Application of Aqueous Biphasic Systems Extraction in Various Biomolecules Separation and Purification: Advancements Brought by Quaternary Systems

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Abstract

Aqueous biphasic systems (ABS) extraction is a simple, selective, efficient, and easy to scale-up technology that, over the years, has attracted a considerable attention from the researcher community as an alternative methodology in downstream processing of a wide variety of biomolecules. This review summarizes and discusses the fundamental features of ABS, as well as its advantages and disadvantages, as a separation and purification technology of biomolecules. Nevertheless, the focus of this review are quaternary ABS formed by the addition of neutral salts and ionic liquids to conventional ABS or those ABS composed by deep eutectic solvents and another phase forming compound. The advantages brought by quaternary ABS in terms of separation and purification of biomolecules, as well as the main issues governing the phase behavior of these systems, are discussed. With examples of application of quaternary ABS as an alternative extraction and purification methodology, it is shown that such ABS are a promising method to improve the effectiveness of biomolecules downstream processing, potentially providing a response to the increasing demand for high purity bioproduct. Furthermore, some of the discussed quaternary ABS have a great potential as a novel, sustainable and cost-effective purification platform for biomolecules downstream processing that can potentially simplify the whole ABS-based purification process due to no need for target bioproduct recovery or phase formers removal. Finally, perspectives of such quaternary ABS are made, and some future challenges pointed out.

Keywords: Aqueous biphasic systems, Additives, Ionic liquids, Deep eutectic solvents, Downstream processing

1. Introduction

Aqueous biphasic systems (ABS) were discovered in 1896, when Beijerinck observed two-phase formation upon mixing an aqueous solution of agar and starch or gelatin [1]. Nevertheless, their widespread recognition has begun with the works of Albertsson in mid-1950s [2-4], where the use of ABS in partitioning of macromolecules, organelles and cells was explored. In general, ABS consist of two immiscible aqueous-rich phases formed by mixing two water-soluble substances in exceeding concentrations [5] and is described by binodal phase diagram (see Fig. 1). Hence, these systems can be potentially constructed with a wide variety of compounds. Nevertheless, the most studied systems are usually composed of two polymers, a polymer, and a salt or two salts combinations. Moreover, since both phases of ABS are mainly composed of water, these systems offer a good environment for separation of biomolecules, without hampering their structural and biological activities [6]. This is the great advantage of this methodology over liquid-liquid extraction based on organic solvents, where poor solubility and denaturation of the biomolecules is a common problem [7]. Furthermore, ABS-based extraction technologies are environment-friendly, easy to scale-up, and offer the possibility of continuous operation mode and integration of several steps into one-unit operation [8-9]. Due to those remarkable features, ABS have been extensively studied as an alternative technology in the extraction and purification of wide range of biomolecules, such as proteins, enzymes, virus and virus-like particles (VLPs), among others [10-18].

Over the years, ABS have been proven to be advantageous in terms of process economics and technical simplicity and the idea of using them in the primary recovery and purification of biological samples was very well received by the scientific community and thereafter the number of publications regarding this topic significantly increased. Although a lot of studies have been dedicated to biomolecules partitioning in ABS (see Fig. 2), the mechanisms that

rule biomolecule separation within the two coexisting phases are still not fully understood. Several factors, such as biomolecule hydrophobicity, charge, structure, or size, influence the preference of the biomolecule to partition to a particular phase of the ABS [5, 10, 15]. Besides biomolecules properties, also ABS features play a crucial role in solute extraction. Parameters that need to be considered are type, molecular weight and concentration of phase forming polymer, type, and salt concentration, tie line length (TLL), temperature, pH, density and viscosity, interfacial tension, and the presence of additives. The influence of each parameter on partitioning for different ABS was discussed in earlier reviews [10-12, 14-16]. Therefore, considering the lack of predictive models for ABS selection envisaging a specific purification problem, considerable number of trial-and-error experiments are necessary, in order to gain sufficient insights to allow the understanding of the separation mechanism of each ABS so that high purification factors and recovery yields can be obtained [19].

Moreover, despite the large number of works that showed the potential of ABS extraction in downstream processing of biomolecules in the batch-scale, until now this methodology was not adopted in the industrial scale. The main reason of that is not only the poor understanding of mechanism that governs phase formation and solute partitioning in the ABS [13], and thus the lack of models that can predict phase splitting behavior, but also the need for high quantities of chemicals used as phase formers [20], which are sometimes very expensive (e.g. high molecular weight polymers [21]), additionally increasing the cost of the process. Thus, throughout the years an effort has been made in order to reduce these constraints mainly through the development of new cheaper, more efficient, recyclable phase forming compounds. Introduction of novel phase forming compounds provided new, interesting schemes for the separation of biomolecules, allowing higher extraction efficiencies (EE%) and recoveries, as well as the integration of several steps of separation

and purification process into one-unit operation [8-9]. Additionally, new approaches using conventional and well-studied ABS were tested. For instance, the addition of a fourth component to ABS, such as salts or ionic liquids (ILs), has shown to be possible solution for overcoming the low selectivity (S) of these systems. These additives act as modifiers and change the properties of the phases in equilibrium, allowing, in many cases, the complete separation between bioproduct and contaminants. Fig. 3 shows the most important landmarks in the ABS history since their origin, with the introduction of polymer-polymer and polymer-salt systems to the first use of deep eutectic solvents (DES) as phase forming compounds.

This paper provides a review on various biomolecules extraction and purification using quaternary ABS. To the best to our knowledge, this is the first review that discusses entirely quaternary ABS. The main objective of this review is to discuss the different types of additives used in formation of quaternary ABS and explain how they can have positive impact on phase splitting behavior and most importantly on separation and purification of high-value biomolecules. Furthermore, short introduction to downstream processes for various biomolecules is made and the need for new cost-effective purification technologies as part of the bioprocess is highlighted. Finally, examples of application of quaternary ABS as an alternative extraction and purification methodology are shown. The advancements made using each type of quaternary ABS are highlighted showing that these systems can ultimately lead to a powerful technology to purify biomolecules with high quality (high EE% and/or final bioproduct purity), while being less expensive and safer to the environment than most conventional ABS, and thus placing them as a promising answer on the questions raised by the industry on conventional ABS. The perspectives quaternary ABS are also pointed out, as well as some future challenges.

2. Additives to conventional ABS: the rise of quaternary ABS

The addition of a fourth component to conventional ABS, composed of polymer-polymer or polymer-salt, is another approach used to enhance selectivity and to decrease some other constraints of these systems. In these quaternary ABS one additive (the 4th component) partitions between the two coexisting phases in equilibrium and, consequently, changes their properties. Very often the use of additives affords lower concentrations of the phase forming compounds needed to promote two-phase formation. Therefore, more cost-effective processes can be developed. Furthermore, some additives can act as adjuvants, not only increasing the separation performance, but also acting as stabilizers for the target biomolecule. The most extensively used additives are electrolytes (such as NaCl, KCl, KI, KNO_3, among others) [22-24], osmolytes (e.g., sucrose, sorbitol, trehalose, urea) [25-26] and ILs [27]. Moreover, in this section, DES will also be included. Due to the solvation by water of DES components in ABS and the breaking of the hydrogen bond between hydrogen bond acceptor (HBA) and hydrogen bond donor (HBD) [28-29], DES components independently partition between both phases[30-31]. In that way, one of the DES components usually acts as additive and the other as ABS phase forming compound [30-31]. The strengths and weaknesses, opportunities and threats of quaternary ABS compared with conventional ABS are presented in Fig. 4.

This section aims to review the most studied families of compounds employed in development of quaternary ABS. A general discussion on how each group of these additives changes the ABS properties in terms of biomolecules separation will be provided in the next subsections. Moreover, special attention will also be given to the effect of the 4th component on the binodal curves of ABS, and some examples will be presented. The name and acronym of investigated compounds employed in quaternary ABS implementation are listed in Table 1.

2.1. Electrolytes and osmolytes

2.1.1. The effect of electrolytes and osmolytes on biomolecules partitioning

The addition of electrolytes, such as neutral salts (salts of strong acid and strong base, which do not hydrolyze and are ionic compounds), are one of the well-known factors which strongly affect the biomolecule partitioning in polymer-polymer [22] and polymer-salt ABS [23-24]. The most widely chosen salt is NaCl, which is considered as being relatively neutral to a large range of biomolecules. Nevertheless, the concentrations of salts used as additives are usually kept in a rather low range of concentrations, from 0.0 to 1.0 M, due to denaturation of proteins in high concentrations of salts [13]. In general, the addition of salts to the conventional ABS increases the ionic strength and the hydrophobic difference due to generation of an electrical potential variation between the phases [32]. An increase in the hydrophobicity leads to the decrease of the amount of water available for the biomolecule solvation [24]. Therefore, the hydrophobic moieties on the biomolecule surface are exposed and enhanced partitioning towards the more hydrophobic polymer-rich phase is observed [32].

Another group of compounds used as additives in ABS are osmolytes. They are naturally occurring compounds found in the cells of many organisms as they help to counteract the effects of environmental stresses, such as temperature and pH variations, high salinity, freezing, and dehydration [33-34]. There are two types of osmolytes, depending upon their action on the proteins, protecting and denaturing. Protecting osmolytes have stabilizing effect on proteins [35], since they bind to the water around the protein surfaces and force protein folding by excluding water molecules from the protein backbone [33]. Different compounds, such as amino acids (AAs), methylamines, polyols and sugars are representants

of protecting osmolytes. On the other hand, denaturing osmolytes, such as urea or guanidine hydrochloride (GuHCl) [34], bind to the protein backbone and lead to protein unfolding [33]. The observation that protecting osmolytes can force protein to fold without binding to its backbone indicates that their addition to the solution might change the properties of solvent. In fact, it has been reported that these molecules change the water structure when in solutions [36-38]. Consequently, the water structure around the biomolecules is controlled by osmolytes. When added to ABS, they can increase the solute separation by exposing the hydrophobic groups on the biomolecule surface in similar way to electrolytes. In general, the partition of biomolecules using ABS in the presence of osmolytes is described as an effect of changes in the osmolyte-induced solvent properties of aqueous media in the coexisting phases and not as a direct osmolyte-biomolecule interactions [25-26].

2.1.2. The effect of electrolytes and osmolytes on phase equilibrium

In what concerns the effect of salt additives on the binodal curves of ABS, it was shown that the two-phase area is not significantly affected when compared to the ABS without additive. In general, the salt addition influences the binodal phase diagrams in a concentration dependent manner [39-40]. It was shown that very small concentrations of salt additive have no major effect on the shape and position of binodal curves and the higher the concentration, the more pronounced is the observed effect. Typically, biphasic region increases with the increasing concentration of salt additive. Moreover, the extent to which each additive enlarges the biphasic area depends on the nature of the cations and anions of the salt added [39-42], as well as on the nature of the compounds used to create ABS. In general, the salts additives composed of the cations and anions with strong salting out ability (e.g., NH_4^+, K^+ or SO_4^{2-}, HPO_4^{2-}) are capable to strongly induce phase formation.

Until now, the major focus of the researchers has been the effect of osmolytes on the partitioning behavior of different solutes in ABS and data on how these compounds affect phase separation is very scarce. The majority of the works in which binodal phase diagrams are presented, studies the effect of addition of urea [43-47] and guanidine hydrochloride [45, 48-49]. To the best of our knowledge there is only one work, which evaluates the effect of amino acids on the phase behavior of IL-salt ABS [50] and one study that evaluates the effect of glycine, betaine and trimethylamine N-oxide (TMAO) [47]. The obtained results showed that the addition of denaturing osmolytes (urea and GuHCl) to the polymer-salt or polymer-polymer ABS usually causes the reduction of the biphasic region and, consequently, increased the concentrations of polymer and salt required to promote phase splitting in comparison to the same systems without denaturing osmolytes. Furthermore, GuHCl has a larger influence on the binodal curves than urea, as it decreases the two-phase region to a greater extent [45]. Also, a shift of the binodal towards high concentrations of the phase forming compounds with the increased concentration of these osmolytes was observed. On the other hand, data reported for protecting osmolytes, such as AAs, indicate that when they are present in ABS, they enhance the ability of the liquid–liquid demixing and thus, the binodal curves become closer to the origin as the concentrations of AAs increase [50]. Similar observation was made in the work of Joshi et al. [47] where the addition of protecting osmolytes (glycine, betaine and TMAO) shifted the binodal curve towards the PEG axis, most probably due to an improved salting-out ability of the citrate-rich phase in presence of osmolytes. Furthermore, in this work the authors used conductivity measurements of the citrate-rich phases to determine the systems tie-lines and tie-lines slope, and compared the changes in the tie-lines slopes due to the addition of osmolytes to the tie-line slope in osmolyte-free ABS [47]. It was reported that betaine and TMAO protecting osmolytes decreased the tie-lines slope, while glycine had only slight negative impact as compared to

the osmolyte-free system [47]. Moreover, the addition of urea resulted in increase of the slope, but the changes in the final phase compositions were insignificant [47]. Overall, TMAO showed a maximum change in the tie-line slope followed by betaine, glycine, and urea [47].

2.2. Ionic liquids

Another strategy proposed in the formation of quaternary ABS is through the use of ILs as additives to polymer-polymer or polymer-salt ABS, where they act as adjuvants [27, 51] or electrolytes [52-53]. ILs are advanced and highly performant solvents, with unique properties such as negligible vapor pressure, low flammability, and tunable solvation ability. Along the years, they have shown to be feasible alternatives to polymer-rich phases [54-56] and to salt-rich phases [57-58]. One of the main advantages of ILs-based ABS is the tailoring of their phase polarities and affinities by a proper manipulation of the cation/anion chemical structure and their combinations [27, 59]. However, some ILs are toxic and expensive, and this is a major critical issue that have prevented the widespread use of these solvents in industry. The idea behind the use of ILs as additives in ABS comes from the desire to further exploit their remarkable properties shown in ABS creation as phase forming compounds while enhancing the biocompatibility, lowering cost and environmental impact of ILs-based systems. This crossover between conventional ABS and ILs-based ABS also allowed to overcome some limitations of polymer-based systems. In particular, polymer-based systems usually display low selectivity and polarity differences between the two phases, which greatly affects the purity of the desired product. However, with the wide range of ILs available and their designer solvent character, it is possible to fine tune the physicochemical properties of the polymer-rich phase for polymer-polymer and polymer-salt ABS, and by proper selection of the IL, the extractability and selectivity of a target biomolecule is greatly improved. The most employed ILs used as additives are imidazolium-based ILs [27, 51-53, 60-

69]. Furthermore, ammonium- [52, 61, 63-64, 66], phosphonium- [61, 64, 66], piperidinium- [60, 64, 66], pyridinium- [62], pyrrolidinium-based ILs [60, 62, 64, 66] or protic ILs [70] were also used. The chemical structures of the most commonly used in ABS IL cations and anions are depicted in Fig. 5.

2.2.1. The effect of ionic liquids on biomolecules partitioning

When ILs are added to ABS, they partition between the coexisting phases and, for the majority, a preferential partitioning to the polymer-rich phase was observed [27, 51, 60-61, 64, 66, 68]. Nevertheless, the migration of ILs to a particular phase of ABS depends on the affinity of ions for water, and the ILs presence in both phases changes their respective physical and chemical properties [71], thus regulating the extractability of ABS. In general, in such quaternary ABS, the specific interactions, in particular hydrogen bonds established between the biomolecules and ILs, play a crucial role in the biomolecules partitioning [27, 51, 60-66, 68]. These specific interactions between biomolecules and ILs are usually more important than the total amount of ILs present in each phase. Furthermore, besides hydrogen bonding interactions also the relative hydrophobicity/hydrophilicity of ILs is a crucial property to manipulate in order to improve system selectivity [63, 65, 67, 70]. Thus, as a rule of thumb we suggest that the selected ILs should have high hydrogen bond basicity (β) in order to maximize EE% of the systems. On the other hand, for improved selectivity also the hydrophobicity/hydrophilicity of ILs should be carefully chosen depending on the nature of target biomolecule and impurities.

2.2.2. The effect of ionic liquids on phase splitting behavior

Apart from the beneficial effect of ILs on the separation and purification performance of conventional ABS, they have also shown to affect the phase separation ability of the systems. ILs can either increase or decrease the two-phase region and there is no current

understanding on the driving forces of phase formation in such quaternary ABS. This is probably due to the lack of information of the compositions of the coexisting phases in quaternary ABS. However, many works state that the ILs cation and anion hydrophobicity, and thus their affinity for water, affects the phase separation [27, 72]. Consequently, the more hydrophobic ILs usually enhance the two-phase region. Nevertheless, some contradicting observations have also been made by in our work [62] and by Yang et al. [69]. In these studies, the opposite trend was observed and the increase in the ILs hydrophobicity, with increased IL cation alkyl chain length, enhanced the mutual solubility between the two phases of polyethylene glycol (PEG) 3350 + $(NH_4)_2SO_4$ ABS [62] and PEG (800, 1000, 2000) + Na_2SO_4 ABS (see Fig. 6 B)) [69]. These results oppose those published for PEG (400, 600) + Na_2SO_4 (see Fig. 6 A)) [27, 69], where the imidazolium-based IL with the longest alkyl chain length leads to an enlargement of the two-phase region. These observations clearly show that beside the ILs properties, also the properties of the phase forming compounds, such as the nature of salt cation and anion or PEG molar mass, should be taken into account when discussing the phase behavior of these quaternary systems [62]. Furthermore, in the systems composed of PEG-salt-IL-H_2O, ILs are enriched in PEG more hydrophobic phase (as discussed earlier), and thus the interactions between the PEG polymer and ILs also influence the phase separation behavior. Given these observations, it is possible to find the most adequate IL adjuvant to a polymer–salt ABS which will result in better performance in terms of phase separation, requiring lower amounts of each solute to form an ABS. In that way, comparatively cheaper and more benign IL–based ABS can be afforded due to lower amounts of IL used.

2.3. Deep eutectic solvents

The most recent compounds studied as phase splitters of ABS are DES. DES were introduced almost two decades ago (in 2003) by Abbott as liquid mixture of two or more

compounds resulting from the hydrogen bond interaction of a HBA and a HBD, typically both solids at room temperature [73]. Thus, this liquid mixture exhibits a significantly lower melting point compared to its pure compounds. When salts are used in the formation of DES, these solvents share with ILs some of the characteristic features such as high solvation ability for a large number of compounds [74], similar to that of ILs [75-76]. However, compared with ILs, DES have some advantageous characteristics such as their easier straightforward preparation, which consists on simply mixing and heating HBDs and HBAs [77]. In addition, the compounds typically used in preparation of DES are abundant, inexpensive, and very often come from natural sources. Furthermore, the large body of them can be considered as green solvents with low toxicity [78]. Fig. 7 summarizes typical combinations of HBAs and HBDs used in DES preparation.

Owning to these remarkable characteristics, DES were studied in highly diverse fields and different applications, including ABS implementation, and they are expected to be applied successfully in large-scale industrial production. The use of DES as phase forming compounds of ABS was proposed in 2014 by Zeng et al. [79] and in recent years more studies were dedicated to ABS in which DES is used as one of the ABS components. DES composed of different HBAs and HBDs were used in ABS formation and applied in extraction and purification of diverse biomolecules, beginning with simple solutes, such as amino acids, dyes, and ending with more complex molecules such as proteins, enzymes, nucleic acids or VLPs [80-81]. DES have shown to be feasible alternatives to polymer-rich [28-29], salt-rich [30-31, 79, 82-86] phases and can also be used in small quantities as adjuvants in conventional alcohol-salt [87] and polymer-salt [88] ABS. However, it should be remarked here, that although the first reports on ABS composed of DES considered them as a new type of ternary ABS, and a DES-rich and a salt-rich phase were considered to coexist [79, 84-86], in the following years it was shown that such ABS should be regarded as quaternary

systems. The reason of that is the solvation of DES components by water leads at large dilutions to the break of the hydrogen bond between HBA and HBD and the solvation of the two (or more) independent components [28-29]. As a result, when a DES is used in ABS formulation, we are in fact not dealing with one component aqueous solution but with two or more solutes depending on the type of DES used. It raises a question whether when dealing with such ABS they can be named as DES-based ABS because in high dilution ranges, the DES characteristics no longer exist. In such situation, DES should be considered as pseudo-component and according to principles for thermophysical and thermochemical property measurements proposed by Bazyleva et al. [89] in phase equilibrium experiments the components of pseudo-component are expected to be unevenly distributed between phases and thus pseudo-component should be considered as mixture. Consequently, in ABS formulation, DES components partition independently to both phases and one of them acts as an additive and another as phase forming component, this last one enables the manipulation of the equilibrium and the phases polarities [30-31], and consequently a conventional ternary ABS is recovered for high dilution of DES. If none of the DES components have phase splitting ability then the formation of ABS will not be possible. Nonetheless, in some cases the creation of pseudo-ternary DES-based ABS, where the initial molar ratio of DES HBA and HBD in both phases in equilibrium is maintained, was also reported [28, 82]. Consequently, in this review, DES are considered as "additives" and ABS composed of DES and another phase forming compound are regarded as quaternary systems.

2.3.1. The effect of deep eutectic solvents on biomolecules partitioning

All of the above-mentioned findings did not prevent the applicability of ABS composed of DES in the extraction and purification of biomolecules. It was observed that the partition of biomolecules is mainly driven by the hydrophobicity difference between the phases and

depends on the biomolecule and DES components nature. Furthermore, the concentration of the DES component which act as an additive influences the partitioning of biomolecules. In summary, DES-based ABS show high versatility in biomolecules separation and purification due to large number of possible HBAs and HBDs to be used in DES preparation. By the change of the nature and molar ratio between HBA and HBD of DES used in ABS formation, it is possible to tune the properties of the phases and separation of different biomolecules. Taking all of this into account, when developing new DES-based ABS, we suggest looking for a DES prepared from starting materials that are commonly used either as excipients or stabilizers of final bioproduct formulation. The use of such a DES in ABS, will most likely enable to achieve high EE%, and furthermore will also allow to avoid the tricky recovery of biomolecule from phase forming compounds. Moreover, further improvement of EE% can be achieved through manipulation of the ratio between HBA and HBD since the concentration of the DES component which act as additive has an impact on biomolecules extraction.

2.3.2. The effect of deep eutectic solvents on phase equilibrium

As discussed earlier, ABS composed of DES are quaternary systems, where usually HBD act as additive and therefore influence the phase properties and biomolecules partitioning. Consequently, in DES-based ABS the HBA and polymer or salt are responsible for the two-phase formation and the HBD may or may not impact the ABS formation, depending on its nature and concentration. For instance, Passos and co-workers studied the effect of the carboxylic acid nature and concentration on the formation of ABS composed of four DES (acetic acid:choline chloride (ChCl), glycolic acid:ChCl, lactic acid:ChCl, and citric acid:ChCl) and polypropylene glycol (PPG) [29]. In general, all DES decreased the binodal region compared to the systems composed with ChCl and PPG only. It was also stated that carboxylic acids with small alkyl side chain have a high liquid−liquid demixing ability [29].

Furthermore, when the carboxylic acid concentration was increased, the biphasic region was decreased. However, the representation of binodal curves as a function of the ChCl concentration revealed that binodal curves are very similar to that of the ChCl without carboxylic acid (see Fig. 8 A)). This indicates that carboxylic acids have only a minor effect on the ABS formation, which is mainly driven by salting-out ability of ChCl. Similar results were reported by Farias at al. for the ABS composed of ChCl:sugars + K_2HPO_4 + H_2O (see Fig. 8 B)), where sugars had also small effect on the formation of DES-salt ABS [31, 83]. However, the opposite results were obtained for ABS composed of ChCl:glucose + PPG + H_2O [28] and ChCl:alcohols + K_2HPO_4 + H_2O [83]. In these systems, the presence of glucose and most of the alcohols clearly enlarged the biphasic region in comparison with the system composed of ChCl alone. As the HBD concentration increased, a lower amount of HBA (ChCl) was needed to induce the phase separation (see Fig. 8 A) and 8 B)). In the case of ABS composed of ChCl:glucose + PPG + water, glucose is acting as a salting-out agent along with ChCl. Furthermore, the authors determined tie-lines and TLLs of such quaternary systems and it was observed that, independently of the HBA:HBD molar ratio used with increased TLL, there was a significant decrease of the amount of water and thus an increase of the ChCl and glucose concentration in ChCl-rich phase [28]. However, the composition of the PPG-rich phase, composed of more than 76% (w/w) and less than 2% (w/w) of ChCl and glucose, was not greatly affected by the change of the starting mixture point[28]. Furthermore, it was observed that the HBA:HBD initial mixture molar ratio was in close agreement with the ratio measured in ChCl-rich phases [28]. On the other hand, for PPG-rich phases, the initial molar ratio was not kept for higher HBA:HBD ratio, and when the molar ratio decreased better results were obtained [28]. On the other hand, in the systems composed of ChCl:alcohols + K_2HPO_4 + H_2O, the formation of an alcohol-rich top phase and salt-rich bottom phase was observed. The HBA (ChCl) in these systems acts as an

adjuvant and is enriched in bottom phase [30, 83]. The study on tie-lines and TLLs further revealed that the HBA:HBD molar ratio of the initial mixture was maintained in the top phase of ethanol- and 1,2-propanediol-based ABS, while in the bottom phase, due to the very low concentration of both HBA and HBD, the initial molar ratio was not kept [30]. Moreover, for glycerol-based ABS, due to a high hydrophilic character of glycerol and thus increased partitioning to the bottom phase, the HBA:HBD molar ratio changed in both phases for 1:1 and 1:2 mixtures [30]. However, at a molar ratio of 1:2 the stoichiometry was maintained in both phases [30]. Furthermore, in case of the n-propanol-based ABS, the HBA:HBD molar ratio in both phases was totally different of that in initial mixture [30]. All these results indicate that different HBDs lead to different phase equilibria, depending mostly on HBD nature and concentration. In summary, it can be concluded that the content and hydrophobicity of HBD, as well as HBA and HBD molecular weights determine phase splitting behavior [81].

3. Application of quaternary ABS in biomolecules separation and purification

Due to their advantageous characteristics, ABS have been seen as an alternative, cost-effective and efficient downstream processing technology, which is suitable for separation and purification of wide variety of biomolecules. Even though, commonly used systems composed of polymer-polymer, polymer-salt or ILs showed real advantages in extraction of biomolecules, very often they were not so effective in terms of bioproduct purity. Therefore, in order to exploit undeniable potential of conventional ABS as extraction platform and to further improve its selectivity, an approach in which the properties of the phase are manipulated through the addition of different additives into the system was proposed. In that way, quaternary ABS have shown real advantages both in phase separation

and purification of biomolecules thus attracting researcher's attention. The advantages of quaternary ABS over ternary can be especially seen in terms of selectivity and purity levels. However, it must be mentioned at this point that besides benefits in the systems extraction and purification efficiency, the addition of fourth compound has made the recovery of extracted biomolecules and recycling of phase forming compounds more difficult and complex. The works gathered from literature regarding different quaternary ABS are discussed below. In the following subsections, the extraction and purification capacity of ABS for several biomolecules is evaluated through the partition coefficients (K), extraction efficiencies (EE%) and selectivity (S) values. K is defined as:

$$K = \frac{C_T}{C_B} \quad (1)$$

where C_T and C_B are the concentrations of the biomolecule in the top and bottom phases, respectively.

The extraction efficiency (EE%) is defined according to:

$$EE\% = \frac{w^T_{biomolecule}}{w^T_{biomolecule} + w^B_{biomolecule}} \times 100 \quad (2)$$

where $w^T_{biomolecule}$ and $w^B_{biomolecule}$ are the total weight of biomolecule in the top phase and in the bottom phase, respectively.

The selectivity (S) of target biomolecules compared to the impurity is represented by equation (3):

$$S = \frac{K_{target}}{K_{impurity}} \quad (3)$$

Where K_{target} and $K_{impurity}$ are the partition coefficients of target biomolecule and major impurity, respectively.

In this section, only biomolecules relevant in food, feed, and pharmaceutical industries, such as amino acids, proteins, enzymes, monoclonal antibodies and virus or virus-like particles, will be revised. On top of that, while reviewing the most important achievements of quaternary ABS in high-value biomolecules partitioning, the focus will be put on quaternary-based systems formed with ILs and DES.

The major reason why ABS technology is widely studied as an alternative method for separation and purification of biomolecules is the fact that the production step in which the cellular product is processed to meet purity and quality requirements (downstream process) is very complex and constitutes the major bottleneck, being a substantial component of total manufacturing costs.

Moreover, most of the currently used methods for separation, concentration and purification of biomolecules have long processing times, difficulties in scaling-up, among others [90]. Thus, short introduction into the currently used methods in purification of each group of biomolecules will be provided and the importance of development of new separation and purification methodology for these biomolecules will be highlighted.

3.1. Amino acids

Amino acids (AAs) are a very important class of biomolecules as they play an important role in metabolism, gene expression, signal transduction, and in cellular and extracellular structures [91]. Thus, AAs are essential in animal and human nutrition and they are used in various applications, such as food additives, feed supplements, components of pharmaceuticals or sweet taste agents [92]. Furthermore, AAs are protein monomers, and their residues determine the surface properties of proteins. Nevertheless, before they can be used, AAs must be obtained in high purity levels. That is why downstream processing (DSP)

of AAs is a complex process and includes several steps, such as cells removal by centrifugation and filtration, ion exchange, chromatography, and crystallization [93-94]. Moreover, all these methods are very difficult to scale-up and expensive [95], and thus the cost related to the AAs downstream processing may account for up to 40% of total production costs [90]. Therefore, efficient and inexpensive methods for AAs separation, concentration and purification are in need, so that industrial and society requirements are met.

To overcome such limitations, research focused on ABS and the suitability of this methodology in extraction of AAs has been widely studied. Moreover, since the details obtained from the study of single AA partition behavior allow a good understanding of the driving forces for the partitioning of more complex proteins, AAs have been extensively used in ABS development as a model biomolecules [11]. The results found in the literature for the extraction and purification of AAs using quaternary ABS are presented in Table 2. For example, the effect of salts and osmolytes as additives on the dinitrophenylated amino acids (DNP-AAs) partitioning in PEG-salt and PEG-dextran ABS was studied by Zaslavsky's group [26, 96-99]. The authors found out that the presence of salts and osmolytes affects the properties of the coexisting phases, especially modifying their hydrophobic, electrostatic differences and the water structure. It was observed that K of AAs were affected by the presence of additives in a solute-specific manner. Furthermore, the changes in K of DNP-AAs in PEG-salt ABS significantly exceeded those observed for PEG-dextran ABS.

Quaternary ABS composed of ILs have been also employed in various AAs extraction. In the work of Pereira et al. [27], PEG 600-Na_2SO_4-H_2O ABS with various imidazolium-based ILs as adjuvants was used to study the partition of L-tryptophan. The studies revealed that salting-in inducing ILs increase the K of L-tryptophan to the PEG-rich phase and in the

485 system where [C_7H_7mim]Cl was added an increase in K_{Trp} from 20.54 to 42.47 was
486 obtained. On the other hand, the addition of small amounts of salting-out inducing ILs
487 decreased the partitioning of this AA. Overall, in this pioneering work was shown that by
488 adding adequate ILs as adjuvants to the PEG-salt ABS, the L-tryptophan partitioning
489 behavior can be controlled and manipulated. Afterwards, other AAs such as L-tyrosine [64,
490 66, 100] and L-phenylalanine [64, 66, 68] were also used as a model biomolecules to test
491 quaternary ABS composed of different salts, PEGs and ILs. In the works of Coutinho's
492 group, ABS composed of PEG 400 and weak (potassium citrate) [66] or strong (ammonium
493 sulfate) [64] salting-out salts with ILs as additives were studied. The partitioning studies
494 showed that AAs extraction in these systems is probably dominated by differences in
495 hydrophobicity between the phases. Moreover, the addition of 5% (w/w) of ILs had a small
496 influence on the AAs extraction. Nevertheless, the results obtained in these works indicate
497 that the intensity of the IL effect on the partitioning behavior is dependent on the chemical
498 nature of the salt and the IL used. The use of ILs as additives in conventional ABS can
499 modulate the extractability according to AAs hydrophobicity. Furthermore, the obtained
500 results suggest that the weaker salting-out agents allow the enhancement of the IL effect as
501 additive, not only in terms of tuning the hydrophobicity of the phases, but also by promoting
502 the occurrence of specific interactions between the ILs and the AAs [64, 66]. The thesis that
503 the chemical nature of the IL plays important role in AAs partitioning seems to be confirmed
504 in the work of Hamzehzadeh et al. [100], where an enhancement in the L-tyrosine extraction
505 into PEG-rich phase of the PEG 600-tripotassium citrate ABS with addition of [C_4mim]Br
506 was observed. This increase in K of L-tyrosine when IL was added has been assigned to
507 specific π···π interactions between IL and aromatic AA [100]. These results are in opposition
508 to what have been noticed for pyridinium and piperidinium ILs in the ABS composed of the

PEG 400 and potassium citrate buffer, where the decrease in the K of L-tyrosine when compared to the system without IL was observed [66].

Amino acids were also used to ascertain the potential applicability of DES-based ABS in separation and purification of biomolecules. The first study was conducted by Farias et al. [28] and the ABS composed of PPG 400 and ChCl:glucose DES was employed in extraction of L-tryptophan, L-phenylalanine and L-tyrosine. The authors showed that the K have a clear agreement with the octanol−water partition coefficients for all the AAs studied, with the exception of L-tryptophan, where specific interactions with ChCl were put forward as the possible extraction mechanism [28]. All AAs partitioned preferentially to the more hydrophilic ChCl-rich phase and EE% between 50% and ~90% were obtained. Furthermore, K increased with the increase of the TLL. This behavior is related with the increase of the amount of DES components in ChCl-rich phase, which results in a more hydrophilic character and consequently higher affinity of the AAs to this phase. On the other hand, the increase of HBD (glucose) concentration was found to have a small effect on the K and only a slight increase of K was observed, with L-tryptophan partitioning being the most affected [28]. Later, the same group studied the partitioning behavior of the same three AAs and glycine now using an ABS composed of ChCl:alcohols DES and K_2HPO_4 [30]. All the AAs, except for glycine, showed high affinity to the top phase, which is mainly composed of ChCl and alcohol. Furthermore, the K order agreement with the octanol−water partition coefficients was observed, with exception of the L-tryptophan, as in the previous work [28]. Moreover, the effect of the nature and concentration of the alcohol used as HBD on K is highly dependent on the AA relative hydrophilic/hydrophobic character. It was shown that the addition of ethanol results in only slight changes in the K [30], while di-alcohols, in particular ethylene glycol, caused a significant decrease in aromatic AAs extraction and the increase in aliphatic glycine partitioning to the top phase, due to

hydrophilic character of di-alcohols. Additionally, the system composed with ChCl:1,2-propanediol (1:1) DES was shown to be highly effective in the separation of aromatic AAs, such as L-tryptophan and L-tyrosine, and the selectivity ($S_{Trp/Tyr}$) of 30.9 was obtained, highlighting the potential of fine HBD tuning for better separation performance. More recently, Chao et al. [101] showed the potential of DES-salt ABS in extraction of L-tryptophan. The DES composed of ChCl and PEG 2000 (mass ratio of 2:3) was combined with different salts, namely $Na_3C_6H_5O_7$, Na_2CO_3, NaH_2PO_4, or K_2HPO_4 [101]. These systems were shown to be highly efficient in this AA extraction and the amount of DES, as well as the salting-out ability of the salt, were the main factors that affected the extraction. Overall, after optimization, EE% of 93.88%, 90.83%, 88.88% and 86.72% for ChCl:PEG 2000 (2:3)-K_2HPO_4, DES-NaH_2PO_4, DES-$Na_3C_6H_5O_7$ and DES-Na_2CO_3 ABS were obtained, respectively [101].

3.2. Enzymes

While most of the commercial applications of enzymes do not require high purity of the final bioproduct, for their usage in food, cosmetics or pharmaceutical industries, purified enzymes preparations are compulsory [102]. There are many conventional purification methods used for recovery and purification of enzymes. They include ammonium sulfate precipitation, ultrafiltration and different chromatography such as size-exclusion, ion exchange, hydrophobic interaction and affinity chromatography; or some combinations of these techniques [103]. However, all these methods do not meet the industrial requirements because they are time-consuming and expensive. For instance, ultrafiltration and hydrophobic interaction chromatography consist of several steps. Moreover, in affinity and ion exchange chromatography, which are commonly used in the purification of recombinant enzymes, a sample pretreatment step is required. As a result, at each step of these multi-step protocols, some quantity of the target enzyme is lost and, thus, low final recovery yields are

559 achieved. Since conventional liquid-liquid extraction based on organic solvents are
560 generally not suitable for the purification of enzymes, due to the irreversible loss of
561 enzymatic activity [104], the research focus has been ABS. ABS were proposed for
562 purification of enzymes as an alternative methodology able to overcome the before
563 mentioned limitations, while being cost-effective, efficient and capable of combining
564 several steps of different downstream processes into one-unit operation [104-106].

565 A summary of quaternary ABS found in the literature for the extraction of enzymes is
566 presented in Table 3.

567 The effect of different salts used as additives in ABS has been largely studied since salts
568 can modify the enzymes partition to one of the phases, changing purification factors and
569 recovery yields, without hampering their activity. For example, in the work of Ooi et al.
570 [107], the addition of 4.5% (w/v) of NaCl to 2-propanol-K_2HPO_4 ABS increased the lipase
571 recovery from 76% to 99% and purity from 6.4 to 13.5-fold in alcohol-rich phase. In another
572 study, conducted by Barbosa and co-workers, although the addition of NaCl did not
573 significantly improve K of lipase, the purification factor increased from 59.93 to 141.65
574 fold when 6% (w/v) NaCl was added to the PEG 8000-potassium phosphate ABS [108].
575 However, the highest concentration of NaCl added, decreased the K of lipase [108]. Similar
576 trend of improved recovery and/or purity was also observed for other enzymes. The
577 recovery of protease from *Calotropis procera* in PEG-rich phase was significantly
578 enhanced, from 23.58% to 107%, with addition of 6% (w/w) NaCl to PEG 4000-$MgSO_4$
579 [109]. Furthermore, increased recovery of invertase and increased purity in PEG-rich phase,
580 from 68% to 90% and 3.3 to 5.5-fold, respectively, were observed when 5% (w/w) KCl was
581 added to PEG 3000-Na_2SO_4 ABS [110]. The addition of 4.5% (w/w) NaCl also allowed to
582 obtained higher K (84.2), purification factor (14.37) and yield (97.3%) of serine protease
583 from mango peel in PEG-rich phase of PEG-dextran ABS[111]. In general, it is evident that

by adding neutral salts to the conventional ABS, improved extraction performance for the target enzymes is achieved. Moreover, very often the changes in phase properties induced by added salt may also result in increased enzyme recovery and purification factor.

The strategy of using ILs as adjuvants in ABS for improved purification of enzymes was also ascertained for lipase [67] and L-asparaginase [65]. The effect of 5% (w/w) of imidazolium-based ILs on lipase from *Bacillus* sp. ITP-001 purification in ABS composed of different PEGs and K_2HPO_4/KH_2PO_4 was first studied. After the optimization procedure, in the PEG 1500-K_2HPO_4/KH_2PO_4 ABS, with [C_6mim]Cl added, the purification factor increased from 175.61 (no IL added) to 245 [67]. This high increase in purity was a result of the favorable interactions between the IL and the contaminant proteins. The presence of [C_6mim]Cl greatly improved the partition of contaminant proteins to the PEG-rich phase, also enriched in IL, while the lipase remained in the salt-rich phase. On the other hand, the favorable effect of ILs on L-asparaginase purification performance of PEG-citrate buffer ABS was also observed by Santos et al. [65]. High recoveries of 87.94%, purification factors of 20.09 and a final specific activity of 3.61 U/mg were observed for ABS composed of PEG 6000 and citrate buffer with 5% (w/w) of [C_4mim][CH_3SO_3] [65]. Due to high affinity of L-asparaginase to the PEG-rich phase, ILs that display low polarity and hydrogen bond basicity, such as [C_4mim][CH_3SO_3] and [C_4mim][CF_3SO_3], were not capable to establish strong specific interactions with the contaminant proteins and, as a consequence, to improve the partitioning of these proteins in salt-rich phase. Furthermore, ILs with high hydrogen bond basicity decreased the L-asparaginase purification factor since they promoted the specific polar interactions between contaminant proteins and ILs in the top phase. The results obtained in those works, indicate that by proper selection of the IL anion it is possible to manipulate the partitioning behavior of contaminant proteins and thus allowing to increase the purification performance of the PEG-salt ABS for two different enzymes.

DES-based quaternary ABS were used, for the first time, in extraction of a model enzyme (trypsin) in the work conducted by Zeng and co-workers [79]. In this study, DES composed of ChCl: urea (1:2) ChCl:methylurea (1:2), [N_{1111}]Cl:urea (1:2) and [N_{3333}]Br:urea (1:2) were used to create ABS with K_2HPO_4 salt. The highest EE% in DES enriched phase of 95.53% was obtained in the system containing ChCl:urea DES. Moreover, slightly lower EE% (95.27%) was achieved using ChCl:methylurea DES, suggesting that the nature of the HBD does not have a major impact on the trypsin partitioning [79]. On the other hand, HBA nature seems to play a crucial role in the extraction of the trypsin since when [N_{1111}]Cl and [N_{3333}]Br were used, a significant decrease in EE% was registered, 81.31% and 36.87%, respectively [79]. Trypsin was also used while evaluating the extraction potential of betaine-based DES ABS [112]. Contrary to the previous work [79], it was shown that the HBD nature greatly affects the EE% of trypsin. Finally, a high EE% of 94.36% in DES enriched phase was achieved for trypsin in ABS composed of ChCl:glycerol and K_2HPO_4 [113]. Moreover, the extraction of papain was studied using ABS composed of ChCl:PEG 2000 DES and NaH_2PO_4, Na_2CO_3, $Na_3C_6H_5O_7$ salts [114]. The papain EE% in DES-enriched phase decreased according to the order of phase forming ability of the salt: Na_2CO_3 > $Na_3C_6H_5O_7$ > NaH_2PO_4. The molar ratio between ChCl and PEG 2000 was shown to highly affect the enzyme partitioning. It was observed that using the ChCl:PEG 2000 (20:1), the EE% increased from 54.42% (molar ratio (1:1)) to 83.50% [114] and after optimization 90.95% efficiency was attained [114]. Furthermore, in our work the extraction of pepsin using DES-based quaternary ABS was studied [115]. We used ABS composed of PPG 425 and DES prepared with betaine hydrochloride (BeHCl) as HBA and fructose, glucose, sucrose, urea as HBDs. Although pepsin extraction studies showed a high affinity of this enzyme to the BeHCl/DES-rich phase, the ternary ABS composed of BeHCl and PPG 425 was the best system in terms of pepsin EE%, with 99.8% [115]. However, when glucose was used as HBD,

an increased recovery activity of 141.9% and EE% of 99.5% were attained [115]. Therefore, the presence of adequate HBD might lead to specific interactions between an enzyme and HBD that yield an increased activity of pepsin.

3.3. Monoclonal antibodies

Antibodies are glycoproteins present in plasma and extracellular fluids that have binding specificity for particular antigens [116]. Nowadays, antibody-based therapies play an important role in the treatment of many infectious diseases, autoimmune disorders, and cancers. Therefore, the demand for antibodies is constantly increasing. However, to use antibodies as therapeutic agents, their production has to meet high safety standards and high levels of purity in the final product [117]. Although, improvements have been made in both upstream and downstream processes of antibodies, the high cost of the currently used purification methods is still the major bottleneck that has been preventing the widespread use of these biopharmaceuticals. Typical downstream processing of antibodies is composed of several steps, which include (i) clarification by removal of cells and cell debris by centrifugation or microfiltration, (ii) concentration by ultrafiltration, (iii) purification by chromatography, (iv) virus inactivation and removal and (v) validation and quality control tests [118]. Furthermore, the purification step can account for up to 90% of the total downstream costs and the whole downstream processing is responsible for 50-70% of total production costs [90]. The major reason for that is the fact that in the purification step very expensive chromatographic methods are usually used, which are also very difficult to scale-up. Thus, in order to solve these shortcomings, the separation and purification using ABS has been proposed. However, the applicability of ABS in downstream processing of antibodies so far has been limited to the academic studies only [24].

The results found in literature for the extraction and purification of monoclonal antibodies using quaternary ABS are presented in Table 4.

The first report, in which the use of salt as additive to ABS for purification of antibodies, dates back to 1996 [119]. In this work, Andrews and co-workers employed PEG 1450-phosphate system for the purification of murine immunoglobulin G (IgG) from a hybridoma supernatant. The developed strategy consisted of two steps - extraction and back extraction. In the extraction step, the addition of 12% (w/w) NaCl promoted the antibody partitioning to the PEG-rich phase, while the impurities preferentially concentrated in the salt-rich phase [119]. The antibodies were then successfully back-extracted using the same ABS but formed with fresh phosphate solution without NaCl. Overall, at the end of the process, IgG purity of 80%, 5.9 purification fold and 100% recovery, were achieved [119]. Over a decade later, Aires-Barros group brought back the idea of using ABS with NaCl as additive for the purification of antibodies in a series of articles [24, 117-118, 120-123]. At first, the authors used PEG–phosphate systems for the purification of antibodies from an artificial mixture of proteins composed of human serum albumin and myoglobin [118]. The same trend of increased antibodies partitioning towards the PEG-rich phase, decreasing the impurities with increased NaCl concentrations was observed, corroborating Andrews et al. results [119]. The highest purification was obtained using the ABS composed of PEG 3350, phosphate at pH 6 and 15% (w/w) NaCl with a recovery yield of 101%, a purity of 99% and a yield of native IgG of 97% [118]. Later on, the same strategy was applied in purification of antibodies from real matrices, such as Chinese hamster ovary (CHO) and hybridoma cell supernatants [117]. Once again, it was shown that high concentrations of NaCl maximize the partition of IgG into the top PEG-rich phase of the PEG 6000-phosphate buffer pH 6 ABS, while the impurities were mostly found in the bottom phase [117]. As a result, recovery yield of 88% and 90% in a PEG-rich phase and a purification factor of 4.3 and 4.1 for IgG from CHO

and a hybridoma cell culture supernatants were obtained, respectively [117]. Later, the phosphate salt was replaced by the biodegradable citrate salt in order to decrease the environmental impact of these ABS. As observed for PEG–phosphate systems, an increase in the concentration of NaCl favored the IgG partitioning to the top phase [120] and, by changing the concentration of NaCl, it was also possible to manipulate the IgG partitioning behavior towards the phase with fewer impurities, also in the PEG–citrate ABS. Moreover, in the same year (2009), the same group proposed a multi-stage approach using PEG-phosphate ABS containing 10% (w/w) NaCl ABS [24]. The authors showed that, by using multi-stage extraction, higher IgG recovery and purity than in single-stage experiment can be obtained, as 89 % of IgG with 75% purity was recovered in PEG-rich multi-stage extraction, while only 61% recovery and 55% purity were achieved in single-stage extraction [24]. In the following years, Aires-Barros's group focused on the possibility of integration of ABS extraction with NaCl as additive in downstream processing of antibodies. The continuous extraction using packed differential contactor was evaluated in the purification of human IgG from CHO cells supernatant, using the PEG 3350-phosphate-NaCl ABS [121]. The PEG-rich phase was continuously dispersed at the bottom of the column through a capillar and the phosphate-rich phase was continuously fed at the top of the column [121]. In this continuous ABS extraction set-up, an improved IgG recovery yield of 85% and a purification factor of 1.84 were obtained compared to the batch extraction, where recovery yield of 61% and purification factor of 1.59 were observed [121]. Moreover, the continuous-flow process in microfluidic device for the extraction of mAbs in the ABS composed of PEG-phosphate with NaCl as additive was also proposed by Silva et al. [123]. A fluorescently tagged IgG was used in the partition studies and the results obtained in this microscale were found to be in agreement with those obtained in batch laboratory scale, while reducing the operation time and allowing the continuous monitoring of the separation

process [123]. Finally, an attempt to replace the low capacity, difficult to scale-up and expensive chromatography by a continuous ABS extraction process, has been made by Rosa et al. [122]. In this study, a continuous process incorporating three different steps (extraction, back-extraction, and washing) was set up and validated in a pump mixer-settler battery. ABS composed of PEG 3350-phosphate buffer at pH 6 with NaCl was used and the IgG from CHO and PER.C6® cell supernatant was purified. The removal of the high molecular weight impurities was observed in the extraction step and the back-extraction and washing allowed further IgG purification and separation from the lower molecular weight impurities and polymer-rich phase, as well as the PEG recycling [122]. Overall, IgG recovery yield of 80% and a final total purity of 97% from CHO supernatants was achieved. Furthermore, 100% recovery yield with a promising host cell protein/IgG ratio was observed for IgG purification from PER.C6® cell supernatant [122]. All these studies clearly show that quaternary ABS, composed of PEG, citrate salt and NaCl as fourth component, constitute an economical and benign alternative methodology for the purification of monoclonal antibodies.

In another vein, polymer-salt ABS, this time using ILs as adjuvants, were also evaluated in extraction and purification of IgG from rabbit serum samples [61]. After optimization, the ABS composed of PEG 400 and citrate buffer at pH 7 was selected and 5% (w/w) of different ILs added. Imidazolium-based, quaternary ammonium and phosphonium ILs were selected allowing the evaluation of the IL cation and anion nature effects, as well as the effect of increase of the alkyl side chain length. The addition of the 5% (w/w) of ILs that combined imidazolium cation and $[CH_3CO_2]^-$, Cl^-, and $[TOS]^-$ anions resulted in an increase IgG EE% from 96% (with no IL added) to 100% in a single-step [61]. It was concluded that specific hydrogen-bonding and $\pi\cdots\pi$ interactions (in the case of $[C_4mim][TOS]$) play a crucial role in the improved IgG extraction to the PEG-rich phase. The ILs with anions with

a higher hydrogen bond basicity were able to induce complete extraction of IgG, while this was not attained with the ILs that display lower hydrogen bond basicity, such as [C_4mim]Br and [C_4mim][$N(CN)_2$]. Furthermore, quaternary ammonium- and quaternary phosphonium-based ILs also led to the complete extraction of IgG to the polymer-rich phase and no major differences on the IgG partitioning as a function of the alkyl chains length of these ILs was observed [61]. The same observation was made for the alkyl side chain length effect of imidazolium-based IL, thus indicating that no significant interactions between the IL cations and the protein surface take place in these systems [61]. Moreover, the advantageous performance of the quaternary ABS with ILs was also confirmed in IgG purification from rabbit serum samples, and the complete extraction and an enhancement of ca. 37% in the IgG purity was obtained by the use of [C_4mim][CH_3CO_2] as adjuvant [61]. This work shows that the addition of small quantities of ILs to the polymer-salt ABS constitute a viable and scalable strategy to extract and purify antibodies from real serum samples to be used as therapeutic agents. Freire's group also studied the effect of addition of chloride-based ILs to PEG 6000-dextran ABS on partitioning of IgG [124]. It was revealed that the partition coefficients increased in following order: [P_{4444}]Cl < no IL ≈ [C_4mim]Cl ≈ [C_4mpyr]Cl < [N_{4444}]Cl < [C_4mpip]Cl, and that in all systems IgG preferred dextran-rich phase (K < 1) [124]. Moreover, 5% (w/w) of [C_4mpip]Cl and [N_{4444}]Cl and 10% (w/w) of [C_4mpip]Cl and [C_4mim]Cl favored IgG migration to the PEG-rich phase mainly as an effect of IL-IgG interactions depending on the chemical structure of the IL [124]. Furthermore, improved selectivity between Cyt C and IgG was obtained with the addition of 5% (w/w) of [C_4mpyr]Cl, [N_{4444}]Cl, and [P_{4444}]Cl compared to the ABS without IL [124]. Also, higher selectivity values between BSA and IgG, compared with the ABS without IL, were observed using 5 % (w/w) of [C_4mim]Cl and [C_4mpip]Cl [124]. Overall, these results

further highlight that using ILs as adjuvants constitute viable approach to improve extraction and purification of antibodies.

Even though the high potential of quaternary ABS in extraction and purification of antibodies have been described using salts and ILs additives, at the moment there is no reports evaluating the use of DES-based ABS in the separation and purification of these therapeutic agents. The reason is most probably the fact that DES as phase former compounds of ABS were introduced only in 2014 and consequently only the extraction of model biomolecules such as amino acids, proteins, alkaloids, among others, were studied. Their true potential in purification of monoclonal antibodies still remains to be proved.

3.4. Proteins

Proteins are larger biomolecules that consist of long chains of amino acid residues. They are present in all living organisms, where they play a vast array of functions. Therefore, proteins have been applied in various industries, such as in food, feed and pharmaceuticals sectors [125]. Downstream processing of proteins consists of several steps and various unit operations due to complexity of starting material [126]. In order to meet the regulatory requirements for purity and quality of proteins for pharmaceutical or food applications, both low- and high-resolution technologies are used. They include tangential flow filtration, ultra/diafiltration and different chromatography methods (IEC, SEC, HIC, RP, multimodal and affinity). Furthermore, mild operation conditions for their recovery and purification are necessary because proteins can be very easily denatured and lose their native structure and function [125]. Chromatography of proteins is very often considered as not scalable and an expensive methodology and, in the large scale, some difficulties such as slow protein diffusion or discontinuity in the process are observed [126]. Moreover, the high cost of protein bioseparation continues to remain a major drawback in manufacturing of proteins, with up

to 80% of total bioprocessing costs for plasma proteins [90]. Therefore, the development of cost-effective and high yielding purification methods for proteins is still an unmet challenge. To overcome these shortcomings, research has been focused on ABS and the quaternary ABS formed with salts, osmolytes, ILs or DES have been shown to be beneficial in the separation of proteins.

A summary of results found in literature for the extraction and purification of proteins using quaternary ABS are presented in Table 5.

The effect of addition of different salts to PEG-dextran and PEG-phosphate ABS was studied by Cascone et al. [127] for the partitioning and purification of thaumatin. Thaumatin is a protein sweetener used as flavor and aroma enhancer [128]. The obtained results showed that, using NaCl in PEG 6000-phosphate ABS, K of thaumatin significantly increased from 0.53 to 33 when 1.5 M of NaCl was added to the system [127]. Moreover, this effect was more pronounced in PEG-phosphate ABS than in PEG-dextran systems. Much lower improvements in the K were found for other salts, such as $(NH_4)_2SO_4$ and $NaClO_4$ [127]. Therefore, the purification of thaumatin from *E. coli* homogenate proteins and BSA was conducted using PEG-phosphate-NaCl ABS. It was shown that K of homogenate proteins and BSA decreases with the addition of NaCl to the systems and the observations made using individual proteins were maintained for the mixture of thaumatin and *E. coli* homogenate proteins, with 90-95% recovery yield and a 20-fold purification in one step [127]. The significant increase of the K by the addition of NaCl to PEG-salt systems seems to significantly affect the protein's hydrophobicity [32]. The authors observed that the addition of NaCl to PEG-phosphate ABS increases the hydrophobic difference between the phases and promotes hydrophobic interaction between the proteins (BSA, lysozyme, conalbumin, α-lactalbumin and α-lactoglobulin A) and PEG [32]. This fact was further confirmed in the work of Franco et al. [52], where two different series of hydrophobically

805 modified proteins were partitioned in PEG-phosphate ABS with addition of NaCl [129]. The
806 authors observed an increase in separation capacity of these systems, when compared with
807 the same systems without NaCl [129]. Moreover, Fan and Glatz reported that T4 lysozyme
808 partitioning in PEG-dextran ABS with salt additives shifts more protein from the bottom to
809 top phase. The authors also observed that the different concentrations of salt also influence
810 electrostatic and non-electrostatic interactions [130]. Furthermore, the addition of different
811 concentrations of NaCl (0.0-1.0 M) increased the K of myoglobin and ovalbumin from 4.20
812 to 15.77 and 2.82 to 5.51, respectively, in the PEG 4000- polyacrylic acid (PAA) ABS [20].

813 In similar context, Zaslavsky's group conducted several studies on the effect of osmolytes
814 on the partitioning of 11 different proteins, namely trypsinogen, α-chymotrypsinogen A,
815 ribonuclease A, ribonuclease B, β-lactoglobulin A, β-lactoglobulin B, papain,
816 chymotrypsin, lysozyme, hemoglobin, and concanavalin in PEG-dextran and PEG-
817 phosphate buffer (0.01 M phosphate buffer, pH 7.4) ABS [25, 99, 131]. They used sorbitol,
818 sucrose, trehalose, and TMAO as additives. It was shown that differences in the K for
819 proteins in the presence of 0.5 M of different osmolytes were exclusively related to solvent
820 properties of the coexisting phase, with no direct interaction between the osmolytes and the
821 proteins [25]. The authors also concluded that proteins responses to the presence of different
822 osmolytes in ABS are governed by the proteins structures and that these effects are less
823 pronounced than those observed in the presence of salts additives [99].

824 The first reports on the use of ILs as additives to the polymer-polymer or polymer-salt ABS
825 date to 2015 [52, 132]. In the work of Santos et al. [52] the ABS composed of PEG 8000 and
826 sodium polyacrylate (NaPA) 8000 with 5% (w/w) ILs was used in extraction of cytochrome
827 c (Cyt c). Several ILs comprising distinct cations and anions, namely imidazolium-based
828 and ammonium-based ILs, were selected. It was seen that Cyt c preferentially partitioned
829 to the bottom, NaPA 8000-rich phase, and the EE% were in most cases higher than those

obtained by the addition of NaCl or Na_2SO_4 and complete extraction of Cyt c was observed. The partition behavior of Cyt c was attributed to the electrostatic interactions between the negatively charged protein and NaPA 8000, which were further increased by additional Cyt c–ILs interactions [52]. In the same year, the partitioning of BSA, lysozyme and myoglobin within PEG 600-potassium phosphate buffer ABS with 2.5, 5 or 7% (w/w) of [C_2mim]Cl and [C_4mim]Cl was investigated [132]. For both ILs used, the K of the proteins increased with the increase of the IL concentration added to the system when compared to the ABS without IL. Moreover, higher K were obtained in the systems containing [C_4mim]Cl than with [C_2mim]Cl, accordingly to the amount of these ILs found in the PEG-rich phase [132]. The authors concluded that the IL decreases the hydrophobic nature of the PEG-rich and therefore enhances the extraction of proteins to this phase [132]. The idea of using ILs as adjuvants in PEG-salt ABS for protein extraction was revisited in the work of our group in 2019, in which the myoglobin partitioning behavior was studied [62]. In our study, the ABS composed of PEG 3350 and $(NH_4)_2SO_4$ was used and the effect of several imidazolium-, pyridinium- and pyrrolidinium-based ILs was evaluated. It was shown that myoglobin EE% and K increased with the IL hydrogen bond basicity (β) and thus, the IL with highest hydrogen bond accepting character, [C_4mim][CH_3CO_2] changed the myoglobin partitioning preferences from salt-rich to the PEG-rich phase [62]. Moreover, the increase of concentration of this IL from 5 to only 7.5% (w/w) allowed to obtain 100% EE% in the PEG-rich phase. Overall, in this work we showed that by appropriate choice of IL it is possible to tune properties of the PEG phase and use PEG-salt-IL-H_2O quaternary ABS in either forward or back-extraction of myoglobin. In the most recent work, ILs were used as adjuvants in polymer-polymer ABS and the extractability of BSA and Cyt c tested [124]. The authors showed that the addition of chloride-based ILs as adjuvants resulted in either an increase or a decrease of protein´s K, meaning that it was possible to tailor the proteins

partitioning between the phases of PEG-dextran ABS [124]. The partitioning experiments showed that in all systems BSA preferentially partitioned to the dextran-rich phase (the most hydrophilic phase in the investigated systems) and that the addition of ILs always increased the protein partitioning to the PEG-rich phase [124]. Furthermore, it was concluded that BSA partitioning essentially depends on the IL content in each phase [124]. On the other hand, Cyt c showed a K close to unity, meaning that there was no preference to any of the phases. However, the addition of 5% (w/w) of $[C_4mim]Cl$, $[C_4mpyr]Cl$, $[P_{4444}]Cl$, and $[N_{4444}]Cl$ increased K values thus Cyt c was more prone to migrate to the PEG-rich phase [124]. Overall, partitioning of Cyt c was ruled by the ILs chemical structure and established interactions [124].

The applicability of DES-based ABS in protein extraction was studied for the first time in the pioneering work regarding the use of DES as phase forming compounds of ABS [79]. The authors evaluated the performance of four DES (ChCl:urea (1:2), $[N_{1111}]Cl$:urea (1:2), $[N_{3333}]Br$:urea (1:2) and ChCl:methylurea (1:2)) with K_2HPO_4 to yield ABS and these systems were used in BSA extraction [79]. In all these systems, the protein preferentially partitioned to the top, enriched in DES components, phase. The obtained EE% ranged from 26.92 to 99.94% for $[N_{3333}]Br$:urea (1:2) and ChCl:urea (1:2), respectively. These results indicate that the nature of the HBA in the DES have a significant impact on the BSA extraction. Furthermore, comparing the results obtained for the ChCl:urea and ChCl:methylurea (EE% of 34.39%) it can be also concluded that the influence of HBD cannot be discarded. The authors further used the ChCl:urea-based ABS in the extraction optimization procedure and the 100% EE% was reached [79]. It was stated that hydrophobic interactions, hydrogen bonding interactions and the salting-out effect played important roles in the BSA partitioning [79]. Later, Li et al. [112] prepared six DES using betaine as HBA, different HBDs (e.g. urea, methylurea, glucose, glycerol, sorbitol, ethylene glycol) and

880 water and combined them with K_2HPO_4 to form ABS. The developed systems were then
881 applied in extraction of BSA and ovalbumin. The obtained results showed that DES formed
882 with different HBDs have distinct capabilities for extraction of these proteins. Much higher
883 EE% were achieved for BSA than ovalbumin, in DES enriched phase [112]. The best system
884 in extraction of BSA was composed with betaine:urea:H_2O (1:2:1) DES, achieving 93.95%
885 efficiency and 99.82% after optimization [112]. On the other hand, for ovalbumin, the highest
886 EE% of only around 60% was obtained with betaine:ethylene glycol:H_2O (1:2:1) DES [112].
887 In another report, ChCl:PEG 2000 DES was used to form ABS with three different salts:
888 NaH_2PO_4, Na_2CO_3, $Na_3C_6H_5O_7$, and these systems were applied in BSA partitioning [114].
889 The authors showed that the EE% decreased, following the order of phase forming ability
890 of the salt: Na_2CO_3 > $Na_3C_6H_5O_7$ > NaH_2PO_4. Furthermore, it was shown that by changing
891 the ratio of ChCl and PEG 2000 it is possible to improve the EE% from 54.42% to 83.50%
892 for (1:1) and (20:1) molar ratios, respectively [114]. Overall, using the systems composed of
893 ChCl:PEG (20:1) and Na_2CO_3, a BSA EE% of 95.16% was obtained, after optimization of
894 extraction conditions (protein concentration, temperature, DES and salt amount, and pH of
895 the system) [114]. Furthermore, DES-K_2HPO_4 ABS composed of binary ([N_{1111}]Cl as HBA
896 and urea, glycerol, ethylene glycol, glucose as HBDs) and ternary DES ([N_{1111}]Cl as HBA,
897 glycerol as HBD combined with different HBDs as urea, ethylene glycol, glucose, sorbitol)
898 were used in the extraction of BSA, lysozyme and Cyt c [133]. The authors showed that the
899 ternary DES have better extraction capability for the studied proteins than the binary DES.
900 For instance, in the system composed of [N_{1111}]Cl:glycerol (1:2) DES, lower EE% were
901 obtained than for the four ternary DES, in which glycerol was one of the two HBDs [133].
902 Moreover, [N_{1111}]Cl:glycerol:ethylene glycol (1:1:1) and [N_{1111}]Cl:glycerol:glucose (2:2:1)
903 ternary DES were more advantageous for the extraction of studied proteins than
904 [N_{1111}]Cl:ethylene glycol (1:2) and [N_{1111}]Cl:glucose (1:1) [133]. The ABS formed with

[N_{1111}]Cl:urea (1:2) and [N_{1111}]Cl:glycerol:urea (1:1:1) were further investigated in BSA extraction optimization procedure and EE% in DES enriched phase of up to 99.31% and 98.95% were obtained, respectively [133]. In another study, four ChCl-based DES, namely ChCl:ethylene glycol (1:2), ChCl:glycerol (1:1), ChCl:glucose (2:1), ChCl:sorbitol (1:1) were used to prepare ABS with K_2HPO_4 and the extraction properties of these systems were evaluated using BSA as a model protein [113]. It was reported that ChCl:glycerol DES was the most suitable extraction solvent and after optimization procedure (the amount of DES, the concentration of salt, the mass of protein, the shaking time, the temperature and pH value) 98.16% BSA EE% was obtained in DES enrich phase in a single-step extraction [113]. Furthermore, it was showed that BSA kept its conformation after the extraction process and that the formation of DES–protein aggregates played a crucial role in the extraction mechanism [113]. Furthermore, the extraction potential of ABS formed with [N_{4444}]Br:glycolic acid (1:1) DES and Na_2SO_4 was studied using lysozyme as a model protein [134]. It was found out that more than 98% of protein was extracted to the DES enriched phase at the optimal conditions [134]. Moreover, the biological activity studies revealed that after the extraction lysozyme still retained 91.73% of initial activity [134]. In general, all these works showed that DES-based ABS have a great potential in extraction of different proteins and by using them we can take advantage of their tunability and prepare green, task-specific extractants with the desirable physicochemical properties.

3.5. Virus and virus-like particles

Virus and virus-like particles (VLPs) are biological therapeutic molecules used in various medical, analytical and scientific applications [135]. In particular, a major growth of interest in viral particles use in biomedical applications, such as vaccination, cancer therapy or as delivery vectors for gene therapy, has been observed [135]. For their use as biomedical agents, highly efficient and effective production (upstream process) and purification (downstream

process) process that will meet all regulatory requirements are essential. In the last decades, the upstream processes of virus and VLPs have been optimized and high yields and harvest volumes are currently obtained [135]. On the other hand, the downstream processing step, where the recovery and purification of the virus or VLPs is achieved, still constitutes the major drawback in overall productivity and cost of the manufacturing process [135]. The downstream processing of VLPs usually consists of different unit operations, essentially due to the complex structure and properties of these bioparticles and also to wide range of impurities generated in the upstream process [135]. The standard purification process of virus and VLPs is composed of several steps, which include (i) clarification with the removal of producer cells, cell debris and large aggregates by low speed centrifugation or microfiltration; (ii) concentration by centrifugation, ultrafiltration or precipitation and flocculation; (iii) purification and polishing by density gradient ultrafiltration or chromatography [135]. However, all these methods do not satisfy the economic requirements because they are time consuming, yield low product recovery, and are very difficult to scale-up [136]. For example, density gradient ultracentrifugation provides low yields, some impurities are still retained, and the implementation of the process is very difficult due to problems with scaling up [137-138]. The precipitation techniques possess low selectivity toward viral particles [139], and in ultra- or microfiltration membrane clogging occurs and, thus, large impurities are also often retained and co-concentrated [140]. Consequently, to reduce these constraints, ABS have been successfully employed in the recovery of virus and VLPs as a promising alternative technique able to operate in a continuous mode.

A summary of results found in literature for the extraction and purification of VLPs using quaternary ABS are presented in Table 6.

The addition of neutral salts to ABS composed of PEG and salt for purification of recombinant VLPs from yeast cells homogenate was first evaluated in 1995 by Andrews et

al. [141]. In this work, cell debris removal was achieved using ABS composed of PEG 400 or 600 and $(NH_4)_2SO_4$, while the separation of VLPs from contaminant proteins was obtained employing PEG 4000 or 8000 and $(NH_4)_2SO_4$ ABS with NaCl or phosphate as additive. The authors showed that the addition of the salts had a significant impact on the effectiveness of these systems [141]. In another report, parvovirus B19 VLPs were successfully recovered from a clarified cell disruptate by interfacial partition using ABS composed of PEG 1000, magnesium sulfate and 800 mM NaCl as additive [142]. It was shown that majority of B19 VLPs preferentially partitioned to the interface, while the non-assembled VP2 proteins and host cell proteins migrated to either top or bottom phase. In general, 95.3% and 33.2% recovery yields of VP1 and VP2 proteins were obtained, respectively [142]. Purification of human B19 parvo-VLPs derived from *Spodoptera frugiperda Sf9* insect cells, using single- or multi-stage ABS extraction have been also reported [143]. The addition of NaCl to the PEG 400-phosphate buffer pH 8.5 ABS resulted in increased interfacial partitioning and precipitation of the VP2-VLPs. However, only about 20% of the viral particles were affected, while the rest VLPs were recovered in the top phase. Remarkably, a significant change in partitioning behavior of DNA from top phase to bottom phase was observed upon addition of NaCl [143]. Furthermore, the addition of NaCl slightly decreased the amount of contaminant proteins in the top phase. Overall, with addition of 7.5% (w/w) NaCl, a removal of up to 99% of DNA was achieved in one step and high selectivities were obtained for both single- and multi-stage ABS extraction [143]. Furthermore, Jacinto et al. [18] evaluated how addition of different concentrations of NaCl affected K of Human Immunodeficiency Virus (HIV) VLPs from CHO supernatants in PEG-dextran and PEG-salt ABS. This was the first report in which enveloped VLPs were purified using ABS. However, no significant improvements of K of HIV-VLPs were observed with the addition of NaCl [18].

Osmolytes were also used as additives to enhance the purification of both virus (porcine parvovirus, PPV) and VLPs (HIV-VLPs) in a work of Heldt's group [47]. The authors used ABS composed of PEG with an average molecular weight of 12,000 Da and citrate buffer and added 0.5M of glycine, betaine, TMAO or urea [47]. It was reported that both glycine and betaine were able to increase the salting-out ability of the citrate-rich phase and the hydrophobicity of the PEG-rich phase, resulting in improved partitioning to the PEG-rich phase of PPV and HIV-VLPs at conditions, where the systems without osmolytes were insufficient to induce preferential partitioning [47]. Furthermore, it was observed that TMAO and urea were not capable to improve the virus and VLPs recovery [47]. Overall, recoveries of 100% for infectious PPV and 92% for the HIV-VLPs, with high removal of the contaminant proteins and more than 60% DNA removal when glycine was added were obtained [47]. Consequently, the following order of osmolytes efficiency to improve virus and VLPs purification was deducted: glycine > betaine > TMAO > urea [47]. The authors concluded that high recovery and purity of viral modalities in the PEG-rich phase in the presence of osmolytes was a result of the higher interfacial interactions for comparatively hydrophobic and rigid viruses compared to the intramolecular interactions of flexible proteins [47].

ILs as adjuvants to polymer-salts ABS were used in our recent work, where an initial high-throughput screening was performed to find the most promising PEG-salt ABS for extraction of Hepatitis C Virus (HCV) VLPs [63]. After the screening and the optimization of the extraction conditions, the ABS composed of PEG 400 and citrate buffer at pH 7 was chosen and the effect of ILs on the extraction of HCV-VLPs was studied. We have selected different imidazolium- and quaternary ammonium-based ILs, allowing to evaluate effect of IL anion and the effect of the alkyl side chain length in the IL cation [63]. The extraction studies revealed that the addition of 5% (w/w) [C_4mim]Cl increased EE% from 87.9% (no

IL) to 91.1%. Moreover, with addition of [C_4mim][CH_3CO_2], the IL with a highest hydrogen bonding basicity and ability to accept protons, higher extraction efficiency (EE_{VLPs}% = 90.3%) was obtained and VLPs recovery yield enhanced from 84.9% (system without IL) to 88.5%. Furthermore, the advantageous effect of ILs was also confirmed in the VLPs purification directly from clarified cell culture supernatants. The obtained results showed that 100% VLPs EE% was attained in a single step. Moreover, with the addition of [C_4mim]Cl, the VLPs purity in the top phase was enhanced by 37% compared to the same system without IL [63]. These promising results show that ILs can be very effective in modulating the phase properties of polymer-salt ABS, achieving high HCV-VLP purification, without hampering their structural and functional properties.

In our following work, we extended the knowledge on the use of quaternary ABS in extraction and purification of VLPs, using ABS composed of carbohydrates-based natural deep eutectic solvents (NADES) [80]. We showed that HCV-VLPs have high affinity to the carbohydrate/NADES enriched phase in most of the studied ABS and that the NADES-based ABS are capable to enhance EE% of VLPs to the bottom phase compared to the systems composed of each one of NADES components alone. The most promising ABS in VLPs extraction was formed using fructose:sucrose (1:1) NADES and EE% of 99.6% were obtained [80]. Furthermore, the potential of these systems in separation of VLPs from BSA (the main contaminant protein in production of VLPs) was also evaluated. The obtained results showed that BSA preferentially partitioned to the carbohydrate/NADES-rich phase and fructose:glucose (1:1) NADES-based ABS extracted BSA with 99.5% efficiency [80]. This system was shown to be the best suited to separate VLPs from BSA contaminants and selectivity of 46.5 was attained, highlighting the potential of NADES-based ABS in VLPs purification. Overall, these results highlight that by proper selection of NADES

components, it is possible to finely tune the extractability and the separation efficiency of ABS for the distinct biomolecules and achieve better selectivity of ABS.

4. Biomolecules recovery, recycling and reusing of phase forming compounds

Having in mind industrial application of ABS for downstream processing and aiming at further decreasing their environmental and economic impact, it is inevitable to advance strategies for target biomolecule recovery and then recycling and reusing of phase forming compounds. Achieving this is very often difficult and complex and it can compromise the advantages of ABS in extraction and purification of biomolecules, such as their simplicity, scalability, and high efficiency. Furthermore, the addition of unit operations for the removal of phase forming chemicals from the product of interest also increases costs and complexity of the process. Moreover, it must be noted that in quaternary ABS, the presence of fourth compound in biphasic mixture turned this task even more complex as sometimes more unit operations are required to achieve this task.

Over the years, different strategies have been studied to conveniently recover target biomolecule from phase forming compounds. Usually, these strategies take advantage of size or chemical differences between the target biomolecule and the ABS phase forming chemicals and they include ultrafiltration, diafiltration, dialysis or precipitation. Moreover, other strategies such as back-extraction or induced phase separation (temperature) were also studied.

Ultrafiltration/diafiltration (UF/DF) has been used to recover target biomolecule from the top [144-146] or bottom [147-150] phase of different ABS. This method enables to separate the target biomolecule from phase forming chemicals by size with the use of a porous membrane. Moreover, UF/DF can be used both in laboratorial and large scale and it also allows to further concentrate the final product by reduction of the volume. Another approach

for the recovery of target biomolecule described in the literature is dialysis. For instance, a successful attempt of BSA recovery from IL-rich phase was described in the work of Pereira et. al [151]. The authors showed that after protein removal by dialysis, IL can be reused for two more extraction cycles without decreasing their high EE% for BSA (ca. 100%) [151]. In another work, dialysis was also used to recover lipase from Bacillus sp. ITP-001 and contaminant proteins from the polymer- and salt-rich phases of PEG-salt-IL quaternary ABS, respectively [67]. The authors showed that after the dialysis of each phase, the enzyme and contaminant proteins can be successfully recovered, and each phase can be reused for new extraction and purification cycles [67]. Another strategy very often used in order to separate proteins from phase forming compounds is precipitation [152]. In particular, in the case of ABS affinity precipitation is used in which the target molecule is connected to a specific ligand added to the system (without being a phase forming chemical) and after the separation of the phases, the complex of target molecule and ligand is precipitated [153-155].

Another alternative strategy is back-extraction. In this approach a top phase of a system where the biomolecules partitioned in a first system is transferred to a second fresh bottom, salt-rich phase with a different composition. In that way, the target biomolecule partitions into the bottom phase of this second system, thus obtaining top phase without target biomolecule that would be reutilized in another extraction cycle [17, 122, 156]. Furthermore, in our work we showed that by using ILs as adjuvants to PEG-salt ABS it is possible to manipulate protein affinity to the phases by using adequate IL and use such systems for extraction and back-extraction, making such quaternary ABS a very versatile extraction platform [62].

Furthermore, thermosensitive polymers were also used in ABS formation. This property of polymers allowed their recover and reuse in the next extraction cycles by increasing the temperature of the system. For example, in the work of Li et. al, the PPG recycling process

was proposed where the increase in the temperature drives PPG from the aqueous solution of cholinium propionate + PPG 400, allowing the concentration of PPG 400 in the polymer-rich phase to circa 90 % (w/w) at 45ºC, achieving the recyclability of 90% of PPG 400 [157]. In another work, ABS composed of thermo-sensitive ethylene oxide–propylene oxide (EOPO) random copolymer and salt was used to extract polyphenols from *Camellia sinensis var. assamica* leaves [158]. After the extraction process the authors proposed the EOPO polymer recovery by thermo-induction of the polymer-rich phase, allowing to recover 95.2% of the EOPO 2500 polymer [158].

However, DES-based quaternary ABS very often do not require the recovery of target biomolecule from ABS phase forming chemicals. Due to the high number of DES starting materials that are non-toxic, biocompatible, and used as excipients in formulation of the final bioproduct, it is possible directly use the phase to which biomolecule partitioned. The examples of such ABS were shown in our works where the extraction and purification of VLPs [80] and pepsin [115] was studied using DES-based ABS. Furthermore, since the polymer used in ABS formation was PPG, it also opens a possibility of recycling of the PPG-rich phase by thermo-induction.

5. Conclusions and outlook

Aqueous biphasic systems extraction has been recognized as an attractive and alternative methodology in downstream processing of various biomolecules, mainly because of its simplicity, selectivity, high capacity, and easy scalability. However, even though much effort has been put into their development and a lot of advantages associated with the use of ABS have been acknowledged, no major advances in introducing this methodology in industries have been made in the last decades. The reason behind this is, primarily, the lack of predictive models due to the poor overall understanding of the partition mechanisms

involved in ABS. Furthermore, the implementation of ABS in large scale may raise some environmental, safety and economic concerns due to the great amount of chemicals (e.g. salts and polymers, ILs) needed to form ABS, further resulting in increased costs of the process. Moreover, the most of biomolecules already have their purification process well established and to replace it by ABS it would be necessary to change the existing infrastructures. In our opinion ABS have higher opportunity to be introduced in industrial downstream processes of newly developed biomolecules which have not well-defined purification process, or for the biomolecules that are currently purified using expensive and time-consuming process. We believe that, perhaps, the reluctancy of the industry may be overcome by introduction of some modification to the conventional ABS. In particular, as discussed in this review, quaternary ABS formed with the addition of different chemicals, such as neutral salts, ILs or those composed of DES, can lead to a powerful technology to purify biomolecules with high quality, while being less expensive and safer to the environment than the conventional systems and thus being the potential answer on the doubts raised by the industry. Throughout this review, we show the advantages of quaternary systems over conventional ABS. For example, the addition of NaCl to polymer-salt was shown to be very effective in enhancing extraction and purification of monoclonal antibodies. Furthermore, the use of osmolytes as additives to polymer-salt ABS or DES composed of osmolytes combined with polymer to yield ABS seems to be an interesting approach to increase the purification of virus and VLPs, while enhancing their stability due to common use of these substances as excipients in vaccine formulation. Overall, due to high number of possible ILs, these chemicals offer the possibility to manipulate extractability of very wide range of biomolecules such as amino acids, enzymes, proteins, monoclonal antibodies, VLPs, among others. However, their use should be well-thought-out due to the toxicity of some of them. Thus, envisaging extraction and purification of

biomolecules relevant in industries, in which biocompatibility is required, DES should be considered as viable alternative to ILs as they were shown as effective but greener than ILs.

Beyond effectiveness of quaternary ABS in the extraction and purification of many different types of biomolecules, these systems are able to decrease the amounts of the phase forming compounds needed for the liquid-liquid demixing. Moreover, the possibility of using natural components, either in the synthesis of ILs or preparation of DES, opened exciting new perspectives to design truly sustainable and biocompatible solvents for the quaternary ABS implementation. Undoubtedly, the new compounds used to create quaternary ABS will result in further reduction of the costs. What is more, quaternary ABS formed using DES offer the possibility of integrated bioprocess, where the recovery of the target biomolecule can be avoided. In that way, even more competitive and sustainable downstream process using ABS than those used nowadays can be developed.

It is expected that, in a near future, the theoretical and experimental knowledge about quaternary ABS will evolve rapidly and these systems will surely gain more importance. Nevertheless, there are still many challenges before ABS could be implemented in industrial processes. For instance, more attention should be paid to the development of large-scale industrial settlements for ABS technology. Also, more studies on the design of integrated and continuous bioprocesses using quaternary ABS should be performed. Furthermore, predictive models for the behavior of ABS and biomolecules partition should still be studied, allowing to estimate K or specific ABS conditions. Moreover, it is believed that ABS will continue to have a significant research interest for biomolecules purification and recovery. In that way, in the near future more effective, sustainable and robust ABS at different scales will be developed, leading to commercial implementation of this technology at large scale recovery of high-value biomolecules.

Disclosure statement

The authors declare that they have no competing interests.

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1684 **Table 1:** Name and acronym of the compounds used in quaternary ABS implementation
1685 considered in this review.

Type	Name	Acronym
Ionic liquids	1-alkyl-3-methylimidazolium acetate	$[C_n mim][CH_3CO_2]$
	1-alkyl-3-methylimidazolium bromide	$[C_n mim]Br$
	1-alkyl-3-methylimidazolium chloride	$[C_n mim]Cl$
	1-alkyl-3-methylimidazolium dicyanide	$[C_n mim][N(CN)_2]$
	1-alkyl-3-methylimidazolium dimethylphosphate	$[C_n mim][(CH_3)_2PO_4]$
	1-alkyl-3-methylimidazolium methanesulfonate	$[C_n mim][CH_3SO_3]$
	1-alkyl-3-methylimidazolium tosylate	$[C_n mim][TOS]$
	1-alkyl-3-methylimidazolium trifluoromethanesulfonate	$[C_n mim][CF_3SO_3]$
	1-benzyl-3-methylimidazolium chloride	$[C_7H_7mim]Cl$
	1-hydroxyalkyl-3-methylimidazolium chloride	$[OHC_n mim]Cl$
	Tetrabutylammonium chloride	$[N_{4444}]Cl$
	Tetrabutylphosphonium chloride	$[P_{4444}]Cl$
	Tetramethylammonium chloride	$[N_{1111}]Cl$
	Tetrapropylammonium bromide	$[N_{3333}]Br$
Osmolytes	Amino acid	AA
	Betaine hydrochloride	BeHCl
	Guanidine hydrochloride	GuHCl
	Trimethylamine N-oxide	TMAO
Polymers	Polyacrylic acid	PAA
	Polyethylene glycol	PEG

	Polypropylene glycol	PPG
	Sodium polyacrylate	NaPA
Salts	Ammonium sulfate	$(NH_4)_2SO_4$
	Potassium chloride	KCl
	Sodium chloride	NaCl
	Sodium perchlorate	$NaClO_4$
	Choline chloride	ChCl

Table 2: Quaternary ABS and representative results reported in literature for the extraction and purification of AAs. EE% = extraction efficiency; K = partition coefficient; S = selectivity.

Amino acid	ABS composition	Main results	Reference
L-tryptophan	PEG 600 + Na_2SO_4 + H_2O + $[C_7H_7mim]Cl$	K - 20.54 (no IL) to 42.47 (with IL)	[27]
	PEG 400 + $(NH_4)_2SO_4$ + H_2O + $[N_{4444}]Cl$	K - 7.4 (no IL) to 14.6 (with IL)	[64]
	PEG 400 + citrate buffer pH 7 + H_2O + $[N_{4444}]Cl$	K - 13.48 (no IL) to 24.39 (with IL)	[66]
	ChCl:glucose (1:1) + PPG 400 + H_2O	EE% - 90.59% in ChCl enriched phase	[28]
	ChCl:1,2-propanodiol (1:1) + K_2HPO_4 + H_2O	EE% - 98.7% in ChCl enriched phase $S_{Trp/Tyr}$ - 30.9	[30]
	ChCl:PEG 2000 (2:3) + K_2HPO_4 + H_2O	EE% - 93.88% in DES enriched phase	[101]
L-tyrosine	PEG 400 + $(NH_4)SO_4$ + H_2O + $[P_{4444}]Cl$	K - 2.3 (no IL) to 3.4 (with IL)	[64]
	PEG 400 + citrate buffer pH 7 + H_2O + $[P_{4444}]Cl$	K - 4.85 (no IL) to 7.23 (with IL)	[66]
	ChCl:glucose (1:1) + PPG 400 + H_2O	EE% - 83.55% in DES enriched phase	[28]
	ChCl:ethanol (2:1) + K_2HPO_4 + H_2O	EE% - 86.7% in ChCl enriched phase	[30]
L-phenylalanine	PEG 400 + citrate buffer pH 7 + H_2O + $[N_{4444}]Cl$	K - 7.58 (no IL) to 13.39 (with IL)	[66]
	ChCl:glucose (1:1) + PPG 400 + H_2O	EE% - 86.69% in DES enriched phase	[28]
	ChCl:ethanol (2:1) + K_2HPO_4 + H_2O	EE% - 94.3% in ChCl enriched phase	[30]

1709 **Table 3:** Quaternary ABS and representative results reported in literature for the extraction

1710 and purification of enzymes. EE% = extraction efficiency; K = partition coefficient.

Enzyme	ABS composition	Main results	Reference
Lipase	2-proponol + K_2HPO_4 + H_2O + 4.5% (w/v) NaCl	Recovery – 76% (no NaCl) to 99% (with NaCl) Purity - 6.4-fold (no NaCl) to 13.5-fold (with NaCl)	[107]
	PEG 8000 + potassium phosphate + H_2O + 6% (w/w) NaCl	Purity - 59.93-fold (no NaCl) to 141.65-fold (with NaCl)	[108]
	PEG 1500 + K_2HPO_4/KH_2PO_4 + H_2O + $[C_6mim]Cl$	Purity175.61-fold (no IL) to 245-fold (with IL)	[67]
Protease	PEG 4000 + $MgSO_4$ + H_2O + 6% (w/w) NaCl	Recovery - 23.58% (no NaCl) to 107% (with NaCl)	[109]
	PEG + dextran + H_2O + 4.5% (w/w) NaCl	K - 84.2 Purity - 14.37-fold Yield - 97.3%	[111]
Invertase	PEG 3000 + Na_2SO_4 + H_2O + 5% (w/w) KCl	Recovery - 68% (no KCl) to 90% (with KCl) Purity - 3.3-fold (no KCl) to 5.5-fold (with KCl)	[110]
L-asparaginase	PEG 6000 + citrate buffer + H_2O + $[C_4mim][CH_3SO_3]$	Recovery - 87.94% Purity – 20.09-fold Specific activity -3.61 U/mg	[65]
Trypsin	ChCl:urea (1:2) + K_2HPO_4 + H_2O	EE% - 95.53% in DES enriched phase	[79]
	Betaine:methylurea (1:2) + K_2HPO_4 + H_2O	EE% - >90% in DES enriched phase	[112]
	ChCl:glycerol (1:1) + K_2HPO_4 + H_2O	EE% - 94.36% in DES enriched phase	[113]
Papain	ChCl:PEG 2000 (20:1) + Na_2CO_3 + H_2O	EE% - 90.95% in DES enriched phase	[114]
Pepsin	BeHCl:glucose (1:1) + PPG 425 + H_2O	Activity recovery - 141.9% EE% - 99.5% in DES enriched phase	[115]

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1715 **Table 4:** Quaternary ABS and representative results reported in literature for the extraction

1716 and purification of monoclonal antibodies. EE% = extraction efficiency.

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Antibody	ABS composition	Main results	Reference
IgG	PEG 1450 + phosphate + H_2O + 12% (w/w) NaCl	Purity - 80% Purification fold - 5.9-fold Recovery yield - 100%	[119]
	PEG 3350 + phosphate buffer pH 6 + H_2O + 15% (w/w) NaCl	Recovery yield - 101% Purity - 99% Yield - 97%	[118]
	PEG 6000 + phosphate buffer pH 6 + H_2O + 15% (w/w) NaCl	Recovery yield - 88% (CHO cells culture supernatants) and 90% (hybridoma cell culture supernatants) Purification factor - 4.3 (CHO cell culture supernatants) and 4.1 (hybridoma cell culture supernatants)	[117]
	PEG 3350 + phosphate buffer pH 6 + H_2O + 10% (w/w) NaCl	Recovery - 89% Purity - 75%	[24]
	PEG 3350 + phosphate buffer pH 6 + H_2O + NaCl	Recovery yield - 80% (CHO cell culture supernatants) and 100% (PER.C6® cell culture supernatants)	[122]
	PEG 400 + citrate buffer pH 7 + H_2O + 5% (w/w) $[C_4mim][CH_3CO_2]$	EE% - 100% in PEG 400-rich phase Purity - 37% enhancement with IL	[61]
	PEG 6000 + dextran 450-650 + H_2O + $[C_4mim]Cl$, $[C_4mpyr]Cl$, $[C_4mpip]Cl$, $[P_{4444}]Cl$, $[N_{4444}]Cl$	K - ↑ with $[C_4mim]Cl$, $[C_4mpyr]Cl$, $[N_{4444}]Cl$, $[C_4mpip]Cl$ $S_{Cyt\ c/IgG}$ - ↑ with $[C_4mpyr]Cl$, $[N_{4444}]Cl$, $[P_{4444}]Cl$ than without IL $S_{BSA/IgG}$ - ↑ with $[C_4mim]Cl$, $[C_4mpip]Cl$ than without IL	[124]

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1724 **Table 5:** Quaternary ABS and representative results reported in literature for the extraction

1725 and purification of proteins. EE% = extraction efficiency; K = partition coefficient.

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Protein	ABS composition	Main results	Reference
BSA	ChCl:urea (1:2) + K_2HPO_4 + H_2O	EE% - 99.94% in DES enriched phase	[79]
	Betaine:urea (1:2) + K_2HPO_4 + H_2O	EE% - 99.82% in DES enriched phase	[112]
	ChCl:glycerol (1:1) + K_2HPO_4 + H_2O	EE% - 98.16% in DES enriched phase	[113]
	ChCl:PEG 2000 (20:1) + Na_2CO_3 + H_2O	EE% - 95.16% in DES enriched phase	[114]
	$[N_{1111}]$Cl:urea (1:2) + K_2HPO_4 + H_2O	EE% - 99.31% in DES enriched phase	[133]
	PEG 6000 + dextran 450-650 + H_2O + $[C_4mim]$Cl, $[C_4mpyr]$Cl, $[C_4mpip]$Cl, $[P_{4444}]$Cl, $[N_{4444}]$Cl	K - ↑ with ↑ IL concentration	[124]
Cytochrome C	PEG 8000 + NaPA 8000 + H_2O + $[C_2mim][(CH_3)_2PO_4]$, $[C_2mim][CH_3SO_3]$, $[C_2mim][CH_3CO_2]$, $[C_2mim]$Cl, $[C_2mim][CF_3SO_3]$, $[OHC_2mim]$Cl	EE% - 100% in the NaPA 8000-rich phase	[52]
	PEG 6000 + dextran 450-650 + H_2O + $[C_4mim]$Cl, $[C_4mpyr]$Cl, $[P_{4444}]$Cl, $[N_{4444}]$Cl	K - ↑ (with IL)	[124]
Lysozyme	PEG 600 + phosphate buffer + H_2O + $[C_2mim]$Cl or $[C_4mim]$Cl	K - ↑ with ↑ IL concentration	[132]
	$[N_{4444}]$Br:glycolic acid (1:1) + Na_2SO_4 + H_2O	EE% - >98% in DES enriched phase	[134]
Myoglobin	PEG 4000 + PAA + H_2O + NaCl	K - 4.20 (no NaCl) to 15.77 (with NaCl)	[20]
	PEG 600 + phosphate buffer + H_2O + $[C_2mim]$Cl or $[C_4mim]$Cl	K - ↑ with ↑ IL concentration	[132]
	PEG 3350 + $NH_4)_2SO_4$ + H_2O + 7.5% (w/w) $[C_4mim][CH_3CO_2]$	EE% - 100% in PEG 3350-rich phase	[62]
Ovalbumin	PEG 4000 + PAA + H_2O + NaCl	K - 2.82 (no NaCl) to 5.51 (with NaCl)	[20]
	Betaine:ethylene glycol (1:2) + K_2HPO_4 + H_2O	EE% - ~60% in DES enriched phase	[112]

Thaumatin	PEG 6000 + phosphate + H_2O + 1.5 M NaCl	K - ↑ (with NaCl) Recovery yield - 90-95% Purification - 20-fold	[127]

Table 6: Quaternary ABS and representative results reported in literature for the extraction and purification of virus or virus-like particles. EE% = extraction efficiency; S = selectivity.

Virus or VLPs	ABS composition	Main results	Reference
Parvovirus B19 VLPs	PEG 1000 + $MgSO_4$ + H_2O + 800 mM NaCl	Recovery yield - 95.3% (VP1 protein) and 33.2% (VP2 protein)	[142]
	PEG 400 + phosphate buffer pH 8.5 + H_2O + 7.5% (w/w) NaCl	DNA removal - 99% S - ↑ (with NaCl)	[143]
Porcine parvovirus	PEG 12000 Da + citrate buffer pH 7 + 0.5M glycine + H_2O	Recovery yield - 100% DNA removal - >60%	[47]
HCV-VLPs	PEG 400 + citrate buffer pH 7 + H_2O + $[C_4mim]Cl$	EE% - 100% in PEG 400-rich phase Purity - 37% enhancement with IL	[63]
	Fructose:glucose (1:1) + PPG 425 + H_2O	EE% - ↑ than for ternary ABS $S_{VLPs/BSA}$ - 46.5	[80]
HIV-VLPs	PEG 12000 Da + citrate buffer pH 7 + 0.5M glycine + H_2O	Recovery yield - 92% DNA removal - >60%	[47]

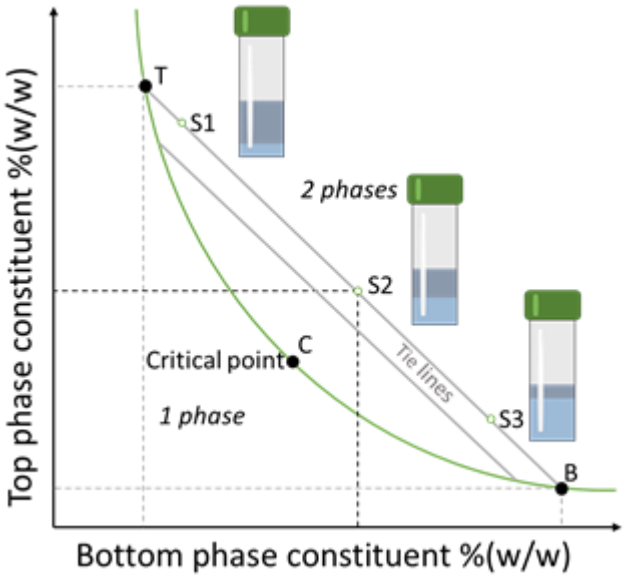
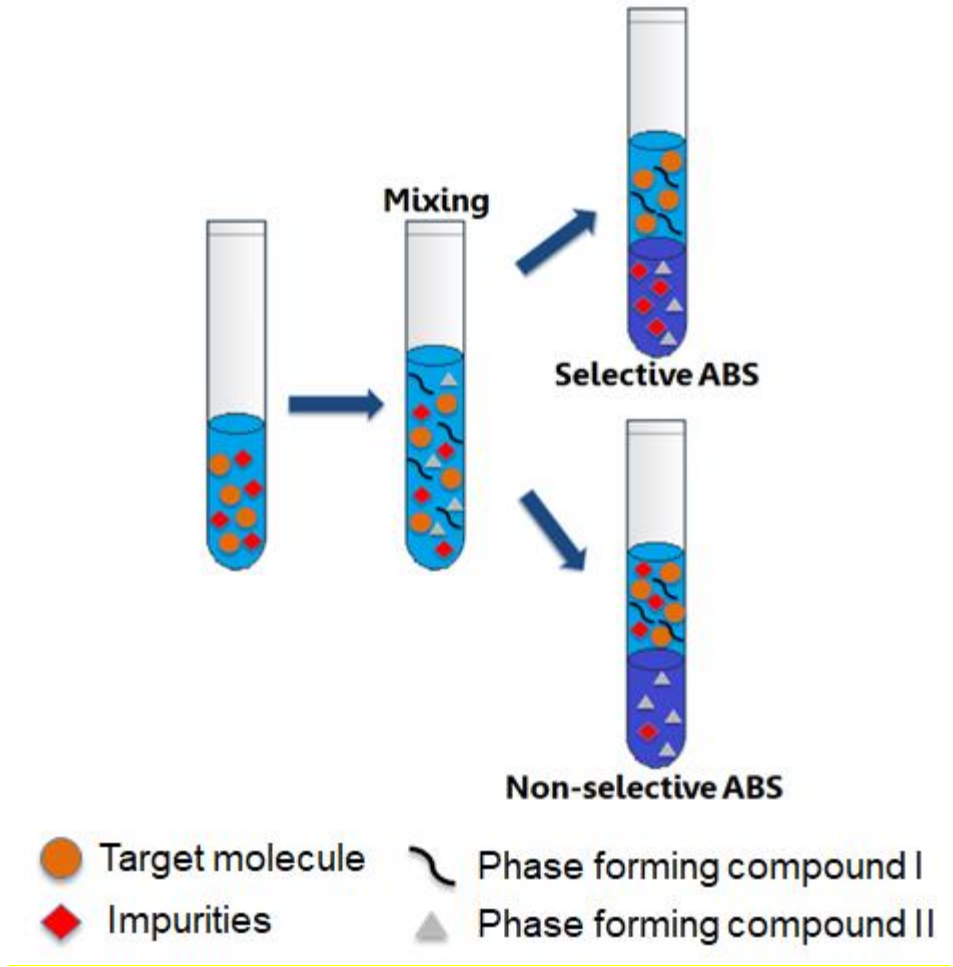


Fig. 1



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1751 Fig. 2

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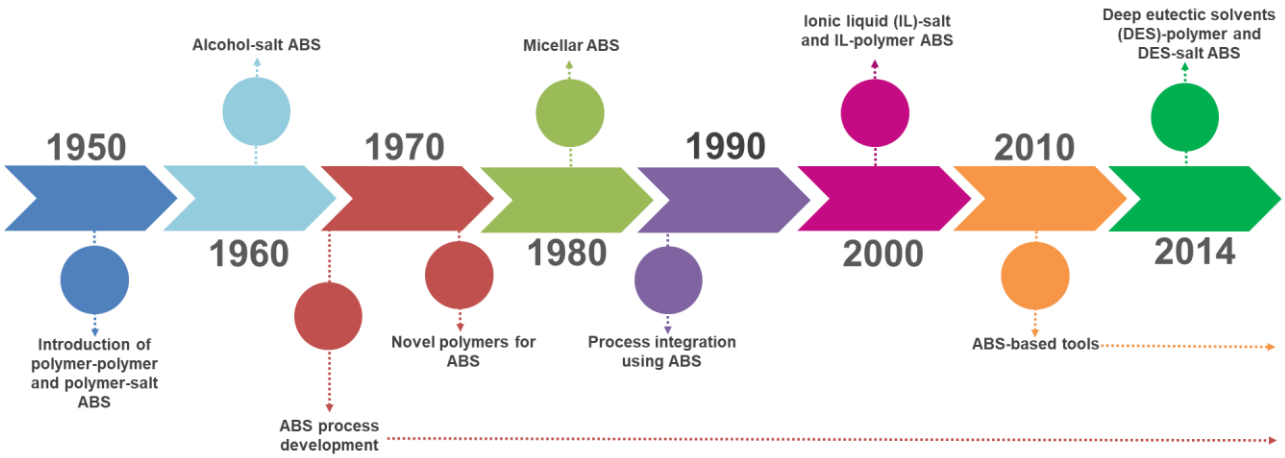


Fig. 3

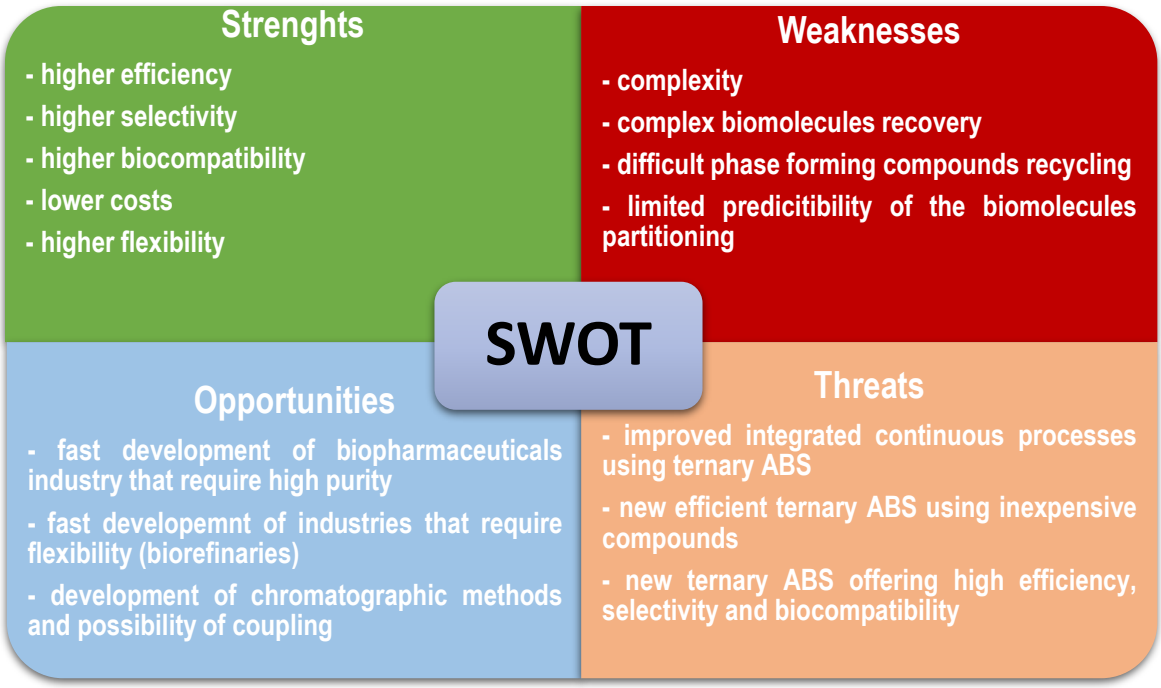


Fig. 4

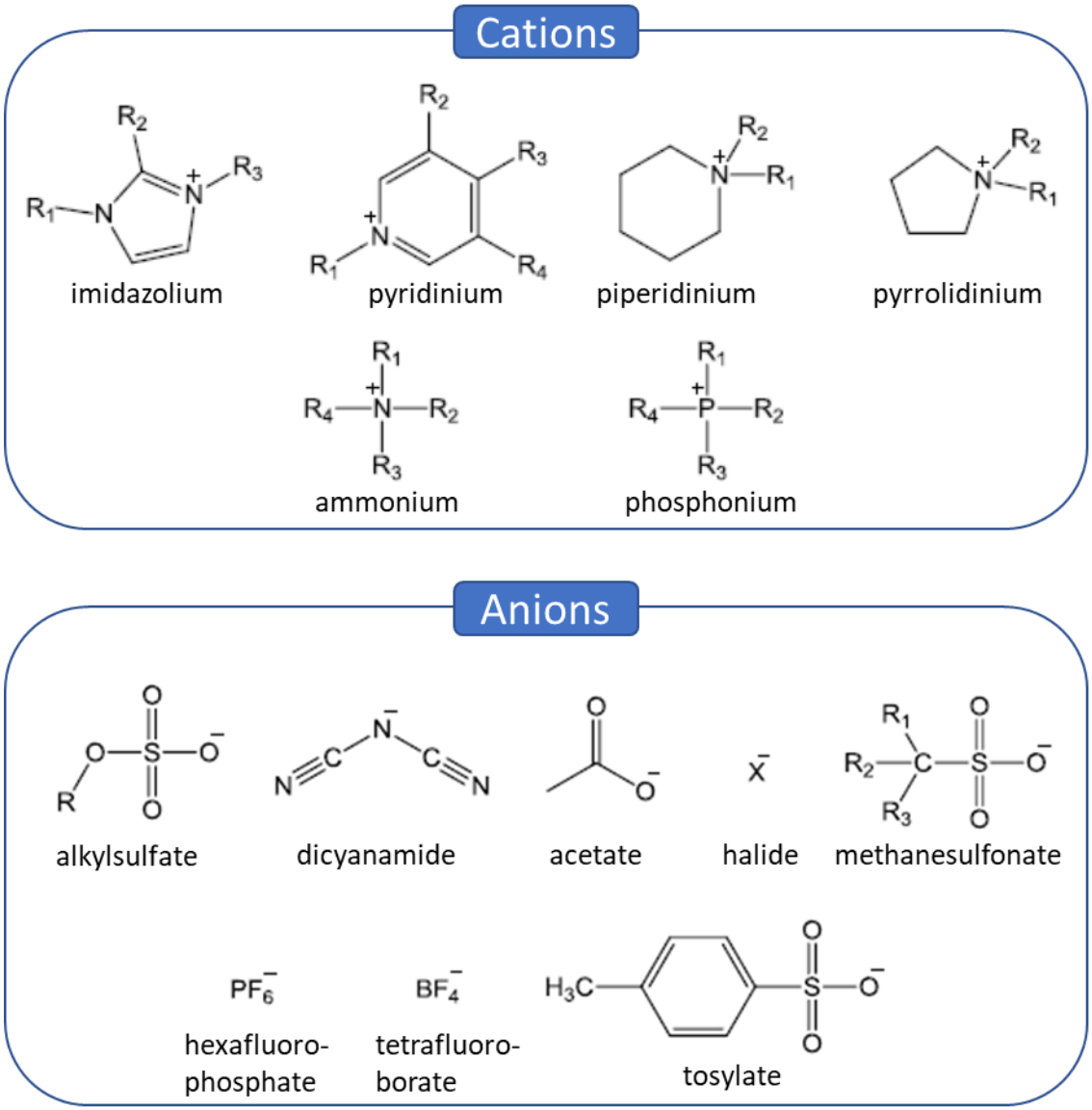


Fig. 5

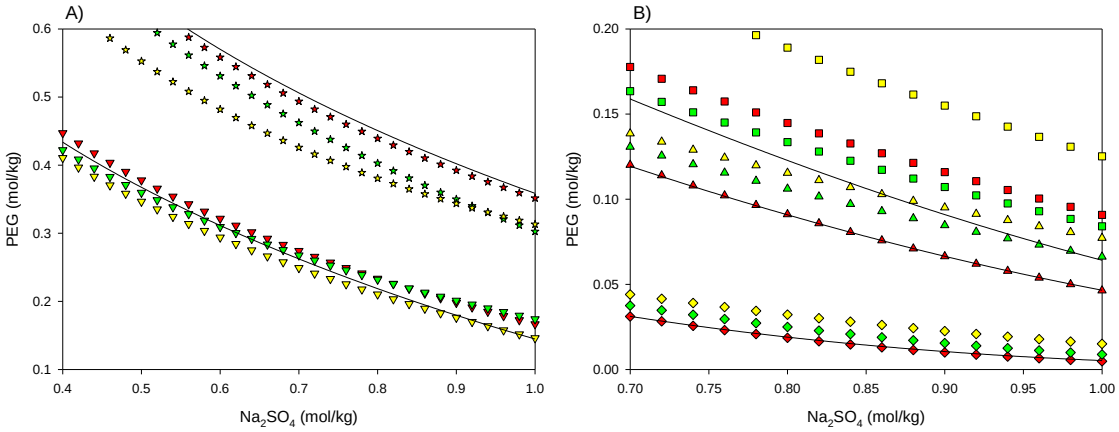
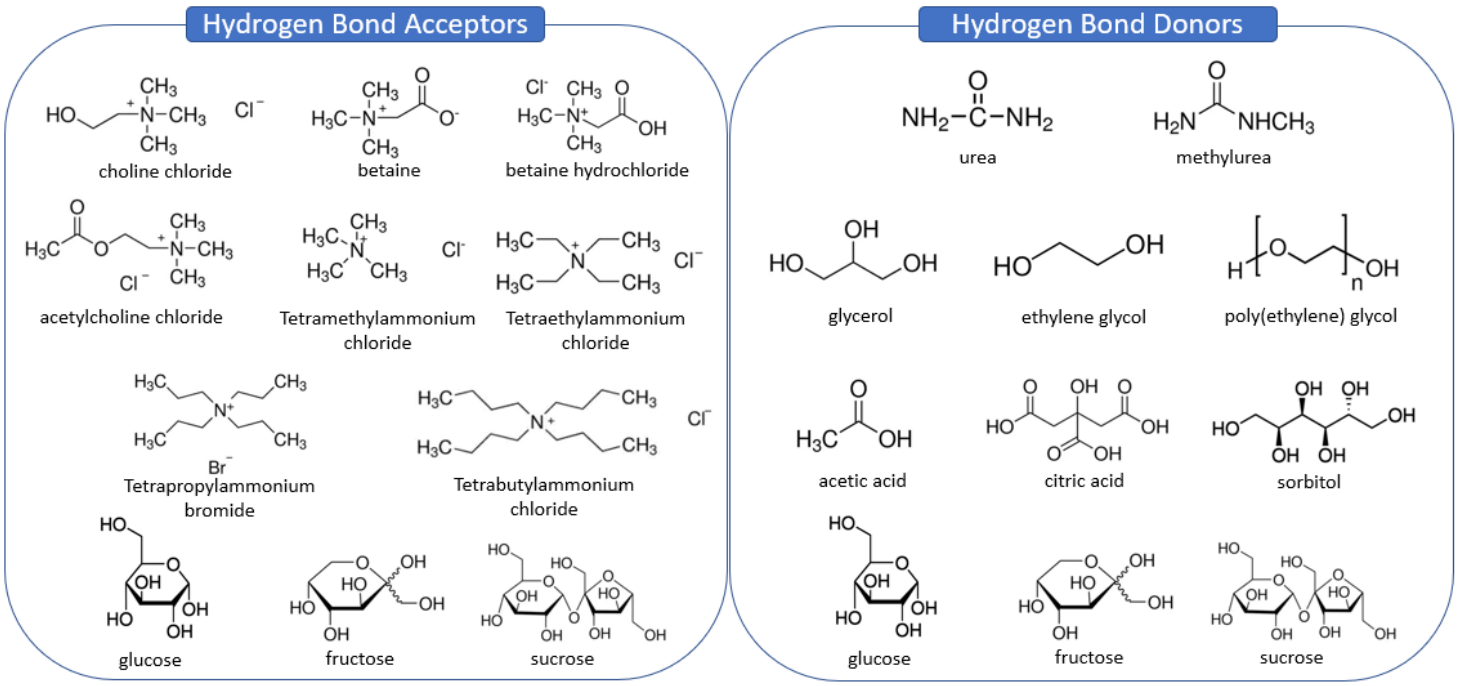


Fig. 6



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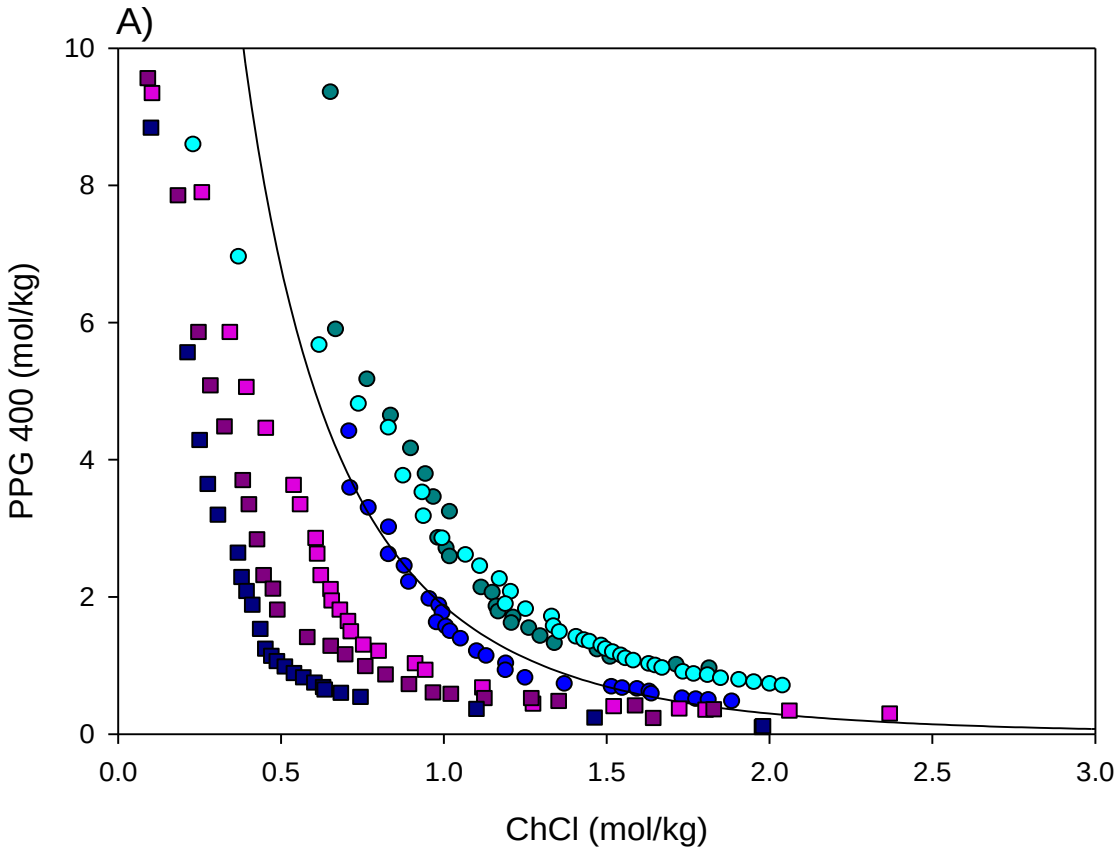

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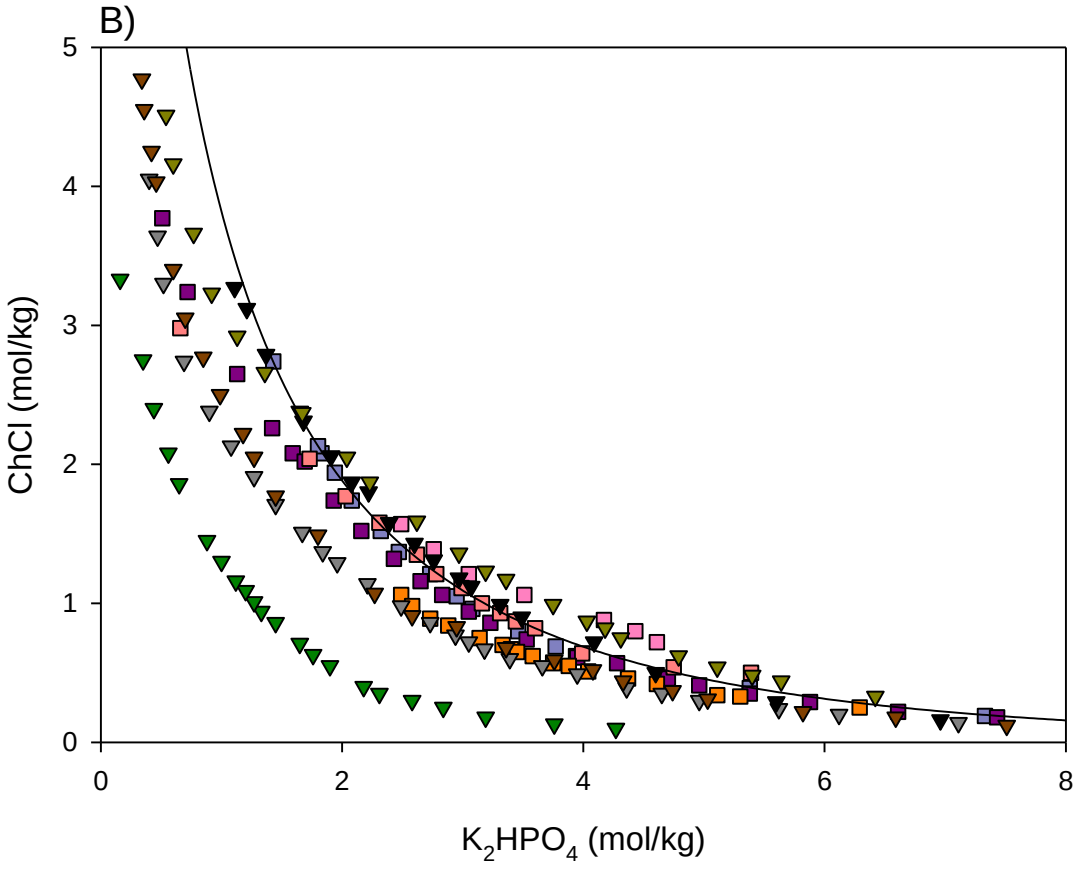

.790

.791 Fig. 8

Fig. 1: Schematic representation of a phase diagram for an aqueous biphasic system. T = top phase composition; B = bottom phase composition; S1, S2, S3 = overall system compositions; C = critical point.

Fig 2: Schematic diagram illustrating ABS formation and partitioning of target molecule and contaminant proteins (impurities) in a ABS.

Fig. 3: Important landmarks in aqueous biphasic systems (ABS) history.

Fig. 4: Summary of the main strengths, weaknesses, opportunities, and threats (SWOT analysis) of the quaternary ABS compared to the conventional ABS for the downstream processing of biomolecules.

Fig. 5: Examples of the chemical structures of cations and anions present in common ionic liquids.

Fig. 6: Binodal curves for the systems composed of: **A)** PEG 400 (☆) or PEG 600 (▽) + Na_2SO_4 + H_2O + 5% (w/w) of [C_2mim]Cl (red), [C_4mim]Cl (green), [C_6mim]Cl (yellow); and **B)** PEG 800 (□), PEG 1000 (△) or PEG 2000 (◇) + Na_2SO_4 + H_2O + 5% (w/w) of [C_2mim]Cl (red), [C_4mim]Cl (green), [C_6mim]Cl (yellow). The lines correspond to the systems composed with PEG and salt only. Data taken from [69].

Fig. 7: Chemical structures of HBAs and HBDs commonly used in DES preparation.

Fig. 8: Phase diagrams of DES-based ABS. **A)** Representation of the binodal curves of acetic acid:ChCl- and glucose:ChCl-based ABS as a function of the ChCl concentration for DES composed of acetic acid:ChCl at 1:2 (●), 1:1 (●), and 2:1 (●) molar ratio and for DES composed of glucose:ChCl at 1:2 (■), 1:1 (■), and 2:1 (■) molar ratio. The line corresponds to the system composed with PPG and ChCl only. Data taken from [28-29]. **B)** Representation of the binodal curves of sugars:ChCl- and alcohols:ChCl-based ABS as a function of ChCl concentration for DES composed of ChCl and fructose (■), glucose (■), sucrose (■), xylose (■), sorbitol (■) at 1:1 molar ratio and for DES composed of ChCl and *n*-propanol (▼), ethanol (▼), 1,2-propanodiol (▼), glycerol

(▼), ethylene glycol (▼) at 1:1 molar ratio.. The line corresponds to the system composed with K_2HPO_4 and ChCl only. Data taken from [30-31, 83].