$, which is the direct order to their ability to induce phase separation as reported in Fig. 4. The conclusion emerging from the K_{IL} data is that the partition of the ILs between the aqueous phases is the main driving force behind the different aptitudes of the ILs to induce ABS formation in polymeric systems. ILs with partition coefficient values closer to the unit represent systems with higher ability to induce phase separation. In this respect their behaviour resembles that of common salts previously described by Johansson et al. [6]. Three ILs appear as exceptions to this general behaviour, namely $[\text{C}_2\text{mim}]\text{Cl}$, $[\text{OHC}_2\text{mim}]\text{Cl}$ and $[\text{C}_2\text{mim}][\text{CH}_3\text{CO}_2]$, suggesting the possibility of additional specific interactions occurring and contributing to the phase split (establishment of additional hydrogen bonds between PEG 8000 and $[\text{CH}_3\text{CO}_2]^-$ and Cl^- anions or extra hydrophilic forces between the $[\text{OHC}_2\text{mim}]^+$ cation and NaPA 8000). Additionally, some of these tendencies are reversed or even faded at different regions of the binodal curves (i.e. when moving from the PEG 8000-richer to the NaPA 8000-richer region). The most evident example is represented by the quaternary system containing $[\text{OHC}_2\text{mim}]\text{Cl}$ that below 1–2 wt% of NaPA 8000 is the one revealing a lower aptitude to induce phase separation, being this inverted at high concentrations of NaPA 8000, sustaining the idea that besides the simple mechanism proposed by Johansson et al. [6] other type of interactions contribute, and may even become dominant, to the two-phase formation at high NaPA 8000 contents. Most studies assessing the impact of the cation core are related with IL/salts-based ABS. When dealing with polymeric ABS containing ILs, the interactions are by far more complex [18,37,38], and

consequently, additional molecular insights are needed for a full picture of the phase separation mechanism in polymer-polymer ABS induced by ILs used as electrolytes.

Because the partition of molecules in general is also influenced by the concentration factor, the electrolyte concentration on the ABS formation was also assessed by varying the amount of either NaCl and $[\text{C}_2\text{mim}][\text{N}(\text{CN})_2]$ (IL with the highest capacity to promote ABS formation) from 0.1 to 5.0 wt% being the respective binodal curves depicted in Fig. 3. By increasing the electrolyte concentration and thus the ionic strength, enhanced capacities to induce ABS formation are achieved as previously shown [6,39] (Table 1). At high concentrations of NaCl, its effect seems to be less relevant, which can be attributed to a saturation effect, described by the non-relevance of the addition of more concentration of electrolyte to promote the phase separation, as discussed elsewhere [6]. This phenomenon of saturation justifies thus the flatter form of the binodal curves, which means that even if more salt is added, the phase separation is not significantly changed. In the specific case of $[\text{C}_2\text{mim}][\text{N}(\text{CN})_2]$, the saturation was not achieved, the results describing thus its significant capacity to promote binodal curves closer to the water-rich region with the increase of the IL content, explaining the “round” shape of the binodal curves described for the various amounts of IL. This different tendency can be attributed to the different hydrophobicity of NaCl vs. $[\text{C}_2\text{mim}][\text{N}(\text{CN})_2]$. In this sense, the stronger capacity of NaCl to induce phase separation compared to $[\text{C}_2\text{mim}][\text{N}(\text{CN})_2]$ is minimized at lower electrolyte concentrations (e.g. 0.1 wt%) and fades at higher concentrations of NaPA 8000.

3.2. Application of the studied ABS as purification platforms

After the study of the binodal curves which showed the ability of the ILs to induce ABS formation in polymeric systems, these are now evaluated for the extraction of cytochrome c and chloranilic acid, and used to gather information about the ILs' partition (K_{IL}) on these systems as well as their pH. Most of ILs preferentially migrate towards the NaPA 8000-rich phase ($K_{\text{IL}} < 1$) likely due to the strong interactions between the ILs' cation with the negatively charged moieties of the polymer (NaPA is negatively charged at neutral pH, due to the presence of carboxylic groups in the main chain); however $[\text{C}_2\text{mim}][\text{N}(\text{CN})_2]$ partitions almost equally between both phases. This parameter is important as the ILs may interact with the target molecules affecting the performance of this technology. Fig. 5

provides information about the results obtained for the extraction efficiency of the two target molecules selected for investigation in this work, as well as about the pH values of both phases. Although these systems are not buffered, the pH is kept practically constant around neutral/slightly alkaline values (only minor variations from 7 to 8 were observed, Fig. 5A), allowing the molecules' charge to be kept during the partition experiments.

After the ABS characterization, partitioning studies of two model molecules, namely CA and Cyt c, were conducted in which the extraction efficiencies were considered for discussion. Those were carried out using ABS composed of 15 wt% PEG 8000 + 4.5 wt% NaPA 8000 + 5.0 wt% of electrolyte (either ILs or inorganic salts). Actually, high extraction efficiencies were attained, ranging from $80.13 \pm 1.45\%$ to $97.85 \pm 1.37\%$ for CA (top phase) and from $86.23 \pm 3.75\%$ to 100% for Cyt c (bottom phase). Carefully analysing the results shown in Fig. 5A and inspecting the visual aspect of the phases (Fig. 5B), it is possible to observe a complete migration of Cyt c and CA towards opposite phases in all systems investigated, which demonstrates the potential of these systems for selective extraction. Although the selectivity is not relevant for the two model compounds here investigated these results suggest that it will be possible, through the proper design of the ABS, to manipulate the selectivity of the extraction of other compounds found in more complex matrices, in which the number of distinct classes of high value compounds is significant. This selectivity may be explained by a balance of interactions acting in each phase and between the main ABS components and the target compounds under investigation. The partition phenomenon of Cyt c seems to be governed by “NaPA 8000–Cyt c” interactions, since Cyt c is positively charged at neutral pH ($pI \approx 10.0$) [40], giving rise to electrostatic interactions with the NaPA 8000 negatively charged species, which seem to be incremented by additional “Cyt c–electrolyte” interactions.

In what concerns the CA, its preferential migration towards the PEG 8000-rich phase seems to be controlled by a balance between the “PEG 8000–CA” favourable hydrophobic interactions and the “NaPA 8000–CA” electrorepulsive effects. If in one hand, CA displays a log P of 1.22 that indicates a preference for more hydrophobic environments (e.g. PEG 8000 layer), on the other hand this compound is negatively charged at neutral pH (pK_a of 0.58 and 3.18) [18], thus having less tendency to favourably interact with the negatively charged compounds at the NaPA-rich phase. While no significant differences with the electrolyte adopted at the level of the extractive performances are observed, the K_{CA} contrarily to the

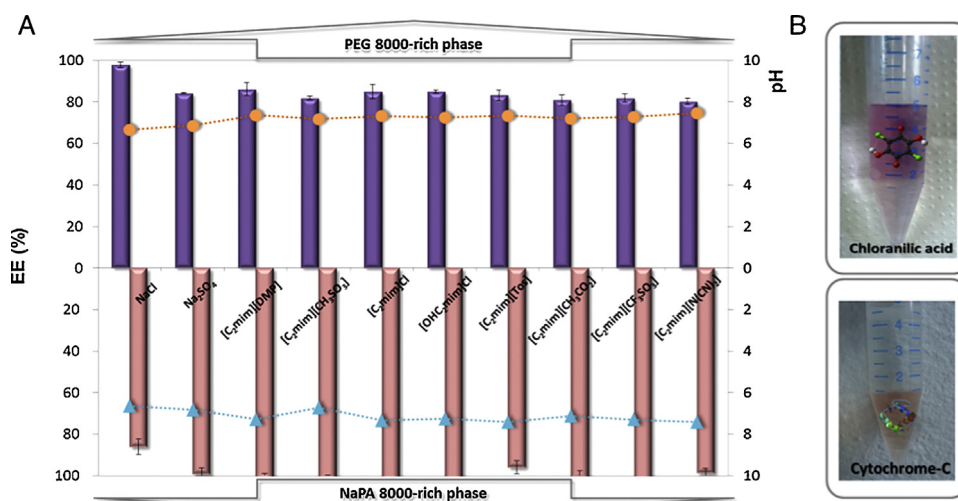


Fig. 5. (A) Representation of the extraction efficiencies (EE, %) for CA (■) and Cyt c (■) in the ABS composed of 15.0 wt% of PEG + 4.5 wt% of NaPA + 5.0 wt% of electrolytes (inorganic salts or $[\text{C}_2\text{mim}]\text{X}$) along with the pH of both PEG 8000-rich (●) and NaPA 8000-rich (▲) phases. (B) The colours of the phases allow the observation of the partition of the compounds. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

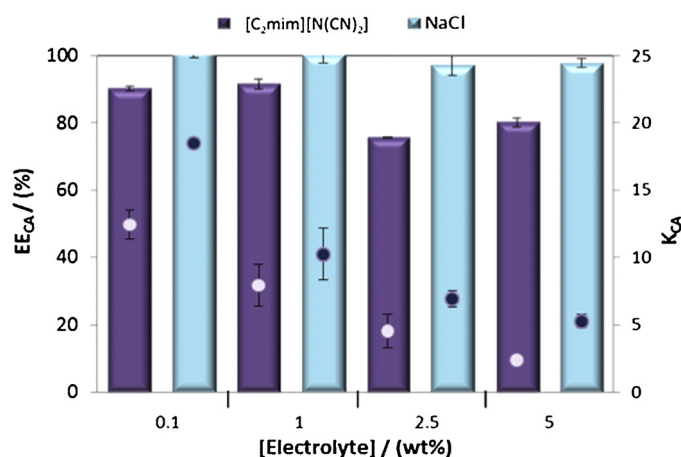


Fig. 6. Effect of electrolyte concentration on the partition coefficients, K_{CA} (dots) and extraction efficiency data, EE_{CA} % (bars) of CA. NaCl data represented in blue and $[C_2mim][N(CN)_2]$ in purple. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

EE_{CA} , is boosted by the use of inorganic salts as electrolytes (maximum K_{CA} of 10.91 ± 0.41 attained for Na_2SO_4) when compared to ILs (maximum K_{CA} of 4.18 ± 0.29 obtained for $[C_2mim][DMP]$). The K_{CA} values determined for this set of ABS along with additional pictures of each coloured/uncoloured phases (for CA and Cyt c) are provided in the Supporting Information file in Table A.5 and Figure A.2, aimed at supporting the observations about the preferential partitions observed.

As an attempt to improve the extractive performance of this class of ABS, the effect of electrolytes' concentration was studied by using either $[C_2mim][N(CN)_2]$ or NaCl at 5.0, 2.5, 1.0 and 0.1 wt%. As shown in Fig. 6 and Table A.6 from Supporting Information, it is clearly noticed that the extractive performance is enhanced by lower electrolyte concentrations. The most auspicious result was achieved using 0.1 wt% of NaCl, where highest K_{CA} value (18.47 ± 0.23 , *circa* 3.5 times higher than that achieved using 5 wt% of NaCl) while keeping levels of 100% of EE_{CA} . Similar evidences are found for the lowest IL concentration (0.1 wt%), which is prompting the CA migration towards the PEG 8000-rich phase (*circa* 5 times more than with the use of 5 wt% of IL regarding the K_{CA} values depicted and 10% more in terms of EE_{CA} data). Hereupon, at this lower IL concentrations, "CA-ILs" interactions are much weakened in the whole system, thus decreasing the screening on the electrorepulsive effect of NaPA 8000 upon the dye forcing its partition to the PEG 8000-rich phase, boosting the K_{CA} values. This effect is depicted in Fig. 6, for the two electrolytes studied. This significant reduction at the level of the ILs' consumption together with the known easy recyclability of both PEG [41] and NaPA [42] polymers is of paramount relevance from the industrial point of view, since not only the costs are reduced due to the fact that these systems can be richer in water and use lower amounts of electrolyte, but also because the reuse of the principal components of the system becomes an easier task. Both cost and environmental impacts linked to the use of ILs can be decreased, due to lower separation requirements in the additional stages aimed at their recovery and reuse processes [43]. This will certainly encourage more studies to understand their behaviour at the molecular level and optimize their extractive performances as well as their implementation at industrial level.

4. Conclusions

New experimental phase diagrams composed of PEG 8000+NaPA 8000+water and several ILs as electrolytes were

determined at $(298 \pm 1)K$ and atmospheric pressure. Firstly, it is important to remark that this class of ABS reveals a greater aptitude to undergo phase separation when compared to other IL-based ABS. The ability of the electrolytes to promote the ABS formation is ruled by the electrolytes' ionic strength, being the inorganic salts (NaCl and Na_2SO_4) more effective than the ILs here investigated, this difference decreasing at lower concentrations of electrolyte. Considering the IL structural features, the anion moiety effect is well-defined, *i.e.* lower hydrogen-bond basicity induces larger biphasic regions; however, the effect of the cation core is more complex. Higher electrolyte concentrations are more beneficial to form ABS. In a biphasic system this result in a PEG 8000-rich (top) phase and a NaPA 8000-rich (bottom) phase, towards which the IL preferentially migrates.

The application of these ABS in the extraction of Cyt c and CA revealed an efficient and selective separation process. While Cyt c accumulated in the NaPA 8000-rich phase, mainly due to electrostatic interactions, the CA preferentially migrated towards the PEG 8000-rich phase as the main result of hydrophobic interactions and electrorepulsive effects. It was further shown that the extraction parameters could be significantly improved by lowering the electrolyte concentration down to 0.1 wt%. In this context, both cost and environmental impacts are significantly reduced, what may lead the creation of more competitive separation processes based on ABS.

Acknowledgments

The authors are grateful for financial support from FEDER through Programa Operacional Fatores de Competitividade – COMPETE and national funds through FCT – Fundação para a Ciência e Tecnologia within CICECO project – FCOMP-01-0124-FEDER-037271 (Ref^a. FCT PEst-C/CTM/LA0011/2013), and the doctoral and post-doctoral grants refs: SFRH/BD/94901/2013 and SFRH/BPD/79263/2011 of F.A. e Silva and S.P.M. Ventura, respectively. The authors also thank the Santander Scholarship granted to J.H.P.M. Santos. This project was also supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) from Brazil. The authors thank the anonymous reviewer for the suggestions concerning the phase formation mechanisms that contributed to enhance the quality of the final manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.procbio.2015.02.001.

References

- [1] Da Silva LM, Meirelles AA. Phase equilibrium in polyethylene glycol/maltodextrin aqueous two-phase systems. *Carbohydr Polym* 2000;42:273–8.
- [2] Szlag DC, Guiliano KA. A low-cost aqueous two phase system for enzyme extraction. *Biotechnol Tech* 1988;2:277–82.
- [3] Asenjo JA, Andrews BA. Aqueous two-phase systems for protein separation: a perspective. *J Chromatogr A* 2011;1218:8826–35.
- [4] Johansson G, Reczey K. Concentration and purification of β -glucosidase from *Aspergillus niger* by using aqueous two-phase partitioning. *J Chromatogr B: Biomed Appl* 1998;711:161–72.
- [5] Gupta V, Nath S, Chand S. Role of water structure on phase separation in polyelectrolyte–polyethyleneglycol based aqueous two-phase systems. *Polymer* 2002;43:3387–90.
- [6] Johansson H-O, Feitosa E, Junior AP. Phase diagrams of the aqueous two-phase systems of poly(ethylene glycol)/sodium polyacrylate/salts. *Polymers* 2011;3:587–601.
- [7] Saravanan S, Rao JR, Nair BU, Ramasami T. Aqueous two-phase poly(ethylene glycol)–poly(acrylic acid) system for protein partitioning: influence of molecular weight, pH and temperature. *Process Biochem* 2008;43:905–11.

- [8] Johansson H-O, Magaldi FM, Feitosa E, Pessoa A. Protein partitioning in poly(ethylene glycol)/sodium polyacrylate aqueous two-phase systems. *J Chromatogr A* 2008;1178:145–53.
- [9] Johansson H-O, Ishii M, Minaguti M, Feitosa E, Penna TCV, Pessoa A. Separation and partitioning of Green Fluorescent Protein from *Escherichia coli* homogenate in poly(ethylene glycol)/sodium-poly(acrylate) aqueous two-phase systems. *Sep Purif Technol* 2008;62:166–74.
- [10] De Lencastre Novaes LC, De Carvalho Santos Ebinuma V, Mazzola PG, Pessoa Jr A. Polymer-based alternative method to extract bromelain from pineapple peel waste. *Biotechnol Appl Biochem* 2013;60:527–35.
- [11] Pereira Alcântara LA, Do Nascimento KS, Mourão CA, Minim VPR, Minim LA. Aqueous two-phase poly(ethylene glycol)–sodium polyacrylate system for amyloglucosidase purification: equilibrium diagrams and partitioning studies. *Sep Purif Technol* 2013;118:888–94.
- [12] Barros KVG, Souza PM, Freitas MM, Filho EXF, Junior AP, Magalhães PO. PEG/NaPA aqueous two-phase systems for the purification of proteases expressed by *Penicillium restrictum* from Brazilian Savanna. *Process Biochem* 2014;49:2305–12.
- [13] Matos T, Johansson H-O, Queiroz JA, Bülow L. Isolation of PCR DNA fragments using aqueous two-phase systems. *Sep Purif Technol* 2014;122:144–8.
- [14] Johansson H-O, Matos T, Luz JS, Feitosa E, Oliveira CC, Pessoa A, et al. Plasmid DNA partitioning and separation using poly(ethylene glycol)/poly(acrylate)/salt aqueous two-phase systems. *J Chromatogr A* 2012;1233:30–5.
- [15] Pereira JFB, Santos VC, Johansson H-O, Teixeira JAC, Pessoa A. A stable liquid–liquid extraction system for clavulanic acid using polymer-based aqueous two-phase systems. *Sep Purif Technol* 2012;98:441–50.
- [16] Freire MG, Cláudio AFM, Araújo JMM, Coutinho JAP, Marrucho IM, Lopes JNC, et al. Aqueous biphasic systems: a boost brought about by using ionic liquids. *Chem Soc Rev* 2012;41:4966–95.
- [17] Galiński M, Lewandowski A, Stepniak I. Ionic liquids as electrolytes. *Electrochim Acta* 2006;51:5567–80.
- [18] Pereira JFB, Rebelo LPN, Rogers RD, Coutinho JAP, Freire MG. Combining ionic liquids and polyethylene glycols to boost the hydrophobic–hydrophilic range of aqueous biphasic systems. *Phys Chem Chem Phys* 2013;15:19580–3.
- [19] Freemantle M. Designer solvents. *Chem Eng News Arch* 1998;76:32–7.
- [20] Leclercq L, Suisse I, Agbossou-Niedercorn F. Biphasic hydroformylation in ionic liquids: interaction between phosphane ligands and imidazolium triflate, toward an asymmetric process. *Chem Commun (Camb)* 2008;3:311–3.
- [21] Hapiot P, Lagrost C. Electrochemical reactivity in room-temperature ionic liquids. *Chem Rev* 2008;108:2238–64.
- [22] Ichikawa T, Yoshio M, Hamasaki A, Mukai T, Ohno H, Kato T. Self-organization of room-temperature ionic liquids exhibiting liquid–crystalline bicontinuous cubic phases: formation of nano-ion channel networks. *J Am Chem Soc* 2007;129:10662–3.
- [23] Ventura SPM, Neves CMSS, Freire MG, Marrucho IM, Oliveira J, Coutinho JAP. Evaluation of anion influence on the formation and extraction capacity of ionic-liquid-based aqueous biphasic systems. *J Phys Chem B* 2009;113:9304–10.
- [24] Merchuk JC, Andrews BA, Asenjo JA. Aqueous two-phase systems for protein separation. Studies on phase inversion. *J Chromatogr B: Biomed Sci Appl* 1998;711:285–93.
- [25] Sintra TE, Cruz R, Ventura SPM, Coutinho JAP. Phase diagrams of ionic liquids-based aqueous biphasic systems as a platform for extraction processes. *J Chem Thermodyn* 2013;77:206–13.
- [26] Egashira M, Okada S, Yamaki JI, Dri DA, Bonadies F, Scrosati B. The preparation of quaternary ammonium-based ionic liquid containing a cyano group and its properties in a lithium battery electrolyte. *J Power Sources* 2004;138:240–4.
- [27] Egashira M, Todo H, Yoshimoto N, Morita M, Yamaki JI. Functionalized imidazolium ionic liquids as electrolyte components of lithium batteries. *J Power Sources* 2007;174:560–4.
- [28] Bridges NJ, Gutowski KE, Rogers RD. Investigation of aqueous biphasic systems formed from solutions of chaotropic salts with kosmotropic salts (salt/salt ABS). *Green Chem* 2007;9:177–83.
- [29] Passos H, Ferreira AR, Cláudio AFM, Coutinho JAP, Freire MG. Characterization of aqueous biphasic systems composed of ionic liquids and a citrate-based biodegradable salt. *Biochem Eng J* 2012;67:68–76.
- [30] Zafarani-Moattar MT, Hamzehzadeh S. Partitioning of amino acids in the aqueous biphasic system containing the water-miscible ionic liquid 1-butyl-3-methylimidazolium bromide and the water-structuring salt potassium citrate. *Biotechnol Prog* 2011;27:986–97.
- [31] Freire MG, Pereira JFB, Francisco M, Rodríguez H, Rebelo LPN, Rogers RD, et al. Insight into the interactions that control the phase behaviour of new aqueous biphasic systems composed of polyethylene glycol polymers and ionic liquids. *Chemistry – Eur J* 2012;18:1831–9.
- [32] Zafarani-Moattar MT, Hamzehzadeh S, Nasiri S. A new aqueous biphasic system containing polypropylene glycol and a water-miscible ionic liquid. *Biotechnol Prog* 2012;28:146–56.
- [33] Freire MG, Louros CLS, Rebelo LPN, Coutinho JAP. Aqueous biphasic systems composed of a water-stable ionic liquid + carbohydrates and their applications. *Green Chem* 2011;13:1536–45.
- [34] Chen Y, Meng Y, Yang J, Li H, Liu X. Phenol distribution behavior in aqueous biphasic systems composed of ionic liquids–carbohydrate–water. *J Chem Eng Data* 2012;57:1910–4.
- [35] Pereira JFB, Lima AS, Freire MG, Coutinho JAP. Ionic liquids as adjuvants for the tailored extraction of biomolecules in aqueous biphasic systems. *Green Chem* 2010;12:1661–9.
- [36] e Silva FA, Sintra T, Ventura SPM, Coutinho JAP. Recovery of paracetamol from pharmaceutical wastes. *Sep Purif Technol* 2014;122:315–22.
- [37] Tomé LIN, Pereira JFB, Rogers RD, Freire MG, Gomes JRB, Coutinho JAP. Evidence for the interactions occurring between ionic liquids and tetraethylene glycol in binary mixtures and aqueous biphasic systems. *J Phys Chem B* 2014;118:4615–29.
- [38] Pereira JFB, Kurnia KA, Cójocarú OA, Gurau G, Rebelo LPN, Rogers RD, et al. Molecular interactions in aqueous biphasic systems composed of polyethylene glycol and crystalline vs. liquid cholinium-based salts. *Phys Chem Chem Phys* 2014;16:5723–31.
- [39] Almeida MR, Passos H, Pereira MM, Lima Álvaro S, Coutinho JAP, Freire MG. Ionic liquids as additives to enhance the extraction of antioxidants in aqueous two-phase systems. *Sep Purif Technol* 2014;128:1–10.
- [40] Bushnell GW, Louie GV, Brayer GD. High-resolution three-dimensional structure of horse heart cytochrome c. *J Mol Biol* 1990;214:585–95.
- [41] Albertsson PA. Partition of cell particles and macromolecules in polymer two-phase systems. *Adv Protein Chem* 1970;24:309–41.
- [42] Ikawa T, Abe K, Honda K, Tsuchida E. Interpolymer complex between poly(ethylene oxide) and poly(carboxylic acid). *J Polym Sci Polym Chem Ed* 1975;13:1505–14.
- [43] Alvarez-Guerra E, Ventura SPM, Coutinho JAP, Irabien A. Ionic liquid-based three phase partitioning (ILTPP) systems: ionic liquid recovery and recycling. *Fluid Phase Equilib* 2014;371:67–74.