Overview on Protein Extraction and Purification Using Ionic-Liquid-Based Processes

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Abstract

Proteins are one the most widely studied biomolecules with diverse functions and applications. Aiming at overcoming the current drawbacks of purification processes of proteins, the introduction of ionic liquids (ILs) has been a hot topic of research. ILs have been applied in the creation of aqueous biphasic systems (IL-based ABS), solid-phase extractions through poly(ionic liquid)s (PILs) and supported ionic-liquid phases (SILPs), and in the crystallization of proteins. In this sense, ILs have emerged as solvents, electrolytes or adjuvants, or as supported materials to tune the adsorption/affinity capacity aiming at developing an efficient, cost-effective, sustainable and green IL-based process for protein extraction. This review discusses different IL-based processes in the extraction and purification of proteins in the past years, namely IL-based aqueous biphasic systems (IL-based ABS), solid-phase extractions through PILs and SILPs, and protein crystallization. The type and structure of ILs applied and their influence in the different processes performance are also discussed.

Keywords Ionic liquids · Aqueous biphasic systems · Solid-phase extractions · Poly(ionic liquid)s · Supported ionic liquid phases · Crystallization · Extraction · Purification · Proteins

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1 Introduction

Proteins are produced in living organisms to perform diverse functions, such as gene expression, signal transduction and metabolism [1]. Due to their relevant roles, proteins are widely applied in the textile, cosmetic, food and pharmaceutical industries [2]. However, the three-dimensional structure of proteins constituted by a proper folding of polypeptide chains, stabilized by non-covalent bonding with merely 21–84 kJ·mol^{-1} (5–20 kcal·mol^{-1}), reveals the fragile structural stability of proteins. Therefore, protein unfolding/denaturation often occurs during extraction and purification processes [3]. Additionally, harsh conditions and toxic volatile organic solvents that may be employed to extract, recover or purify proteins, commonly lead to the loss of biological activity. On the other hand, extraction and separation processes of proteins are usually multi-step and complex, increasing the cost of the final protein product [4]. To overcome these drawbacks, the development of cost-effective techniques, while employing "greener" and more sustainable solvents, for proteins extraction, purification and recovery is of high relevance. In this context, ionic liquids (ILs) have gained increased popularity due to their unique properties.

ILs are typically composed of a large and unsymmetrical organic cation and an organic or inorganic anion. By general definition, ILs are molten salts at temperatures below 100 °C, and may be designed to display excellent chemical, thermal and electrochemical stabilities, nonflammability, and negligible volatility [4, 5]. Moreover, ILs have been recognized as designer solvents due to the large number of ion combinations and the possibility of designing task-specific fluids, overcoming the limited selectivity of common volatile organic solvents [4]. ILs are also regularly recognized by their extraordinary solvation ability for a wide range of compounds and materials, and as good stabilizing media for proteins [6]. Taking into account all of these features, several works describing the use of ILs as alternative platforms for proteins extraction and purification have been reported.

This review summarizes the use of ILs in the extraction and purification of proteins. Different IL-based processes are herein overviewed, namely IL-based aqueous biphasic systems (IL-based ABS), solid-phase extractions through poly(ionic liquid)s (PILs) and supported ionic liquid phases (SILPs), and protein crystallization achieved using ILs. The type and structure of ILs applied and their performance, and influence in the different processes are discussed.
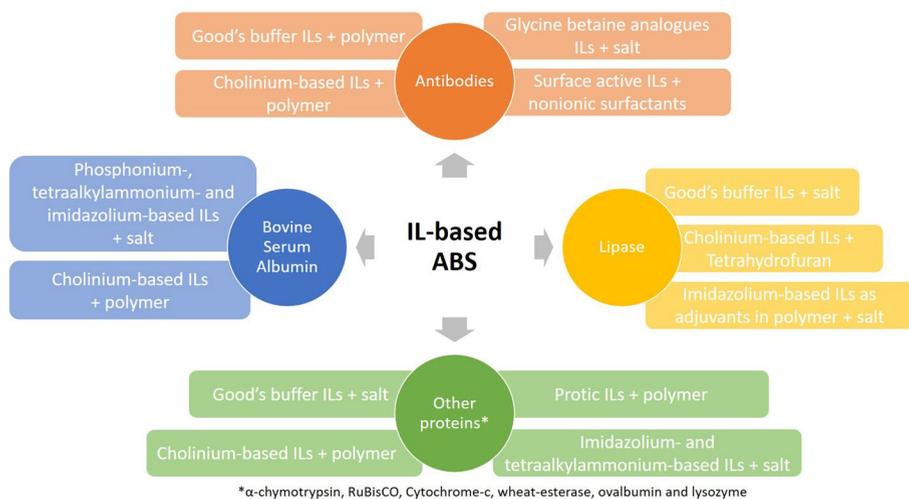
2 Extraction and Purification of Proteins Using Ionic Liquids

2.1 Ionic-Liquid-Based Aqueous Biphasic Systems

Liquid–liquid extraction (LLE) involves a classic ternary system which separates solutes from one solution into another, based on their solubility in both solutions [7]. IL-based ABS fall within LLE and are formed by the addition of salts, polymers, carbohydrates, or amino acids to IL aqueous solutions [8], corresponding at least to ternary systems. ABS are mainly composed of water and offer many relevant advantages when dealing with proteins, such as high extraction efficiency, high selectivity, short equilibration time, mild operating conditions, and a biocompatible environment, if properly designed [4, 8]. Due to these advantages, and since ILs lead to high extraction performances and selectivity, IL-based ABS have been extensively studied, as described in several reviews articles [3, 4, 8–10].

Thus, in this section, only research articles from the last five years regarding IL-based ABS for the extraction and purification of proteins are presented and discussed. These are summarized in Fig. 1, while Table 1 summarizes the main results of each work. Additionally, the most relevant works from 2000 to 2015 are summarized in Table 1. The influence of the IL's structure and concentration, salt, pH and temperature are the main parameters studied in the extraction and purification of proteins, such as bovine serum albumin (BSA), lipase, cytochrome C (cyt-c) and immunoglobulin G (IgG), among others.

BSA is one of most studied model proteins used to prove the extraction efficiency of IL-based ABS. Pereira et al. [11] reported the extraction of BSA using ABS composed of tetraalkylphosphonium- or tetraalkylammonium-based ILs, combined with a buffered aqueous solution of potassium citrate/citric acid (pH 7.0). The obtained results reveal that, with the exception of ABS composed of more hydrophobic ILs, the systems investigated allow the complete extraction of BSA to the IL-rich phase in a single-step [11]. Hydrophobic ILs led to the precipitation and/or denaturation of BSA at the ABS interface, which may be due to changes in the protein structure from specific interactions occurring between the protein and the IL [11]. The composition of the biphasic systems, namely the amount of phase-forming components, was also investigated, with the extraction efficiencies of BSA being maintained at 100% up to high protein concentrations (at least up to 10 kg·m^{-3}) [11]. For the best identified ABS, the preservation of the protein native conformation after recovery from the IL-rich phase was confirmed by size exclusion high-performance liquid chromatography (SE-HPLC) and by Fourier transform infra-red spectroscopy (FT-IR) [11]. IL-based ABS were also used to separate BSA and saccharides, in a work carried out by Wang et al. [12]. Novel ether-functionalized ILs (imidazolium-based) combined with K_2HPO_4 were applied to create ABS, and the influence of the IL's chemical structure, amount of salt, and effect of different saccharides (glucose, sucrose and dextran) was investigated [12]. The results showed that 76.1–94.3% of BSA was enriched into the IL-rich phase whereas almost all the saccharides were extracted into the salt-rich phase in a one-step separation process. Moreover, it was found that the amount of salt and the nature of

*α-chymotrypsin, RuBisCO, Cytochrome-c, wheat-esterase, ovalbumin and lysozyme

Fig. 1 Overview on the ionic-liquid-based aqueous biphasic systems (IL-based ABS) applied in the extraction and purification of different proteins

Table 1 Summary of ionic-liquid-based aqueous biphasic systems (IL-based ABS) applied in protein extraction, studied proteins and main results obtained

IL	Other ABS phase forming compounds	Proteins	Main results	Ref
Selected relevant reports from 2000 to 2015				
1-Butyl-3-methylimidazolium chloride	K_2HPO_4	Proteins from human body fluids	The target proteins were mainly concentrated in the IL-rich phase, while most contaminant proteins remained in the salt-rich layer By increasing the amount of inorganic salt, the authors observed an increase in the extraction efficiency (90% to 100%) of BSA [structural homologue of human serum albumin (HSA)] to the IL-rich phase A maximum enrichment factor of 20 (attained by a second phase separation) was also observed	[35]
Ammonium cation + chloride anion (Ammoeng™110)	K_2HPO_4/ KH_2PO_4 buffer	Lysozyme Myoglobin BSA Hemoglobin	A combination of different interactions between the proteins and the IL were found Among these, the protein's charge as well as the molecular weight are of major importance	[36]
Imidazolium cations + bromide anion	K_2HPO_4	BSA Trypsin Cyt-c Ɣ-globulins	75–100% of the proteins could be extracted into the IL-rich phase in a single-step extraction Extraction efficiency of cyt-c slightly changed with the increase of pH values Extraction efficiencies of proteins were found to increase with increasing temperature and increasing alkyl chain length of IL cations	[37]

Table 1 (continued)

IL	Other ABS phase forming compounds	Proteins	Main results	Ref
1-Butyl-3-methylimidazolium dicyanamide	K_2HPO_4 K_3PO_4	BSA saccharides	82.7%–100% of BSA migrates into the IL–rich phase Almost quantitative saccharides (arabinose, glucose, sucrose, raffinose or dextran) were preferentially extracted into the bottom-rich phase in a single-step extraction The extraction efficiency of BSA from the aqueous saccharide solutions was influenced by the molecular structure of saccharides	[38]
Imidazolium cations + bromide anion N-ethyl-N-butylmorpholinium bromide and N,N,N',N'-tetramethylbutylguanidinium bromide	NaH_2PO_4 K_2HPO_4	Lysozyme BSA Hemoglobin Trypsin	Proteins migrate to the IL-rich phase As the pH value of the phosphate solution was close to the isoelectric point, the protein carried the minimal charge and was transferred from the phosphate-rich phase into the IL-rich phase by hydrophobic interaction between the exposed amino residues and the imidazole ring The endothermic process was controlled by the entropy change. Undoubtedly, while the transfer process was determined by hydrophobic interaction, the salting out effect and electrostatic interaction also played important roles in the intrinsic factors that were related to the extraction efficiency	[39]

Table 1 (continued)

IL	Other ABS phase forming compounds	Proteins	Main results	Ref
Reports from 2015				
Tetrabutylammonium, tetrabutylphosphonium and cholinium cations + Good's buffers anions (MOPSO, BES, TAPSO and CAPSO)	$K_3C_6H_5O_7$	Lipase from *Pseudomonas cepacia*	Lipase preferentially partition to the GB IL–rich phase Partition coefficients between 29.3 ± 1.9 and 119.5 ± 7.0 Recovery yields ranges from 95.7 ± 0.3 (%) to 99.2 ± 0.0 (%)	[23]
Cholinium-based cations + chloride, bitartrate and dihydrogencitrate anions	Tetrahydrofuran	Lipase from *Bacillus* sp. ITP-001	Purification factor of 130.1 ± 11.7 fold Lipase yield of $90.0 \pm 0.7\%$ Partition coefficient of enzyme for IL-rich phase of 0.11 ± 0.01 Partition coefficient of contaminant proteins for THF-rich phase of 1.16 ± 0.1	[25]
Tetrabutylammonium, tetrabutylphosphonium and cholinium cations + Good's buffer anions (MOPSO, BES and TAPSO)	K_3PO_4, K_2CO_3, and $(NH_4)_2SO_4$ PEG, PPG, and PEG-PPG copolymers	Lipase from *Burkholderia cepacia*	Lipase preferentially partition towards the GB IL–rich phase purification factor of 22.4 ± 0.7 Recovery yield of $94.0 \pm 0.2\%$	[24]
Imidazolium cations + chloride anion (ILs as adjuvants in polyethylene glycol-based ABS)	K_2HPO_4/ KH_2PO_4 buffer pH 7	Lipase from *Bacillus* sp. ITP-001	Purification factor of 245	[26]
Cholinium cation + Good's buffers anions (MES, Tricine, CHES, TES, CHES)	PPG 400	IgY from egg yolk	Extraction efficiencies, of the water-soluble fraction of proteins, ranging between 79 and 94%	[28]
Imidazolium cation + tetrafluoroborate anion	NaH_2PO_4 K_2HPO_4 $(NH_4)_2SO_4$ $MgSO_4$	Wheat-esterase	Wheat esterase preferentially partition into the IL-rich phase Wheat esterase was purified with a yield of 88.93%	[14]

Table 1 (continued)

IL	Other ABS phase forming compounds	Proteins	Main results	Ref
Phosphonium or ammonium cations + chloride, bromide, tosylate and methylsulfate anions	$K_3C_6H_5O_7/C_6H_8O_7$ buffer pH 7	BSA	Except for the more hydrophobic ILs, most of the systems allow the complete extraction of BSA for the IL-rich phase in a single-step. Extraction efficiencies of BSA are maintained at 100% up to high protein concentrations (at least up to 10 g·L⁻¹). The preservation of the protein native conformation was confirmed after recovery from the IL-rich phase	[11]
Cholinium cation + chloride, dihydrogen citrate, bitartarate, acetate, dihydrogen phosphate, propanoate, glycolate, butanoate, lactate anions	PPG 400	BSA	Extractions efficiencies of BSA ranging between 92 and 100% for the IL-rich phase were obtained in a single step. The stability of BSA at the IL-rich phase was ascertained	[18]
Cholinium cations + chloride and Good's buffers anions (HEPES, Tricine, CHES, MES, TES)	PPG 400	BSA	BSA preferentially migrates for the GB IL-rich phase, with extraction efficiencies of 100%. The thermal stability of BSA in aqueous solutions of cholinium-based GB ILs was shown to be greater than in the other aqueous solutions of the remaining phase-forming components. After extraction, BSA is stable and keeps its α-helical secondary structure in all studied systems	[20]

Table 1 (continued)

IL	Other ABS phase forming compounds	Proteins	Main results	Ref
Cholinium cation + β-alanine, serine L-lysine and glycine anions	PPG 400	BSA Trypsin	The partition behavior of BSA was governed by the hydrophobicity of the anion When the pH of the system is greater than the isoelectric point of the model proteins and the anion, the model proteins are found to be mainly partitioned into the bottom phase At a pH below the isoelectric pint of the model proteins and the isoelectric point of the anion, the partitioning of model proteins favored the polymer-rich top phase	[19]
Imidazolium cations + chloride anion	K_3PO_4 K_2HPO_4	BSA and saccharides mixture	76.1 – 94.3% of BSA was enriched into the IL-rich top phase and almost all the saccharides were extracted into the salt-rich bottom phase	[12]
Imidazolium, tetraalkylammonium and cholinium cations + chloride, dimethyl-phosphate, methanesulfonate, tosylate, acetate, triflate, dicyanamide and bromide anions (ILs as electrolytes in polyethylene glycol-based ABS)	Sodium poly(acrylate), NaPA	Cyt-c	The extraction efficiencies show that cyt-c is recovered in the NaPA-rich phase ($EE_{cyt-c} > 96.13 \pm 3.22\%$)	[16]
Ammonium cations + chloride, carboxylic acid anions	PPG 400	Cyt-c Azocasein	The two proteins almost completely migrate to the IL-rich phase in a single-step	[33]
Tetrabutylphosphonium cation + Good's buffers anions (TAPS, MOPS, EPPS, CAPS, and BICINE)	Na_2SO_4	α-chymotrypsin	α-chymotrypsin extraction efficiency of 100% was observed	[22]

Table 1 (continued)

IL	Other ABS phase forming compounds	Proteins	Main results	Ref
Tetraalkylammonium cation + Good's buffers anions (TAPS, MOPS, EPPS, CAPS, BICINE)	Na_2SO_4	α-chymotrypsin	GB–ILs provide a greater stabilizing effect on the activity of α chymotrypsin GB–ILs completely extract enzyme from the aqueous solution	[21]
1-Butyl-3-methylimidazolium acetate (polyethylene glycol as adjuvant)	K_2HPO_4	α-amylase	It was observed that polyethylene glycol increased the partition coefficients	[17]
1-Butyl-3-methylimidazolium bromide	K_2HPO_4	BSA and mannan mixture	Extraction efficiency of BSA ranged from 92 to 97% Extraction efficiency of mannan reached values from 95% to about 100%	[13]
Cholinium cation + bicarbonate D-(+)-galacturonic acid, chloride and acetate anions	PPG 400	Commercial IgG IgG from rabbit serum	The complete extraction of IgG to the IL–rich phase was achieved in a single-step With pure IgG a recovery yield of 100% was obtained while with rabbit serum this value slightly decreased to ca. 85% A 58% enhancement in the IgG purity was achieved when compared with its purity in serum samples IgG retained its native structure, without degradation or denaturation effects after extraction	[29]

Table 1 (continued)

IL	Other ABS phase forming compounds	Proteins	Main results	Ref
Cholinium cation + chloride, bitartrate, dihydrogen citrate, dihydrogen phosphate, acetate, lactate, glycolate, propanoate, butanoate, caffeate, syringate, vanillate and gallate anions	PPG 400	Commercial IgG IgG from rabbit serum	Extraction efficiencies ranging between 93 and 100%, and recovery yields ranging between 20 and 100%, were obtained for commercial IgG. Extraction efficiencies of 100% and recovery yields > 80% were obtained IgG from rabbit serum. IgG with a purity level of 49% was obtained in a single-step. After an ultrafiltration step applied to the best ABS, the I IgG purity level increased to 66%	[30]
1-Methyl-3-tetradecylimidazolium chloride Tributyltetradecylphosphonium chloride Benzyldodecyldimethylammonium bromide Cholinium tetradecanoate	Triton X-114 Tergitol 15-S-7	IgG Human serum albumin	The AMTPS at pH 8.0 improved the simultaneous separation of both proteins to the opposite phases. IgG purification of 1.14-fold in the surfactant-poor phase and HSA purification of 1.36-fold in the surfactant-rich phase were obtained	[32]
Triethyl[(4-ethoxy-4-oxobutyl]ammonium bromide Tri(n-propyl)[4-ethoxy-4-oxobutyl] ammonium bromide Tri(n-butyl)[(4-ethoxy-4-oxobutyl]ammonium bromide N-(1-methylpyrrolidyl-4-ethoxy-4-oxobutyl)ammonium bromide Tetra(nbutyl)ammonium bromide 1-Butyl-3-methylimidazolium bromide	K_2HPO_4/KH_2PO_4 buffer pH 7	Anti-interleukin-8 monoclonal antibodies (mAbs)	mAbs preferentially partition to the IL-rich phase, with recovery yields up to 100% and purification factors up to 1.6. A precipitate enriched in mAbs is obtained at the ABS interface, yielding 41.0% of IgG with a purification factor of 2.7 (purity of 60.9%). The best results were obtained with the hybrid process combining three-phase partitioning and ultrafiltration, allowing to obtain mAbs with a purity greater than 60%	[31]

Table 1 (continued)

IL	Other ABS phase forming compounds	Proteins	Main results	Ref
Imidazolium cations + chloride, acetate, dicyanamide, triflate anions	K_3PO_4 K_2HPO_4/KH_2PO_4 buffer pH 7	Polysaccharides and Isochrysis galbana proteins mixtures	The system with the best results obtained an extraction efficiency for the carbohydrates of $71.21 \pm 5.21\%$ to the salt-rich phase, and an extraction efficiency for the proteins of 100% to the IL-rich phase	[15]
Iolilyte 221PG Cholinium dihydrogen phosphate	PEG 400 $K_3C_6H_5O_7$	RuBisCO	Extraction efficiencies between 80 and 100% were obtained. Iolilyte 221PG–$K_3C_6H_5O_7$, was found to be the most efficient ABS in RuBisCO separation	[27]
Tetraalkylammonium cations + bromide and chloride anions	K_3PO_4 K_2HPO_4/KH_2PO_4 buffers	Ovalbumin Lysozyme	At pH 7, the complete extraction and recovery of lysozyme to the IL-rich phase are achieved in all systems. Low recovery yields of ovalbumin are obtained with ABS formed by ILs with longer alkyl side chains. The proteins recovery from the IL-rich phase by ice cold ethanol precipitation, where up to 99%	[34]

the saccharide are the main factors affecting the separation process [12]. Čížová et al. [13] reported the effective separation of mannan–BSA mixtures using ABS formed by 1-butyl-3-methylimidazolium bromide combined with K_2HPO_4. The influences of the salt amount and protein concentration on the extraction efficiency of mannan and BSA were explored [13]. The extraction efficiency of BSA ranged from 92 to 97%, while the extraction efficiency of mannan reached values from 95 to close 100%, depending on phase and/or model sample composition [13]. Additionally, the authors compared the results obtained with an extensively used method (lectin affinity purification with concanavalin A–triazine bead cellulose), which displayed a lower efficiency of BSA removal (73–84%) [13].

Besides BSA, other proteins have been extracted using imidazolium-based ILs. Jiang et al. [14] evaluated the extraction and purification of wheat esterase by applying IL-based ABS composed of 1-butyl-3-methylimidazolium tetrafluoroborate and different salts (NaH_2PO_4, K_2HPO_4, $(NH_4)_2SO_4$ and $MgSO_4$). The effect of IL concentration, salts, pH and temperature on wheat esterase partitioning was studied [14]. The obtained data indicated that wheat esterase preferentially migrates into the IL-rich phase; however, increasing the salt concentration and pH may result in recovery of the enzyme but with a lower activity. Under the optimum conditions {ABS composed of 20 wt% of IL and 25 wt% of NaH_2PO_4 (pH 4.8)), wheat esterase was purified by 4.23-fold with a yield of 88.93% [14].

Santos et al. [15] also proposed an efficient purification process by applying IL-based ABS composed of imidazolium-based ILs and inorganic salts to separate proteins from arabinose-rich and glucose-rich polysaccharides. For this purpose, the nature of the inorganic salt (K_3PO_4, K_2HPO_4 and K_2HPO_4/ KH_2PO_4 buffer), the IL structural features (anion and alkyl chain length) and the extraction mixture point (using different K_3PO_4 concentrations) were the conditions optimized, allowing an extraction efficiency of polysaccharides of 71.2% to the salt-rich phase and an extraction efficiency of proteins of 100% to the IL-rich phase [15]. The authors explained the migration of polysaccharides to the salt-rich phase as due to preferential interactions between the polysaccharide's–OH groups and the more hydrophilic phase, while proteins preferentially migrate to the IL-rich phase because of the salting-out nature of the inorganic salt used and by the π–π interactions occurring between the IL cation aromatic ring and the protein's aromatic amino acids [15]. Moreover, it was found that the extraction efficiencies are dependent on the hydrophobic/hydrophilic nature of the IL anions, with distinct interactions taking place between the proteins, polysaccharides and both phases' phase-forming components [15]. In addition to the use of ILs as one of the main phase-forming components in ABS, Santos et al. [16] applied imidazolium- and ammonium-based ILs as a new class of electrolytes in ABS formed by polyethylene glycol (PEG) and sodium poly(acrylate) (NaPA). Most ILs preferentially migrate towards the NaPA-rich phase, likely due to the strong interactions between the IL's cation with the negatively charged moieties of the polymer (NaPA is negatively charged at neutral pH, due to the presence of carboxylic groups in the main chain) [16]. This parameter is important as the ILs may interact with the target molecule, namely cyt-c, affecting the separation performance. The main results on the extraction efficiencies show that cyt-c is recovered in the NaPA-rich phase, the phase in which the IL is enriched [16]. Contrarily, Vahidnia et al. [17] developed IL-based ABS with PEG as adjuvants for the separation α-amylase. Different PEGs and polymer concentrations in ABS formed by 1-butyl-3-methylimidazolium acetate and K_2HPO_4 were investigated. The data obtained reveal that PEG preferentially migrates to the IL-rich phase and the addition of small amounts of polymer improved the partitioning coefficient of α-amylase [17].

More biocompatible ABS than those formed by ILs with imidazolium cations, have also been investigated for the extraction and purification of proteins, namely ABS formed by

cholinium-based ILs combined with poly(propylene glycol) (PPG) proposed by Quental et al. [18], Song et al. [19] and Taha et al. [20]. Taha et al. [20] showed the preferential migration of BSA to the IL-rich phase, with extraction efficiencies of 100% achieved in a single-step. Furthermore, the authors demonstrated that the BSA partition to the IL-rich phase is ruled by the protein interactions with the surrounding media/molecules through hydrogen-bonding, electrostatic interactions and dispersive forces. After the extraction with the investigated ABS, the absence of changes in the BSA conformation was confirmed through circular dichroism (CD) and the protein's secondary structure was maintained [20]. Quental et al. [18] also reported high BSA extraction efficiencies to the IL-rich phase, varying between 92 and 100%. The authors reported that the BSA partition does not depend on the hydrophilicity of the IL-rich phase provided by the water content or by the IL anion [18]. On the other hand, Song et al. [19] suggested that the BSA partition is governed by the hydrophobicity of the IL anion, whereby the BSA showed a greater affinity toward the IL-rich phase in systems formed by ILs comprising a less hydrophobic anion [19]. Furthermore, the effect of pH on the BSA partition was investigated, it being found that BSA mainly migrates to the IL-rich phase at pH values higher than the protein's isoelectric point (pI) [19]. In contrast, in assays at a pH below the pI of the protein and the pI of the IL anion, BSA preferentially partitions to the polymer-rich phase [19].

Foreseeing the development of additional biocompatible IL-based ABS for the extraction of proteins, several authors resorted to ILs composed of anions derived from Good´s biological buffers (GB), i.e. self-buffering ILs. Gupta et al. [21, 22] studied the extraction of α-chymotrypsin from bovine pancreas using ABS composed of sodium sulfate and Good's buffer ionic liquids (GB-ILs), synthesized by the combination of GB anions (TAPS, [tris(hydroxymethyl)methylamino]propanesulfonic acid; MOPS, 3-(*N*-morpholino) propanesulfonic acid; EPPS, 3-[4-(2-hydroxyethyl)piperazin-1-yl]propane-1-sulfonic acid; CAPS, *N*-cyclohexyl-3-aminopropanesulfonic acid; and BICINE, *N*,*N*-bis(2-hydroxyethyl) glycine) and tetrabutylammonium, tetraethylammonium, tetramethylammonium and tetrabutylphosphonium cations. Both works indicated that GB ILs are able to maintain the required pH of the medium, while allowing the complete extraction of α-chymotrypsin to the IL-rich phase with enhanced activity [21, 22]. Ying et al. [23], using similar ILs, reported the extraction of lipase from *Pseudomonas cepacia* using ABS composed of potassium citrate and GB ILs synthesized by the combination of GB anions (MOPSO, 2-hydroxy-3-morpholinopropanesulfonic acid; BES, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; TAPSO, 2-hydroxy-3-[tris(hydroxymethyl)methylamino]-1-propanesulfonic acid; and CAPSO, 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid) and tetrabutylammonium, tetrabutylphosphonium, and cholinium cations [23]. First, the lipase activity and stability in aqueous solutions of these GB ILs were evaluated, showing the advantages of GB ILs as media for enzymatic reactions when compared to conventional phosphate buffers [23]. Additionally, ABS comprising these GB ILs were investigated, showing them to be highly effective and selective for the partitioning of lipase into the GB IL-rich phase [23]. The authors described the protein partitioning as a surface-dependent phenomenon that is mainly due to hydrophobic interactions established between the proteins and the hydrophobic groups of GB ILs [23].

Lee et al. [24] applied ABS composed of GB ILs comprising tetrabutylammonium, tetrabutylphosphonium and cholinium cations paired with GB anions (MOPSO, BES and TAPSO) for the extraction and purification of lipase produced via submerged fermentation by *Burkholderia cepacian* ST8. The effect of different salts (K_3PO_4, K_2CO_3, and (NH_4)_2SO_4), polymers (PEG, PPG and PEG–PPG copolymers) on the partition of a commercial *P. cepacia* lipase was firstly addressed [24]. For the ABS formed by

tetrabutylammonium- or tetrabutylphosphonium-based GB ILs and ammonium sulfate, lipase is completely concentrated in the GB IL-rich phase. However, the lipase partition was not only predominantly driven by hydrophobic interactions or salting-out effects, which was proven by the preferential migration of lipase to the bottom hydrophilic GB IL-rich phase for systems composed of PPG 400 and cholinium-based GB ILs [24]. Considering the results obtained for the lipase partition, the ABS composed of [tetrabutylammonium][BES] and ammonium sulfate were selected to be applied for the purification of lipase produced via fermentation [24]. Different phase compositions with similar tie-line length (TLL) and at fixed pH (ca. 7.0) were studied, with the results revealing that when the protein solvation capacity of the GB IL-rich phase was restricted by its concentration/volume, the respective system demonstrates high selectivity to lipase thus increasing its purity [24]. Lipase has also been extracted using ABS formed by cholinium-based ILs and tetrahydrofuran (THF) [25], aiming the purification of lipase from *Bacillus* sp. ITP-001 produced by submerged fermentation. The partition of lipase from *B. cepacia* (commercially obtained) was firstly studied, with the results suggesting a preferential migration of lipase to the IL-rich phase, driven by the hydrophobicity of the ILs' anions [25]. The concentration of THF and the influence of temperature on the lipase extraction were then investigated. It was observed that 40% of THF is the limiting concentration at which the best activity recovery of lipase in the THF-rich phase is attained, with intermolecular interactions between THF and water controling the enzyme partition. On the other hand, an increase in temperature slightly favors the migration of lipase to the IL-rich phase, until 25 °C [25]. These systems were then evaluated for the separation and purification of lipase from the fermentation broth, revealing a high performance in the purification of the lipolytic lipase with a purification factor increasing from 12.7- to 136.8-fold, comparing the steps of pre-purification (by use of dialysis) with the proposed purification method (using ABS) [25]. The same authors [26] used ABS comprising imidazolium-based ILs as adjuvants for the purification of lipase produced by submerged fermentation from *Bacillus* sp. ITP-001. The initial optimization using ABS composed of PEG, K_2HPO_4/KH_2PO_4 buffer, water and IL (adjuvant) was carried out with the commercial *Candida antarctica* lipase B (CaLB), and the IL effect was studied [26]. The results showed the partition of CaLB and of the contaminant proteins towards opposite phases, with the enzyme being concentrated in the salt-rich phase. These results demonstrated that, although hydrophobic interactions are controlling the impurities partitioning to the polymer-rich phase, also electrostatic interactions, van der Waals forces and hydrogen-bonding play a significant role due to the presence of ILs in this phase [26]. Based on the previous screening, an ABS containing 5 wt% of ILs as adjuvants was chosen as the most promising, allowing an increase in the purification factor of lipase from 175.6 (using the ABS without IL) to 245.0 times (using the ABS with IL) [26].

Beyond the relevant works published on the purification of lipase from complex media, IL-based ABS have been used for the extraction and purification of other proteins, namely D-ribulose 1,5-diphosphate oxygenase/carboxylase (RuBisCO) and antibodies from complex mixtures (Fig. 2). Ruiz et al. [27] proposed biocompatible ABS formed by Iolilyte 221PG-potassium citrate and PEG 400–cholinium dihydrogen phosphate to extract RuBisCO from spinach. In this study, the effect of the TLL, pH and type of phase-forming components on Rubisco extraction efficiency was analyzed. Extraction efficiencies between 80 and 100% were reported, with the Iolilyte 221PG-citrate ABS being the most efficient for RuBisCO separation, this being justified by the higher salting-out potential in this system and the higher hydrophobicity of the IL [27]. Furthermore, it was found that

Fig. 2 Proteins extracted and purified from complex mixtures using ionic-liquid-based aqueous biphasic systems (IL-based ABS)

electrostatic interactions between the IL cation and negatively charged amino acid residues at the surface of RuBisCO positively influence the extraction efficiency [27].

Taha et al. [28] reported the extraction and attempted the purification of immunoglobulin Y (IgY) from chicken egg yolk using ABS composed of cholinium ([Ch]$^+$)-based GB ILs (MES, 2-(*N*-morpholino)ethanesulfonic acid; Tricine, *N*-[Tris(hydroxymethyl)methyl] glycine; TES, 2-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; and CHES, 2-(cyclohexylamino)ethanesulfonic acid, with GBs as anions) combined with PPG 400. For all the investigated systems, a preferential partitioning of proteins into the GB IL-rich phase was observed, with the ABS composed of PPG 400 and [Ch][Tricine] or [Ch][HEPES] leading to the highest extraction efficiencies, above 90%, in a single step [28]. Similar IL-based ABS, composed of cholinium-based ILs and PPG 400, were applied by Mondal et al. [29] and Ramalho et al. [30] for the extraction and purification of immunoglobulin G (IgG) from rabbit serum. Both works described a significant effect of the IL anion on the IgG partitioning and on keeping its native structure, with the best systems achieving IgG purities around 50% and recovery yields higher than 80%, in a single step. Capela et al. [31] also investigated the extraction and purification of IgG, namely anti-interleukin-8 monoclonal antibodies (anti-IL-8 mAbs) from Chinese hamster ovary (CHO) cell culture supernatants, using ABS formed by biocompatible ILs (glycine–betaine analog ionic liquids) and K$_2$HPO$_4$/KH$_2$PO$_4$ buffer at pH 7.0. With the studied ABS, mAbs preferentially partitioned to the IL-rich phase, with recovery yields up to 100% and purification factors up to 1.6 [31]. The best systems were optimized in what concerns the IL concentration, allowing to take advantage of IL-based three-phase partitioning approaches, where a precipitate enriched in mAbs was obtained at the ABS interface, yielding 41.0% of IgG with a purification factor of 2.7 (purity of 60.9%) [31].

Vicente et al. [32] similarly investigated the simultaneous separation of IgG and human serum albumin (HSA) from human expired plasma. Aqueous micellar two-phase systems (AMTPS) composed of nonionic surfactants (Triton X-114 or Tergitol 15-S-7) combined with several surface-active ionic liquids (SAILs) were applied [32]. The mixed AMTPS composed of Tergitol 15-S-7 as the nonionic surfactant and tributyltetradecylphosphonium chloride as the cosurfactant at pH 8.0 improved the simultaneous separation of both proteins to opposite phases, and an IgG purification of 1.14-fold in the surfactant-poor phase

and HSA purification of 1.36-fold in the surfactant-rich phase were obtained [32]. The composition of both phases and the pH influence on the separation of both proteins were evaluated. It was found that the pH was not the driving force on the partitioning of both proteins to different phases in AMTPS. Instead, the separation of the proteins in AMTPS was governed by the phases' hydrophobicity/hydrophilicity, specific interactions established between each protein and the surfactant and/or water, and the protein's molecular weight [32].

Other less common IL-based ABS have been investigated for the extraction of proteins, e.g. thermoreversible IL-based ABS composed of protic ILs in the extraction of cyt-c and azocasein [33]. Passos et al. [33] reported the complete extraction of these two proteins, achieved in a single-step, by the applicability of temperature-induced phase switching. It was demonstrated that these temperature-induced mono(bi)phasic systems are significantly more versatile than classical binary liquid-liquid systems which are constrained by their critical temperatures.

More recently, Belchior et al. [34] reported the use of ABS composed of tetraalkylammonium-based ILs and potassium phosphate solutions at different pH values (pH 7, 8, 9 and 13, using K_2HPO_4/KH_2PO_4 buffer or K_3PO_4) to extract and recover ovalbumin and lysozyme (egg white proteins). At pH 7, the complete extraction and recovery of lysozyme in the IL-rich phase was achieved in all systems; however, low recovery yields of ovalbumin were obtained with ABS formed by ILs with longer alkyl side chains [34]. Furthermore, it was found that an increase in the pH above the proteins pI is deleterious for their recovery in the IL-rich phase [34]. Molecular docking studies were carried out showing that ILs that preferentially establish hydrophobic interactions with these proteins are those that lead to their aggregation and lower recovery yields. Finally, it was shown the proteins' recovery from the IL-rich phase by ice cold ethanol precipitation, where up to 99% of lysozyme can be recovered [34].

From the works summarized above, two distinct types of reports were found: the first reporting the use of IL-based ABS in the partition of model proteins (BSA, cyt-c, among others) and the second in which ABS were applied to extract and purify proteins (lipase, antibodies, etc.) from real and complex matrices. By the results obtained in the partition of model proteins, we cannot draw strong conclusions about the applicability and/or success of ABS, since for these systems to be used as downstream processes, the partition behavior of both target proteins and remaining impurities should be evaluated. Regarding the purification capacity of IL-based ABS from real matrices, the majority of the works were focused on the effect of the IL's chemical structure and mixture compositions in the partition for the target proteins; however, the primary impurities removal from the complex and original medium seems to make the IL-based ABS optimization easier. Taking in account all the works reviewed in this section, the use of more biocompatible ILs, namely GB ILs, seems to be very promising. In fact, using these ILs, there is no need to add extra buffer solutions; however, further optimization studies are required. Finally, for the application of IL-based ABS as downstream processes, more optimization investigations are required as well as complementary strategies in order to increase the worst results in terms of purification, namely the use of sequential purification steps.

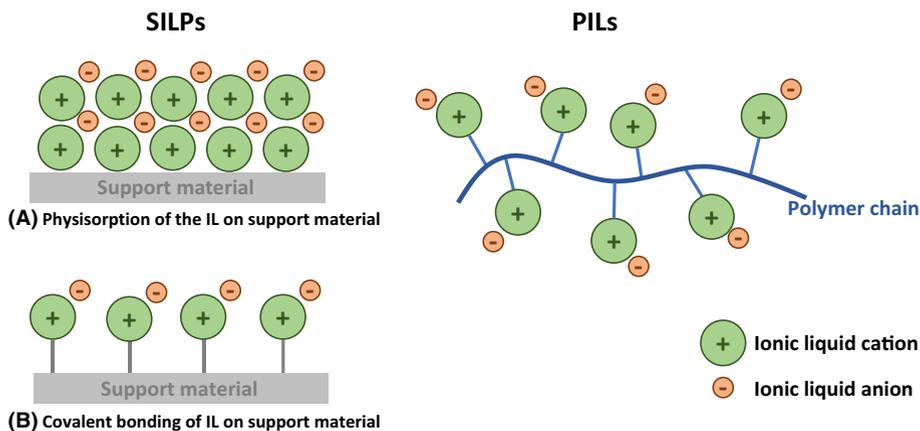## 2.2 Poly(ionic liquid)s and Supported Ionic Liquid Phases

Solid-phase extraction (SPE), also known as liquid–solid extraction, is a standard method for the separation and purification of target compounds from a liquid medium [40]. This

technique includes the use of a solid (adsorbent) phase, allowing the enrichment and purification of the target compound onto the solid adsorbent through adsorption from the solution [41]. Since SPE is a quick, low reagent consuming and highly selective method, it is widely applied in the separation of the most diverse compounds, including proteins [42]. Due to the high extraction performance and selectivity that can be afforded by ILs, they have been applied in SPE of proteins, namely as poly(ionic liquid)s and supported ionic liquid phases [42, 43].

Supported ionic liquid phases (SILPs) are alternative materials for SPE. SILPs have been produced through the chemical or physical immobilization of ILs onto silica or polymeric solid supports [44]. In these, suitable ILs, supports and extraction conditions must be identified and selected to improve the separation of proteins [44]. Additionally, a novel polymer class of materials, entitled polymeric ionic liquids (PILs), also named poly(ionic liquid)s, has emerged and also been applied in the separation of proteins. PILs are comprised of IL species in repeat monomer units linked together in order to shape its macromolecular architecture. PILs have at least one ionic center, analogous to the structure of the IL [45–48]. Figure 3 shows a schematic representation of SILPs and PILs.

In this section published works from the last decade regarding SPE through SILs and PILs for protein purification will be presented and discussed. Extraction materials and conditions, related ILs, studied proteins (BSA, ovotransferrin (OVT), hemoglobin (Hb), bovine hemoglobin (BHb), lysozyme, cyt-c, among others) and the main results obtained concerning the extraction and desorption efficiencies will be addressed. These are summarized in Table 2.

Within the last ten years, several works about SPEs using SILPs and PILs for protein extraction and purification have been published. BSA extraction through SILPs, such as silica-coated ammonium IL modified magnetic Fe_3O_4 nanoparticles [N_{0011}]Cl MNPs [43] and imidazolium-functionalized nanoparticles ([C_1C_1im]Cl NPs) [49], has been reported. [N_{0011}]Cl MNPs were produced for the first time, in 2014, by Chen et al. [43], via modifying its surface with hydroxy functional ILs, achieving a BSA-extraction efficiency of 86.92% (60 mg of [N_{0011}]Cl MNPs and 2.0 mL of 0.5 mg·mL^{-1} protein solution at pH 6 at 30 °C) and BSA-desorption efficiency of 95.34% (NaCl > 1.1 mol·L^{-1}). Additionally,

SILPs

(A) Physisorption of the IL on support material

(B) Covalent bonding of IL on support material

PILs

Polymer chain

(+) Ionic liquid cation

(–) Ionic liquid anion

Fig. 3 Illustration of supported ionic liquids phase, SILPs (A: IL film dispersed onto the support and B: IL acts as a surface modifier for the support, being covalently attached), and poly(ionic liquid)s, PILs

Table 2 Overview of the solid-phase extraction materials applied in protein extraction using supported ionic liquid phases (SILPs) and poly(ionic liquid)s (PILs), studied proteins and main results obtained

Materials	ILs	Studied Proteins	Main Results	Ref
Silica-coated imidazolium IL modified magnetic Fe_3O_4 nanoparticles ([C_1C_3im]Cl MNPs)	1-methyl-3-(triethoxy) silypropyl-imidazolium chloride	BSA OVT BHb	High BSA-extraction efficiency (86.92%)* High BSA-desorption efficiency (95.34%)* High BSA-extraction efficiency ($\approx$ 84.35–82.64%) during 4 cycles*	[43]
Silica-coated ammonium IL modified magnetic Fe_3O_4 nanoparticles ([N_{0011}]Cl MNPs)*	(2-hydroxyethyl)-N,N-dimethyl-3-(triethoxy) silypropyl-ammonium chloride			
Silica-coated morpholinium IL modified magnetic Fe_3O_4 nanoparticles ([C_2C_3mp] Cl MNPs)	N-ethyl-N-[3-(triethoxy) silypropyl]-morpholinium chloride			
Silica-coated guanidinium IL modified magnetic Fe_3O_4 nanoparticles ([$C_1C_1C_1$g] Cl MNPs)	N,N,N',N'-tetramethyl-3-(triethoxy) silylpropyl-guanidinium chloride			
Silica-coated pyrrolium IL modified magnetic Fe_3O_4 nanoparticles ([C_1C_3pyr]Cl MNPs)	N-methyl-N-[3-(triethoxy) silypropyl]-pyrrolium chloride			
Imidazolium-functionalized nanoparticles ([C_1C_1im]Cl NPs)	1-(3-trimethoxysilylpropyl)-3-methylimidazolium chloride	BSA	Maximum BSA-immobilization capacity (Q_e: 23.1 mg·g^{-1}) obtained with pH7.0 Main driving force for BSA-binding: electrostatic interactions 5 cycles with similar immobilization capacity	[49]
1-vinyl-3-octylimidazolium bromide [VOIM] [Br] polymer material ([$C_{vi}C_8$im]Br–AAM)** 1-allyl-3-butylimidazolium chloride [ABIM] [Cl] polymer material ([$C_{al}C_4$im]Cl–AAM)***	1-vinyl-3-octylimidazolium bromide 1-allyl-3-butylimidazolium chloride	BSA BHb Cyt-c	High BSA-adsorption capacity (804.7 mg·g^{-1})** High BHb-adsorption capacity (828.5 mg·g^{-1})***	[50]

Table 2 (continued)

Materials	ILs	Studied Proteins	Main Results	Ref
2-acrylamido-2-methylpropane sulfonic acid imidazole derivative methylenebisacryla-mide (MBA) polymer material ([C$_1$C$_3$S] [Rim] MBA)	2-acrylamido-2-methylpropane sulfonic acid imidazole derivative	BSA OVT BHb Cyt-c Casein	High adsorption capacities toward distinct proteins at diverse pH values BSA (842.2 mg·g^{-1}) and OVT (861.5 mg·g^{-1}) at pH4.0 BHb (983.4 mg·g^{-1}) and cyt-c (882.9 mg·g^{-1}) at pH7.0 Casein (605.2 mg·g^{-1}) at pH8.0 Effectively removing Hb from real bovine blood	[47]
Poly(1-vinyl-3-aminopropyl imidazolium bromide) ([VAPIM][Brl] modified cellulose aerogels ([C$_{vi}$C$_{3NH2}$im]Br–CAs)	Poly(1-vinyl-3-aminopropyl imidazolium bromide)	BSA OVA Hb Lysozyme Papain	High BSA-adsorption capacity (918 ± 8 mg·g^{-1}) after 350 min at pH6 Optimal BSA adsorption concentration: 1.5 mg·mL^{-1} Selective BSA separation from a real bovine serum sample, reaching high purity levels (> 98%) High operational stability, allowing 6 cycles with the loss of just 8.67% of its maximum adsorption capacity	[51]
Hydrophilic IL–polyvinyl chloride (PVC) hybrids ([C$_1$im]Cl–PVC)	N-methylimidazole chloride	Hb Lysozyme Cyt-c	High Hb (94%), lysozyme (97%) and cyt-c (98%) adsorption efficiencies N-mim grafting ratio: 15.1% Effective suppression of non-specific protein adsorption High recovery efficiencies of lysozyme, cyt-c and Hb after elution with phosphate buffer (87%), carbonate buffer (89%) and SDS solution (84%) Biocompatibility enhancement Selective isolation of Hb from human whole blood	[52]

Table 2 (continued)

Materials	ILs	Studied Proteins	Main Results	Ref
Imidazolium IL modified magnetic Fe_3O_4 microspheres ([C_1im]Cl–MMS)	N-methylimidazole chloride	Hb Lysozyme	Selective affinity to proteins with distinctive charges High Hb-adsorption capacity (2.15 g of Hb per gram of beads) Short processing time (15 min) Direct target isolation with high purity from a human blood sample Easy magnetic microspheres recyclability	[53]
N-methylimidazole chloride chloromethyl polystyrene ([C_1im]Cl–CMPS)	N-methylimidazole chloride	Hb Cyt-c $\log_{10}G$ Transferrin BSA	High Hb (91%) and cyt-c (93%) adsorption efficiencies Hb-adsorption capacity: 23.6 $\mu g \cdot mg^{-1}$ Hb-efficiency ($\approx$ 80%) of elution by 0.5% (m/v) SDS, retaining $\approx$ 90% of its activity Successful Hb isolation from human whole blood	[54]
Poly(1-vinylimidazolium-3-n-dodecyl) bromide azobis(2-methylpropionitrile) ([$C_{v1}C_{12}$im]Br–AIBN)	Poly(1-vinylimidazolium-3-n-dodecyl) bromide	Hb	High Hb adsorption (93.8%) Positive sorption capacity (205.4 $mg \cdot g^{-1}$) High adsorbed protein recovery (86.3%) Successful Hb isolation from human whole blood	[55]
ViBuIm$^+$Cl$^-$/acrylamide (AAM) macroporous polymer material ([$C_{v1}C_4$im]Cl AAM)	1-vinyl-3-butylimidazolium chloride	Lysozyme BSA BHb Myoglobin (MB) Cyt c	High binding ability for several proteins, particularly lysozyme (maximum binding capacity: 755.1 $mg \cdot g^{-1}$)	[42]

Table 2 (continued)

Materials	ILs	Studied Proteins	Main Results	Ref
Tetra-*n*-butylphosphonium *p*-styrene sulfonate poly(ethylene glycol) diacrylate membrane ([P$_{4444}$][SS]–PEG)	Tetra-*n*-butylphosphonium *p*-styrene sulfonate Analogous IL monomer composed of a cation with a longer alkyl chain	Cyt-c MB Horseradish peroxidase	Ability to control proteins partition through lower critical solution temperature (LCST) behavior Maximum extraction efficiency values (EE) were achieved for cyt-c (99.7% at 30 °C) Selective proteins concentration deprived of a substantial loss of their higher-order structures	[56]
Thiol graphene (TG) doped 1-vinyl-3-oc-tylimidazolium bromide boronate affinity monolithic material ([C$_{vi}$C$_8$im]Br–TG)	1-vinyl-3-octylimidazolium bromide	BSA BHb MB Cyt-c Lysozyme Ovalbumin Horseradish peroxidase	Capture specificity regarding cis-diol-contain-ing catechol and glycoproteins over wide pH range High binding capacities for ovalbumin (11.54 mg·g^{-1}) and for horseradish peroxi-dase (10.82 mg·g^{-1}) Selective glycoproteins separation and enrich-ment from human serum and egg white	[57]

*, ** and *** indicate the results obtained (Main Results column) with the respective material

[C$_1$C$_1$im]Cl–NPs prepared through a one-step grafting reaction, reached a maximum BSA-immobilization capacity (Q_e) of 23.1 mg·g^{-1} at pH 7, whose main driving force, detected through experimental studies and theoretical calculation by density functional theory (DFT), were electrostatic interactions [49]. On the other hand, BSA extraction through PILs, such as 1-vinyl-3-octylimidazolium bromide polymer material ([C$_{vi}$C$_8$im]Br–AAM) [50], 2-acrylamido-2-methylpropane sulfonic acid imidazole derivative methylenebis-acrylamide polymer material ([C$_1$C$_3$S][Rim] MBA) [47] and poly(1-vinyl-3-aminopropyl imidazolium bromide) modified cellulose aerogels [C$_{vi}$C$_{3NH2}$im]Br–CAs [51], have also been described. [C$_{vi}$C$_8$im]Br–AAM was developed through the use of 1-vinyl-3-octylim-idazolium bromide and acrylamide (AAM) as co-functional monomers, plus *N*,*N*'-methylene bisacrylamide (MBA) as a cross-linker [50]. [C$_{vi}$C$_8$im]Br–AAM exhibited a high BSA-adsorption capacity of 804.7 mg·g^{-1}, whose absorption was driven by multiple interactions, namely electrostatic and hydrophobic interactions, hydrogen bonds, salting-out and size-exclusion effects [50]. [C$_1$C$_3$S][Rim]–MBA (R: –H, –C$_6$H$_5$, or –(CH$_2$)$_3$CH$_3$) was produced in 2017 by Dang et al. [47] via the use of 2-acrylamido-2-methylpropane sul-fonic acid imidazole derivative as the functional monomer. The results showed that a high adsorption capacity toward BSA (842.2 mg·g^{-1}) at pH 4.0 was achieved [47]. [C$_{vi}$C$_{3NH2}$im]Br–CAs were developed in 2019 by Qian et al. [51] using poly(1-vinyl-3-aminopropyl imi-dazolium bromide) to change cellulose aerogels through the Schiff base reaction, reach-ing a BSA-adsorption capacity of 918 ± 8 mg·g^{-1}. Following 350 min at pH 6, an optimal BSA adsorption concentration of 1.5 mg·mL^{-1} was attained [51]. Among all the presented imidazolium-based PILs for BSA extraction, [C$_1$C$_3$S][Rim]–MBA [47] and [C$_{vi}$C$_8$im]Br–AAM [50] showed high BSA-adsorption capacities (804.7 mg·g^{-1} and 842.2 mg·g^{-1}, respectively), with the highest result (918 ± 8 mg·g^{-1}) being obtained with [C$_{vi}$C$_{3NH2}$im]Br–CAs [51]. When comparing the performance of SILPs and PILs, it is with PILs that a higher BSA-immobilization capacity was reported, namely 918 ± 8 mg·g^{-1} vs 23.1 mg·g^{-1} [51]. Furthermore, [C$_{vi}$C$_{3NH2}$im]Br–CAs [51] exhibited the selective BSA separation from a real bovine serum sample, displaying their potential for real applications.

Beyond BSA, Hb extraction through SILPs, such as hydrophilic IL–polyvinyl chloride (PVC) hybrids ([C$_1$im]Cl–PVC) [52], imidazolium IL modified magnetic Fe$_3$O$_4$ micro-spheres ([C$_1$im]Cl–MMS) [53] and *N*-methylimidazole chloride chloromethyl polystyrene ([C$_1$im]Cl–CMPS) [54], has also been reported. [C$_1$im]Cl–PVC allowed a Hb-adsorption efficiency of 94% with an N-mim grafting ratio of 15.1% [52]. Due to a non-specific pro-tein adsorption, Hb recovery efficiency of 89% after elution with phosphate buffer, car-bonate buffer and SDS solution, along with a biocompatibility enhancement characterized by the Hb activity maintenance was attained after adsorption and elution. [C$_1$im]Cl–MMS exhibited a high Hb-adsorption capacity (2.15 g of Hb per gram of beads) in a short pro-cessing time (15 min) because of their high specific surface area, along with their cova-lent coordination binding (among the imidazolium cation and the iron atom in the heme group) with Hb [53]. [C$_1$im]Cl–CMPS was produced via surface modification of chlorome-thyl polystyrene resin (CMPS) and showed a selective Hb adsorption [54]. An adsorption efficiency of 91% along with a Hb-adsorption capacity of 23.6 μg·mg^{-1}, and an 80% Hb-efficiency of elution using a 0.5% (m/v) SDS solution with the maintenance of 90% of its activity was achieved [54]. Among all the SILPs applied in the Hb extraction, it should be remarked that the same IL was always applied, namely *N*-methylimidazole chloride.

Hb extraction through PILs, namely 1-allyl-3-butylimidazolium chloride polymer material ([C$_{al}$C$_4$im]Cl–AAM) [50], [C$_1$C$_3$S][Rim]–MBA [47] and poly(1-vinylimidazo-lium-3-*n*-dodecyl) bromide azobis(2-methylpropionitrile) ([C$_{vi}$C$_{12}$im]Br–AIBN) [55], has also been described. Regarding bovine Hb (BHb) extraction, while [C$_{al}$C$_4$im]Cl–AAM [50]

allowed a BHb-adsorption capacity of 828.5 mg·g^{-1}, [C$_1$C$_3$S][Rim]MBA [47] permitted an even higher adsorption of 983.4 mg·g^{-1} at pH 7.0. Additionally, [C$_{vi}$C$_{12}$im]Br–AIBN, produced in 2015 by Wang et al. [55] through solution polymerization, displayed a high Hb adsorption (93.8%) under pH 8.0, likely due to the coordination between the cationic [C$_{vi}$C$_{12}$im]$^+$ and the iron atom in heme group of BHb, whose efficiency is practically not affected by the ionic strength variation. An adsorption capacity of 205.4 mg·g^{-1} due to a reduction of non-specific protein adsorption and a high adsorbed protein recovery (86.3%) after an elution step with 0.5% (m/v) SDS solution were also reached. BHb extraction through PILs [C$_{vi}$C$_4$im]Cl–AAM and [C$_1$C$_3$S][Rim]–MBA reached a higher adsorption capacity (983.4 mg·g^{-1}) than the one obtained for Hb extraction (205.4 mg·g^{-1}) with [C$_{vi}$C$_{12}$im]Br–AIBN. Hb extraction through PILs attained a higher adsorption capacity (205.4 mg·g^{-1}) than the one achieved with SILPs, namely [C$_1$im]Cl–CMPS (23.6 µg·mg^{-1}) [54]. Nevertheless, the SILPs ([C$_1$im]Cl–PVC [52], [C$_1$im]Cl–MMS [53] and [C$_1$im] Cl–CMPS [54]) along with the PILs ([C$_{vi}$C$_{12}$im]Br–AIBN [55]) showed the ability to provide the selective isolation of Hb from a complex biological sample matrix, namely human whole blood.

Another commonly used model protein, namely lysozyme, was also extracted through SILPS, specifically [C$_1$im]Cl–PVC [52], and PILs, namely C$_{vi}$C$_4$im$^+$Cl$^-$/acrylamide (AAM) macroporous polymer material ([C$_{vi}$C$_4$im]Cl–AAM) [42]. Applying [C$_1$im] Cl–PVC [52], a lysozyme adsorption efficiency of 97% and a lysozyme recovery efficiency after elution with phosphate buffer, carbonate buffer and SDS solution of 87%, were obtained [42]. When [C$_{vi}$C$_4$im]Cl–AAM was used, a maximum lysozyme binding capacity of 755.1 mg·g^{-1} under optimum adsorption conditions was reached [42].

The cyt-c extraction has been described by the use of SILPs, such as [C$_1$im]Cl–PVC [52] and [C$_1$im]Cl–CMPS [54], and PILs, such as [C$_1$C$_3$S][Rim]–MBA [47] and tetra-*n*-butylphosphonium *p*-styrene sulfonate poly(ethylene glycol) diacrylate membrane ([P$_{4444}$][SS]–PEG) [56]. By applying [C$_1$im]Cl–PVC [52], a cyt-c adsorption efficiency of 98% and a cyt-c recovery efficiency of 89% after elution with phosphate buffer, carbonate buffer and SDS solution were achieved. Similarly, using [C$_1$im]Cl–CMPS, a cyt-c adsorption efficiency of 93% was accomplished [54], while applying [C$_1$C$_3$S][Rim]–MBA a higher adsorption capacity towards cyt-c of 882.9 mg·g^{-1} at pH 7.0 was attained [47]. On the other hand, employing PILs, and specifically a transparent, and flexible thermoresponsive [P$_{4444}$][SS]–PEG, manufactured by Kohno et al. [56] via radical photo-cross-linking of two IL monomers (tetra-*n*-butylphosphonium *p*-styrene sulfonate and an analogous composed of a cation with a longer alkyl chain) and using PEG diacrylate as a cross-linker and 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone as a radical photo-initiator, it was achieved a maximum cyt-c extraction efficiency of 99.7% at 30 °C. Upon small temperature changes, a hydration/dehydration transition behavior was detected, yielding the selective proteins concentration deprived of a substantial loss of their higher-order structures, due to the absence of direct interactions among proteins and the membrane [56]. Although SILPs exhibited very high cyt-c adsorption efficiencies (between 93 and 98%), the highest adsorption efficiency (99.7%) was obtained with the PIL [P$_{4444}$][SS]–PEG [56].

The PIL [C$_1$C$_3$S][Rim]–MBA [47], previously discussed and applied in the extraction of BSA, BHb and cyt-c, also allows high adsorption capacities toward other proteins at diverse pH values, namely ovotransferrin (861.5 mg·g^{-1}) at pH 4.0 and casein (605.2 mg·g^{-1}) at pH 8.0, reinforcing its wide applicability.

Beyond simpler proteins, glycoproteins (vital in several biological functions [58, 59]) were captured by a thiol graphene (TG) doped 1-vinyl-3-octylimidazolium bromide boronate affinity monolithic material ([C$_{vi}$C$_8$im]Br–TG), synthetized by Liu et al. [57] through

a simple one-step synthesis method, by a "thiol–ene" click reaction and free radical polymerization. In fact, a demonstrated capture specificity regarding cis-diol-containing catechol and glycoproteins at a wide pH range was observed, suitable for a large number of applications [57]. $[C_{vi}C_8im]Br$–TG with its large specific surface area (133.64 $m^2 \cdot g^{-1}$) also led to high binding capacities, reaching 11.54 $mg \cdot g^{-1}$ for ovalbumin and 10.82 $mg \cdot g^{-1}$ for horseradish peroxidase. Furthermore, the selective glycoproteins separation and enrichment from human serum and egg white shows the separation potential of this material using complex biological samples [57].

In summary, several works concerning the SPE of proteins through SILs and PILs were presented, highlighting the enhancement of extraction and desorption efficiencies, operational stability, and biocompatibility. In fact, model proteins, namely BSA, Hb, lysozyme and cyt-c are the most applied proteins to prove the efficiency of SILPs and PILs, with extraction efficiencies reaching values higher than 85%. However, there is a lack of studies concerning the application of SILPs and PILs in the extraction and purification of proteins from complex sources, and therefore, further investigations are required.

2.3 Crystallization with Ionic Liquids

Protein crystallization is a cost-effective purification step, since it allows the reduction of several cost-intensive chromatographic steps [60]. ILs are potential additives or precipitation agents for the crystallization of proteins due to their ability to participate in ionic, hydrophobic, and hydrogen bonding interactions [61–65]. Therefore, for more than two decades, several authors have been investigating the influence of distinct ILs in the crystallization of model proteins, mainly lysozyme [60, 61, 65–73], but also, glucose isomerase (GI) [61, 71, 74, 75], trypsin [61, 71, 73], ribonuclease A (RNase A) [72, 73], myoglobin [61, 73], xylanase [61, 75], β-lactoglobulin B [75], canavalin [75], catalase [61], Hb [73], proteinase K [73], and thaumatin [69]. The main results achieved, as well as proteins and ILs investigated are summarized in Table 3.

In 1999, Garlitz et al. [67] successfully reported, for the first time, the IL ethylammonium nitrate, $[N_{0002}][NO_3]$, as a crystallization agent for lysozyme. Two crystal forms were achieved, namely monoclinic (pH 4.5 ± 4.6) and tetragonal (pH 5.4 ± 5.6) lysozyme crystals [67]. Li et al. [68] later proposed the mixture 1-butyl-3-methylimidizolium tetrafluoroborate ($[C_4C_1im][BF_4]$)–water for lysozyme crystallization. Larger and more perfect single crystals were obtained, with adaptable morphologies due to the control of the crystal growth velocity and solution supersaturation [68]. The same authors [69] also applied $[C_4C_1im][BF_4]$, $[C_4C_1im]Cl$ and $[C_4C_1im][PO_4]$ to the crystallization of lysozyme and thaumatin, but only $[C_4C_1im][BF_4]$ revealed to be a suitable precipitant or union-precipitant ($[C_4C_1im][BF_4]$–NaCl). Since $[C_4C_1im][BF_4]$ is a water-soluble IL, it leads to moderate losses of water molecules by the protein and an enhancement of the binding forces between protein molecules was observed [69]. Furthermore, $[C_4C_1im][BF_4]$ allowed slow protein precipitation and high quality production of single crystals due to its specific characteristics, namely lower ionic strength because of its large cation radius, and greater density and lower vapor pressure than the solution, which might help mimic gels and oils roles in protein crystallization [69]. Chen et al. [70] also succeeded by using 1,3-dibutylimidazolium chloride ($[C_4C_4im]Cl$) as an additive in the direct crystallization of lysozyme, but directly from a complex real sample matrix (i.e. egg white). These findings open the path for the application of ILs in the purification of proteins by crystallization from raw samples.

Table 3 Summary of protein crystallization studies using ionic liquids (ILs), respective model proteins used and main results

Ionic liquid(s)	Model proteins	Main results	Ref
Ethylammonium nitrate ([N$_{0002}$][NO$_3$])	Lysozyme	2 crystal forms (300 ± 400 mmol·L^{-1}) monoclinic (pH 4.5 ± 4.6) lysozyme crystals tetragonal (pH 5.4 ± 5.6) lysozyme crystals keep their native structures	[67]
1-Butyl-3-methylimidizolium tetrafluoroborate ([C$_4$C$_1$im][BF$_4$])	Lysozyme	Novel system for lysozyme crystallization, [C$_4$C$_1$im][BF$_4$]–water larger and more perfect single crystals development with adaptable morphologies	[68]
1-Butyl-3-methylimidizolium tetrafluoroborate ([C$_4$C$_1$im][BF$_4$]) 1-Butyl-3-methylimidizolium chloride ([C$_4$C$_1$im]Cl) 1-Butyl-3-methylimidizolium phosphate ([C$_4$C$_1$im][PO$_4$])	Lysozyme Thaumatin	Only [C$_4$C$_1$im][BF$_4$] revealed to be suitable precipitants or union-precipitants ([C$_4$C$_1$im][BF$_4$]–NaCl) enhancement of the binding force between protein molecules [C$_4$C$_1$im][BF$_4$] allowed slow protein sedimentation high quality single crystals	[69]
1,3-Butylimidazolium chloride ([C$_4$C$_4$im]Cl)	Lysozyme	1,3-butylimidazolium chloride as an additive in direct cratallization of lysozyme from a complex sample matrix (egg-white) major breakthrough for the future of purification of protein of interest	[70]

Table 3 (continued)

Ionic liquid(s)	Model proteins	Main results	Ref
1-Ethyl-3-methylimidazolium tetrafluoroborate ($[C_2C_1im][BF_4]$) 1-Butyl-3-methylimidazolium chloride ($[C_4C_1im]Cl$) 1-Hexyl-3-methylimidazolium chloride ($[C_6C_1im]Cl$) 1-Ethyl-3-methylimidazolium trifluoromethanesulfonate ($[C_2C_1im]$ $[CF_3SO_3]$) Triisobutyl (methyl) phosphonium p-toluenesulfonate ($[P_{1444}]$ [TOSl]) Tetraethylammonium bromide ($[N_{2222}]Br$) n-Butylpyridinium chloride ($[C_4py]Cl$) Tetrabutylphosphonium bromide ($[P_{4444}]Br$) Benzyltriethylammonium chloride ($[N_{B222}]Cl$) 1-Ethyl-3-methylimidazolium chloride ($[C_2C_1im]Cl$) 1-Butyl-2,3-dimethylimidazolium tetrafluoroborate ($[C_4C_1C_1im]$ $[BF_4]$) 1,3-Dimethylimidazolium methyl sulfate ($[C_1C_1im][CH_4SO_4]$) 1-Butyl-3-methylimidazolium methyl sulfate ($[C_4C_1im][CH_4SO_4]$) 1-Butyl-3-methylimidazolium trifluoroacetate ($[C_4C_1im][C_2F_3O_2]$) 1-Butyl-3-methylimidazolium tetrafluoroborate ($[C_4C_1im][BF_4]$) 1-Butyl-3-methylimidazolium octyl sulfate ($[C_4C_1im]$ $[(CH_2)_7CH_3SO_4]$)	Lysozyme Catalase Myoglobin Trypsin Glucose Isomerase (GI) Xylanase	ILs applied, mostly as crystallization additives, but also as precipitation agents protein crystals grown in their presence display an equal or improved X-ray diffraction resolution Only trypsin and lysozyme yield crystals ILs mode of action looks to be via changes in solution features	[61]
1-Butyl-3-methylimidazolium tetrafluoroborate ($[C_4C_1im][BF_4]$) 1-Butyl-3-methylimidazolium chloride ($[C_4C_1im]Cl$) 1-Butyl-3-methylimidazolium bromide ($[C_4C_1im]Br$) 1,3-Dimethylimidazolium iodine ($[C_1C_1im]I$)	Lysozyme	4 studied ILs as lysozyme crystallization additives enabled the production of larger crystals or diverse crystal morphologies $[C_4C_1im][BF_4]$, $[C_4C_1im]Cl$ and $[C_4C_1im]Br$ led to platelike lysozyme crystals $[C_1C_1im]I$ generated needlelike lysozyme crystals	[62]
1-Butyl-3-methylimidazolium chloride ($[C_4C_1im]Cl$) 1,3-Dimethylimidazolium iodine ($[C_1C_1im]I$)	Lysozyme	Determination of the nucleation induction time and evaluation of nucleation parameters Critical free energy change, size, and molecular number of critical nuclei declined and the nucleation rate augmented decrease of attractive interactions between the lysozyme molecules	[76]

Table 3 (continued)

Ionic liquid(s)	Model proteins	Main results	Ref
1,3-Dimethylimidazolium dimethylphosphate [C$_1$C$_1$im][(CH$_3$)$_2$ PO$_4$] 1-Ethyl-3-methylimidazolium chloride ([C$_2$C$_1$im]Cl) 1-Ethyl-3-methylimidazolium bromide ([C$_2$C$_1$im]Br) 1-Ethyl-3-methylimidazolium thiocyanate ([C$_2$C$_1$im][SCN]) 1-Ethyl-3-methylimidazolium acetate ([C$_2$C$_1$im][C$_2$H$_3$O$_2$]) 1-Ethyl-3-methylimidazolium ethanesulfonate ([C$_2$C$_1$im] [CH$_2$CH$_3$SO$_3$]) 1-Ethyl-3-methylimidazolium butylsulfonate ([C$_2$C$_1$im] [(CH$_2$)$_3$CH$_3$SO$_3$]) 1-Butyl-3-methylimidazolium chloride ([C$_4$C$_1$im]Cl) 1-Butyl-3-methylimidazolium acetate ([C$_4$C$_1$im][C$_2$H$_3$O$_2$]) 2-Hydroxyethyl-trimethylammonium chloride ([N$_{2OH 111}$]Cl) 2-Hydroxyethyl-trimethylammonium acetate ([N$_{2OH 111}$][C$_2$H$_3$O$_2$]) 2-Hydroxyethyl-trimethylammonium methanesulfonate ([N$_{2OH 111}$] [C$_1$SO$_3$]) 2-Hydroxyethyl-trimethylammonium lactate ([N$_{2OH 111}$][C$_3$H$_5$O$_3$])	Lysozyme Ribonuclease A (RNase A)	Systematic rationale regarding the impact of the previously pre-sented complex IL ions effect on protein crystallization intrinsic hydration features of IL solutions favor binding of the IL ions to the protein surface stabilize the protein in solution through protein-water interfacial tension decrease Novel reports on hydration modification of protein charged sections	[72]
1-Butyl-3-methylimidazolium tetrafluoroborate ([C$_4$C$_1$im][BF$_4$]) 1-Butyl-3-methylimidazolium chloride ([C$_4$C$_1$im]Cl) 1-Butyl-3-methylimidazolium bromide ([C$_4$C$_1$im]Br) 1,3-Dimethylimidazolium iodine ([C$_1$C$_1$im]I)	Lysozyme	ILs concentration affected specific intermolecular interactions strength between proteins or IL and individual protein Lysozyme crystals formed from [C$_4$C$_1$im]Cl were sharp and steady under 1% IL [dmim][I] varied from plate-like and needle-like mixture to pure needle-like crystals when the IL concentration increases from 1 to 3%	[65]
1-Ethyl-3-methylimidazolium bromide ([C$_2$C$_1$im]Br) 1-Ethyl-3-methylimidazolium tetrafluoroborate ([C$_2$C$_1$im]BF$_4$) 1-Butyl-3-methylimidazolium bromide ([C$_4$C$_1$im]Br)	Lysozyme	Three studied ILs helped to improve lysozyme crystal morphology More compacted and highly ordered lysozyme molecules displaying fewer defects particularly with 0.01 mol·L^{-1} [C$_2$C$_1$im]Br	[66]

Table 3 (continued)

Ionic liquid(s)	Model proteins	Main results	Ref
1-Butyl-3-methylimidizolium chloride ($[C_4C_1im]Cl$) 1-Butyl-3-methylimidizolium 2(2-methoxyethoxy)ethylsulfate ($[C_4C_1im][MDEGSO_4]$) 1-Butyl-1-methylpyrrollidinium dihydrogenphosphate ($[C_4C_1pyrr][H_2PO_4]$)	Canavalin β-lactoglobulin B Xylanase GI	All protein–IL combinations generated, at least, one crystal All proteins showed distinctive responses to each IL Potential mechanisms for the impact of ILs on protein crystallization were proposed Helped slow down the vapor transfer Increased the protein solubility or lower nonspecific binding among hydrophobic sites	[75]
1-Ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide ($[C_2C_1im][NTf_2]$) 1-Hexyl-3-methylimidazolium Bis(trifluoromethylsulfonyl)imide ($[C_6C_1im][NTf_2]$) 1-Dodecyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide ($[C_{12}C_1im][NTf2]$)	Lysozyme RNase A Trypsin Proteinase K Myoglobin Hemoglobin (Hb)	Hydrophobic ILs immobilized at the surface of barium sulfate ($BaSO_4$) aid induce myoglobin and Hb nucleation at inferior concentrations reduce the lysozyme and myoglobin nucleation time enhance myoglobin and Hb crystal morphology Myoglobin crystallization may be induced at protein concentrations up to 80% lower in the presence of $BaSO_4$ particles	[73]
Ethylammonium formate ($[N_{0002}][HCO_2]$) Ethylammonium propionate ($[N_{0002}][C_3H_5O_2]$) Ethylammonium methanesulfonate ($[N_{0002}][CH_3O_3S]$) Ethylammonium pivalate ($[N_{0002}][C_5H_9O_2]$) Ethylammonium trifluoroacetate ($[N_{0002}][C_2F_3O_2]$) Ethylammonium acetate ($[N_{0002}][C_2H_3O_2]$) Ethylammonium nitrate ($[N_{0002}][NO_3]$) Ethanolammonium nitrate ($[N_{0002}][NO_3]$) Triethanolammonium nitrate ($[N_{020H20H20H}][NO_3]$) Diethanolammonium nitrate ($[N_{020H20H}][NO_3]$)	Lysozyme Trypsin GI	Ten studied protic ILs suitably applied as additives in protein crystallization aid increasing protein crystals size and quality boost the reproducibility of protein crystallization events protic ILs impact on protein crystallization is protein specific	[71]
Ethanolammonium formate ($[N_{0002OH}][HCO_2]$) Ethylammonium nitrate ($[N_{0002}][NO_3]$) Bis(2-methoxyethyl)ammonium acetate ($[N_{002(OCH3)2(OCH3)}][CH_3CO_2]$) N,N-dimethylethanolammonium glycolate ($[N_{0112OH}][C_2H_3O_3]$) Cholinium dihydrogenphosphatea ($[Ch][H_2PO_4]$)[a]	Lysozyme	Potential of biocompatible, water-soluble ILs as crystallization additives lead to larger crystals with less polymorphism	[60]

Table 3 (continued)

Ionic liquid(s)	Model proteins	Main results	Ref
1-Butyl-3-methylimidazolium hexafluorophosphate ([C$_4$C$_1$im][PF$_6$])	GI	ILs applied as additives in crystallization of GI (a model protein for polymorphic studies) as IL hydrogel composite membranes (IL–HCMs) elements promote protein crystallization, while keeping crystal properties IL–HCMs influence the selection process of GI polymorphs remaining active throughout crystallization and sustaining crystals following their growth	[74]
1-Ethyl-3-methylimidazolium bromide ([C$_2$C$_1$im]Br) 1-Ehyl-3-methylimidazolium tetrafluoroborate ([C$_2$C$_1$im][BF$_4$]) 1-Butyl-3-methylimidazolium bromide ([C$_4$C$_1$im]Br)	Lysozyme	ILs help to improve lysozyme crystal morphology induce more compacted and highly ordered lysozyme molecules	[66]

^{a}Liquid at room temperature when diluted with 20% (v/v) H$_2$O [60]

Judge et al. [61] investigated the use of sixteen ILs (Table 3) composed of three distinctive cations, namely imidazolium, phosphonium and ammonium and five different anions, namely borate, halide, sulfate, acetate and sulfonate, for the crystallization of six model proteins, viz. lysozyme, catalase, myoglobin, trypsin, GI and xylanase. This study showed that ILs can be applied, mostly as crystallization additives, but also as precipitation agents since the protein crystal grown in their presence displayed an equal or improved X-ray diffraction resolution than those acquired without ILs. Furthermore, the mode of IL action seemed to be by promoting changes in solution features instead of through protein binding as proven by X-ray analyses. However, among all the studied proteins, only lysozyme and trypsin yielded crystals [61].

Xiao et al. [62] applied $[C_4C_1im][BF_4]$, $[C_4C_1im]Cl$, $[C_4mim]Br$ and $[C_1C_1im]I$ as lysozyme crystallization additives, which enabled the production of large crystals or diverse crystal morphologies. Actually, while $[C_4C_1im][BF_4]$, $[C_4mim][Cl]$ and $[C_4mim][Br]$ led to plate-like lysozyme crystals, $[C_1C_1im]I$ generated needle-like lysozyme crystals, probably due to the differences between the cations $[C_4C_1im]$ and $[C_1C_1im]$ in aqueous solution [62]. Furthermore, the lysozyme solubility increased with increasing $[C_4C_1im][BF_4]$ and $[C_4C_1im][Cl]$ concentrations, whereas the opposite effect was observed with $[C_1C_1im]I$. Wang et al. [76] explored the impact of $[C_4C_1im][Cl]$ and $[C_1C_1im]I$ on the nucleation kinetics of lysozyme through the determination of the nucleation induction time and evaluation of nucleation parameters. Following the introduction of $[C_4C_1im][Cl]$, the critical Gibbs energy change, size, and molecular number of critical nuclei declined and the nucleation rate were augmented, suggesting the decrease of attractive interactions between the lysozyme molecules [76]. Following the introduction of $[C_1C_1im]I$, the critical Gibbs energy change, size, and molecular number of critical nuclei was increased and the nucleation rate declined, suggesting an improvement of the attractive interactions between the lysozyme molecules and the formation of hydrophobic sites with the salt [65, 76].

To the best of our knowledge, Kowacz et al. [72] has given, for the first time, in 2012, a systematic rationale regarding the impact of the IL ions effect on protein crystallization. The intrinsic hydration features of IL solutions favor the IL ions binding to the protein surface, which increase according with the ions' negative/hydrophobic hydration and aid in protein nucleation, disclosed via electrostatic charge assessment, whereas stabilizing of proteins in solution through the protein–water interfacial tension decreases [72]. Furthermore, novel reports on hydration modification of protein charged sections uncovered their significance in crystallization, e.g. in systems with limited solubility [72]. Wang et al. [65] pointed out that an IL's concentration affects specific intermolecular interactions strength between proteins or IL and an individual protein, which will probably change with concentration. In fact, lysozyme crystals formed from $[C_4C_1im]Cl$ are sharp and steady under 1% IL concentration and become smaller when the IL concentration increases. On the other hand, lysozyme crystals formed from $[C_1C_1im]I$ solution vary from plate-like and needle-like mixtures to pure needle-like crystals when the IL concentration increases from 1 to 3%, and become thinner when the IL concentration increases to 5% [65]. Yu et al. [66] found that $[C_2C_1im]Br$, $[C_2C_1im][BF_4]$ and $[C_4C_1im]Br$ help improve lysozyme crystal morphology, inducing more compact and highly ordered lysozyme molecules displaying fewer defects, particularly with 0.01 mol·L^{-1} $[C_2C_1im]Br$, supported by a high α-helix % (38.1%) content in the lysozyme's secondary structure. A crystal shape change due to the presence of $[BF_4]^-$ was identified, since this IL anion has a larger volume and a smaller negative charge density than Cl$^-$, which is the principal anion binding at lysozyme molecule surfaces in IL-free solutions. Consequently, the lysozyme surface charge distribution adjusts due to a higher amount of anion binding, blocking crystal face growth. Furthermore, since the hydrophobicity of $[BF_4]^-$ is greater than for

Br⁻, hydrophobic interaction between lysozyme molecules in solution are weakened, increasing its solubility [66].

The influence of ILs in the crystallization of other proteins has also been studied. Pusey et al. [75] reported several protein–IL combinations (Table 3) and, at least one crystal was formed under conditions that had formerly not resulted in crystallization. Besides that, all proteins studied (canavalin, β-lactoglobulin B, xylanase and GI) showed distinctive responses to each IL, suggesting that ILs do not influence all proteins in an analogous way [75]. Although, a unique, specific mechanism for the impact of ILs on protein crystallization is still not known, some potential mechanisms have been proposed, namely: ILs help slow down the vapor transfer, enabling a slower nucleation and crystal growth, and ILs increase the protein solubility or lower nonspecific binding among hydrophobic sites, slowing down the self-association process [75].

Kowacz et al. [73] demonstrated that hydrophobic non-water soluble ILs, namely $[C_2C_1im]$ $[NTf_2]$, $[C_6C_1im][NTf_2]$ and $[C_{12}C_1im][NTf_2]$, immobilized at the surface of barium sulfate $(BaSO_4)$, induce myoglobin and Hb crystallization. Acidic proteins with higher functional groups are predisposed to interact with the mineral's pore wall, leading to nucleation at concentrations lower to those which lead to crystal growth under control conditions [73]. In fact, myoglobin crystallization may be induced at protein concentrations up to 80% lower in the presence of $BaSO_4$ particles with and without ILs $([C_{12}C_1im][NTf_2])$ [73].

Kennedy et al. [71] reported that ten protic ILs (Table 3) may be suitably applied as additives in three protein's (lysozyme, trypsin, GI) crystallization. Although protic ILs boost the reproducibility of protein crystallization events, no significant change in lysozyme's structure was observed [71]. Furthermore, it was found that the protic ILs impact on protein crystallization is protein specific, since the crystallization process was distinctive for each studied protein as proved by pH, density and solutions viscosity variations [71]. Hekmat et al. [60] also showed the potential of biocompatible and water-soluble ILs as crystallization additives using lysozyme, since they led to larger crystals with less polymorphism.

Belviso et al. [74] reported the use of ILs as additives and IL hydrogel composite membranes (IL–HCMs) elements in GI crystallization, a model protein for polymorphic studies. IL–HCMs promote protein crystallization, while retaining crystal properties since protein crystals grow fully immersed in the IL hydrogel layer due to the strong forces established between protein and IL monomers [74]. Moreover, it was proved that IL–HCMs influence the selection process of GI polymorphs, remaining active throughout crystallization and sustaining crystals following their growth. Nevertheless, IL–HCMs did not directly interact with residues since X-ray diffraction analyses exhibit that IL ions do not bind to the protein [74].

Overall, the works described above show the potential of ILs as additives and precipitation agents for protein crystallization, emphasizing the improvement of intermolecular interaction strength and crystal morphology through the formation of more compacted and highly ordered molecules.

3 Conclusions and Future Perspectives

An overview of recent applications of ILs for the extraction and purification of proteins by the application of IL-based ABS, solid-phase extraction through PILs and SILPs and protein crystallization were herein presented and discussed. ILs were either applied as phase-forming, electrolytes or adjuvants in ABS, in the modification of supported materials to tune the adsorption/affinity capacity of silica and polymers, to create PILs, or as agents to

induce the crystallization of proteins. Generally, if properly selected, IL-based processes are able to afford higher extraction yields and purification factors and maximize adsorption capacities when compared to traditional solvents and materials. Furthermore, they also are potential additives or precipitation agents allowing the crystallization of proteins due to their ability to participate in ionic, hydrophobic, and hydrogen bonding interactions.

The advantageous features of IL properties (non-volatility, non-flammability and high thermal stability) combined with the benefits of ABS, solid–liquid extraction and crystallization, make IL-based processes powerful processes in protein separation and purification technology. Moreover, it should be noted that recently IL-based processes with IL's having environmentally benign behaviors and biocompatibilities have gained more interest with the aim at developing an efficient, cost-effective, sustainable and green IL-based process for protein extraction. However, the use of these techniques is still mainly be applied with model proteins, restricting the conclusions about their applicability and/or success in downstream processes. Therefore, further investigations envisaging the purification of proteins from complex sources, through an IL chemical structure tailoring for the target proteins/enzymes and/or main contaminants, are strongly recommended.

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