Research Article

Received: 10 December 2017 Revised: 22 January 2018 Accepted article published: 31 January 2018 Published online in Wiley Online Library: 5 March 2018

(wileyonlinelibrary.com) DOI 10.1002/jctb.5594

Separation of immunoglobulin G using aqueous biphasic systems composed of cholinium-based ionic liquids and poly(propylene glycol)

Catarina C Ramalho, Catarina MSS Neves, Maria V Quental, João AP Coutinho and Mara G Freire*

Abstract

BACKGROUND: The use of antibodies, such as immunoglobulin G (IgG), has seen a significant growth in recent decades for biomedical and research purposes. However, antibodies are high cost biopharmaceuticals, for which the development of alternative and cost-effective purification strategies is still in high demand.

RESULTS: Aqueous biphasic systems (ABS) composed of poly(propylene glycol) (PPG) and cholinium-based ionic liquids (ILs) were investigated for the separation of IgG. The ABS phase diagrams were determined whenever required. Initial studies with commercial IgG were carried out, followed by IgG separation from rabbit serum. In all ABS, IgG preferentially partitions to the IL-rich phase, unveiling preferential interactions between IgG and ILs. Extraction efficiencies ranging between 93% and 100%, and recovery yields ranging between 20% and 100%, were obtained for commercial IgG. Two of the best and two of the worst identified ABS were then evaluated for their performance to separate IgG from rabbit serum, where extraction efficiencies of 100% and recovery yields >80% were obtained. Under the best conditions studied, IgG with a purity level of 49% was obtained in a single-step. After an ultrafiltration step applied to the best ABS, allowing the IgG recovery from the IL-rich phase, the IgG purity level increased to 66%. This purity level of IgG is higher than those previously reported using other IL–polymer ABS.

CONCLUSION: IgG preferentially migrates to the IL-rich phase in ABS formed by ILs and polymers, allowing the design of effective separation systems for its recovery from serum samples.

Supporting information may be found in the online version of this article.

Keywords: aqueous two-phase systems; bioseparations; downstream; liquid–liquid extraction; pharmaceuticals

INTRODUCTION

In recent years, we have been facing a high demand for proteins present in the blood of mammals, specifically for immunoglobulin G (IgG) due to its potential as an alternative biopharmaceutical. Antibodies, also known as immunoglobulins, are large proteins secreted by cells of the immune system. Their function is to identify and eliminate pathogens, such as viruses, bacteria or fungi, through a recognition mechanism of high specificity. The growing interest in IgG is a result of the expanding number of biomedical applications in which this plasma-derived protein can be used.[1] IgG is currently administered to prevent infections in patients with immune deficiencies,[2,3] providing passive immune protection, and in the treatment of inflammatory and autoimmune diseases.[4,5]

Commercially available serum antibodies are typically obtained through chromatographic techniques, following a series of steps for the removal of impurities.[6–8] These methods yield high purity antibodies but result in highly expensive products, therefore limiting their widespread use as recurrent biopharmaceuticals. There is thus a need for the development of competitive cost-effective purification methods. Among the several possibilities, aqueous biphasic systems (ABS), also known as aqueous two-phase systems (ATPS), emerge as a possible alternative for the extraction and purification of IgG. ABS were first proposed as an alternative protein extraction technique in 1958 by Albertsson.[9] They are composed of two water-soluble solutes, which above a given concentration form two immiscible aqueous-rich phases, each enriched in one of the phase-forming components. Given their high water content, ABS may be seen as biocompatible media for the extraction and purification of a wide range of biomolecules.[9,10] Furthermore, being liquid–liquid systems, ABS enable the combination of clarification, concentration and purification steps in a single stage.[11] Most work dealing with ABS and IgG have been

* *Correspondence to: MG Freire, CICECO - Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal. E-mail: maragfreire@ua.pt*

Department of Chemistry, University of Aveiro, Aveiro, Portugal

focused on its recovery from cell culture media.[12–15] However, Vargas *et al.*[16] proposed the use of ABS for the purification of IgG from human plasma. Studies on the partitioning of pure IgG, obtained from human serum, have also been reported,[17,18] as well as on the purification of IgG from transgenic plants.[19]

The combinations of solutes typically used for ABS formation are polymer–polymer, polymer–salt and salt–salt.[20] Initial studies were chiefly focused on polymer-based ABS, consisting of two polymers or a polymer and a salting-out inducing salt in aqueous media.[20] Nevertheless, this type of ABS has some downsides. Most polymer-based ABS display high viscosity and are associated with a slow phase separation.[21] Moreover, when two polymers are used to create ABS, both phases display similar properties, such as hydrophobicity or polarity. Thus, polarity differences between the coexisting phases in polymer-based ABS display a limited range,[21] accounting for their reduced applicability and lack of selectivity for separation purposes. To overcome this limitation, the use of salts as adjuvants and the addition of affinity ligands to polymer-based ABS have been proposed.[22,23]

In the past decade, a new type of ABS composed of ionic liquids (ILs) and inorganic salts in aqueous media was proposed.[24] ILs are organic salts with low melting temperatures.[25] IL-based ABS display a reduced viscosity and a fast phase separation rate, representing thus potential alternatives to polymer-rich systems.[26,27] ILs have also been proposed as greener alternatives to volatile organic solvents commonly applied in liquid–liquid separations, mainly due to their non-flammability and negligible vapor pressure under atmospheric conditions,[28] although these features may also be displayed by polymers and salts. The main advantage of IL-based ABS is the possibility to tailor the phases' polarities and affinities, leading to improvements in selectivity, product solubility and extraction efficiency.[29–36] In addition to the widely studied salt–IL ABS, more recently, ILs have been proposed as alternatives to common salts, leading to the creation of IL–polymer ABS.[37–40] These systems were suggested as improved media to separate proteins, since these can bind to the metal ions of the salts commonly used and thus affect protein stability.[41] When dealing with antibodies with the potential to be applied as biopharmaceuticals, it is of significant relevance to preserve their native structure during the extraction and purification procedure, aiming at maintaining their function and activity. Shifts in the medium composition, pH and temperature can also cause modifications to the three-dimensional structure of proteins.[42] Most IL–polymer ABS previously reported are formed by imidazolium-based ILs,[20] which may present some degree of toxicity.[43] To overcome this drawback, cholinium-based ILs, less toxic ILs,[44–46] were recently proposed to create IL–polymer ABS, and were shown to provide high extraction efficiencies for biologically active molecules.[39,47,48]

Based on the potential of IL–polymer ABS formed by cholinium-based ILs, in this work, their performance for the extraction and purification of IgG from rabbit serum was evaluated. ABS composed of cholinium-based ILs and the polymer polypropylene glycol with an average molecular weight of 400 g mol^{-1} (PPG 400) were investigated. PPG was chosen due to its stronger hydrophobic character, requiring thus low amounts of polymer and IL to create ABS, while providing a high water content at the IL-rich phase. Some of the systems tested herein were previously investigated for the extraction of BSA (bovine serum albumin).[39] The remaining ABS are composed of a set of cholinium-based ILs with antioxidant activities,[45] and for which novel ternary phase diagrams have been determined in this work. These ILs not only display higher antioxidant activity than the respective precursors, but also higher solubility in water, higher anti-inflammatory activity and thermal stability, and lower toxicity.[45]

MATERIALS AND METHODS

Materials

The cholinium-based ILs used for the preparation of ABS for the IgG extraction assays were cholinium chloride ([Chol]Cl, 98 wt% pure) and cholinium bitartrate ([Chol][Bit], 97 wt% pure), both from Acros Organics, cholinium dihydrogen citrate ([Chol][DHCit], 99 wt% pure) from Sigma, and cholinium dihydrogen phosphate ([Chol][DHPh], > 98 wt% pure) and cholinium acetate ([Chol][Ac], 98 wt% pure) from Iolitec. Cholinium lactate ([Chol][Lac]), cholinium glycolate ([Chol][Gly]), cholinium propanoate ([Chol][Prop]), and cholinium butanoate ([Chol][But]) were synthesized in our laboratory following previously described protocols.[39,49] The antioxidant cholinium-based ILs cholinium caffeate ([Chol][Caf]), cholinium syringate ([Chol][Syr]), cholinium vanillate ([Chol][Van]), and cholinium gallate ([Chol][Gal]) were also synthesized in our laboratory, as described in previous work.[45] All the cholinium-based ILs synthetized by us showed purity values >97 wt%, which were confirmed by ^{1}H and ^{13}C NMR. The polymer used was polypropylene glycol with an average molecular weight of 400 g mol^{-1} (PPG 400), acquired from Aldrich. The chemical structures of the ILs investigated and PPG 400 are depicted in Fig. 1.

For the preparation of IgG aqueous solutions, a lyophilized powder of IgG obtained from rabbit serum (reagent grade, ≥ 95%) was used, purchased from Sigma-Aldrich, and kept in storage at 5 °C. The solutions of IgG were prepared in a buffer solution composed of phosphate salts (PBS, pH = 7.4) pellets acquired from Sigma. The rabbit serum used was purchased from Sigma-Aldrich, and kept at −20 °C until use.

Determination of the ABS phase diagrams

Some ternary phase diagrams tested for extraction purposes were determined in previous work,[39] while others were determined in this work, particularly for the systems formed by [Chol][Caf], [Chol][Syr], [Chol][Van] and [Chol][Gal] + PPG 400 + water. These were determined through the cloud point titration method at 25 ± 1 °C and atmospheric pressure. Aqueous solutions of PPG 400 at ≈ 90 wt% and aqueous solutions of the ILs with concentrations varying between 45 wt% and 70 wt% were prepared gravimetrically (±10^{-4} g). The repetitive drop-wise addition of the aqueous solution of IL to the PPG–water mixture was carried out until a cloudy biphasic mixture was discerned. Subsequently, double distilled water was added drop-wise until the mixture became limpid, entering the monophasic region. This procedure was repeatedly performed under constant stirring, until no more cloud points were observed. The reverse method, consisting of the addition of the PPG 400 aqueous solution to the IL mixture was also performed to obtain more solubility points which could better describe the binodal curve. The composition of the systems after the addition of each component was determined by weight quantification (± 10^{-4} g). The experimental binodal curves were fitted by Equation (1):

$$[\mathrm{PPG}] = A \exp\left[\left(B\,[\mathrm{IL}]^{0.5}\right) - \left(C\,[\mathrm{IL}]^{3}\right)\right] \quad (1)$$

where [PPG] and [IL] correspond to the weight fraction percentages of PPG 400 and IL, respectively, while *A*, *B* and *C* are the fitting parameters. This equation was used since it has been commonly employed for similar systems comprising PPG 400 and

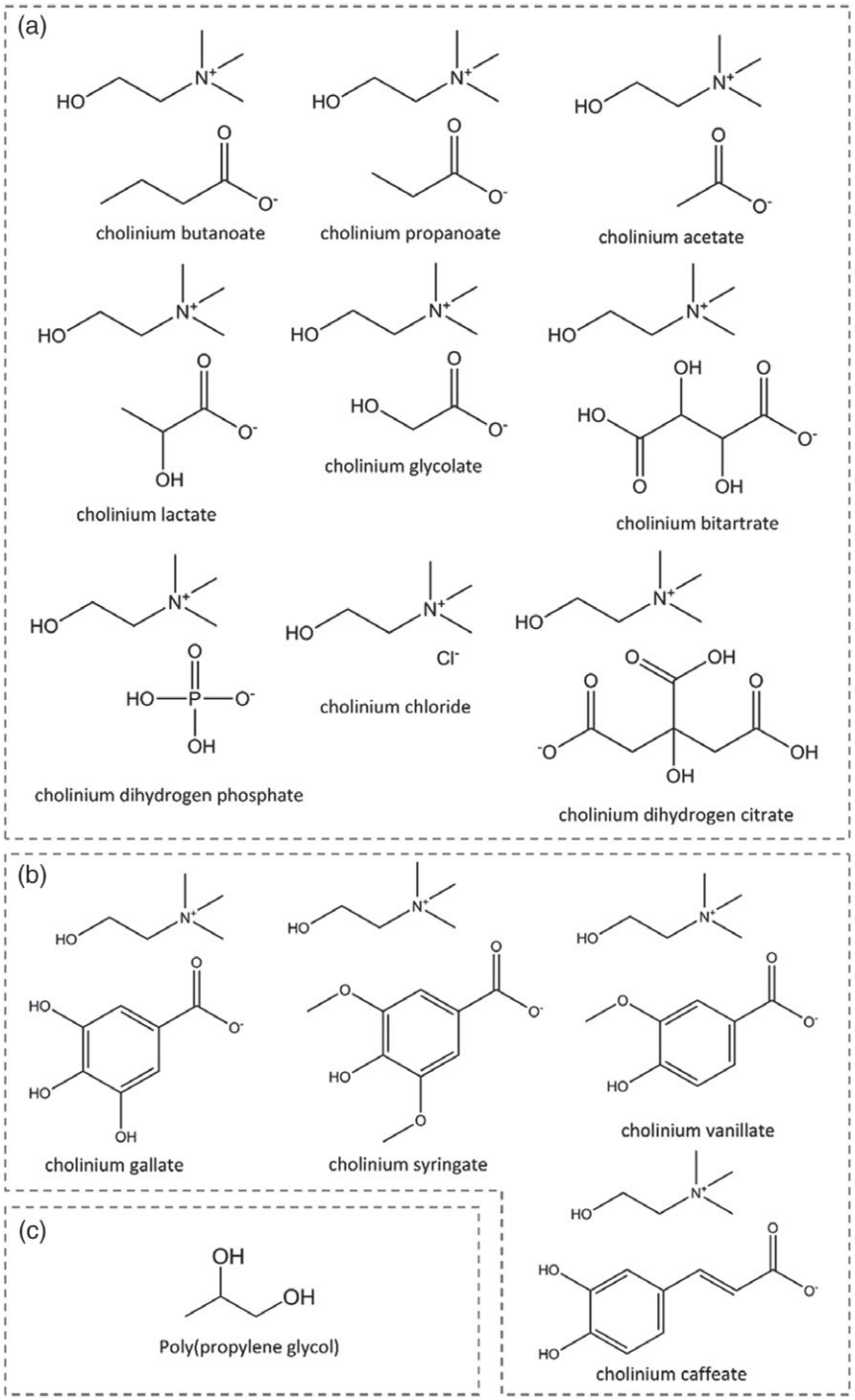

Figure 1. Chemical structure of the compounds used in this work: (a) cholinium-based ILs; (b) antioxidant cholinium-based ILs; (c) polymer.

cholinium-based ILs.[39] Tie-lines (TLs) of each phase diagram, i.e. the compositions of each phase for a common mixture composition, as well as the tie-line lengths (TLLs), were determined according to the method reported by Merchuk *et al.*,[50] and used elsewhere to describe polymer-IL ABS.[38,39,51,52]

Extraction and separation of IgG using ABS

After gathering knowledge on the mixture compositions required to form two-phase systems by determination of the ABS phase diagrams, a mixture point which guarantees that all systems are within the biphasic region was selected for the commercial IgG partition assays. The overall mixture composition used was 45 wt% of PPG 400, 25 wt% of IL and 30 wt% of a buffered PBS (pH = 7.4) aqueous solution containing IgG at 1 g L^{-1}. At least three individual experiments were prepared for each system. Each ABS was prepared by weighing the appropriate amount of each component, within $\pm 10^{-4}$ g, and mixing until all components were dissolved. All systems were centrifuged for 10 min at 1000 rpm and left to equilibrate for a further 10 min at 25 ± 1 °C to promote phase separation and IgG partitioning between phases. 100 µL

were collected from both the top and bottom phases of each ABS and diluted in a phosphate buffer solution (1:10 (v:v)), used as the mobile phase for quantification. The IgG content in each phase was determined by size exclusion high-performance liquid chromatography (SE-HPLC). The equipment used was a Chromaster HPLC system (VWR Hitachi) equipped with a binary pump, column oven, temperature controlled auto-sampler, DAD detector and an analytical column Shodex Protein KW- 802.5 (8 mm × 300 mm). A 100 mmol L^{-1} phosphate buffer pH 7.0 with NaCl 0.3 mol L^{-1} was run isocratically with a flow rate of 0.5 mL min^{-1} and the injection volume was of 25 µL. The wavelength was set at 280 nm. The calibration curve was established with commercial IgG from rabbit serum.

The ABS performance to extract and recover IgG to the IL-rich phase was evaluated by their extraction efficiency (EE_{IgG}%) and yield (Y_{IgG}%), according to the following equations:

$$EE_{IgG}\% = \frac{W_{IgG}^{IL}}{W_{IgG}^{polymer} + W_{IgG}^{IL}} \times 100 \quad (2)$$

$$Y_{IgG}\% = \frac{W_{IgG}^{IL}}{W_{IgG}^{Initial}} \times 100 \quad (3)$$

where W_{IgG}^{IL}, $W_{IgG}^{polymer}$ and $W_{IgG}^{Initial}$ represent the weight of IgG in the IL-rich phase, in the polymer-rich phase, and in the initial solution, respectively.

After the identification of favorable systems for IgG extraction to the IL-rich phase, new experiments were performed for the extraction and purification of IgG, directly from rabbit serum. The ABS chosen are composed of 45 wt% PPG 400, 25 wt% IL and 30 wt% rabbit serum diluted at 1:50 (v:v) in a PBS aqueous solution, with the ILs [Chol][DHPh], [Chol][Lac], [Chol][Van] and [Chol][Gly]. Each mixture composition was weighed and mixed, centrifuged for 10 min at 1000 rpm, and left to equilibrate for 10 min at 25 ± 1 °C. Then, 100 µL of each phase were collected and diluted (1:10 (v:v)) in the mobile phase used for the analysis by SE-HPLC, as described earlier. In the systems containing [Chol][DHPh] and [Chol][Lac] a large amount of proteins precipitated at the interface was observed. In both cases the systems were centrifuged for 20 min at 3500 rpm, left to equilibrate under the same conditions, and the precipitate collected and diluted in 600 µL of the PBS aqueous solution for further analysis. All assays were performed at least in triplicate. The percentage purity of IgG was calculated dividing the HPLC peak area of IgG by the total area of the peaks corresponding to all proteins present in the IL-rich phase.

Ultrafiltration for IgG recovery and IL removal

The bottom phases of the ABS with the ILs [Chol][DHPh] and [Chol][Lac], identified as the best systems to purify IgG, were subjected to ultrafiltration aiming at recovering IgG from the IL-rich phase. 400 µL of the IL-rich phase were placed in a vial with a cut-off filter of 30 kDa, and then centrifuged at 14 000 × g for 15 min. After each centrifugation cycle the filtrated solution was collected, and 300 µL of a buffer phosphate aqueous solution was added. This procedure was repeated until the IL was completely removed, which in this work corresponded to two cycles. To recover the concentrated IgG, 50 µL of a phosphate buffer aqueous solution was added to the filter, inverted, and centrifuged at 1000 × g for 2 min. The collected samples, the filtrated solution, and the concentrated IgG, were analyzed by SE-HPLC.

RESULTS AND DISCUSSION

ABS ternary phase diagrams

To identify the mixture compositions that can be used in the separation of IgG from rabbit serum, the respective ABS ternary phase diagrams were determined whenever required at 25 °C and atmospheric pressure. Some phase diagrams were taken from the literature,[39] while those for the systems composed of the cholinium-based ILs with antioxidant properties were determined in this work, namely for [Chol][Caf], [Chol][Syr], [Chol][Van] and [Chol][Gal]. In all studied ABS the bottom phase corresponds to the IL-rich phase, while the top phase is mainly composed of PPG 400 and water. The respective phase diagrams are depicted in Fig. 2, in weight fraction and in molality units (mol kg^{-1}, moles of PPG or IL per kg of IL + water or PPG + water). The representation in mass fraction is important for the design of separation processes, while molality units allow a better comparison of the ILs' impact in the two-phase formation since their molecular weight discrepancies are overwhelmed. The detailed experimental weight fraction data are given in Table S1 in the Supporting information. The regression parameters (*A*, *B* and *C*) were obtained through the least squares regression method using Equation (1).[50] Their values and the corresponding standard deviations, as well the experimental TLs, TLLs, and volume ratio (V_r), are provided in Tables S2 and S3 in the Supporting information.

Above each binodal curve, all mixtures are biphasic. Hence, in systems with larger biphasic regions, the ability of the IL to promote liquid–liquid demixing is higher. The cholinium cation is common to all ILs; therefore, the differences observed in the phase diagrams result mainly from the IL anion nature. Contrarily to salt-based ABS, where the liquid–liquid demixing is driven by a salting-out effect of the high charge density ions over ILs,[20] in IL–polymer ABS the phenomenon is more complex since the driving mechanism for the phase separation is mainly ruled by the strength of the interactions occurring between the three system components: PPG 400, IL and water.[53] It was previously demonstrated that there is competition between PPG 400 and the IL ions for water molecules, and also between themselves, where the IL anion is more prone to form hydration complexes than the corresponding IL cation.[53]

According to the phase diagrams depicted in Fig. 2, the ability of an IL to form an ABS at a fixed molality of IL, e.g. at 0.6 mol.kg^{-1}, decreases as follows: [Chol][DHPh] > [Chol][Gly] > [Chol][Ac] > [Chol][Bit] > [Chol][Lac] > [Chol][Prop] ≈ [Chol][DHCit] ≈ [Chol][But] > [Chol]Cl > [Chol][Gal] > [Chol][Syr] > [Chol][Van] > [Chol][Caf]. This comparison was performed in molality units to avoid the contribution of the IL's molecular weight. [Chol][DHPh] is the strongest IL in terms of liquid–liquid demixing, thus requiring lower amounts of IL or PPG to form ABS. $[DHPh]^-$ is the IL anion with the highest polar surface, improving the IL ability to form a second phase when combined with a polymer, namely poly(ethylene glycol) or PPG, as demonstrated in the literature.[39,54] This IL has therefore more affinity with water and a higher ability to exclude PPG to a second liquid phase.[39] In general, ILs with shorter aliphatic moieties are more prone to form ABS, as verified, for instance, with the series of ILs derived from aliphatic carboxylic acids ([Chol][Ac] > [Chol][Prop] ≈ [Chol][But]). Furthermore, the addition of hydroxyl groups to the ILs promotes phase separation ([Chol][Gly] > [Chol][Ac] and [Chol][Lac] > [Chol][Prop]). The correlation between the chemical structure of each IL and their relative tendency to promote phase demixing has been discussed in detail by Quental *et al.*[39] These systems display larger biphasic regions when compared with the new systems composed

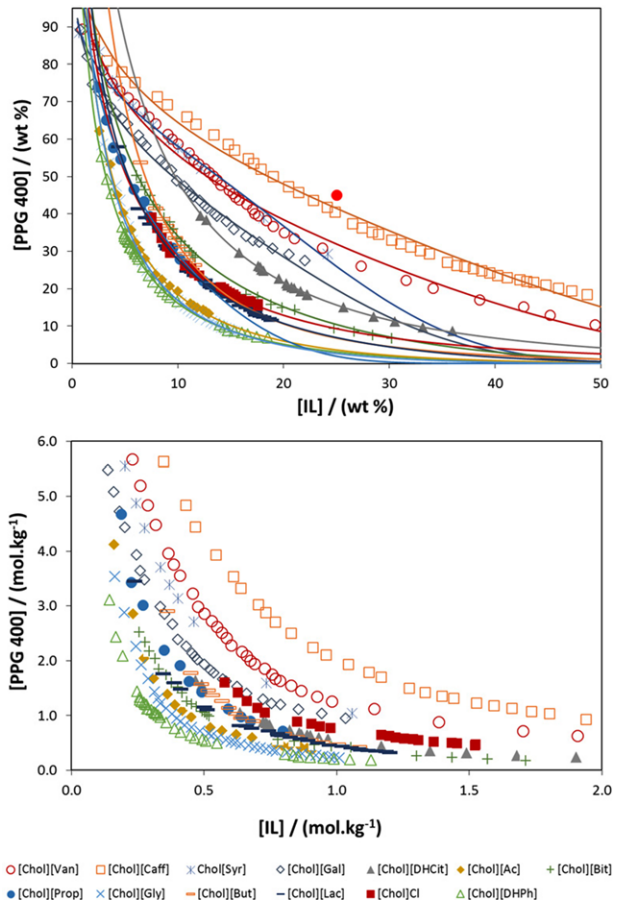

Figure 2. Ternary phase diagrams of ABS composed of cholinium-based ILs + PPG 400 + H_2O. The ABS formed by [Chol][DHPh], [Chol][Gly], [Chol][Ac], [Chol][Lac], [Chol]Cl, [Chol][Prop], [Chol][But], [Chol][Bit] and [Chol][DHCit] have been previously reported in the literature.[39] The lines correspond to the fitting of the experimental data using Equation (1). The mixture composition used in the extraction experiments is represented by ●.

of the antioxidant ILs herein characterized, namely [Chol][Gal], [Chol][Syr], [Chol][Van] and [Chol][Caf], which follow the trend of the octanol–water partition coefficients (K_{ow}) of the corresponding phenolic acids.[55] The higher the K_{ow} of the phenolic acids the more difficult it is to create ABS with PPG 400. In general, the size of the biphasic regions increases with increase in the hydrophilicity of the cholinium-based ILs.

Extraction and separation of IgG using ABS

All ABS herein studied were evaluated in terms of their performance to extract and recover IgG to the IL-rich phase in a single-step. ABS constituted by the ILs [Chol][Bit], [Chol][Lac], [Chol][DHCit], [Chol][DHPh], [Chol]Cl, [Chol][Prop], [Ch][Gly], [Chol][Ac], [Chol][Caf], [Chol][Syr], [Chol][Van], [Chol][Gal], and [Chol][But] at 25 wt%, 45 wt% of PPG and 30 wt% of an aqueous solution of PBS containing IgG at 1 g L^{-1} were prepared. The common mixture point used is depicted in Fig. 2, along with the phase diagrams for all the ABS tested. The TLs, which correspond to the composition of each phase at the common mixture point, are provided in Table S3, while the pH of the coexisting phases is given in Table S4, in the Supporting information. The extraction efficiencies and yields for IgG are present in Fig. 3; cf. detailed data in Table S5 in the Supporting information.

Under the chromatographic conditions used, the retention time of IgG was found to be ≈ 15.6 min, within an analysis time of 45 min. It should be noted that some minor peaks are detected at lower retention times, even for the pure, commercial IgG, indicating the presence of a few IgG aggregates. Figure S1 in the Supporting information shows some examples of chromatograms corresponding to commercial IgG in the PBS aqueous solution and to the bottom phase of one of the ABS tested.

In all ABS, the antibody preferentially migrates to the more hydrophilic IL-rich phase, which corresponds to the bottom phase. In fact, in the SE-HPLC chromatograms of most systems, no IgG at the top (polymer-rich) phase was identified. The extraction efficiencies of all the investigated ABS to the IL-rich phase are higher than 93%, denoting the preference of IgG towards the IL-rich phase. Notably, all the new ABS containing the antioxidant cholinium-based ILs display high extraction efficiencies (> 96% up to complete extraction in a single-step), meaning that IgG has a high affinity for these ILs. All the antioxidant cholinium-based ILs display an aromatic group and several hydroxyl groups, which seem to be beneficial to the extraction of IgG. Other ILs allowed extraction efficiencies of 100%, namely [Chol][Lac], [Chol][But], [Chol][Bit], [Chol][Prop] and [Chol][Gly]. The ABS investigated are formed by ILs and PPG and thus no significant salting-out effects dominate the exclusion of IgG to the IL-rich phase. Therefore, it seems that the IgG partitioning is dominated by specific interactions established between the IL and the amino acids at the antibody surface.

Conventional polymer-based ABS formed by polyethylene glycol (PEG) of high molecular weight and phosphate-based salts have been used for the purification of IgG from the supernatants of Chinese Hamster Ovary (CHO) and hybridoma cell cultures.[13,14] The systems investigated led to extraction efficiencies to the polymer-rich phase of 90%.[13,14] Therefore, the IL-based ABS tested in the present work display higher extraction efficiencies, in addition to making use of polymers of lower molecular weight, with subsequent advantages in terms of viscosity, mass transfer, and phase demixing rate. An additional difference is the preferential migration of IgG to the polymer-rich phase observed in the literature,[13,14] where salting-out effects seem to dominate the partition, in contrast to what is observed in the systems investigated here. As a way of tailoring and improving the partition of IgG to a given phase, ILs were also recently used as adjuvants in PEG–salt-based ABS.[22] The use of ILs allowed the achievement of extraction efficiencies of 100% to the PEG-rich phase (the phase to which the IL preferentially partitions) in most systems investigated.[22] Specific interactions between ILs and IgG were previously proposed,[22] corroborating the preferential partitioning of the antibody to the IL-rich phase in the ABS studied here.

Although the ABS extraction efficiencies achieved in a single-step for IgG are >93%, a significant effect of the IL chemical structure was observed in the recovery yields. The IgG recovery yields are depicted in Fig. 3, and range from 20% to 100%. Detailed data are given in Table S5 in the Supporting information. The ABS formed by [Chol][Gly] and [Chol][Prop] do not lead to any losses of the protein, with recovery yields of IgG at the IL-rich phase of 100%. [Chol][Van] and [Chol]Cl, although with a small loss of IgG, also lead to high recovery yields (> 94%). Since these ABS also present high extraction efficiencies (100% in a single-step), these ILs may be considered as promising phase-forming components of ABS for the purification of IgG from real matrices. ABS formed by [Chol][Ac], [Chol][Caf] and [Chol][Bit] show yields ranging from 87% to 90%. On the other hand, ABS constituted by [Chol][Lac]

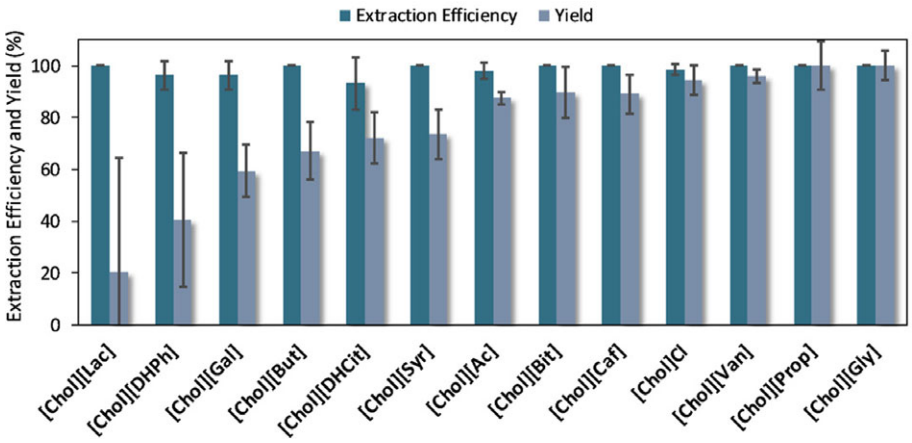

Figure 3. Average extraction efficiencies (EE_{IgG} %) and yields (Y_{IgG} %) of IgG to the IL-rich phase in the ABS composed of 25 wt% of IL + 45 wt% of PPG 400 + 30 wt% of a buffered aqueous solution containing pure IgG at 1 g L^{-1}.

and [Chol][DHPh] provide the lowest recovery yields of IgG (20% and 41%, respectively). These ABS lead to the precipitation of IgG at the interface as confirmed by SE-HPLC, showing that the low recovery yield is due to antibody precipitation.

The partitioning of proteins in ABS is driven by several interactions established between the protein surface and the phase-forming components, where steric effects may also be involved.[39] These interactions comprise dispersive forces, hydrogen-bonding and electrostatic interactions.[39] According to the recovery yields obtained, it seems that the IL anion has a significant influence on the IgG partitioning and on keeping its native structure. The isoelectric point of IgG is 8.6[56]; therefore, the overall charge of the protein in the studied systems at pH 7.4 is positive and may justify the impact exerted by the IL anion. However, a study involving IgG using IL–salt systems reported the preferential migration of the antibody to the IL-rich phase,[57] as observed in this work with polymer–IL systems, meaning that electrostatic interactions between the IL anion and the positively charged protein do not play a major role. These results support the idea that hydrogen-bonding and dispersive forces play a more significant role.

In the literature, polymer-based ABS have been described to allow recovery yields of 89% for IgG from the supernatant of CHO cell cultures.[14] In a similar work, IgG from the supernatant of CHO and hybridoma cell cultures was extracted with recovery yields of 88% and 90%, respectively.[13] More recently, polymer–polymer ABS were developed and tested for the purification of IgG from a hybridoma cell culture, with a recovery yield of 84%.[58] Dhadge *et al.*[59] obtained a 92% recovery yield of IgG from cell culture supernatants, using PEG–dextran ABS combined with magnetic nanoparticles. PEG–salt-based ABS using ILs as adjuvants provide recovery yields around 50%.[22] Considering the results found in the literature, it can be concluded that some of the systems tested here have great potential to extract IgG and to be exploited as separation systems of IgG from real matrices.

Two of the most promising ABS, with high extraction efficiencies and recovery yields, namely those formed by [Chol][Gly] and [Chol][Van], were then subjected to further tests regarding the extraction of IgG from rabbit serum. In addition to these, ABS formed by [Chol][Lac] and [Chol][DHPh], which correspond to the systems with the lower recovery yields and formation of large amounts of precipitate, were also investigated. The formation of an interface of precipitated proteins in these ABS could be a potential strategy to purify IgG from complex matrices within a three-phase partitioning (TPP) approach, where the target compound is enriched at the interface of two liquid phases.[60] The *t*-butanol and ammonium sulfate aqueous solutions are often used to induce the precipitation of biomolecules at the interface,[60] and recently, Alvarez *et al.*[61,62] showed that this approach is also feasible with ILs – ionic-liquid-based three-phase partitioning (ILTPP).

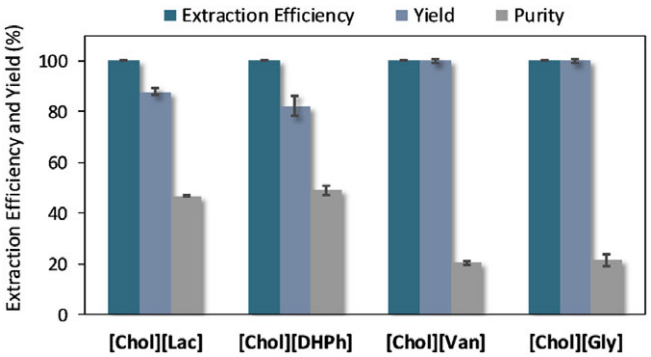

Figure 4. Average extraction efficiencies (EE_{IgG} %), recovery yield (Y_{IgG} %) and purity (%) of IgG at the IL-rich phase in ABS composed of 25 wt% of IL + 45 wt% of PPG 400 + 30 wt% of a buffered aqueous solution of rabbit serum (diluted at 1:50 (v:v)).

The extraction efficiencies, yields and IgG purity at the IL-rich phase, where ABS were applied to separate IgG from rabbit serum samples, are displayed in Fig. 4. Detailed data are given in Table S6 in the Supporting information. In these assays, a peak corresponding to IgG was observed at a retention time of ≈ 15.6 min, which is in agreement with the experiments using commercial IgG. As observed with pure IgG, additional peaks at lower retention times were identified in the SE-HPLC chromatograms, which correspond to protein aggregates. In the assays carried out with serum a larger peak at ≈ 16.8 min is identified, corresponding to albumin, the main serum protein. Examples of the chromatograms corresponding to rabbit serum and to the bottom phase of ABS are given in Fig. S2 in the Supporting information.

For the ABS containing [Chol][Gly] and [Chol][Van], excellent results were obtained. In both cases, the extraction efficiency and recovery yield were maintained at 100%, meaning that no losses of the target protein occurred. The IgG is completely extracted

to the IL-rich phase in a single step, even in the presence of a more complex, real matrix. This behavior is in agreement with the results obtained with pure IgG, and further indicates that the remaining proteins and other compounds present in the serum have no significant influence on the partition of IgG. These systems increase the purity of IgG from 18.1% (in the original rabbit serum) to 21–22% at the IL-rich phase. However, this is not a significant increment in the purity of IgG, and is mainly due to the large amount of albumin that migrates to the bottom phase along with IgG – cf. the chromatograms shown in Fig. S2 in the Supporting information. Nevertheless, while absolutely no IgG is detected in the top phase, small peaks corresponding to albumin and to the protein aggregates are observed in this phase, justifying the small improvement in the IgG purity.

Similarly to what occurred in the assays with pure IgG, in the assays with rabbit serum the antibody is present at the IL-rich phase and absent at the polymer-rich phase of the ABS formed by [Chol][Lac] and [Chol][DHPh], with extraction efficiencies of 100%. Surprisingly, and in contrast with the studies with pure IgG, a significant increase in the IgG recovery yield of the systems formed by [Chol][Lac] (88% vs 20%) and [Chol][DHPh] (82% vs 41%) when using rabbit serum was observed. Furthermore, these systems also led to higher purity levels of IgG at the IL-rich phase (47% purity with [Chol][Lac]; 49% purity with [Chol][DHPh]) than those composed of [Chol][Gly] and [Chol][Van]. In order to investigate the presence of IgG in the precipitate as well as the possibility of using these systems as an ILTPP approach, the precipitated proteins were recovered and examined by SE-HPLC. Most of the protein found in the precipitate is albumin, leading thus to significant improvements in the IgG purity at the IL-rich phase – cf. Fig. S3 in the Supporting information. Although a significant amount of albumin is still present at the IL-rich phase, these systems allow a more significant enhancement in the purity of IgG at the IL-rich phase. Although the ILTPP approach does not induce the recovery of IgG at the interface, it however allows the precipitation of the major contaminant protein from serum (albumin), leading to an increase in the IgG purity at the IL-rich phase. Mondal *et al.*[63] previously applied PPG–IL ABS for the separation of IgG from serum samples. Albeit high recovery yields were reported, the purification percentages were considerably lower than those obtained in this work (< 30%).[63] Further optimization studies aiming at both the complete partitioning of IgG to the IL-rich phase and the precipitation of albumin at the interface may be thus envisioned with IL-based ABS to confirm their potential as alternative purification systems for antibodies.

Envisaging the IgG recovery and the IL removal, an ultrafiltration step was finally applied to the IL-rich phase of the ABS formed by [Chol][DHPh] and [Chol][Lac] – the most promising systems identified. With this step it was possible to completely remove the IL, which can be further reused in new separation steps, and recover IgG in a PBS buffer aqueous solution. Remarkably, with this step the purity level of IgG increased to 62% when recovered from the ABS comprising [Chol][Lac], and up to 66% when recovered from the system with [Chol][DHPh]. Detailed data and respective SE-HPLC chromatograms are given in Fig. S4 in the Supporting information.

CONCLUSION

ABS formed by cholinium-based ILs and PPG 400 were evaluated as alternative systems for the separation of IgG. Novel ABS phase diagrams were determined at 25 °C to infer the mixture compositions required to form liquid–liquid systems. Initial assays with pure IgG showed the preferential partitioning of IgG to the IL-rich phase, with extraction efficiencies ranging between 93% and 100%, and recovery yields ranging between 20% and 100%. [Chol][Gly] and [Chol][Prop] allow recovery yields of 100%, while [Chol][Van], [Chol][Caf] and [Chol]Cl allow recovery yields higher than 89%, which are quite promising when compared with literature values using conventional polymer–salt and polymer–polymer ABS. On the other hand, ABS formed by the remaining ILs led to lower recovery yields, with higher protein losses and precipitated protein, observed in particular in the ABS constituted by [Chol][Lac] and [Chol][DHPh].

Two of the most promising ABS, formed by [Chol][Gly] and [Chol][Van], were then tested for the purification of IgG from rabbit serum samples. The ABS comprising [Chol][Lac] and [Chol][DHPh] were tested as well. Good results were obtained with the ABS containing [Chol][Gly] and [Chol][Van], with extraction efficiencies and recovery yields kept at 100%, even for a real matrix. These systems do not allow, however, a significant increase in the purity of IgG. In the ABS formed by [Chol][Lac] and [Chol][DHPh], extraction efficiencies for IgG of 100% were obtained. However, and in contrast with the studies with pure IgG, a significant increase in the IgG recovery yield when using rabbit serum was observed. These systems led to purity levels of IgG at the IL-rich phase of 47% for [Chol][Lac] and 49% for [Chol][DHPh] in a single-step, which is mainly due to the precipitation of the major serum protein, albumin. The purity levels obtained here are significantly higher than those previously reported using IL–polymer ABS. By applying a final ultrafiltration step to the IL-rich phase of the best identified ABS, the IgG purity increased to 66%. These systems have thus potential for the purification of IgG, and should be further investigated for the extraction and purification of other proteins.

ACKNOWLEDGEMENTS

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 co-financed by FEDER under the PT2020 Partnership Agreement. Neves and Quental thank FCT for the postdoctoral (SFRH/BPD/109057/2015) and PhD (SFRH/BD/109765/2015) grants, respectively. The authors acknowledge financial support from the European Union Framework Programme for Research and Innovation HORIZON 2020, under the TEAMING Grant agreement No 739572 – The Discoveries CTR. MG Freire thanks the European Research Council (ERC) for the Starting Grant ECR-2013-StG-337753.

Supporting Information

Supporting information may be found in the online version of this article.

REFERENCES

1 Buchacher A and Iberer G, Purification of intravenous immunoglobulin G from human plasma – aspects of yield and virus safety. *Biotechnol J* **1**:148–163 (2006).
2 Stiehm ER, Keller MA and Vyas GN, Preparation and use of therapeutic antibodies primarily of human origin. *Biologicals* **36**:363–374 (2008).
3 Zeitlin L, Cone RA, Moench TR and Whaley KJ, Preventing infectious disease with passive immunization. *Microbes Infect* **2**:701–708 (2000).
4 Lemieux R, Bazin R and Néron S, Therapeutic intravenous immunoglobulins. *Mol Immunol* **42**:839–848 (2005).

5 Kivity S, Katz U, Daniel N, Nussinovitch U, Papageorgiou N and Shoenfeld Y, Evidence for the use of intravenous immunoglobulins – a review of the literature. *Clin Rev Allergy Immunol* **38**:201–269 (2009).
6 Hoppe HH, Mester T, Hennig W and Krebs HJ, Prevention of Rh-immunization. Modified production of IgG anti-Rh for intravenous application by ion exchange chromatography (IEC). *Vox Sang* **25**:308–316 (1973).
7 Friesen AD, Bowman JM and Bees WC, Column ion exchange chromatographic production of human immune serum globulin for intravenous use. *Vox Sang* **48**:201–212 (1985).
8 Teng SF, Sproule K, Husain A and Lowe CR, Affinity chromatography on immobilized 'biomimetic' ligands: synthesis, immobilization and chromatographic assessment of an immunoglobulin G-binding ligand. *J Chromatogr B Biomed Sci Applic* **740**:1–15 (2000).
9 Albertsson PÅ, Partition of proteins in liquid polymer – polymer two-phase systems. *Nature* **182**:709–711 (1958).
10 Gündüz U, Optimization of bovine serum albumin partition coefficient in aqueous two-phase systems. *Bioseparation* **9**:277–281 (2000).
11 Asenjo JA and Andrews BA, Aqueous two-phase systems for protein separation: a perspective. *J Chromatogr A* **1218**:8826–8835 (2011).
12 Andrews BA, Nielsen S and Asenjo JA, Partitioning and purification of monoclonal antibodies in aqueous two-phase systems. *Bioseparation* **6**:303–313 (1996).
13 Azevedo AM, Rosa PAJ, Ferreira IF and Aires-Barros MR, Optimisation of aqueous two-phase extraction of human antibodies. *J Biotechnol* **132**:209–217 (2007).
14 Rosa PAJ, Azevedo AM, Sommerfeld S, Mutter M, Aires-Barros MR and Bäcker W, Application of aqueous two-phase systems to antibody purification: a multi-stage approach. *J Biotechnol* **139**:306–313 (2009).
15 Espitia-Saloma E, Vâzquez-Villegas P, Rito-Palomares M and Aguilar O, An integrated practical implementation of continuous aqueous two-phase systems for the recovery of human IgG: from the microdevice to a multistage bench-scale mixer-settler device. *Biotechnol J* **11**:708–716 (2016).
16 Vargas M, Segura A, Herrera M, Villalta M, Angulo Y, Gutiérrez JM *et al.*, Purification of IgG and albumin from human plasma by aqueous two phase system fractionation. *Biotechnol Prog* **28**:1005–1011 (2012).
17 Wu Q, Lin DQ and Yao SJ, Evaluation of poly(ethylene glycol)/hydroxypropyl starch aqueous two-phase system for immunoglobulin G extraction. *J Chromatogr B Analyt Technol Biomed Life Sci* **928**:106–112 (2013).
18 Chow YH, Yap YJ, Show PL, Juan JC, Anuar MS, Ng EP *et al.*, Characterization of partitioning behaviors of immunoglobulin G in polymer-salt aqueous two-phase systems. *J Biosci Bioeng* **122**:613–619 (2016).
19 Lee JW and Forciniti D, Purification of human antibodies from transgenic corn using aqueous two-phase systems. *Biotechnol Prog* **26**:159–167 (2010).
20 Freire MG, Cláudio AFM, Araújo JMM, Coutinho JAP, Marrucho IM, Canongia Lopes JN *et al.*, Aqueous biphasic systems: a boost brought about by using ionic liquids. *Chem Soc Rev* **41**:4966–4995 (2012).
21 Pereira JFB, Rebelo LPN, Rogers RD, Coutinho JAP and Freire MG, Combining ionic liquids and polyethylene glycols to boost the hydrophobic–hydrophilic range of aqueous biphasic systems. *Phys Chem Chem Phys* **15**:19580–19583 (2013).
22 Ferreira AM, Faustino VFM, Mondal D, Coutinho JAP and Freire MG, Improving the extraction and purification of immunoglobulin G by the use of ionic liquids as adjuvants in aqueous biphasic systems. *J Biotechnol* **236**:166–175 (2016).
23 Ruiz-Ruiz F, Benavides J, Aguilar O and Rito-Palomares M, Aqueous two-phase affinity partitioning systems: current applications and trends. *J Chromatogr A* **1244**:1–13 (2012).
24 Gutowski KE, Broker GA, Willauer HD, Huddleston JG, Swatloski RP, Holbrey JD *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* **125**:6632–6633 (2003).
25 Wilkes JS, A short history of ionic liquids – from molten salts to neoteric solvents. *Green Chem* **4**:73–80 (2002).
26 Cláudio AFM, Ferreira AM, Shahriari S, Freire MG and Coutinho JAP, Critical assessment of the formation of ionic-liquid-based aqueous two-phase systems in acidic media. *J Phys Chem B* **115**:11145–11153 (2011).
27 Freire MG, Neves CMSS, Canongia Lopes JN, Marrucho IM, Coutinho JAP and Rebelo LPN, Impact of self-aggregation on the formation of ionic-liquid-based aqueous biphasic systems. *J Phys Chem B* **116**:7660–7668 (2012).
28 Earle MJ, Esperança JMSS, Gilea MA, Canongia Lopes JN, Rebelo LPN, Magee JW *et al.*, The distillation and volatility of ionic liquids. *Nature* **439**:831–834 (2006).
29 Rogers RD and Seddon KR, Ionic liquids – solvents of the future? *Science* **302**:792–793 (2003).
30 Louros CLS, Cláudio AFM, Neves CMSS, Freire MG, Marrucho IM, Pauly J and Coutinho JAP, Extraction of biomolecules using phosphonium-based ionic liquids + K3PO4 aqueous biphasic systems. *Int J Mol Sci* **11**:1777–1791 (2010).
31 Deive FJ, Rodríguez A, Pereiro AB, Araújo JMM, Longo MA, Coelho MAZ *et al.*, Ionic liquid-based aqueous biphasic system for lipase extraction. *Green Chem* **13**:390–396 (2011).
32 Deive FJ, Rodríguez A, Rebelo LPN and Marrucho IM, Extraction of *Candida antarctica* lipase A from aqueous solutions using imidazolium-based ionic liquids. *Sep Purif Technol* **97**:205–210 (2012).
33 Ventura SPM, De Barros RLF, Barbosa JMP, Soares CMF, Lima ÁS and Coutinho JAP, Production and purification of an extracellular lipolytic enzyme using ionic liquid-based aqueous two-phase systems. *Green Chem* **14**:734–740 (2012).
34 Ding X, Wang Y, Zeng Q, Chen J, Huang Y and Xu K, Design of functional guanidinium ionic liquid aqueous two-phase systems for the efficient purification of protein. *Anal Chim Acta* **815**:22–32 (2014).
35 Taha M, E Silva FA, Quental MV, Ventura SPM, Freire MG and Coutinho JAP, Good's buffers as a basis for developing self-buffering and biocompatible ionic liquids for biological research. *Green Chem* **16**:3149–3159 (2014).
36 Pereira MM, Pedro SN, Quental MV, Lima ÁS, Coutinho JAP and Freire MG, Enhanced extraction of bovine serum albumin with aqueous biphasic systems of phosphonium- and ammonium-based ionic liquids. *J Biotechnol* **206**:17–25 (2015).
37 Pereira JFB, Lima ÁS, Freire MG and Coutinho JAP, Ionic liquids as adjuvants for the tailored extraction of biomolecules in aqueous biphasic systems. *Green Chem* **12**:1661–1669 (2010).
38 Freire MG, Pereira JFB, Francisco M, Rodríguez H, Rebelo LPN, Rogers RD and Coutinho JAP, 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* **18**:1831–1839 (2012).
39 Quental MV, Caban M, Pereira MM, Stepnowski P, Coutinho JAP and Freire MG, Enhanced extraction of proteins using cholinium-based ionic liquids as phase-forming components of aqueous biphasic systems. *Biotechnol J* **10**:1457–1466 (2015).
40 Taha M, Almeida MR, E Silva FA, Domingues P, Ventura SPM, Coutinho JAP *et al.*, Novel biocompatible and self-buffering ionic liquids for biopharmaceutical applications. *Chem Eur J* **21**:4781–4788 (2015).
41 Good NE, Winget GD, Winter W, Connolly TN, Izawa S and Singh RM, Hydrogen ion buffers for biological research. *Biochemistry* **5**:467–477 (1966).
42 Patel R, Kumari M and Khan AB, Recent advances in the applications of ionic liquids in protein stability and activity: a review. *Appl Biochem Biotechnol* **172**:3701–3720 (2014).
43 Petkovic M, Seddon KR, Rebelo LPN and Pereira CS, Ionic liquids: a pathway to environmental acceptability. *Chem Soc Rev* **40**:1383–1403 (2011).
44 Ventura SPM, E Silva FA, Gonçalves AMM, Pereira JL, Gonçalves F and Coutinho JAP, Ecotoxicity analysis of cholinium-based ionic liquids to *Vibrio fischeri* marine bacteria. *Ecotoxicol Environ Saf* **102**:48–54 (2014).
45 Sintra TE, Luís A, Rocha SN, Lobo Ferreira AIMC, Gonçalves F, Santos LMNBF *et al.*, Enhancing the antioxidant characteristics of phenolic acids by their conversion into cholinium salts. *ACS Sustain Chem Eng* **3**:2558–2565 (2015).
46 Santos JI, Gonçalves AMM, Pereira JL, Figueiredo BFHT, E Silva FA, Coutinho JAP *et al.*, Environmental safety of cholinium-based ionic liquids: assessing structure – ecotoxicity relationships. *Green Chem* **17**:4657–4668 (2015).
47 Li Z, Liu X, Pei Y, Wang J and He M, Design of environmentally friendly ionic liquid aqueous two-phase systems for the efficient and high activity extraction of proteins. *Green Chem* **14**:2941–2950 (2012).

48 Taha M, Quental MV, Correia I, Freire MG and Coutinho JAP, Extraction and stability of bovine serum albumin (BSA) using cholinium-based Good's buffers ionic liquids. *Process Biochem* **50**:1158–1166 (2015).
49 Muhammad N, Hossain MI, Man Z, El-Harbawi M, Bustam MA, Noaman YA *et al.*, Synthesis and physical properties of choline carboxylate ionic liquids. *J Chem Eng Data* **57**:2191–2196 (2012).
50 Merchuk JC, Andrews BA and Asenjo JA, Aqueous two-phase systems for protein separation. *Studies on phase inversion*. *J Chromatogr B Biomed Sci Applic* **711**:285–293 (1998).
51 Santos JHPM, E Silva FA, Coutinho JAP, Ventura SPM and Pessoa A, Ionic liquids as a novel class of electrolytes in polymeric aqueous biphasic systems. *Process Biochem* **50**:661–668 (2015).
52 Sousa RCS, Neves CMSS, Pereira MM, Freire MG and Coutinho JAP, Potential of aqueous two-phase systems for the separation of *Levodopa* from similar biomolecules. *J Chem Technol Biotechnol* https://doi.org/10.1002/jctb.5553 (2017).
53 Neves CMSS, Shariari S, Lemus J, Pereira JFB, Freire GM and Coutinho JAP, Aqueous biphasic systems composed of ionic liquids and polypropylene glycol: insights into their liquid–liquid demixing mechanisms. *Phys Chem Chem Phys* **18**:20571–20582 (2016).
54 Pereira JFB, Kurnia KA, Cojocaru 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* **16**:5723–5731 (2014).
55 ChemSpider | Search and share chemistry. Available: http://www.chemspider.com/. [8 July 2016].
56 Bollen LS and Hau J, Comparison of immunospecific antibody response in young and old chickens immunized with human IgG. *Lab Anim* **33**:71–76 (1999).
57 Desai RK, Streefland M, Wijffels RH and Eppink MHM, Extraction and stability of selected proteins in ionic liquid based aqueous two phase systems. *Green Chem* **16**:2670–2679 (2014).
58 Silva MF, Fernandes-Platzgummer A, Aires-Barros MR and Azevedo AM, Integrated purification of monoclonal antibodies directly from cell culture medium with aqueous two-phase systems. *Sep Purif Technol* **132**:330–335 (2014).
59 Dhadge VL, Rosa SA, Azevedo A, Aires-Barros R and Roque AC, Magnetic aqueous two phase fishing: a hybrid process technology for antibody purification. *J Chromatogr A* **1339**:59–64 (2014).
60 Dennison C and Lovrien R, Three phase partitioning: concentration and purification of proteins. *Protein Expr Purif* **11**:149–161 (1997).
61 Alvarez-Guerra E and Irabien A, Ionic liquid-based three phase partitioning (ILTPP) for Lactoferrin recovery. *Sep Sci Technol* **49**:957–965 (2014).
62 Alvarez-Guerra E and Irabien A, Ionic liquid-based three phase partitioning (ILTPP) systems for whey protein recovery: ionic liquid selection. *J Chem Technol Biotechnol* **90**:939–946 (2015).
63 Mondal D, Sharma M, Quental MV, Tavares APM, Prasad K and Freire MG, Suitability of bio-based ionic liquids for the extraction and purification of IgG antibodies. *Green Chem* **18**:6071–6081 (2016).

