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Original Article

Tailoring the partitioning of proteins using ionic liquids as adjuvants in polymer-polymer aqueous biphasic systems

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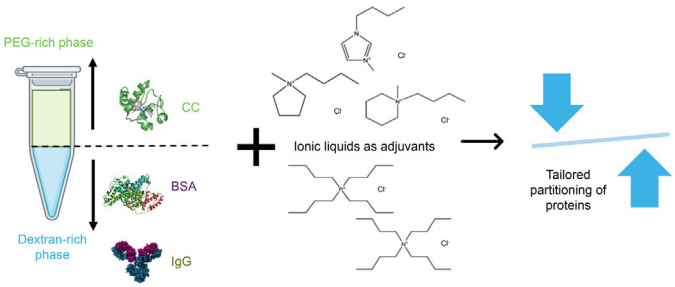
HIGHLIGHTS

- Ionic liquids as adjuvants allow tailoring the phase diagrams of aqueous biphasic systems.
- Ionic liquids as adjuvants allow tailoring the partition of proteins in aqueous biphasic systems.
- Different ionic liquids can increase or decrease the partition coefficient of proteins.
- The partition of proteins depends on the ionic liquid content, ionic liquid chemical structure, and established interactions.
- Ionic liquids allow increasing the selectivity of the systems to separate pairs of proteins.

GRAPHICAL ABSTRACT



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ABSTRACT

Aqueous biphasic systems (ABS) are promising for proteins purification; however, when dealing with samples comprising several proteins, the selectivity towards a target protein is difficult to achieve. In this work, the addition of ionic liquids (ILs) as adjuvants (5 wt% and 10 wt%) in ABS composed of polyethylene glycol (PEG) and dextran was investigated to tailor proteins partitioning between the coexisting phases. The liquid-liquid phase diagrams were determined at 298 K, followed by partition studies of three proteins (bovine serum albumin (BSA), immunoglobulin G (IgG), and cytochrome C (Cyt C)). Partition coefficients of IgG and BSA indicate the preference of both proteins to the dextran-rich phase, whereas Cyt C has no preferential partitioning between the phases. The addition of chloride-based ILs as adjuvants allows to increase or decrease the partition coefficients, thus tailoring the proteins partitioning between the phases. BSA partitioning essentially depends on the IL content in each phase, whereas Cyt C and IgG partitioning is ruled by the ILs chemical structure and established interactions. Molecular docking was carried out to address the ILs effect on the proteins partitioning, supporting experimental observations, while identifying the specific interactions occurring. The partition of each protein in polymer-salt ABS with ILs as adjuvants was determined, demonstrating the higher tailoring ability of polymer-polymer ABS when adding ILs. Finally, the partition of each protein in presence of the remaining ones was determined, as well as the selectivity of the studied systems to separate each pair of proteins, paving the way for their use in liquid-liquid chromatography.

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1. Introduction

Proteins are biological molecules with multiple applications, *e.g.* in therapeutic fields for which a high purity level is required [1]. Protein isolation and purification are usually achieved by precipitation, ultrafiltration, and chromatography [2]. However, depending on the technique, sample complexity and target protein nature, these methods may be of limited selectivity, of high-cost, and apply solvents or salts that lead to proteins denaturation and loss of stability [3–5]. To overcome some of these drawbacks, other approaches such as liquid-liquid extraction (LLE) have been investigated to purify proteins. Aqueous biphasic systems (ABS) are formed by the combination of water and at least two phase-forming components (*e.g.* polymers, salts, ionic liquids (ILs), surfactants, among others [6]), above which certain concentrations there is phase separation [7]. Due to their water-rich nature, ABS have been applied in the separation of biologically active compounds, *e.g.* amino acids [8–10], DNA [11] , RNA [12], antibodies [13], and other proteins [14–19]. The selective partition of biomolecules between the two phases in equilibrium, which in turn reflects the ABS efficiency to separate a target product when dealing with complex mixtures, depends on several parameters inherent to the product and ABS phase-forming components and composition [20].

Traditional polymer-polymer-based ABS have been applied to extract and separate several proteins, such as lipase [21], cytochrome C (Cyt C) [21], lysozyme [21], bovine serum albumin (BSA) [22], and immunoglobulin G (IgG) [23]. However, these systems show limitations in the separation of target proteins in samples presenting a pool of proteins with similar properties, which is due to the limited polarity difference between the phases. To overcome this drawback and improve selectivity, salt additives [24] and affinity ligands [25–27] have been investigated as components of polymer-polymer-based ABS.

A new approach for creating ABS composed of ILs as phase-forming components were proposed by Rogers and co-workers in 2003 [28], widening the range of applications of the ABS technology [29]. In addition to other relevant characteristics, the most important feature in the ABS field is the ILs tailoring ability, by which the IL can be chemically modified to fulfill the requirements of a given separation [30–32]. Over the years, the use of ILs has been proposed to overcome the limitations of conventional polymer-based ABS to separate and purify a wide range of compounds [33–35], including proteins [8,36]. However, ILs have a high solvation ability and allow a variety of interactions, often leading to complete protein extractions in one-step when using IL-salt or IL-polymer ABS, which may compromise their selectivity [30]. However, when used as adjuvants, *i.e.* in lower amounts, improved selectivity has been identified in traditional salt ABS for several biomolecules [8,10,18], such as amino acids [8–10], antioxidants [9,10,37], and dyes [38].

In addition to the published works carried out with small molecules, studies involving proteins have been investigated as well. The partition of BSA, lysozyme, and myoglobin were studied in ABS composed of PEG 600, phosphate salts, and imidazolium-based ILs (2.5 wt%, 5 wt%, and 7 wt%) [39]. In the same line, Marchel et al. [18] studied the extraction of myoglobin using ABS composed of PEG 3350, ammonium sulphate, and 5 wt% of IL (ILs based on imidazolium, pyrrolidinium or pyrydinium). Despite these works carried out with model proteins, the extraction of β-mannase from its biological medium was performed with PEG-salt ABS using 3 wt% of ILs as adjuvants [14]. Ferreira et al. [13] studied the IgG separation from rabbit serum using ABS formed by PEG 400 and potassium citrate buffer, using 5 wt% of ILs as adjuvants, observing an increase in the purity of IgG by 37% when compared to systems without IL. ABS comprising PEGs and potassium phosphate buffer (pH 7.0) with 5 wt% of imidazolium-based ILs as ABS adjuvants were also applied for lipase purification [15], leading to an increase in the lipase purification factor from 176 to 245. More recently, the separation of crude papain extract from papaya latex using ABS composed of PEG 400 and ammonium sulphate with quaternary ammonium ILs as adjuvants were investigated, with an improvement in the papain purification factor from 2.13 to 13.51 [16].

Overall, in the described works using ILs as adjuvants in polymer-salt ABS [13–16,18,39], a strong salting-out effect promoted by the inorganic salts exists, which may mask the tailoring ability of ILs. To address this question while trying to improve the ILs ability to tailor the proteins separation performance, ABS formed by two polymers, namely PEG and dextran, using ILs as adjuvants were investigated in this work.

2. Materials and methods

2.1. Materials

Dextran with a molecular weight of 450,000–650,000 g mol^{-1} (dextran 450–650), PEG with molecular weight of 400 and 6000 g mol^{-1} (PEG 400 and PEG 6000, respectively), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS, > 98%) and hydrogen peroxide solution (H_2O_2 30% (w/w) in water) were acquired from Sigma-Aldrich. Ammonium sulphate ($(NH_4)_2SO_4$, > 99% pure) and sodium phosphate dibasic (Na_2HPO_4 > 99% pure) were acquired from Merck. Citric acid ($C_6H_8O_7{\cdot}H_2O$ > 99%) was purchased from Fisher Scientific. The ILs 1-butyl-3-methylimidazolium chloride ([C_4mim]Cl, > 99% pure), 1-butyl-1-methylpyrrolidinium chloride ([C_4mpyr]Cl, > 99% pure) and 1-butyl-3-methylpiperidinium chloride ([C_4mpip]Cl, > 99% pure) were acquired from Iolitec. Tetrabutylammonium chloride ([N_{4444}]Cl, > 97% pure) was purchased from Sigma-Aldrich and tetrabutylphosphonium chloride ([P_{4444}]Cl, 96% pure) was supplied by Cytec Ind. The chemical structures of the ILs studied as adjuvants are presented in Table 1. Double distilled water was employed, passed across a reverse osmosis system, and finally treated with a Milli-Q plus 185 water purification apparatus.

The proteins immunoglobulin G (IgG, > 95% pure) from human serum and cytochrome C (Cyt C, > 95% pure) from equine heart were supplied by Sigma-Aldrich. Bovine serum albumin (BSA, > 98% pure) was purchased from Fisher Scientific. Table 2 summarizes the properties of the investigated proteins.

2.2. Determination of ABS phase diagrams

For the determination of the liquid-liquid phase diagrams, water and aqueous solutions of PEG 6000 and dextran 450–650 containing 5 wt% or 10 wt% of each IL were used, allowing to keep the IL concentration constant in all regions of the phase diagram. The binodal curves were obtained by the cloud point titration method at 298 K and atmospheric pressure. Repetitive drop-wise addition of the PEG solution to the dextran solution was carried out until the recognition of a cloudy solution, followed by the drop-wise addition of water until the detection of a clear solution. This procedure was carried out under constant stirring and temperature (298 K). Further details are provided in the literature [9].

The experimental data corresponding to the binodal curves were fitted using Eq. (1), proposed by Neves et al. [9]:

$$[PEG] = Aexp\left[\left(B[dextran]^{0.5}\right) - \left(C[dextran]^{3}\right)\right] \quad (1)$$

where *[PEG]* and *[dextran]* are PEG and dextran weight percentages (wt %) and *A*, *B* and *C* are fitting parameters obtained by the non-linear regression of the experimental data.

2.3. Quantification and partition coefficients of ionic liquids

The partition coefficient of each IL (K_{IL}) in each ABS was determined to address their effect on the biomolecules partitioning between the two phases. These studies were conducted in two mixture points: 5 wt% PEG + 10 wt% dextran + 5 wt% IL + 80 wt% H_2O and 5 wt% PEG + 10 wt% dextran + 10 wt% IL + 75 wt% H_2O. The IL concentration in each phase was quantified using a Metrohm 904 Titrando ion chloride electrode. A

Table 1
Chemical structure and predicted hydrogen-bond acidity of ionic liquids using the COnductor-like Screening MOdel for Realistic Solvents (COSMO-RS) [40].

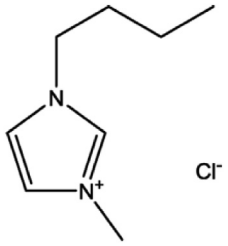
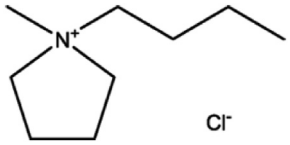
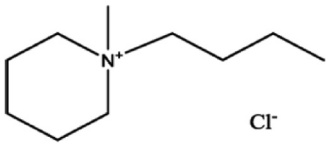
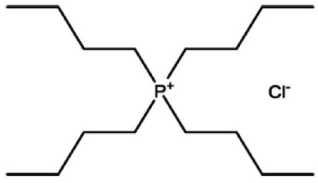
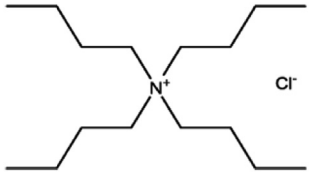
IL	Chemical structure	α [40]
[C_4mim]Cl		0.986
[C_4mpyr]Cl		0.537
[C_4mpip]Cl		0.527
[P_{4444}]Cl		0.428
[N_{4444}]Cl		0.423

Table 2
Molecular weight (MW) and isoelectric point (pI) of the proteins investigated.

Protein	MW/kDa	pI
IgG	150 [41]	6.6–7.2 [41]
BSA	65 [42]	4.7 [42]
Cyt C	12 [43]	10 [44]

calibration curve was made using a stock aqueous solution of potassium chloride (KCl, 1 mol·L^{-1}) and diluted at appropriate concentrations. To maintain the ionic strength during the measurements, a total ionic strength adjustment buffer (TISAB) was used. TISAB contains aqueous solutions of potassium nitrate (KNO_3), acetic acid ($C_2H_4O_2$) and sodium acetate ($C_2H_3NaO_2$) at 0.1 mol·L^{-1}. The partition coefficient of each IL, K_{IL}, was determined according to Eq. (2):

$$K_{IL} = \frac{[IL]_T}{[IL]_B} \quad (2)$$

where $[IL]_T$ and $[IL]_B$ are the concentrations (g L^{-1}) of the IL in the top and bottom phases, respectively. The bottom phase corresponds to the dextran-rich phase in all systems investigated, whereas the top phase is majorly enriched in PEG.

2.4. Quantification and partition coefficients of proteins

Three types of assays were performed to analyse the partition behaviour of the studied proteins in the investigated ABS: (i) partition of each protein in the studied polymer-polymer ABS with ILs as adjuvants; (ii) partition of each protein in polymer-salt ABS with ILs as adjuvants (for comparative purposes); and (iii) partition of each protein in presence of all proteins in polymer-polymer ABS with ILs as adjuvants. Partition studies of IgG, BSA, and Cyt C were carried out using ILs as adjuvants in each polymer-polymer ABS, using two different amounts of IL, at the following mixture compositions: 5 wt% PEG 6000 + 10 wt% dextran + 5 wt% IL + 80 wt% aqueous solution containing the target protein and 5 wt % PEG 6000 + 10 wt% dextran + 10 wt% IL + 75 wt% aqueous solution containing the target protein. ABS without IL (control) were prepared as well at the following mixture composition: 5 wt% PEG 6000 + 10 wt% dextran + 85 wt% aqueous solution containing the target protein.

Partition studies of each protein in polymer-salt ABS using ILs as adjuvants were additionally carried out for comparison purposes. The liquid-liquid phase diagrams of these ABS were taken from the literature [9]. According to their phase diagrams and biphasic region, the following mixture composition was used: 22 wt% PEG 400 + 18 wt% $(NH_4)_2SO_4$ + 5 wt% IL ([C_4mpip]Cl or [N_{4444}]Cl) + 55 wt% aqueous solution containing each protein. ABS without IL (control) were prepared as well at the following mixture composition: 22 wt% PEG 400 + 18 wt% $(NH_4)_2SO_4$ + 60 wt% aqueous solution containing each protein.

To address the partition of each protein in presence of all proteins investigated, partition studies were carried out in polymer-polymer ABS at the following mixture composition: 5 wt% PEG 6000 + 10 wt% dextran +5 wt% IL ([C_4mpip]Cl or [N_{4444}]Cl) + 80 wt% aqueous solution containing the mixture of the three proteins. These ILs were chosen due to their extreme hydrogen-bond acidity values (Table 1). ABS without IL (control) were prepared as well at the following mixture composition: 5 wt% PEG 6000 + 10 wt% dextran + 85 wt% aqueous solution containing the mixture of the three proteins.

All ABS studied used aqueous solutions of each protein at 1 g L^{-1} and the proteins mixture at a final concentration of 1 g L^{-1}. All components were weighted and vigorously stirred until obtaining a homogeneous solution. ABS were left to equilibrate at 298 K for 10 min and centrifuged at 10,000 rpm for 10 min at the same temperature. The top and bottom phases were carefully separated and weighed, and the proteins were quantified in each phase, as described below.

The quantification of IgG and BSA was carried by size-exclusion high-performance liquid chromatography (SE-HPLC) (Chromaster, VWR Hitachi) with a Shodex Protein KW-802.5 (8 mm × 300 mm) column. The mobile phase contained 100 mM phosphate buffer with NaCl 0.3 M, previously degassed by ultrasonication. The mobile phase was run isocratically at 0.5 mL min^{-1}. The column and autosampler were kept at 313 K and 283 K, respectively. Each phase was diluted at a 1:10 (v:v) ratio in the mobile phase before injection with a volume of 25 μL. The wavelength was set at 280 nm using a DAD detector. At these conditions, IgG and BSA presented retention times of 12.7 and 13.8 min, respectively. The quantification of Cyt C was carried out by HPLC-DAD (Shimadzu, model PROMINENCE), with an analytical C18 reversed-phase column (250 × 4.60 mm), kinetex 5 μm C18 100 A, from Phenomenex. The mobile phase used was a gradient system of 0.1% of trifluoracetic acid (TFA)-ultra pure water (phase A) and 0.1% TFA-acetonitrile (phase B), previously degassed by ultrasonication. The separation was conducted using the following gradient mode: 0 min 25% of B, 42 min 42% of B, 45 min of B, and then returning to initial conditions during 20 min to ensure the column stabilization. The flow rate used was 0.8 mL min^{-1} with an injection volume of 40 μL. The wavelength was set at 204 nm using a DAD detector. The column and the autosampler operated at a controlled temperature of 298 K. At these conditions, Cyt C presented a retention time of 3.4 min.

The partition coefficient (K_{Prot}) of each protein was determined according to Eq. (3):

$$K_{Prot} = \frac{[Prot]_T}{[Prot]_B} \quad (3)$$

where $[Prot]_T$ and $[Prot]_B$ are each protein concentration in the top and bottom phases, respectively.

The selectivity (S_{Prot}) of each protein (i) in respect to a second one (j) was determined according to Eq. (4):

$$S_{Prot} = \frac{K_{Prot\ i}}{K_{Prot\ j}} \quad (4)$$

2.5. Determination of Cyt C activity

To assess the biological activity and stability of Cyt C in the ABS studied, the peroxidase activity of Cyt C was determined using ABTS as substrate in the presence of H_2O_2. The oxidation of ABTS is catalysed by the Cyt C and the green ABTS $^+$ radical formed was monitored by the changes in the absorption at 420 nm using a UV–Vis spectrophotometer (Shimadzu UV1800 Spectrometer). The enzymatic reaction was started by mixing 100 μL of each sample, 200 μL of ABTS solution (15 mM), 100 μL H_2O_2 and 600 μL of citrate/phosphate buffer (0.1 M), pH 4.5. The relative enzyme activity in the top and bottom phases of the ABS comprising ILs was calculated against the enzymatic activity of the ABS without IL.

2.6. Molecular docking

The interactions of Cyt C, BSA, and IgG with the IL cations ($[C_4mim]^+$, $[C_4mpyr]^+$, $[C_4mpip]^+$, $[N_{4444}]^+$, $[P_{4444}]^+$), and anion (Cl^-) were identified using AutoDock Vina 1.1.2 [45]. The crystal structure of Cyt C (PDB: 1hrc), BSA (PDB: 3v03), and IgG (PDB: 1hzh) were used in the molecular docking as receptor. All PDB files were downloaded from Protein Data bank and uploaded in ProteinPrepare [46]. The pK_a calculation was performed at pH 6.0 (average pH of all ABS experimentally studied), without water molecules. AutoDockTools (ADT) [47] was used to prepare the protonated proteins input files. Ligands 3D atomic coordinates were created by using Discovery Studio, v20 (Accelrys, San Diego, CA, USA), and applied to Chem3D-MM2 protocol for energy minimization [48]. Ligands rigid root was generated using AutoDockTools (ADT), setting all possible rotatable bonds defined as active by torsions. The grid center at the center of mass (x-, y-, and z-axes) for Cyt C, BSA, and IgG were 46.735 × 23.231 × 5.546, 40.479 × 22.698 × 41.262, and 79.989 × 11.705 × 137.356, respectively. The grid dimension of Cyt C, BSA and IgG were 50 Å × 50 Å × 50 Å, 90 Å × 70 Å × 90 Å, and 120 Å × 120 Å × 120 Å, respectively. The binding model that has the lowest binding free energy was searched out from 9 different conformers for each ligand (IL cations).

3. Results and discussion

3.1. ABS phase diagrams determination

Due to the novelty of the ABS investigated, the corresponding binodal or solubility curves were first determined. The phase diagrams obtained for ABS formed by PEG 6000, dextran 450–650 and 5 wt% or 10 wt% of each IL are illustrated in Fig. 1a and b, respectively. The respective detailed data are reported in the Supporting Information (Tables S1–S3). Eq. (1) was fitted to the experimental data, being also given in Fig. 1a and b. The corresponding parameter values are given in the Supporting Information (Tables S4–S5). Phase diagrams are shown in an orthogonal representation. Each IL is kept at a constant concentration (5 wt% or 10 wt%) in the complete phase diagram.

The binodal curves in Fig. 1 provide information regarding the monophasic and biphasic regions (below and above each solubility curve), in which it is shown that ILs exert an effect on the phase separation. However, contrarily to what was reported in previous works with other ABS formed by PEG, salt and chloride-based ILs [9,37], not all systems have an increase in the biphasic region with the addition of ILs as adjuvants. This difference is a result of using dextran instead of a salt, and consequent IL affinity for each phase. When evaluating the curves at a fixed content of 10 wt% of dextran, the ability of ILs (at 5 wt%) to induce the formation of ABS (Fig. 1 a) follows the order:

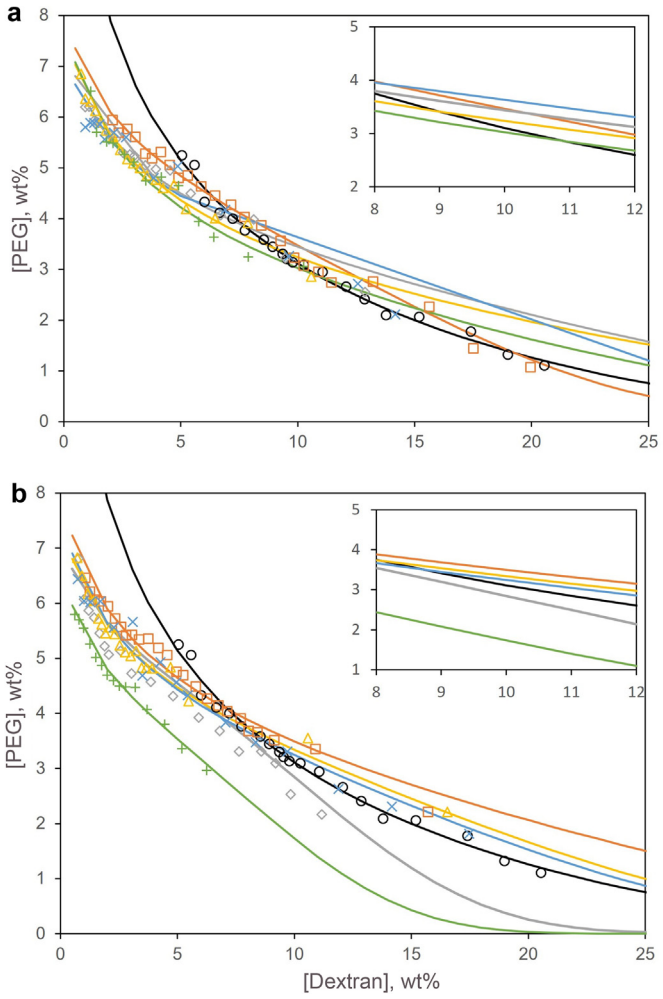


Fig. 1. Phase diagrams of ABS composed of: (a) 5 wt% PEG + 10 wt% dextran + 5 wt% IL + 80 wt% H_2O and (b) 5 wt% PEG + 10 wt% dextran + 10 wt% IL + 75 wt% H_2O at 298 K. Continuous lines (−) correspond to non-linear regressions by Eq. (1) applied to the experimental data. (○) ABS without IL; (□) ABS with $[C_4mim]Cl$; (◇) ABS with $[C_4mpyr]Cl$; (△) ABS with $[C_4mpip]Cl$; (×) ABS with $[N_{4444}]Cl$; (+) ABS with $[P_{4444}]Cl$.

$$[P_{4444}]Cl > \text{no IL} > [C_4mpip]Cl > [C_4mpyr]Cl \approx [C_4mim]Cl > [N_{4444}]Cl$$

On the other hand, in presence of 10 wt% of IL as adjuvant (Fig. 1 b), it follows the order:

$$[P_{4444}]Cl > [C_4mpyr]Cl > \text{no IL} > [C_4mpip]Cl \approx [N_{4444}]Cl > [C_4mim]Cl$$

These results show that the addition of 5 wt% of $[C_4mpip]Cl$, $[C_4mpyr]Cl$, $[C_4mim]Cl$ and $[N_{4444}]Cl$ in ABS leads to a slight decrease in the biphasic region compared to the ABS without the addition of IL. When the IL has increased to 10 wt% for $[C_4mim]Cl$, $[C_4mpip]Cl$ and $[N_{4444}]Cl$, the biphasic region also decreases compared with the ABS without IL.

According to results reported in the literature for PEG-salt ABS with ILs as adjuvants [8–10], the biphasic region of the binodal curve increases with the increase of the PEG-rich phase hydrophobicity, the phase for which ILs preferentially migrate, and due to strong salting-out

effects induced by the salt. However, in the present work, a strong salting-out phenomenon induced by salts does not occur since ABS are composed of two polymers. With the exception of the [P_{4444}]Cl IL, a close correlation between the hydrophobicity of ILs (given by the ILs hydrogen-bond acidity, Table 1) and the formation of two phases was found (cf. Fig. S1 in the Supporting Information). In the studied systems, the higher the hydrogen-bond acidity of a given IL, and thus its ability to interact with water, the higher is the amount of PEG or dextran required to create two-phase systems.

It should be remarked that the composition of each phase varies with the PEG and dextran composition at the chosen mixture point, which in turn slightly affects the density and viscosity of each phase and consequently phase separation. Accordingly, a common mixture composition was investigated to appraise the partition coefficients of the three proteins in the investigated ABS, as shown below.

3.2. Partition of ILs in PEG-dextran ABS

To better understand the partition behaviour of proteins due to their interactions with each IL, it is important to know the partition behaviour of each IL in the ABS studied. All ILs investigated in this work present K_{IL} values higher than 1.0 (ranging between 1.08 and 1.48), showing that there is a preferential migration of ILs to the PEG-rich phase. The respective data are provided in the Supporting Information (Table S6 and Fig. S2). The K_{IL} values increase in the following order: [C_4mim]Cl ≈ [C_4mpip]Cl ≈ [C_4mpyr]Cl < [P_{4444}]Cl < [N_{4444}]Cl. Furthermore, K_{IL} is similar when comparing the two concentrations of ILs, proving that no IL aggregation or saturation of the ABS phases is occurring. In general, the lower the IL hydrogen-bond acidity and thus IL ability to donate protons, the more the IL migrates to the PEG-rich phase.

Other works using chloride-based ILs show similar behaviour of K_{IL} [9,10]. In ABS composed of PEG 400 and salts with the addition of 5 wt% ILs, ILs have a preferential migration to the PEG-rich phase (K_{IL} ranging between 3 and 7) [9,10], with the more hydrophobic ILs ([P_{4444}]Cl and [N_{4444}]Cl) presenting a higher K_{IL} or preferential migration to the PEG-rich phase [9]. Although the ILs trend is similar, higher K_{IL} values are observed in PEG-salt than in PEG-dextran ABS, which is a consequence of the lower polarity difference of the phases in these systems.

3.3. Effect of ILs in the partitioning of proteins in PEG-dextran ABS

The partition coefficient of each protein (K_{Prot}), namely IgG, BSA, and Cyt C was determined in ABS composed of 5 wt% of PEG and 10 wt% of dextran, with 5 wt% or 10 wt% of IL, and 80 wt% or 75 wt% of each protein aqueous solution. These results are shown in Fig. 2 and compared with results for the ABS without IL. The respective data are given in the Supporting Information (Table S7). When using 10 wt% of [P_{4444}]Cl with IgG and BSA, the proteins precipitate, making unfeasible the calculation of K_{Prot}. This trend, also known as three-phase partitioning (TPP) in ABS [49], has been reported before for BSA with the same IL, yet in ABS formed by ILs and salts [50]. In these systems, the BSA precipitation occurs due to the high hydrophobicity of one of the phases combined with a strong salting-out ability induced by the salt. In this work, it is shown that the salt used is not essential to achieve TPP, and polymer-polymer ABS comprising ILs could be also studied to separate target proteins by TPP.

The K_{Prot} of IgG and BSA are below 1 for all systems, revealing the preference of both proteins for the dextran-rich phase (the most hydrophilic phase in the investigated systems). Cyt C presents a partition coefficient close to 1, showing thus no preference to any given phase. This trend applies to the system without IL and all systems with ILs as adjuvants. However, an increase or decrease in the partition coefficient of each protein can be induced by different ILs, further depending on the IL chemical structure and IL content. The partition coefficient of Cyt C in the various ABS with 5 wt% of IL increases in the following trend:

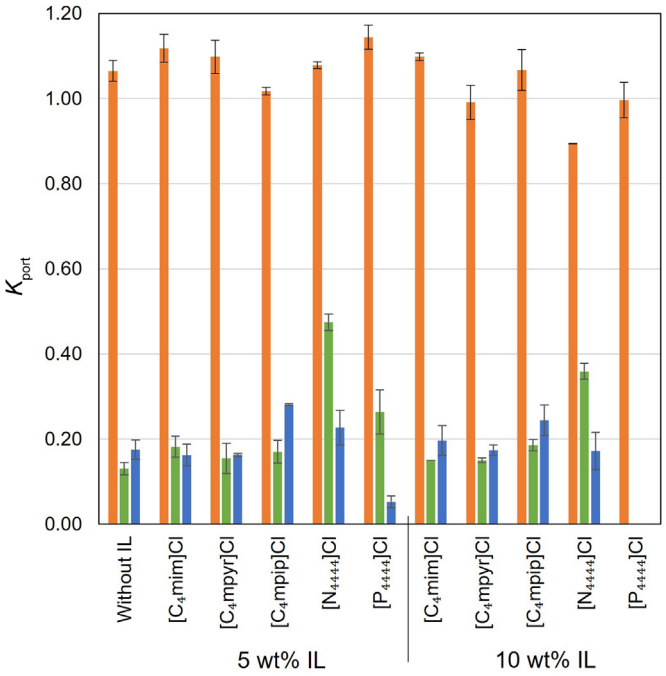


Fig. 2. Partition coefficient of each protein (K_{Prot}) in ABS composed of 5 wt% PEG + 10 wt% dextran + 5 wt% or 10 wt% IL + 80 wt% or 75 wt% protein aqueous solution: Cyt C (■); BSA (■); IgG (■).

[C_4mpip]Cl < no IL < [C_4mpyr]Cl ≈ [N_{4444}]Cl ≈ [C_4mim]Cl < [P_{4444}]Cl

For BSA, these values increase in the following order:

no IL < [C_4mpyr]Cl ≈ [C_4mpip]Cl ≈ [C_4mim]Cl < [P_{4444}]Cl < [N_{4444}]Cl

Finally, for IgG, the partition coefficients increase as follows:

[P_{4444}]Cl < no IL ≈ [C_4mim]Cl ≈ [C_4mpyr]Cl < [N_{4444}]Cl < [C_4mpip]Cl

Overall, for BSA, the introduction of ILs always promotes the protein partitioning to the PEG-rich phase, whereas with IgG only the ILs [C_4mpip]Cl and [N_{4444}]Cl at 5 wt% and [C_4mpip]Cl and [C_4mim]Cl at 10 wt% favour these proteins migration to the PEG-rich phase. With Cyt C, the ILs [C_4mim]Cl, [C_4mpyr]Cl, and [P_{4444}]Cl at 5 wt% and [N_{4444}]Cl at 5 wt% favour this protein migration to the PEG-rich phase. When representing K_{Prot} as a function of K_{IL} for BSA (cf. Fig. S3 in the Supporting Information), it is visible a close correlation between these parameters at both concentrations studied; therefore, BSA partition depends essentially on the IL content in the PEG-rich phase. However, the same correlation is not observed for IgG and Cyt C, meaning that the partitioning of these two proteins depends more strongly on the IL chemical structure than on the IL content – as discussed below.

The phases of the studied ABS have pH values between 5.5 and 6.5 (measured in this work). At this pH range, IgG (pI = 6.6–7.2) and Cyt C (pI = 10) are positively charged, whereas BSA (pI = 4.7) is negatively charged (Table 2). Given that ILs are enriched in the PEG-rich phase, and that opposite partitioning is shown for positively charged proteins such as Cyt C and BSA, it seems that electrostatic interactions between the IL and proteins are not key interactions ruling these proteins partitioning.

To better understand the role played by ILs in polymer-polymer and polymer-salt ABS, partition studies of each protein in polymer-salt ABS with ILs as adjuvants were carried out. The mixture composition was chosen according to the respective phase diagrams taken from the literature [9], corresponding to: 22 wt% PEG 400 + 18 wt% $(NH_4)_2SO_4$ + 5 wt% IL + 55 wt% protein aqueous solution. [C_4mim]Cl and [N_{4444}]Cl were the ILs chosen in this set of assays due to their extreme α values

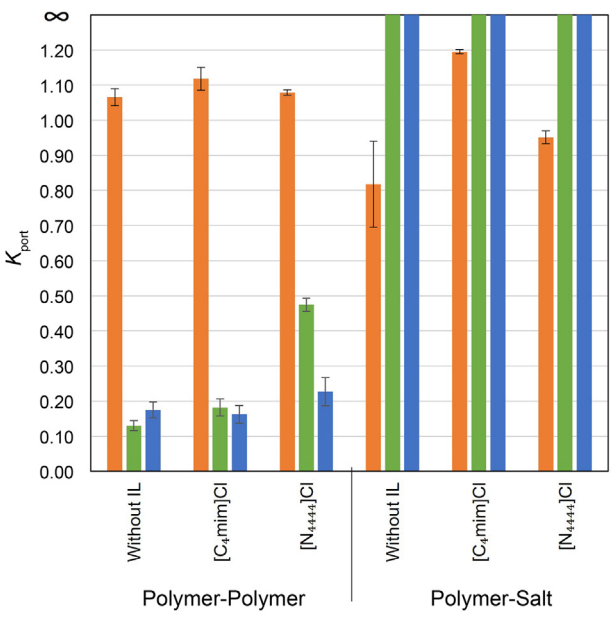


Fig. 3. Partition coefficient of each protein (K_{Prot}) in ABS composed of 5 wt% PEG 6000 + 10 wt% dextran + 5 wt% IL + 80 wt% protein aqueous solution (Polymer-Polymer ABS) and 22 wt% PEG 400 + 18 wt% $(NH_4)_2SO_4$ + 5 wt% IL + 55 wt% protein aqueous solution (Polymer-Salt ABS): Cyt C (■); BSA (■); IgG (■). ∞ means total partition of a given protein to the top (PEG-rich) phase.

(Table 1). The obtained results are shown in Fig. 3 and the respective data are provided in the Supporting Information (Table S8).

The partition of Cyt C is highly similar between polymer-salt and polymer-polymer ABS, both with and without ILs. The main difference with Cyt C is shown in the systems with no IL added as an adjuvant, where Cyt C has a preferential partition to the salt-rich phase in polymer-salt ABS and no preferential partitioning (K_{Prot} close to 1) in systems comprising PEG and dextran, followed by a slight improvement or decrease in the partition coefficient in polymer-salt ABS when using $[C_4mim]Cl$ and $[N_{4444}]Cl$. Regarding BSA and IgG, both proteins were not detected in the bottom-phase (salt-rich phase) in polymer-salt ABS, indicating a complete partition of both proteins to the top phase (PEG-rich phase). This behaviour is completely different to that afforded by polymer-polymer ABS, where both proteins have a preferential partition to the dextran-rich phase (bottom phase) and where different partition coefficients are obtained. Furthermore, in polymer-salt ABS, the addition of IL as adjuvant does not affect the partition behaviour of BSA and IgG. These results reinforce that the partition and selectivity of ABS towards proteins are more effective when using polymer-polymer ABS comprising ILs as adjuvants.

In addition to our results with PEG-salt ABS, Bezold et al. [39] applied 3 concentrations (2.5 wt%, 5.0 wt% and 7.5 wt%) of two ILs, namely 1-ethyl-3-methylimidazolium chloride ($[C_2mim]Cl$) and $[C_4mim]Cl$, as adjuvants in PEG-salt ABS and studied the partitioning of BSA, lysozyme and myoglobin. The authors showed that the partitioning of proteins to the PEG-rich phase increases directly with the overall amount of IL used and K_{Prot} increases in the following protein order: myoglobin < lysozyme < BSA. However, the partition of the ILs was not evaluated. Comparing to our results, PEG-dextran ABS have a different behaviour since the correlation between the IL concentration or K_{IL} and K_{Prot} is not verified with all proteins, only with BSA. Furthermore, results from the literature [13–16,18,39,51] demonstrate that the addition of ILs in PEG-salt ABS changes the proteins behaviour, with their partitioning being mainly related to salting-out effects and hydrophobicity of the phases. In this work, where PEG-dextran ABS are investigated, the partition of proteins is not influenced by a strong salting-out effect (since no salt is used). These differences show that the influence of ILs in polymer-polymer ABS is not straightforward (several interactions may rule the proteins partitioning) and could thus allow a more efficient tailoring and improvement of selectivity in these systems.

The wide structural variety of ILs as well as of proteins is responsible for a multiplicity of solvent-protein interactions. To better understand the proteins partition behaviour at the molecular level, molecular docking analysis was carried out, particularly to identify the main interactions occurring between the IL ions and Cyt C, BSA or IgG. All ILs studied are composed of the chloride anion (Cl^-). From the molecular docking studies, Cl^- only presents significant electrostatic interactions with the amino acid residues of BSA, the protein for which the partition coefficient is dependent on the IL content in polymer-polymer ABS. These results show that IL cations are the species that position themselves close to the proteins, independently of their charge. These results are in agreement with the literature, where it has been shown that the concentration of IL cations is higher than that of anions at the protein surface, regardless of the protein charge [52–54]. For proteins in aqueous media, as is the case, this can be explained by the preferential hydrogen-bonded network established between the IL anions and water in the bulk phase [55].

The docking affinity was then calculated for the IL cations ($[C_4mim]^+$, $[C_4mpyr]^+$, $[C_4mpip]^+$, $[N_{4444}]^+$, and $[P_{4444}]^+$) to better appraise differences in the K_{Prot} values. The bind poses with lowest absolute value of affinity (kcal mol^{-1}) for Cyt C, BSA, and IgG with the IL cations are displayed in Fig. 4. 3D diagrams of molecular interactions (interacting amino acid residues) are displayed in the Supporting Information (Figs. S4–S6), as well as the docking affinities of each binding pose for all proteins and ILs cations, interacting amino acids residues, type of interaction and geometry distance (Å) of all ILs cations (cf. the Tables S9–S11 in the Supporting Information).

Docking affinities of IL cations with Cyt C increase in the following sequence:

$$[N_{4444}]^+ < [C_4mim]^+ < [P_{4444}]^+ < [C_4mpyr]^+ < [C_4mpip]^+$$

The binding energies of the cyclic cations with Cyt C increase with the number of carbons in the cation ring. Furthermore, $[C_4mim]^+$ and $[C_4mpyr]^+$ interact through hydrogen bonding and electrostatic interactions with Cyt C. On the other hand, hydrophobic interactions occur between Cyt C amino acid residues and $[C_4mim]^+$ and $[C_4mpip]^+$. The quaternary ammonium- and phosphonium-based cations bind to Cyt C through electrostatic interactions. However, the binding energy of $[P_{4444}]^+$ is higher in comparison with $[N_{4444}]^+$, which may justify the higher partition coefficient of Cyt C observed with the former. Overall, no direct trend with the IL binding energies is observed for Cyt C, showing that with this protein there are other factors also ruling its partitioning, such as the IL content. Furthermore, the binding affinities strength are lower than the ones observed with the remaining two proteins, as discussed below, justifying the effect of other factors taking place. Despite not being the major force, the IL cations establish hydrogen-bonding, electrostatic and hydrophobic interactions with Cyt C.

IL cation docking affinities with BSA decrease in the following order:

$$[N_{4444}]^+ > [C_4mpip]^+ > [C_4mim]^+ > [C_4mpyr]^+ > [P_{4444}]^+$$

All IL cations establish hydrophobic interactions with BSA, being the only type of interactions established, with the exception of $[C_4mpyr]^+$ that also interacts through electrostatic interactions. Furthermore, all cyclic cations have similar binding affinities with BSA (−4.7 to −5.0 kcal mol^{-1}), reinforcing the higher relevance of the IL content as depicted from the experimental data. The ammonium cation displays a higher binding affinity (−5.8 kcal mol^{-1}) than the phosphonium counterpart (−4.1 kcal mol^{-1}), which may be favorable to improve the partition of BSA towards the PEG-rich phase in polymer-polymer ABS as experimentally observed. Overall, with BSA, the major interactions ruling the partition of the protein in the studied PEG-dextran ABS, comprising ILs as adjuvants, are hydrophobic interactions; however, they

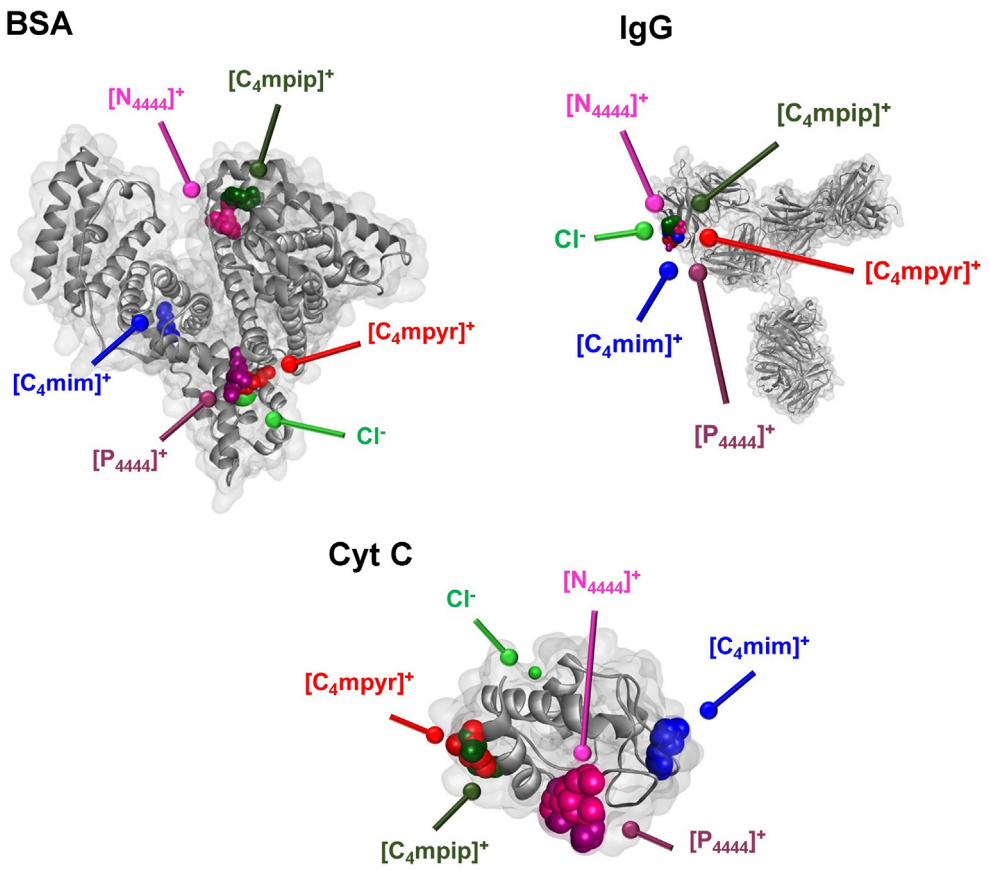


Fig. 4. Docking pose with the lowest absolute value of affinity (kcal mol^{-1}) for BSA, IgG, and Cyt C with the IL cations $[C_4mim]^+$, $[C_4mpyr]^+$, $[C_4mpip]^+$, $[N_{4444}]^+$ and $[P_{4444}]^+$, and IL anion Cl^-.

have similar strength, thus reinforcing the relevance of the IL content in each phase.

The bind affinity of all IL cations studied with the IgG protein increase in the following order:

$$[P_{4444}]^+ < [C_4mim]^+ < [N_{4444}]^+ = [C_4mpyr]^+ < [C_4mpip]^+$$

This trend closely follows the IL effect in promoting the K_{Prot} increase, *i.e.* the protein preferential migration to the PEG-rich phase, thus demonstrating the relevance of the IL chemical structure and its interactions with IgG as experimentally observed. For the cyclic ILs, several IgG amino acid residues interact with $[C_4mim]^+$ through hydrophobic and hydrogen bond interactions, with $[C_4mpip]^+$ through hydrophobic interactions, and with $[C_4mpyr]^+$ by electrostatic interactions. Quaternary cations, such as $[N_{4444}]^+$ and $[P_{4444}]^+$ establish hydrophobic interactions with IgG residues. Based on the provided analysis, together with the gathered experimental data, it is clear that the IL chemical structure and the respective hydrophobic and hydrogen-bonding interactions play a dominant role in the IgG partitioning to the PEG-rich phase.

The selectivity of the ABS to separate each pair of proteins was finally determined, whose results are shown in Fig. 5 (cf. Table S13 in the Supporting Information). The closeness to 1 the selectivity values are, the more challenging is the separation of two proteins. The studied polymer-polymer ABS display adequate selectivity to separate proteins, which may be increased by the appropriate choice of the IL chemical structure and content. For instance, to separate Cyt C from a mixture with IgG and BSA, all of the ILs employed as adjuvants allow a good selectivity, ranging from 2.3 (selectivity between Cyt C and BSA in ABS composed of 5 wt% $[P_{4444}]Cl$) to 21.7 (selectivity between Cyt C and IgG in ABS composed of 5 wt% $[P_{4444}]Cl$). Compared with the ABS without IL, there is selectivity improvement using 5 wt% of $[C_4mpyr]Cl$, $[N_{4444}]Cl$, and $[P_{4444}]Cl$. The higher selectivity values are highlighted in bold in Table S13 in the Supporting Information. When analyzing the ABS selectivity for mixtures comprising BSA and IgG, selectivity values ranging from 0.2 (selectivity between BSA and IgG in ABS composed of 5 wt% $[P_{4444}]Cl$) and 1.8 (selectivity between BSA and IgG in ABS composed of 5 wt% $[C_4mim]Cl$) are obtained. Compared with the ABS without IL, there is an increase in the selectivity using 5 wt% of $[C_4mpyr]Cl$ and $[C_4mpip]Cl$. Therefore, and in general, good selectivity was observed with the studied polymer-polymer ABS, which is even improved by using some ILs as adjuvants, thus opening the door to these ABS application in liquid-liquid chromatography under continuous mode.

Comparing the selectivity values of polymer-polymer ABS with those afforded by polymer-salt ABS composed of PEG 400 and $(NH_4)_2SO_4$ in the separation of the same pairs of proteins there is a high difference, with the first type of ABS showing a better performance. Selectivity values for polymer-salt ABS with and without ILs as adjuvants are provided in Fig. S7 and Table S14 in the Supporting Information. All values are ≥ 1. Since both BSA and IgG are completely extracted to the PEG-rich phase (top-phase), these proteins cannot be separated using polymer-salt ABS and ILs as adjuvants. As for these proteins and Cyt C, they present a value close to 0 since Cyt C partition in a similar way to both phases.

Polymer-polymer ABS with 5 wt% of the two ILs with extreme hydrogen-bond acidity ($[C_4mim]Cl$ and $[N_{4444}]Cl$; Table 1) were investigated in the separation of each protein in presence of the remaining proteins, *i.e.* from the three proteins pool. This set of results allows to confirm how the investigated ABS behave when dealing with real and more complex matrices. The K_{Prot} of each protein in the assays with the single protein and the proteins mixture is provided in Fig. 6 (respective data given in Table S15 in the Supporting information, as well as the

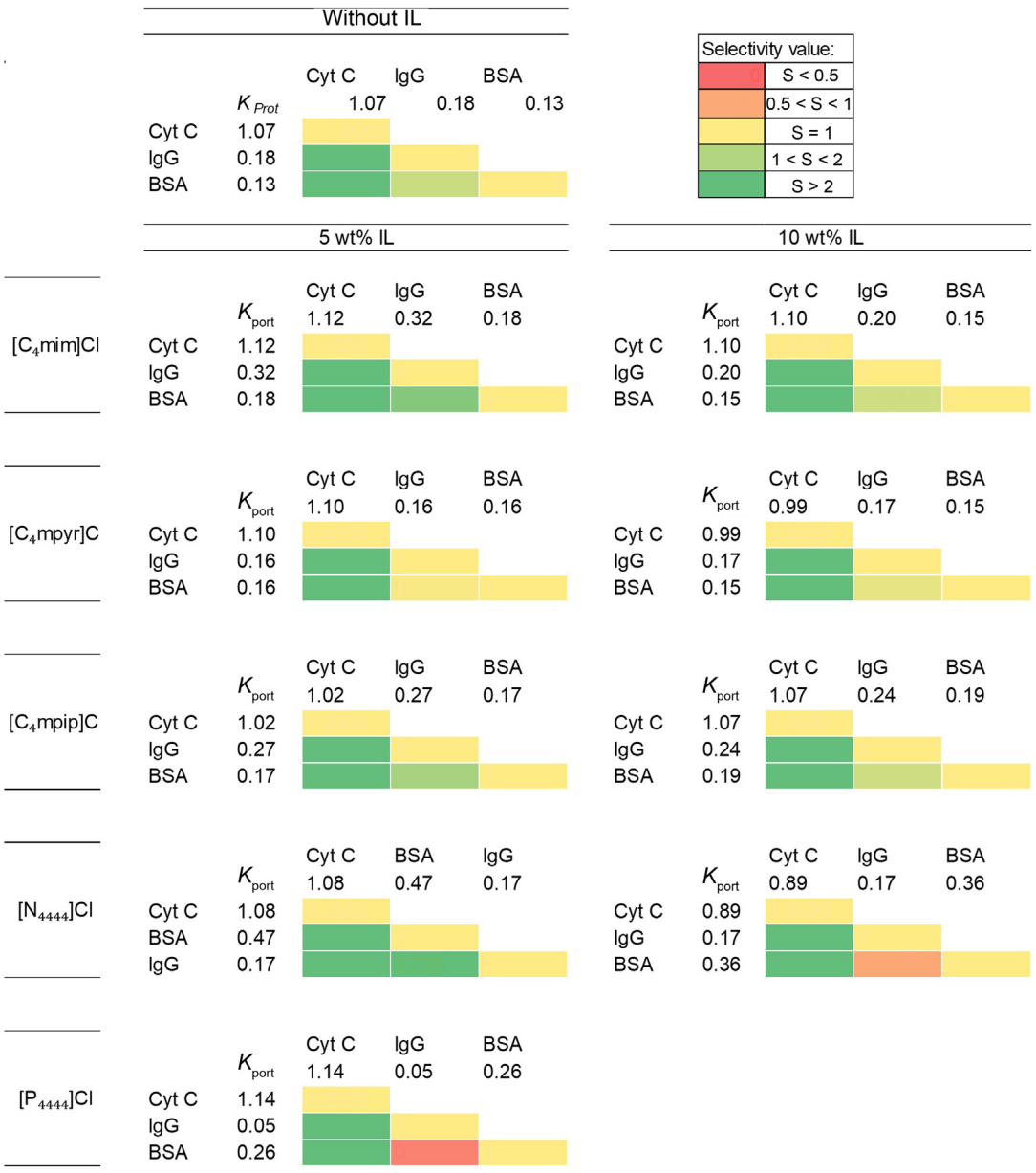


Fig. 5. Selectivity of proteins in ABS composed of composed of 5 wt% PEG 6000 + 10 wt% dextran +5 or 10 wt% IL + 80 or 75 wt% protein aqueous solution.

results of extraction efficiency – Table S16 and Fig. S8).

Cyt C continues to present a K_{Prot} close to 1. In ABS without IL and when using $[N_{4444}]Cl$ a slight increase in K_{Prot} is observed. BSA and IgG continue to prefer the bottom phase (dextran-rich phase). Without the use of IL, the K_{Prot} of both proteins shows a slight decrease, while using 5 wt% of $[C_4mim]Cl$ allows an increase in K_{Prot}. Using $[N_{4444}]Cl$ allows an increase in K_{Prot} of IgG and a decrease in K_{Prot} of BSA. By using a mixture of proteins, the complexity of ABS increases. In this assay, it is possible to verify that this affects slightly the protein partitioning. However, no significant differences in the K_{Prot} values in ABS with individual proteins and the mixture of the proteins were observed, meaning that protein-protein interactions do not play a major role ruling their partitioning, at least at the studied conditions.

Finally, the integrity of the proteins after the extraction step was evaluated. BSA and IgG were quantified by SE-HPLC, in which the retention time of each protein allows to conclude on their fragmentation or formation of protein aggregates [56]. Examples of chromatograms obtained for BSA and IgG are displayed in the Supporting Information (Figs. S9–S12). The chromatograms of the different phases in ABS with 5 wt% of IL with IgG and BSA show the respective peaks (retention time 12.6 and 14.0 min for IgG and BSA, respectively), with insignificant variations observed. These results indicate that IgG and BSA do not form aggregates or suffer fragmentation in a significant extent when using ILs as adjuvants in the studied systems. The effect of the phase-forming components over Cyt C after the extraction step was evaluated by its biological activity, *i.e.*, by determining its capacity to catalyse the oxidation of ABTS into $ABTS^{+}$ radical. The results of relative enzyme activity are presented in the Supporting Information - Fig. S13. The biological activity of Cyt C presents significant differences among the 5 ILs used. The usage of $[C_4mim]Cl$ in both concentrations improves the enzymatic activity in the PEG-rich phase (top-phase) while maintaining its activity in the dextran-rich phase (bottom-phase). On the other hand,

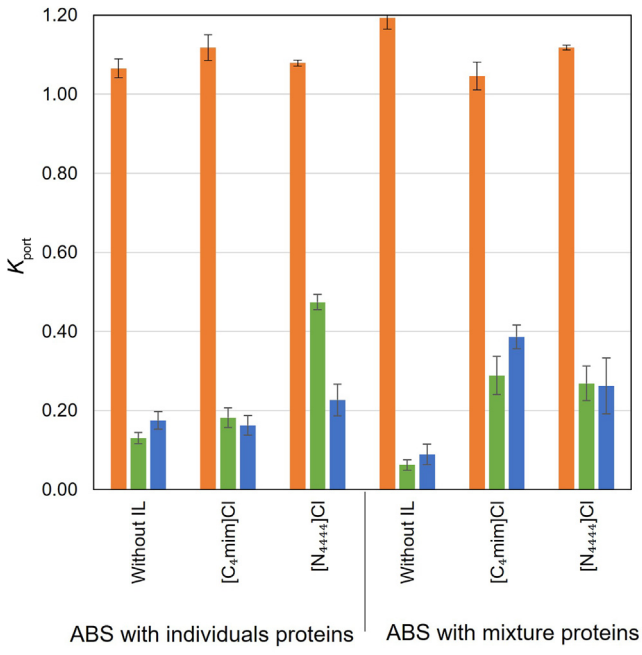


Fig. 6. Partition coefficient of each protein (K_{Prot}) in ABS composed of 5 wt% PEG 6000 + 10 wt% dextran + 5 wt% IL + 80 wt% protein aqueous solution (individual protein and mixture of the three proteins): Cyt C (■); BSA (■); IgG (■).

the use of [C_4mpyr]Cl and [C_4mpip]Cl lead to a better enzymatic activity in the dextran-rich phase when compared to the ABS without IL. However, for the PEG-rich phase, a decrease of about 50% in the enzymatic activity was observed. The same behaviour was observed for ABS comprising [N_{4444}]Cl, where the activity decreases in both phases, while for [P_{4444}]Cl a relevant activity decrease occurs. According to these results, the use of [P_{4444}]Cl and [N_{4444}]Cl is not appropriate to design processes including Cyt C since its biological activity is mandatory. However, the use of [C_4mim]Cl is an excellent option to increase the enzymatic activity of Cyt C.

4. Conclusions

The use of chloride-based ILs as adjuvants to tailor the partitioning of proteins in polymer-polymer-based ABS was investigated in this work. The respective liquid-liquid phase diagrams were determined at 298 K, and their efficiency to separate proteins was determined at given mixture composition. In PEG-dextran ABS, chloride-based ILs as adjuvants allow to increase or decrease the biphasic region, depending on the IL chemical structure and IL content. The higher the hydrogen-bond acidity of a given IL and thus its ability to interact with water, the higher is the amount of PEG or dextran required to create two-phase systems.

In the PEG-dextran ABS, the partition coefficients of IgG and BSA are less than 1, showing that these proteins have preference towards the dextran-rich phase, whereas Cyt C presents a partition coefficient close to 1 and showing no preferential partitioning for a given phase. Nevertheless, it was found that the addition of different ILs in polymer-polymer ABS allow to modulate the proteins partition coefficients and thus tailor their partitioning between the phases, unlike polymer-salt ABS. Selectivity data provided for the two type of systems confirm this behaviour. Experimental data combined with molecular docking studies showed that BSA partition majorly depends on the IL content in each phase, whereas Cyt C and IgG partition are majorly ruled by the IL chemical structure and established interactions. When using a mixture of proteins, no significant changes in the partition coefficients occur, reinforcing that protein-protein interactions do not rule their partition at the studied conditions.

Overall, higher selectivity is obtained when using ILs as adjuvants in polymer-polymer ABS when compared with the polymer-salt counterparts, paving the way for their use in the separation of proteins by liquid-liquid chromatography.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gce.2021.11.004.

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