Chapter 11
On the Hunt for More Benign and Biocompatible ABS

Jorge F.B. Pereira, Rudolf Deutschmann, and Robin D. Rogers

Abstract The late appearance of more environmentally friendly and biocompatible liquid-liquid extraction systems has triggered a shift in research interest toward aqueous biphasic systems (ABS), including those composed of ionic liquids (ILs). Although most ILs were originally considered as green solvents due to their negligible volatility, the release/escape of these materials into the environment from operations employing IL-water solvent systems rose several concerns. As a consequence, recent studies started to focus on finding more benign and biocompatible ILs for new biocompatible ABS (Bio-ABS) formulations. One of the most thoroughly studied Bio-ABS to date employs cholinium-based ILs combined with various polymers or high-melting inorganic salts; however, other Bio-ABS were also suggested and tested, like those utilizing amino-acid-based ILs as ABS promoters. Although naturally resourced and biodegradable ILs obviously improve the biocompatibility and sustainability of a given ABS, similar attention has to be paid to the selection of the other coexisting phase-forming agents (polymers, biopolymers, sugars, and/or biodegradable high-melting salts) in order to achieve a truly environmentally conscious system. Starting from this basis, in this chapter we (a) review and summarize the available information on Bio-ABS published so far, (b) highlight the most important differences between IL-based Bio-ABS and non-Bio-ABS, (c) show examples of nontoxic and biodegradable phase-forming agents, and (d) evaluate the applicability and future industrial perspectives of IL-based Bio-ABS.

Keywords Aqueous biphasic systems • Ionic liquids • Biocompatible • Biodegradable • Nontoxic • Environmentally friendly • Polymers • Cholinium-amino acid salts

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Abbreviations

AA-IL	Amino acid-based ionic liquid
ABS	Aqueous biphasic systems
AIL	Aprotic ionic liquid
BHb	Bovine hemoglobin
Bio-ABS	Biocompatible ionic liquid-based aqueous biphasic systems
BSA	Bovine serum albumin
Ch-ABS	Cholinium-based aqueous biphasic systems
Ch-IL	Cholinium-based ionic liquid
FLS	Fluorescence spectroscopy
FT-IR	Fourier transform infrared spectroscopy
GB	Good's buffer
IL	Ionic liquid
LCST	Lower critical solution temperature
MW	Molecular weight
OECD	Organization for the Economic Co-operation and Development
OVA	Ovalbumin
PEG	Polyethylene glycol
PEO	Polyethylene oxide
PIL	Protic ionic liquid
PPG	Polypropylene glycol
PVA	Polyvinyl alcohol
TEM	Transmission electron microscopy
TLL	Tie line length
VOC	Volatile organic compounds

11.1 Introduction

The energy crisis in the 1970s has changed the mindset of leaders in politics, technology, and research equally to look for sustainable resources. At the same time, major environmental concerns urged the introduction of stricter legislations about industrial processing technologies worldwide. These factors have opened up the path toward the search for new chemicals, processes, and technologies based on renewable (mostly bio) resources [1]. Solvent-based separation and purification technologies were among the first large-scale operations affected by the new line of environmentally conscious thinking. The high volume of solvents used in these processes was mostly composed of volatile organic compounds (VOCs), and their escape/disposal into the environment represented various risks. They were also shown to be incompatible with many biological products and processes, sometimes even decreasing the quality of the final product [2].

During the race to overcome these environmental issues and satisfy regulations, the end of the last century has seen the rediscovery of aqueous biphasic systems (ABS) as an ideal solution to help design water-rich, more sustainable liquid-liquid extraction processes for the separation and/or extraction of biomolecules [3]. ABS have also shown to be capable of maintaining the native conformation and biological activities/properties of several biomolecules, such as enzymes [4], alkaloids [5], antibiotics [6, 7], and biopharmaceuticals [8], among others [9]. The first studies have focused on the use of traditional ABS based on polymer-polymer and polymer-salt [9] systems, but in 2003, Rogers and coworkers [10] proposed the use of imidazolium-based ILs as ABS promoters, demonstrating their ability to form ABS in the presence of inorganic salts (K_3PO_4). One of the major advantages of ILs over traditional VOC-based solvents is their negligible vapor pressure that could prevent gas phase leaks in a wide range of chemical processes [11]. It was also highlighted, however, that the application of ILs in ABS could be disadvantageous in some cases (costly cation-anion combinations, ILs composed of fluorinated anions exhibiting non-benign character, etc.) [10]. Despite these limitations, the development of the first IL-salt ABS started a "boom" in the study of new IL-based ABS and their possible applications as alternative extraction and purification systems to efficiently separate a wide range of biomolecules [12].

In a recent compilation of the work done so far on ABS composed of ILs, Freire et al. [12] observed that almost all of IL-based ABS are formed with imidazolium-, pyridinium-, piperidinium-, or pyrrolidinium-based ILs. These IL families, however, should not be considered as sustainable and nontoxic compounds, especially those based on imidazolium and pyridinium cations [13–15]. Therefore, there is a strong ongoing motivation to develop and study new ABS incorporating environmentally friendly ILs. Recently, new classes of ILs (cholinium- [16] and amino-acid-based ILs [17]) have been synthesized using bio-precursors through sustainable and easy procedures, exhibiting lower toxicities, higher rate of biodegradation, and higher biocompatibility than conventional ILs [18, 19].

These two classes of ILs were already used and considered as more benign ABS promoters; however, if an IL is more "benign" or more "biocompatible" is an ongoing question. How can one define the benign character of an IL-based ABS? In order to answer this question, we attempt to summarize all recent advances in the field of sustainable and biocompatible IL-based ABS, briefly reviewing the environmental character of some families of ILs and examining if they are capable of promoting an immiscibility region when combined with other compounds (e.g., polymers, salts, or surfactants). However, as the benign behavior of an IL-based ABS is not only dependent on the IL used, it will also be discussed how coexisting compound(s) can be considered in the ABS formulation to increase the "greener" character of ABS. Thus, this chapter will review what has been done to date and discuss what research should be carried out in the future in the liquid-liquid extraction field using ILs.

11.2 Biocompatible and Benign ABS Forming Agents

By definition, an ABS consists of two immiscible water-rich phases formed by the mixing of two or more water-soluble solutes, which, above a certain concentration level, are separated into two coexisting phases [9]. Nowadays, several types of compounds can be used as phase-forming agents, from the more traditional polymers, salts, and surfactants to the more recently reported ILs, peptides, or amino acids. Although there is a wide range of phase-forming agents to choose from, the number of water-soluble solutes that have been used to create ABS in combination with ILs is significantly lower, though it seems that this number will increase in the next few years. Although the recent review by Freire et al. [12] shows that the majority of IL-based ABS are composed of ILs and inorganic salts, other less studied combinations of ILs and other solutes, such as polymers, carbohydrates, amino acids, and surfactants, have also been reported. Herein, in the following subsection, we will go through the concept of benign and biocompatible ILs first and then show other ABS forming agents that have been used (or could be used) in order to increase the biocompatibility of IL-based ABS.

11.2.1 More Benign and Biocompatible ILs

ILs are complex fluids which can express interesting physicochemical properties, such as negligible vapor pressure, nonflammability, large liquid temperature range, high thermal conductivity, and a wide range of electrochemical potential [20, 21]. Moreover, with appropriately chosen synthetic methodologies and the proper manipulation of the ions involved, ILs can be tailor-made for the desired applications, like separation processes, where their polarities [12], safety, and environmentally friendly can be designed to suit certain requirements [22].

According to Seddon's recent claim [23, 24], more than 10^{18} ILs are theoretically possible, although this number includes binary, ternary, and higher mixtures of ILs. With that in mind, it is easy to see that there should be compounds exhibiting complete biocompatibility and benign characteristics on one end of the spectrum and, equally possible, ILs with high toxicity (even higher than in case of many conventional solvents) on the other, among the large array of ILs that have been prepared and characterized to date. So far, the majority of ILs are manufactured synthetically from other chemicals, and this fact questions whether one can consider even the most biocompatible ones as "green" solvents [25]. Usually, the entire life cycle of these chemical compounds is not considered; however, a thorough environmental risk assessment requires one to consider every industrial process involved in the production, application, and ultimate disposal of these ILs, used as phase-forming promoters in different ABS [26]. According to the 12 Green Chemistry principles [27], an IL should have negligible accumulation and persistence in the environment and, for that purpose, its biodegradability is assumed to be a crucial parameter when assessed as "environmentally friendly compound."

A huge number of different ILs have been synthesized and classified according to three generation stages during the last two decades: the first generation of ILs were synthesized in order to obtain specific physicochemical properties; the compounds of the second generation were produced to achieve specific behaviors, suitable to certain final industrial applications; and the third generation of ILs (advanced ILs) is the more recent development and aims to produce ILs with desired biological features [28]. These third-generation potentially environmentally friendly ILs are the central subject of this chapter as they are prepared using more environmentally friendly starting materials, produced by low-energy and easy procedures, composed of ions that are more stable, biodegradable, readily available, and designed to exhibit lower toxicities [29]. ILs of the third generation are mainly composed of anions such as sugars, amino or organic acids, alkylsulfates, or alkylphosphates and cations derived from choline or amino acids.

The late emergence of environmental concerns has pushed a large part of the IL research community toward the search for compound families that are biodegradable and can also be obtained from natural starting materials with well-characterized biodegradability and toxicological properties in order to satisfy the principles of the third IL generation [26]. These new classes of ILs, completely derived from biomaterials, have also been called natural ILs, Bio-ILs [16], or bio-renewable ILs [25]. In addition to being bio-resourced and biodegradable, these ILs have to comply with all the other criteria (mainly toxicity and biocompatibility) themselves as well in order to be considered truly benign and environmentally friendly.

The ecotoxicological features of ILs have been described in numerous studies in the last few years, and a significant number of IL families are already well characterized [30]; however, the concept of biocompatibility is broad and hard to define. By a simplified definition, biocompatibility is a "quality of not having toxic or injurious effects on biological systems" [31]. Thus, if applied to IL research, it should be defined and considered in accordance with the context of its application [32]. Consequently, when it is applied to ILs as ABS promoters, it dictates that only anions and cations that exhibit low toxicities and negligible effects over the target biomolecule to be extracted should be employed. The main criteria that an IL should satisfy in order to be considered as a "green" solvent for separation processes are summarized in Table 11.1.

Several papers highlighted the necessary design and synthetic strategies needed to follow in order to obtain families of ILs that could satisfy the "Green Chemistry" criteria described above [11, 15, 30, 33, 34]. With these strategies in mind, it is possible to form close to ideal Bio-ILs (biodegradable, nontoxic, biocompatible, sustainable) as it was demonstrated through real examples [35–37]. Even though the search for these "Bio-ILs" has been expanding, the number of successful examples obtained from natural sources that fulfills all the "green" requirements is not yet significant. The majority of works executed in this direction so far focused on the synthesis of cholinium- and amino acid-based ILs. A few other classes of

Table 11.1 Summary of the Green Chemistry criteria for an IL to be used in separation processes

Criteria	Standard
Low toxicity	All ILs should exhibit low toxicities against organisms of different trophic levels, using parameters according to the Organization for the Economic Co-operation and Development (OECD) standard tests
Biodegradability	All ILs and derivatives should reach 60 % biodegradation after 28 days of biological treatment to be classified as "readily biodegradable," using parameters defined, for example, according to the closed bottle test (OECD 301D)
Sustainability	All precursor materials used in manufacture should be available from sustainable and renewable feedstocks
Biocompatibility	All compounds should be compatible with the biological compounds to be extracted, maintaining their biological structures and activities

protic ILs have also been suggested as possible environmentally friendly alternative promoters for different ABS formulation; however, generalizations in claiming entire families of ILs as "green" should be avoided since even small changes in the chemical structure of the cation or/and anion can completely modify their character and properties, possibly reversing the environmentally friendly nature of an IL belonging to an assumed bio-family. Moreover, any change that improves one aspect, like biodegradability, might degrade other properties (toxicity and/or biocompatibility) and vice versa.

11.2.1.1 Cholinium-Based ILs

Cholinium-based ILs (Ch-ILs) are derived from choline chloride, *N,N,N*-trimethylethanolammonium chloride, a naturally occurring essential nutrient frequently used as a food additive [38]. Cholinium and other quaternary ammonium salts play very important roles in living systems, especially in the synthesis of vitamins and enzymes needed in various steps of the carbohydrate metabolism [39, 40]. The biological importance and availability of cholinium chloride moved scientists toward the synthesis of its derivatives, especially those with low melting points [39]. The resulting ILs were termed as "benign" [15], "biocompatible" [18, 41–44], "biodegradable" [18, 19, 22], "environmentally friendly," and/or "nontoxic" [18, 19, 22, 34, 39, 45].

Nockeman et al. [45] were one of the first research groups, in 2007, to examine the toxicity of two Ch-ILs, cholinium saccharinate and cholinium acesulfamate, to the crustacean organism *Daphnia magna*. In that preliminary work, the authors observed that both ILs exhibited very low ecotoxicity, being at least two orders of magnitude less toxic than other traditional families of ILs, such as the imidazolium- and pyridinium-based ones [45]. On the other hand, Pernak et al. [41] synthesized homologous series of Ch-ILs with alkoxy groups containing 2–12 carbons combined with distinct anions and verified that the derivatives were increasingly active against some microorganisms.

The influence of the anion on toxicity was also evaluated by Petkovic et al. [18] using filamentous fungi for a series of nine cholinium-alkanoate ILs. The authors [18] demonstrated that all the alkanoate ILs tested were less toxic than the corresponding sodium salts, and, in addition, it was observed that the cholinium cation can also be digested by the respective fungi strain [18]. Sekar et al. [42] showed that Ch-ILs, like cholinium lactate, cholinium saccharinate, cholinium dihydrogen phosphate, choline lactate, and choline tartrate, can even be employed as co-substrate and carbon source to digest organic dye contaminants with the salt-tolerant bacterium *S. lentus*.

In 2009, Vrikkis et al. [43] performed an extensive study on the biocompatibility/cytotoxicity and protein stabilizing capabilities of choline dihydrogen phosphate and choline saccharinate. The measurement of their effect on metabolic activity of a mouse macrophage cell line using the reduction of resazurin (used as an indicator of activity/viability) has proved both compounds to be relatively benign and generally nontoxic. Lysozyme formulations in 80 wt % choline dihydrogen phosphate were also prepared to investigate the short- and long-term effect of the IL on the thermal stability and activity of the enzyme [43]. The results have showed that the thermal stability and activity of the enzyme were higher than that in an aqueous buffer even after one month of storage [43]. The biocompatibility of cholinium-based salts and ILs was also evaluated for different collagenous biomaterials by Vijayaraghavan and coworkers [44]. It was observed that all cholinium-based compounds tested exhibited good cell viability and adhesion properties fulfilling the requirements of the World Health Organization (WHO) for biomedical applications. These data seem to indicate that ABS prepared with Ch-ILs can be efficient tools to extract, separate, and even store enzymes and proteins [44].

Although, the majority of the literature supports the benign nature of the Ch-ILs, not all cholinium-based salts can be considered harmless as Ventura et al. have pointed out [39]. While eight variants of cholinium-based salts could be classified as “practically harmless” or “harmless” for the bioluminescent marine bacteria *Vibrio fischeri*, in the case of cholinium dihydrogencitrate and cholinium bitartrate, showed moderate toxicities against the same organism [39]. In another work of the same research group, they investigated the ecotoxicological effects of 17 monocationic and eight dicationic cholinium (or cholinium derivative) ILs conjugated with bromide anion on the same microbe (*V. fischeri*). It was observed that the structural features of the cation have a high influence on the harmlessness of the IL, and again, just belonging to the family of cholinium compounds does mean the salts will be nontoxic; long alkyl side or linkage chains, high number of CH_2CH_3OH groups, and multiple covalent bonds can increase toxicity significantly [22]. This evidence reinforces how important the role of structural design is in making “greener” ILs [22], and this is in agreement with the initial assumptions of Wood and Stephens [46], suggesting that “ILs have the potential to make a big difference to the environmental impact of chemical manufacturing processes,” but a potential that will be dependent on a judicious selection of the least toxic IL structures.

In order to obtain even more benign IL structures, some researchers [47, 48] have synthesized cholinium-amino acid ILs entirely composed of renewable biomaterials. These new classes of ILs can combine the advantages of two naturally occurring materials, cholinium chloride and amino acids, making them promising candidates as environmentally friendly solvents. The next subsection will provide further details about novel ILs derived from amino acids.

11.2.1.2 Amino-Acid-Based ILs

Amino acids have an enormous structural diversity. They contain both an amino group and a carboxylic acid residue in a single molecule [17, 25, 49]. Additionally, they are one of the most abundant organic compounds in nature and can be produced in large quantities using relatively easy processes. All of these attributes can make amino acids an excellent choice for being used as low-cost feedstock to synthesize new classes of environmentally friendly ILs [17].

Fukomoto et al. [50] prepared the first prototype series of amino acid-based ILs (-AA-ILs) in 2005, combining 1-ethyl-3-methylimidazolium with 20 different amino acids. The preparation of ILs from natural amino acids is not an easy process without significant challenge, since amino acids are zwitterionic species and are stabilized by electrostatic interaction between the carboxylate anion and the protonated amino group [17]. The first successful synthesis of imidazolium-based ILs employing amino acids as the corresponding anion was soon followed by the synthesis of phosphonium- [17] and cholinium-based ILs [46, 51, 52]. Based on a similar approach, a whole new generation of ILs started to emerge, in which the cations are simply derived from natural amino acids or amino acid ester salts [25, 53]. Since the beginning, these ILs were proclaimed as one of the greenest [51] and most biocompatible [25] solvents, mainly due to their natural origin. Nevertheless, in recent years, research has been started to pursue the environmentally friendly (toxicology, biodegradability, etc.) of these new classes of bio-renewable ILs [19, 54, 55].

In order to evaluate the toxic/nontoxic character of AA-ILs, Gouveia et al. [54] prepared 14 ILs using imidazolium, pyridinium, and cholinium cations. Their toxicities were assessed against organisms of various trophic levels, namely, crustacean *Artemia salina*, human cell line HeLa (cervical carcinoma), and Gram-negative and Gram-positive bacteria (*Escherichia coli* and *Bacillus subtilis*, respectively). As expected, the toxicity of each IL appeared to be highly dependent on the chemical nature of both the cation and the anion, as well as the organism tested [54]. Interestingly, however, none of the amino-acid-based ILs exhibited significant toxicity for any of the bacteria strains tested, but it was found to be directly related to the cation used when evaluated against the crustacean and human cell culture. The AA-ILs with imidazolium and pyridinium cations have shown remarkable higher toxicity (10 times higher) than those with the cholinium cation [54]. These data might indicate that ILs produced from bio-materials, such as choline and amino acids, could represent low toxicity to both humans and the environment.

Similarly, Hou et al. [19] assessed the environmental fate of 18 novel ILs prepared with cholinium as cation and amino acids as the anions. The aim of the research was to select and be able to design truly environmentally friendly ILs that are not only produced from bio-resources but are also less toxic and readily biodegradable in the natural environment. For that purpose, the authors [19] examined the toxicity of these novel ILs to enzymes and four strains of representative bacteria and then determined their biodegradability using wastewater microorganisms. Their results showed that the inhibitory effects of cholinium-amino acid ILs were at least an order of magnitude weaker than that of the conventional 1-butyl-3-methylimidazolium tetrafluoroborate, and with the exception of two ILs, these ILs exhibited similar or even lower toxicities than the cholinium chloride salt [19]. According to the biodegradability tests performed by the authors [19], all of the cholinium-amino acid ILs complied with the requirements set by the Organization for the Economic Co-operation and Development (OECD) and thus can be considered as readily biodegradable, since all the compounds tested reached 60 % biodegradation after 28 days of treatment. Hou et al. [19] demonstrated, in general, that the majority of the cholinium *R. satius* was especially sensitive to based ILs are probably "greener," but, as highlighted by the authors as well, other ecotoxicological tests against organisms of different trophic levels are necessary to help design and identify truly "green" chemicals.

Egorova et al. [55] recently proved that the application of amino acids in IL compositions is not always beneficial nor always helps increase the ecological attributes. The authors [55] tested and compared the cytotoxicity of several AA-ILs (incorporation of amino acids either as the cation or the anion) and conventional imidazolium-based ILs on NIH/3 T3 (mouse fibroblasts) and CaCo-2 (colorectal adenocarcinoma) cell cultures [55]. Surprisingly, the presence of an amino acid did not necessarily lower toxicity; especially in the cases when amino acids were employed as the anion, the observed toxicities were comparable with those of the conventional 1-butyl-3-methylimidazolium chloride or 1-butyl-3-methylimidazolium L-lactate [55]. On the other hand, the research group [55] also observed that both the selected conventional and AA-ILs induced the apoptosis of mouse fibroblasts cells. These findings show again the importance of careful design and thorough environmental analysis in the development and application of biomaterials like bio-ILs.

11.2.1.3 Other Classes of Environmentally Friendly ILs

The previous subsections described two of the most important third-generation classes of ILs that are usually claimed as very benign and biocompatible compounds. However, for standard industrial separation or extraction processes, other compounds have also been considered to form environmentally friendly ILs. Brønsted or protic ILs (PILs), as having a proton available for hydrogen bonding, can be also included in this last generation of ILs [56]. These ILs differ from the classic, aprotic ones (AILs), in having both cationic and anionic counterparts formed by low-molecular-weight organic compounds. They usually contain substituted (or polysubstituted) amines as cations and organic acids as anions and were designed exclusively to minimize their environmental impact [56]. PILs have been tested and used for numerous applications, but as the majority of them are fairly new or underdevelopment, ecological assessments of their properties are scarce yet [57].

Peric et al. [56, 57] have been actively studying the ecotoxicological effects and biodegradability of PILs. When they tested the effects of three short aliphatic PILs on soil microbial functions (C and N mineralization) and terrestrial plants (*Lolium perenne*, *Allium cepa*, and *Raphanus sativus*), they found that the PILs were not nontoxic in general for either the plants tested or the soil microbiota and were biodegraded by the soil matrix; plants showed higher sensitivity than microorganisms. *R. satius* was especially sensitive to the 2-hydroxytriethanolamine pentanoate IL which appeared to be somewhat toxic. It was concluded that the more complex molecular structure the ILs has (longer alkyl side chain), the higher rate of inhibition in the organisms is observed [56].

In another study, the same authors [57] examined the toxicity and biodegradability of a wider range of PILs (ten ILs based in three different amines and six organic acids) and AILs (two imidazolium- and two pyridinium-based ILs). The experiments were carried out on various test subjects: three different aquatic organisms (*Vibrio fischeri*, *Pseudokirchneriella subcapitata*, and *Lemna minor*), one enzyme (acetylcholinesterase), and leukemia rat cells (IPC-81). Tests were also performed to assess the potential biodegradability of PILs in water according to the OECD guideline 301. The data showed that the PILs tested were generally nontoxic or at least less toxic than the AILs (various orders of magnitude lower than the aprotic ILs) against most of the organisms studied (aquatic, enzymes, or cells), except for *Lemna minor* that happened to be very sensitive for three of the PILs. The most exciting results obtained by the authors [57], however, were the good rates of biodegradation of the PILs in water, especially if compared with the negligible biodegradation observed for AILs.

Another type of biocompatible ILs was synthesized recently by Taha et al. [36]. The authors developed a series of novel self-buffering and biocompatible ILs using anions derived from biological buffers (designated as "Good's buffers" (GBs)). These GBs are zwitterionic amino acid derivatives, usually acting as strongly hydrated molecules [36]. Twenty GB-based ILs were produced by combining 5 different GBs (N-[tris(hydroxymethyl)methyl]glycine, Tricine; 2-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethanesulfonic acid, TES; (2-cyclohexylamino)ethanesulfonic acid, CHES; 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, HEPES; 4-morpholineethanesulfonic acid, MES) with four hydroxide bases (1-ethyl-3-methylimidazolium, tetramethylammonium, tetraethylammonium, and tetrabutylammonium). Their capability to extract and stabilize proteins was tested using bovine serum albumin (BSA), and their toxicity was evaluated against *V. fischeri*. All tests provided very promising information; the majority of GB-ILs seemed to bear high self-buffering capacity, showed great potential to extract and stabilize proteins (better than the corresponding buffer solution and conventional ILs), and proved to be nontoxic against *V. fischeri* [36].

All these encouraging results on ecotoxicology, biodegradation, and protein friendliness of PILs and alternative APILs, even despite the scarcity of data available, combined with their low production costs and simple synthesis, make it easy to envision their great potential as alternative and more benign phase-forming agents in the formation of ABS.

11.2.2 Environmentally Friendly Coexisting Phase-Forming Agents

Besides the enormous research effort devoted to the development of more environmentally friendly ILs, the scientific community has also been seeking biocompatible species that can promote the formation of an immiscible region when mixed with aqueous solutions of different ILs. Since the first report of an ABS formed by mixing an aqueous solution of 1-butyl-3-methylimidazolium chloride ([C_4mim]Cl) and a concentrated aqueous solution of K_3PO_4 [10], more than 50 papers have described possible scenarios to form an ABS combining several ILs (mainly conventional ILs) and different inorganic/organic salts [12]. The use of salts with high charge density, normally applied as salting-out agents in the formation of IL-ABS, however, should be avoided and rather replaced with more biodegradable organic salts, carbohydrates, polymers, or amino acids, as pointed out by Freire et al. [12].

A large portion of the literature focuses on IL-based ABS employing inorganic salts with anions such as phosphates, sulfates, or carbonates, compounds that raise environmental concerns when released into aqueous streams [58]. One of the most used inorganic salts, K_3PO_4, for instance, introduces potassium and phosphate ions into the aqueous system generating a highly alkaline medium that can be harmful to a large number of pH-sensitive biomolecules (such as proteins, enzymes, pharmaceutical active compounds, etc.) and microorganisms, while, on the other hand, its dissolution in the aqueous phase complicates the recycling processes of the ILs [59]. Recent studies, however, presented new formulations of IL-based ABS based on biodegradable and nontoxic organic salts with citrate, tartrate, and acetate anions [58, 60–63]. These systems can increase the benign character and biocompatibility of extraction processes through the use of high-melting salts that are more biodegradable and less toxic than those applied in traditional IL-based ABS formulations. In addition to the organic salts, other benign phase-forming agents, such as amino acids [64, 65], carbohydrates [59, 66–70], and different polymers [3, 12], have also been tested lately to create IL-based ABS.

Amino acids were previously referred as interesting starting materials for the synthesis of new amino acid-based ILs and consequently being applied as more benign phase-forming ILs, but, on the other hand, amino acids can simply be used as phase separation promoters. As described in the earlier sections, amino acids are zwitterionic and, in general, environmentally friendly molecules, and their addition to aqueous solutions of different ILs, above certain concentrations, can lead to the formation of a biphasic region. The application of amino acids in separation processes will produce a more benign extractive environment for biomolecules, as well as help during the recovery of the IL from aqueous effluents [12]. Despite the promising future of these systems, only two publications included ternary phase diagrams for IL-amino acid-water systems [12]. In 2007, Zhang et al. [64] proved that glycine, L-serine, and L-proline are all able to create ABS when combined with water and the hydrophilic IL 1-butyl-3-methylimidazolium tetrafluoroborate. Domínguez-Pérez et al. [65] also demonstrated the formation of ABS combining imidazolium-based ILs with three different amino acids (L-lysine, D,L-lysine HCl, and L-proline). These ABS, however, are contradictory, claiming to be more environmentally friendly due to the use of amino acids as phase-forming agents, while still using ILs based on non-environmentally friendly ions, such as imidazolium or tetrafluoroborate.

Carbohydrates are non-charged, usually nontoxic, and readily biodegradable compounds, almost always obtained from renewable feedstocks, which make them promising candidates to play a leading role in environmentally friendly processes [59]. Carbohydrates are present in different forms in nature: mono-, di-, oligo-, and polysaccharides with sugar alcohols being the most common ones. Regular monosaccharides (simple sugars) are polyhydroxy aldehydes or ketones with –OH groups attached to every available carbon atom, thus capable of forming several hydrogen bonds both as donor or acceptor. Not surprisingly, these molecules have high affinity toward water and can even displace compounds from aqueous solution. The sugar alcohols (low-molecular-weight polyols) are derivatives of simple sugars where the aldehyde/carbonyl group is hydrogenated, resulting in an even higher water affinity [59]. In the few publications to date, the following monosaccharides, disaccharides, and sugar alcohols were tested to create a biphasic region by mixing them with aqueous solutions of ILs: sucrose [59, 66–69], glucose [59, 67, 69], fructose [59, 67, 70], xylose [59, 67, 69], maltose [59, 68, 69], mannose [59], arabinose [59], maltitol [59], sorbitol [59], and xylitol [59]. It is important to draw attention to the fact that the majority of the ILs tested were of the most conventional and non-benign classes of ILs.

Organic salts, amino acids, and simple sugars can all be considered as environmentally friendly phase-forming agents. However, the most biocompatible, benign, and biodegradable compounds with the best applicability can probably be found among oxygen-rich (potential for forming multiple hydrogen bonds) hydrophilic polymers, like polyethylene glycol (PEG), polypropylene glycol (PPG), polyvinyl alcohol (PVA), polyethylene oxide (PEO), and polysaccharides. They were firstly used in the formulation of ABS in the mid-1950, when Albertsson demonstrated the formation of two immiscible water-rich phases combining two hydrophilic polymers, dextran and PEG [71]. Studies afterward tried to identify other suitable ABS forming candidates among various polymers [72, 73], salts [9, 71], surfactants [74], carbohydrates [75], and ILs [3, 76].

The majority of the abovementioned hydrophilic polymers are less toxic (or nontoxic) and more biocompatible than most of the other materials of choice; some can even be obtained from natural resources. One of the most promising examples is dextran, a naturally occurring complex, branched glucose-based polysaccharide. It is water-soluble, completely biodegradable and biocompatible, and can be produced from renewable sources as it is synthesized from sucrose by certain lactic acid bacteria, such as *Leuconostoc mesenteroides*, *Lactobacillus brevis*, and *Streptococcus mutans* [77, 78]. Despite these advantages the high cost associated with its production represents a major obstacle in the way of widespread industrial application [79].

The most extensively used oxygen-rich polymers are PEGs and PPGs. They are chemically similar compounds; their lower molecular weight variants are soluble in water and capable of creating two immiscible water-rich phases with almost all the ABS phase-forming agents reported so far. PEGs have a long history of employment in industrial processes, from traditional chemical procedures to the newest technologies in life sciences [80]. Their potential biodegradability, high water solubility, low vapor pressure, low melting point, and low production cost make these materials favorable for many applications [74]; they can have important roles in the formulation of polymer surfactants or polymer electrolytes [81], in drug delivery processes [82], and can act as solvents for several separation or purification approaches [71] or as protein stabilizing and partitioning agents [83]. The popularity of PEG in biotechnological applications and the requirement for more sustainable ILs have brought the idea to employ it in IL-based ABS [3, 84–87]. PPGs, although less frequently used, are thermosensitive, biodegradable, and nontoxic materials having similar properties to those of PEGs and can be easily recovered from an ABS by convenient heating [84]. These examples might well illustrate the future potential of oxygen-rich polymeric materials in the formulation of IL-based ABS.

11.3 Biocompatible Ionic-Liquid-Based Aqueous Biphasic Systems

Although authors frequently termed many of IL-based ABS as “biocompatible,” “benign,” “biodegradable,” or “greener,” only a handful of ILs and phase-forming agents exhibit true environmentally friendly characteristics. According to the previously described considerations, it is evident that environmentally friendly and biocompatible IL-ABS will only result from a proper combination of already benign (nontoxic and biodegradable) ILs and phase-forming compounds preferably obtained from renewable resources.

This section will review the recently published IL-based ABS that are the closest ones to an ideal, truly benign, and biocompatible system. First the IL-ABS, employing frequently studied Ch-ILs, will be discussed; then, the systems formulated with AA-ILs will be summarized; and finally other potentially highly benign and biocompatible IL-based ABS will be evaluated. Moreover, the main physicochemical properties and some interesting thermodynamic and/or modeling properties will also be presented for each type of IL-based ABS.

11.3.1 Cholinium-Based ABS

Since 2003, a large number of IL-based ABS were published, but the first work about the use of Ch-ILs (or salts) in the formulation of ABS was only reported recently in 2012, where Wang and coworkers [35] demonstrated the possibility to form ABS combining different Ch-ILs and PPG-400. Other researchers then confirmed the formation of ABS using IL-based ABS (Ch-ABS) as demixing promoters [2, 63, 88–90]. Table 11.2 shows the types of Ch-ABS assessed to date with the temperature at which stable ABS were observed.

In contrast with more conventional IL-based ABS, the majority of Ch-ABS were obtained with oxygen-rich polymers (PPG, PEG, PEG-PPG copolymers) instead of inorganic/organic salts. There were only two exceptions where liquid-liquid demixing was achieved with either aqueous solutions of K_3PO_4 according to Shahriari et al. [89] or when two cholinium-based salts (benzyldimethyl (2-hydroxyethyl)ammonium chloride, [BCh]Cl, and cholinium salicylate, [Ch][Sal]) were mixed with aqueous solutions of potassium citrate buffer ($C_6H_5K_3O_7$/$C_6H_8O_7$), potassium phosphate buffer (KH_2PO_4/K_2HPO_4), and potassium carbonate (K_2CO_3) in the results provided by Sintra et al. [63].

In the case of [Ch]Cl-based ABS, formation was observed with practically all of the polymers, regardless of the molecular weight, as well as with K_3PO_4. This behavior is worth searching for as having the potential for giving more flexibility to the design of future ABS. With this in mind, the systems with [Ch][OAc], [Ch][Gly], [Ch][Lac], [Ch][Glu], and [Ch][Suc] look promising; however, they are not thoroughly tested yet. [Ch][Sal] and [BCh][Cl] were shown to be able to form ABS with various salts and buffers, but were not assessed with PEG- or PPG-based systems.

Regarding the temperature effect on phase diagrams, the majority of the Ch-ABS were determined at 15 °C or 25 °C. A more complete evaluation of the temperature influence was only carried out for some particular examples of Ch-ABS, namely, [Ch]Cl + PPG-400 + H_2O, [Ch][Gly] + PPG-400 + H_2O, [Ch][Lac] + PPG-400 + H_2O, [Ch][Pro] + PPG-400 + H_2O, [Ch]Cl + PEG-600 + H_2O, and [Ch][DHph] + PEG-600 + H_2O. Although detailed information regarding the effect of temperature on ABS is scarce, some examples suggest that, depending on the IL used, elevated temperatures mostly enhance the immiscibility region [88, 90].

Shahriari et al. [89] used a series of Ch-ILs created with anions derived from organic acids or HCl to promote ABS in the presence of K_3PO_4. The trend in capability of forming ABS was observed in the following order:

Table 11.2 List of Ch-ABS studied to date. Each Ch-IL (or salt) is correlated with the phase forming agents studied[a]

	Coexisting phase forming agents								
	Polymers							Salts	
	Polypropylene glycol		Polyethylene glycol				Copolymer		
Ionic liquids	PPG-400	PPG-1000	PEG-400	PEG-600	PEG-1000	PEG-4000	$EO_{10}PO_{90}$	K_3PO_4	Other
Cholinium acetate [Ch][OAc]	At 25 °C [35]	–	At 25 °C [90]	At 25 °C [90]	At 25 °C [90]	–	–	At 25 °C [89]	–
Cholinium benzoate [Ch][Benz]	At 25 °C [35]	–	–	–	–	–	–	–	–
Cholinium bicarbonate [Ch][Bic]	–	–	At 25 °C [90]	At 25 °C [90]	At 25 °C [90]	–	–	–	–
Cholinium bitartrate [Ch][Bit]	–	–	At 25 °C [90]	At 25 °C [90]	At 25 °C [90]	–	–	–	–
Cholinium butyrate (or butanoate) [Ch][But]	At 25 °C [35]	–	N/A [90]	N/A [90]	N/A [90]	–	–	–	–
Cholinium chloride [Ch]Cl	At 5 °C [88]	At 15 °C [88]	At 25 °C [90]	At 25 °C [90]	At 25 °C [90]	–	At 15 °C [88]	At 25 °C [89]	–
	At 15 °C [88]			At 50 °C [90]					
	At 25 °C [88]								
	At 35 °C [88]								
	At 45 °C [88]								
Cholinium dihydrogencitrate [Ch][DHcit]	–	–	At 25 °C [90]	At 25 °C [90]	At 25 °C [90]	At 25 °C [2]	–	N/A [2]	–

(continued)

Table 11.2 (continued)

	Coexisting phase forming agents								
	Polymers							Salts	
	Polypropylene glycol		Polyethylene glycol				Copolymer		
Ionic liquids	PPG-400	PPG-1000	PEG-400	PEG-600	PEG-1000	PEG-4000	$EO_{10}PO_{90}$	K_3PO_4	Other
Cholinium dihydrogenphosphate [Ch][DHph]	–	–	At 25 °C [90]	At 25 °C [90] At 50 °C [90]	At 25 °C [90]	–	–	–	–
Cholinium formate [Ch][For]	At 25 °C [35]	–	–	–	–	–	–	–	–
Cholinium fumarate [Ch][Fum]	–	–	–	At 25 °C [2]	–	At 25 °C [2]	–	N/A [2]	–
Cholinium glutarate [Ch][Glu]	–	–	–	At 25 °C [2]	–	At 25 °C [2]	–	At 25 °C [89]	–
Cholinium glycolate [Ch][Gly]	At 15 °C [35] At 25 °C [35] At 35 °C [35]	At 15 °C [88]	At 25 °C [90]	At 25 °C [90]	At 25 °C [90]	–	At 15 °C [88]	–	–
Cholinium lactate [Ch][Lac]	At 15 °C [35] At 25 °C [35] At 35 °C [35]	At 15 °C [88]	N/A [90]	At 25 °C [90]	At 25 °C [90]	–	At 15 °C [88]	–	–

Cholinium levulinate [Ch][Lev]	–	–	–	N/A [2]	–	–	–	At 25 °C [89]	–
Cholinium L-malate [Ch][L-Ma]	–	–	–	At 25 °C [2]	–	At 25 °C [2]	–	N/A [2]	–
Cholinium malonate [Ch][Mal]	–	–	–	At 25 °C [2]	–	At 25 °C [2]	–	N/A [2]	–
Cholinium oxalate [Ch][Ox]	–	–	–	At 25 °C [2]	–	–	–	N/A [2]	–
Cholinium propionate (or propanoate) [Ch][Pro]	At 15 °C [35] At 25 °C [35] At 35 °C [35]	At 15 °C [88]	N/A [90]	N/A [90]	N/A [90]	–	At 15 °C [88]	–	–
Cholinium salicylate [Ch][Sal]	–	–	–	–	–	–	–	At 25 °C [89]	At 25 °C with: $C_6H_5K_3O_7$/ $C_6H_8O_7$ KH_2PO_4/ K_2HPO_4 K_2CO_3 [63]
Cholinium succinate [Ch][Suc]	–	–	–	At 25 °C [2]	–	At 25 °C [2]	–	At 25 °C [89]	–
Di-cholinium oxalate $[Ch]_2[Ox]$	At 25 °C [35]	–	–	–	–	–	–	–	–
Tri-cholinium citrate $[Ch]_3[Cit]$	At 25 °C [35]	–	–	–	–	–	–	–	–

(continued)

Table 11.2 (continued)

	Coexisting phase forming agents								
	Polymers							Salts	
	Polypropylene glycol		Polyethylene glycol				Copolymer		
Ionic liquids	PPG-400	PPG-1000	PEG-400	PEG-600	PEG-1000	PEG-4000	$EO_{10}PO_{90}$	K_3PO_4	Other
Benzyldimethyl(2-hydroxyethyl)ammonium chloride [BCh]Cl	–	–	–	–	–	–	–	At 25 °C [89]	At 25 °C with:
									$C_6H_5K_3O_7$/ $C_6H_8O_7$
									KH_2PO_4/ K_2HPO_4
									K_2CO_3 [63]

[a] N/A – ABS formation was not observed

[Ch][Sal] > [BCh]Cl > [Ch][Lev] > [Ch][Glu] ≈ [Ch][Suc] ≈ [Ch][Ac] > [Ch]Cl [89]. This is in a good agreement with the findings for more conventional ILs families, such as imidazolium-based ILs, where the more hydrophobic ILs are more easily salted out by K_3PO_4 [91, 92]. The phase formation is driven by the competition of the IL and salt species for water molecules, and the parameters of the immiscible region were determined by the relative hydrophilic/hydrophobic character of the IL compared to the high charge density salt [89]. Accordingly, ILs with anions having more aliphatic or aromatic moieties form ABS more readily (salted out), while the ones with more –OH groups less likely induce phase separation [89].

Sintra et al. [63] studied the formation of ABS by mixing members of several IL families, including the cholinium-based [Ch][Sal] and [BCh]Cl, with appropriate aqueous solutions of $C_6H_5K_3O_7/C_6H_8O_7$, KH_2PO_4/K_2HPO_4, and K_2CO_3. Although the authors [63] showed the expected two-phase regions at 25 °C, their aptitude to form ABS was found to be lower than that of the conventional ILs [63]. When the authors compared the effect of each salt/buffer solution on the phase-forming ability of [Ch][Sal], the following tendency was found: $KH_2PO_4/K_2HPO_4 > C_6H_5K_3O_7/C_6H_8O_7 > K_2CO_3$. Since this was somewhat different from the expectations, where the formation of an ABS is directly related with the affinity of the salt component toward being hydrated, it was deduced that the dominating interactions in these systems are more complex and a more comprehensive model is needed for the future to explain the liquid-liquid demixing [63].

Ch-ILs are capable of forming ABS in the presence of inorganic/organic salts, but, as observed by Shahriari et al. [89], even when strong salting-out species, such as K_3PO_4, are applied, the phase separation potential of the given system could be lower than in the case of other, more conventional IL families. The presence of –OH groups and/or aliphatic/aromatic regions in the ILs has a large influence on their ability to form two phases with salt solutions as pointed out earlier. However, this attribute can be exploited to use Ch-ILs as tunable phase promoting agents. This idea was first presented by the Wang group [35] in 2012, when they intended to design novel, more environmentally friendly ABS systems for protein extraction by combining Ch-ILs with the less hydrophilic PPG-400 polymer instead of an inorganic salt. In this work, the Ch-ILs acted as the phase-forming/salting-out agent. During the study of nine Ch-ILs conjugated with several organic acid anions, the phase-forming potential of the ILs was found to decrease in the following order: $[Ch]_3[Cit] > [Ch]_2[Ox]$ > [Ch][Gly] > [Ch][Pro] ≈ [Ch][Lac] ≈ [Ch][OAc] > [Ch][For] > [Ch][But] [35]. The hydration capacity of the IL anion, and so its capability to salt out PPG-400, appeared to be the main driving force behind the ABS formation and thus establishing the observed trend. Consequently, the ILs with citrate and oxalate anions, the ones with the strongest salting-out character, appeared to be the most potent to induce the formation of an immiscibility region with aqueous solutions of PPG-400 [35]. Additionally, the authors [35] obtained the phase diagrams for three specific systems, [Ch][Pro]/PPG-400/H_2O, [Ch][Gly]/PPG-400/H_2O, and [Ch][Lac]/PPG-400/H_2O, at 15, 25, and 35 °C in order to assess the influence of temperature on the phase behavior. The results were in agreement with previous studies on conventional ABS composed of PPG and inorganic salts [93–95]; an increase of temperature enhances the liquid-liquid demixing region. As highlighted by the authors [35], these findings are in accordance with the salting-out mechanism of the IL over PPG-400, since PPG becomes more and more hydrophobic at elevated temperatures and, consequently, water will be drawn from the polymer-rich phase to the IL-rich phase [35].

Later, the same group of researchers [88] performed a more detailed study in order to have a better understanding of the phase equilibrium of ABS formed by Ch-ILs and PPG [88]. Phase diagrams of four different Ch-ILs in combination with PPGs of different molecular weight (MW 400 and 1000 $g{\cdot}mol^{-1}$) or a PEG-PPG copolymer ($EO_{10}PO_{90}$) were measured; this way, the effects of (1) the type of anion present in the IL, (2) the hydrophilic/hydrophobic nature of the polymers, and (3) the temperature could be analyzed. The chemical makeup of the anion had an effect on phase separation similar to those previously mentioned; more hydrophilic anions had a more pronounced ability to induce ABS ([Ch][Lac] $\approx$ [Ch][Gly] $>$ [Ch][Pro] $>$ [Ch]Cl) in the presence of PPG-1000 [88]. The change in the MW of the polymer also strongly influenced the phase equilibrium as the ones with longer chains ($EO_{10}PO_{90}$ and PPG-1000) were salted out more easily with even less IL due to their more hydrophobic character [88]. The effect of temperature variation on the Ch-ABS closely followed the trend observed in their earlier work with PPG-400 [35], where an increase in temperature enhanced the immiscibility region [88].

Both studies performed by Wang and coworkers [35, 88] clearly demonstrated how effectively Ch-ILs can salt out PPG polymers and form ABS. However, in order to develop liquid-liquid extraction systems with coexisting aqueous-rich phases, it would be desirable to use more hydrophilic polymers. Accordingly, at the beginning of this decade, Coutinho and coworkers [3, 87, 90] started to study how the formation of ABS can be initiated using different combinations of ILs and polyethylene glycol polymers. Contrary to the simple salting-out effect of cholinium-based ILs in Ch-IL/PPG ABS reported by the Wang group [35, 88], the studies on IL-PEG ABS using conventional ILs (such as imidazolium, piperidinium, etc.) suggested that the molecular phenomenon behind the formation of an immiscible regime is more complex and intricate [3]. In order to gain deeper understanding of the IL/PEG ABS, recently, phase diagrams for several ternary systems composed of water, Ch-ILs (or salts), and PEGs (PEG-400, PEG-600, and PEG-1000) at different temperatures were determined [90]. The cholinium-based compounds were distinguished according to their melting points (mp): five salts with high mp (in the range of 103–302 °C), two ILs with mp below 100 °C but above room temperature, and three that are liquid at room temperature [90]. Interestingly, this broad study showed that two of the liquid salts, [Ch][Pro] and [Ch][But], were unable to promote phase demixing when combined with any of the PEGs tested (PEG-400, PEG-600, and PEG-1000), while [Ch][Lac] only forms ABS when mixed with the higher MW PEGs (600 and 1000 $g{\cdot}mol^{-1}$) as highlighted in Table 11.2 [90]. It was observed that all the other cholinium salts could form ABS with any of the PEGs tested and, in general, exhibited the following trend in phase separation potential: [Ch][DHph] > [Ch][Bit] > [Ch][Bic] > [Ch][DHcit] ≈ [Ch][OAc] ≈ [Ch][Lac] ≈ [Ch][Gly] > [Ch]Cl [90].

Based on the equilibrium phase diagrams, as well as on the solubility of each cholinium-based compound in PEG or water, the water activities of the species studied, and the binary and ternary excess enthalpies, the ability to promote an ABS seems to be quite different for higher melting cholinium-based salts than for the lower melting or liquid Ch-ILs. It was demonstrated that in case of the cholinium-based salts with high mp, the formation of an ABS is controlled by the solvation of the salt anion (its salting-out aptitude over PEG), similarly to the phase demixing of the conventional PEG/inorganic salt or IL/inorganic salt ABS [90]. On the other hand, aqueous solutions containing Ch-ILs with mp lower than 100 °C and PEG go through phase separation driven by a more complex mechanism, which is related to the balance of competition between all the components in equilibrium to form hydrogen bonds [90]. It became evident that hydrogen bonding interactions between the IL anion and the terminal –OH groups of the polymer are more predominant, allowing a fine adjustment of the biphasic systems [90].

Later on, Mourão et al. [2] investigated the formation of ABS employing hydrophilic Ch-ILs (or salts) in combination with PEG polymers. The authors measured the ternary phase diagrams at 25 °C for seven Ch-ILs with anions derived from naturally occurring organic acids and thus evaluated the influence of different substituent groups and alkyl chains with various lengths [2]. After the evaluation of the influence that different alkyl chains embedded into cholinium IL dicarboxylate anions can have on ABS formation, the following order was observed: [Ch][Glu] > [Ch][Suc] > [Ch][Ox] > [Ch][Mal] [2]. In general, the longer the alkyl chain of the IL anion, the larger is the biphasic region. Initially, the authors [2] assumed this trend as consequence of the salting out of the more hydrophobic ILs by the more hydrophilic PEG; however, the results of water activities of these Ch-ILs urged the authors to revise this conclusion, since the most hydrophilic [Ch][Glu] was the IL that showed the highest capability to form ABS [2]. The effect of the substituent groups attached to the anion was probed with the [Ch][Fum] and [Ch][L-Ma] systems, and their phase diagrams were compared with those obtained for [Ch][Bit] and [Ch][Suc]. The data provided the following ABS forming tendency: [Ch][Fum] > [Ch][Bit] > [Ch][L-Ma] > [Ch][Suc] [2]. [Ch][Bit] and [Ch][L-Ma] are two high-melting salts; however, in contrast to previous findings observed by Pereira et al. [90], no differences were observed in their binodal curves. It is important to note that Mourão et al. [2] presented the binodal curves in molality units (mol/kg), while in Pereira's work [90] they were expressed as initial, total weight fraction of PEG (wt%) instead of initial, total molality of salt (mole of salt per kg of H_2O). We have been trying to alert the scientific community in the field, that the binodal curves should be displayed in units that do not hide trends due to the high MW of polymers when compared with the MW of ILs or water. Thus, although the authors observed similar shapes in the binodal curves, some intricate phenomena could have stayed hidden.

The authors [2] compared the results from the phase equilibria to the ones on water activities for those cholinium-based compounds, but could not observe any relationship. Thus, the authors [2] assumed the predominance of the PEG-600-IL interactions over the water-IL and water-PEG-600 interactions as a possible explanation [2]. In agreement with our previous work [90], Mourão et al. [2] affirmed that the mechanism behind phase splitting in systems containing very hydrophilic Ch-ILs and hydrophilic PEG polymers is not always obvious; ABS formation can be a result of a delicate balance between all possible interactions (PEG-water, PEG-ion, water-ion). Additionally, the authors also evaluated how MW increase of PEG chains affects the phase equilibria by testing all of the seven Ch-ILs (or salts) in combination with PEG-4000. Behavior and trends, similar to those for PEG-600, were observed [2].

Phase separation was also investigated by Mourão et al. for Ch-ILs/K_3PO_4 systems as well, to gain more mechanistic insight [2]. As presented in Table 11.2, five of the Ch-ILs tested were not able to induce a two-phase system when mixed with the strong salting-out K_3PO_4; the two exceptions, [Ch][Glu] and [Ch][Suc] [2], were in agreement with the earlier description of these systems [89]. On the other hand, the majority of Ch-ILs studied did promote two immiscible phases in the presence of aqueous PEG solutions [89]. Similarly to our earlier remarks [90], however, it was noted by the authors that the majority of the cholinium monocarboxylate ILs did not undergo phase separation with PEGs (especially with the ones having low MW), while the IL anions derived from di- and tricarboxylic acids exhibited an aptitude for biphase formation [2].

Table 11.3 summarizes the main conclusions drawn from the published phase diagrams of Ch-IL/K_3PO_4, Ch-IL/PPG, and Ch-IL/PEG systems based on the

Table 11.3 Summary of Ch-ABS and the suspected mechanisms behind phase separation

Ch-ABS type	Ability to induce ABS formation	Mechanism
K_3PO_4 [89]	[Ch][Sal] > [BCh]Cl > [Ch][Lev] > [Ch][Glu] ≈ [Ch][Suc] ≈ [Ch][Ac] > [Ch]Cl	Salting-out aptitude of K_3PO_4 over Ch-ILs
PPG-400 [35]	$[Ch]_3[Cit] > [Ch]_2[Ox] > [Ch][Gly] >$ [Ch][Pro] ≈ [Ch][Lac] ≈ [Ch][OAc] > [Ch][For] > [Ch][But]	Salting-out aptitude of Ch-ILs over PPG-400
PPG-1000 [88]	[Ch][Lac] ≈ [Ch][Gly] > [Ch][Pro] > [Ch]Cl	Salting-out aptitude of Ch-ILs over PPG-400
PEG-600 [90]	[Ch][DHph] > [Ch][Bit] > [Ch][Bic] > [Ch][DHcit] ≈ [Ch][OAc] ≈ [Ch][Lac] ≈ [Ch][Gly] > [Ch]Cl	*Ch-salts:* solvation of salt anion (salting-out aptitude)
		Ch-ILs: complex hydrogen bonding competition between all the components of the system
PEG-600 [2]	[Ch][Glu] > [Ch][Suc] > [Ch][Ox] > [Ch][Mal] – effect of alkyl chain length	Complex interactions between PEG-600 and ILs
	[Ch][Fum] > [Ch][Bit] > [Ch][L-Ma] > [Ch][Suc] – effect of substituent groups	

discussions presented in this section and in previously published research. Although the number of phase diagrams obtained for cholinium-based compounds is not yet significant, tendencies in the mechanism of phase separation can be observed in Table 11.3. When Ch-ILs are mixed with K_3PO_4, the preferential solvation of the inorganic salt appears to be the driving force toward ABS formation. On the other end of the spectrum, the mixture of Ch-IL and PPG, the most hydrophobic of the tested phase inducers, will separate due to the higher affinity of the IL ions toward water molecules. However, when the systems incorporating PEG polymers are studied, the mechanism controlling the phase splitting at the molecular level becomes far more complex since all the dissolved components can contribute significantly to the solvation processes. The most accepted mechanism describing the behavior of these systems is based on a balance of competition for hydrogen bonding between the ternary phase components.

Despite the complexity of the ABS composed of Ch-ILs and the abovementioned polymers, these systems are among the most interesting and promising ones to be utilized as a sustainable alternative in several industrial processes, mainly due to their environmentally friendly characteristics, including high biodegradability, low toxicity, high biocompatibility, and low cost.

11.3.2 Amino-Acid-Based ABS

Amino acids are one of the most abundant groups of natural compounds. Their applicability in the synthesis of new amino acid-based ABS, both as ILs and/or phase-forming agents, has been meticulously studied by several research groups (see Sects. 11.2.1 and 11.2.2). The introduction of amino acids in these complex materials is hoped to increase the sustainability and environmentally friendly of the related synthesis and separation processes.

The first successful syntheses of ILs from 20 natural amino acids were published in 2005 by Fukumoto et al. [50]. Shortly after that, as noted in Sect. 11.2.1.2, several other classes of AA-ILs were obtained, some of them with high biodegradability and biocompatibility. Additionally, the majority of these IL families are easy to obtain in large quantities at low cost, which also makes them promising candidates for separation and extraction processes [96, 97]. Since their first report about amino acid ILs [50], Ohno group [96, 97] has focused their studies on the ability of these ILs to promote two immiscible phases when mixed with water above certain concentrations and at an appropriate temperature. They found [96, 97] that mixtures of water and ILs composed of phosphonium or ammonium cations and various anions derived from amino acids showed phase separation with a lower critical solution temperature (LCST), suggesting that these IL-water mixtures can later be applied as functional fluids for reversible phase transitions (i.e., can be reversed by temperature change or bubbling gases) and used as a rapid separation method for extraction of water-stable proteins. However, these LCST-type IL-water systems, despite the large number of systems published by the

Ohno group [96, 97] and their foreseeable future applications, cannot be considered as true ABS; ABS are composed of two non-mixing yet water-rich phases, while one of the phases of these IL-water mixtures contains mainly hydrophobic ILs with only traces of water. Nevertheless, attempts to obtain true ABS with AA-ILs have been carried out by other researchers.

In 2011, Wu et al. [98] published the first ternary phase diagrams for mixtures of AA-ILs, K_3PO_4, and water. The authors studied the phase equilibria of the obtained ABS in order to gain more insight onto the impact of the structural diversity of the IL components on the relative hydrophobicity and immiscibility of the phases. The extractive capabilities of these systems for proteins were also examined by measuring the partition coefficients as a function of pH and hydrophobicity of the IL for cytochrome-c as a model protein. In their first study, they prepared a series of ILs with $[C_4mim]^+$ as cation and derivatives of four amino acids, L-serine ($[Ser]^-$), glycine ($[Gly]^-$), L-alanine ($[Ala]^-$), and L-Leucine ($[Leu]^-$), as the corresponding anion. The solubility curves of these AA-IL/K_3PO_4 ABS at 25 °C showed that the capability to form two distinct phases decreased in the following order: $[C_4mim][Leu] > [C_4mim][Ala] > [C_4mim][Gly] > [C_4mim][Ser]$, a trend resulted from the hydrophobic nature of the amino acid anions [98]. This suggests that the mechanism driving the phase separation is similar to the one hypothesized for Ch-ILs/K_3PO_4 ABS [89]; the liquid-liquid demixing is a consequence of the competition between the IL ions and the strong salting-out inorganic salt for hydration, where the more hydrophobic IL is easier to be salted out by K_3PO_4 to form another phase [98].

In order to clarify the relationship between relative hydrophobicity of the phases in the AA-ILs ABS and the ability to induce phase separation, the authors [98] calculated the amount of Gibbs free energy required to transfer a nonpolar $–CH_2$ group from the bottom salt-rich phase to the top AA-IL-rich phase ($\Delta G_T(CH_2)$). The $\Delta G_T(CH_2)$ values measured by Wu et al. [98] are negative, indicating the favorable transfer of CH_2 from the bottom phase to the top phase and consequently the more hydrophobic nature of the AA-ILs-rich phase [98]. The observed relative hydrophobicity values showed the same trend among the systems tested as the one obtained while analyzing the phase separation efficiency [98]. Interestingly, it was also found that the relative hydrophobicities of these AA-IL/salt ABS are equivalent to hydrophobicities of polymer/salt ABS and higher than those obtained for polymer/polymer ABS.

Despite their capability to induce liquid-liquid demixing, this series of AA-ILs exhibits some non-benign and non-environmentally friendly characteristics, due to the $[C_4mim]^+$ cation employed. In order to overcome these environmental issues, the same research group proposed alternative solutions in which amino acids are combined with ammonium or phosphonium cations, thus being less toxic for aquatic environments than the imidazolium- (or pyridinium)-based ILs [99]. They synthesized five glycine-based ILs, four of them were prepared with ammonium-based cations (tetramethylammonium glycine, $[N_{1111}][Gly]$; tetraethylammonium glycine, $[N_{2222}][Gly]$; tetra-*n*-butyl-ammonium glycine, $[N_{4444}][Gly]$; tetra-*n*-pentylammonium, $[N_{5555}][Gly]$) and one with tetra-*n*-butylphosphonium cation

([P_{4444}][Gly]). In this manner, they were able to gain information about how the structure of the IL cation affects phase formation, phase hydrophobicity, and the phase equilibrium after mixing these AA-ILs with aqueous solutions of K_2HPO_4 at 25 °C. The results indicated that, with the exception of the [N_{1111}][Gly]/K_2HPO_4 system, immiscible phases were successfully promoted and the phase-forming ability decreased in the following order: [N_{5555}][Gly] > [P_{4444}][Gly] > [N_{4444}][Gly] > [N_{2222}][Gly] [99]. As expected from previous observations on other IL/salt ABS, the formation of ABS was controlled by the relative hydration of the ions in equilibrium, especially that of K_2HPO_4 being the provider of the most competitive ions, thus salting out the IL into the other phase. The analysis of the relative hydrophobicity between the coexisting phases showed the same trend found to illustrate the ability to induce phase separation; the system containing the [N_{5555}][Gly] IL exhibited the highest hydrophobicity while the one with [N_{2222}][Gly] the lowest [99].

Although the synthesis of AA-ILs opened a wide window of new applications, their use in the formation of ABS is quite limited yet. The two works considered in this section proved that this new class of ILs should be taken into account as an interesting alternative to the conventional ILs that are already heavily studied for different ABS-based separation processes. We would like to emphasize again, however, how important it is to consider the toxicity, sustainability, and environmentally friendly of both the IL cations and anions during their design and application in ABS. A good example might be the class of new cholinium-amino-acid ILs, members of which were recently synthesized and characterized [19].

11.3.3 Other Biocompatible IL-ABS

In two previous subsections, we provided examples of biocompatible ABS formulations based on cholinium- and various amino acid-based ILs. Besides these, other new classes have been recently discovered and reported using benign, coexisting phase-forming agents like biodegradable organic salts, carbohydrates, and bio-friendly polymers. We will briefly go through these classes of ABS discussing their phase equilibria and the possible mechanisms behind their formation.

One of the classes of new, more biocompatible ABS are based on ILs generated with biological buffers (Good's buffers (GBs)) as described in Sect. 11.2.1.3. [36]. Taha et al. [36] synthesized a series of 20 new ILs conjugating five GBs (Tricine, TES, CHES, HEPES, and MES) with four hydroxide bases (1-ethyl-3-methylimidazolium, [C_2mim]$^+$; tetramethylammonium, [N_{1111}]$^+$; tetraethylammonium, [N_{2222}]$^+$; tetrabutylammonium, [N_{4444}]$^+$) and then analyzed their environmental impact and biocompatibility [36]. As a next step, they tried to apply some of the GB-ILs in ABS formulations by mixing the obtained ILs with aqueous solutions of inorganic/organic salts. They were able to achieve phase separation with both Na_2SO_4 and potassium citrate ($K_3C_6H_5O_7$) for every

$[N_{4444}]^+$-based GB-ILs they tested [36]. The binodal curves of the ternary phase diagrams clearly displayed that the ILs with more hydrophobic buffer anions, such as CHES or MES, are more easily salted out than the GB-ILs having more hydrophilic anions (HEPES, TES, or Tricine) [36]. These observations are in parallel with the results of earlier IL/salt ABS experiments, thus the mechanism controlling the phase equilibria at a molecular level is most probably the same competition between the ions in equilibrium for hydration. The differences in the phase diagrams of systems with Na_2SO_4 and $K_3C_6H_5O_7$ were also pointing in the same direction; the inorganic salt with high charge density is a stronger salting-out agent and consequently induced two-phase formation much easier than the weakly salting-out organic salt [36]. Even though the ABS composed of GB-ILs and potassium citrate exhibited lower capability to promote liquid-liquid demixing, the more environmentally friendly nature of the organic salt employed, compared to Na_2SO_4, still makes their application preferable.

Taha et al. [36] also compared the phase equilibria of these new GB-ILs-based ABS with the more conventional ABS composed of $[N_{4444}]Cl$ IL and potassium citrate, a system previously examined by Passos et al. [58]. All the $[N_{4444}][GB]$ ILs exhibited higher ability to form ABS than $[N_{4444}]Cl$, since these GB-ILs are easier to salt out by $K_3C_6H_5O_7$ than the ILs comprising Cl^-. Thus, these biological buffer ILs/organic salt systems can be effective and bio-friendly phase formers with an additional benefit of delicate pH control in the separation media, a useful process parameter when proteins or biopharmaceuticals have to be handled in a more biocompatible manner. However, biocompatibility can be enhanced even in the cases of more conventional ABS, like the ones tested by Passos et al. [58] containing $[N_{4444}]Cl$ and $[P_{4444}]Cl$ ILs, by the environmentally conscious selection of the coexisting phase-forming agents.

Efforts have been made in order to improve the "greener" character of frequently used ABS through the application of less toxic and more environmentally friendly phase-forming agents. As recently reviewed by Freire et al. [12], many of the approaches, however, were initiated from a false starting point by adding biocompatible phase formers into systems composed of highly toxic IL families. Two studies, for example, confirmed the formation of ABS for the combination of ILs and amino acids, but unfortunately the ILs tested (imidazolium- and tetrafluoroborate-based ILs) were members of nonbiodegradable families and considered to be highly toxic to aquatic life forms. Similar works were also published describing the combination of carbohydrates or biocompatible polymers and not very environmentally friendly ILs, with the exception of Ch-ILs, to form aqueous biphasic systems.

Another halfway approach was recently published by Wang and coworkers [37, 100]. They suggested the use of guanidinium-based ILs, as more biocompatible phase-forming ABS promoters, supposedly suitable for protein extractions. Their shorter synthesis time and better designability could be advantageous over traditional ILs; however, as we highlighted in Sect. 11.2, more information is needed about their biodegradability and aquatic toxicity. The authors analyzed the phase equilibria of 1,1,3,3-tetramethylguanidinium acrylate [TMG][Acr] systems in the presence of inorganic salts, concluding salting-out strength in the following order: $K_3PO_4 > K_2HPO_4 > KH_2PO_4 > K_2CO_3$ [100]. They also evaluated the phase-forming ability of nine guanidinium-based ILs when mixed with aqueous solutions of K_2HPO_4 and observed a trend that follows: acetate > sorbate > itaconate > acrylate > methacrylate > lactate > cinnamate > maleate [100]. Based on this trend, Wang et al. [100] proposed that the IL viscosity, which depends on the IL anion, may play a key role in the phase separation mechanism. The same group then went further down this road by synthesizing another series of guanidinium-based ILs and testing their ABS forming efficiency with aqueous solutions of K_2HPO_4 [37]. The ternary phase diagrams showed differently shaped curves for the guanidinium-based ILs, which could be related to many complex factors, such as hydration capacity (IL components with higher charge density are strongly hydrated and consequently decrease the number of water molecules available for hydration of the salt ions) or longer alkyl chain length that leads to easier liquid-liquid demixing [37].

Sintra et al. [63] obtained phase diagrams for systems formed by cholinium-, phosphonium-, or ammonium-based ILs (or salts) and aqueous solutions of biodegradable salts, in particular, potassium citrate buffer. It was concluded that, in general, phase separation was controlled by the pH and the length of the alkyl chains appended to the IL ions, that is, the hydrophobic/hydrophilic nature of the IL, and their susceptibility toward being salted out [63].

Herein we summarized the major recent advancements in creating biocompatible ABS, highlighting the most important classes of ILs able to create them and showing the main effects governing the liquid-liquid demixing. However, as we have seen, judging results and chemical products can be troublesome when even the concepts of "biocompatibility" and "environmentally friendly" are too ambiguous themselves.

11.4 Applications of Biocompatible Aqueous Biphasic Systems

The major applications of IL-based ABS, proven and/or predicted, could be the extraction and/or purification of a wide range of solutes from simple alcohols to complex proteins and enzymes and the recovery of the ILs themselves from aqueous media [12]. Bio-ABS should be able to fulfill the same or similar roles; however, in most cases, experimental results describing phase equilibria and, in some reports, intended applications tested on model compounds are the only data yet available.

Proposed applications of biocompatible ABS are summarized in Table 11.4. The ABS considered were prepared using new biocompatible ILs families, cholinium-, amino-acid-, and guanidinium-based ILs; guanidinium-based ILs were included as

Table 11.4 Applications of biocompatible ABS

Type of Bio-ABS	Ternary system	Application
Ch-ILs-based ABS	Salt $+K_3PO_4+H_2O$	Separation of antibiotics: *tetracycline, tetracycline-HCl, ciprofloxacin-HCl* [89]
		Recovery of *saponins* and *polyphenols* from two matrixes (tea and mate) [103]
	Salt $+K_2HPO_4+H_2O$	Extraction of proteins: *bovine serum albumin (BSA)* [101]
	Salt + PEG-600 $+H_2O$	Extraction of antibiotics from fermentation broth: *tetracycline* [102]
		Extraction/separation of antioxidants: *tert-butylhydroquinone (TBHQ)* [2]
	Salt + PPG-400 $+H_2O$	Partitioning of proteins: *lysozyme, papain, trypsin, BSA* [35]
AA-ILs-based ABS	IL $+K_3PO_4+H_2O$	Partitioning of proteins: *cytochrome-c* [98]
	IL $+K_2HPO_4+H_2O$	Partitioning of proteins: *cytochrome-c* [99]
Guanidinium-ILs-based ABS	IL $+K_2HPO_4+H_2O$	Extraction of proteins: *lysozyme, BSA, ovalbumin (OVA), bovine hemoglobin (BHb)* [100]
		Purification of proteins: *lysozyme, trypsin, OVA, BSA* [37]
GB-ILs-based ABS	IL $+K_3C_6H_5O_7+H_2O$	Extraction of proteins: *BSA* [36]

benign classes, since these ABS are suggested to be able to extract proteins with low degradation rates [37]. Other ABS that use conventional ILs with more benign coexisting phase-forming agents (polymers, organic salts, carbohydrates, etc.) were recently reviewed by Freire et al. [12].

As it can be seen from Table 11.4, the majority of Bio-ABS, 7 out of the 11 systems shown, are intended for extraction and/or separation of various proteins, while other possible extractive applications were also suggested (extraction of antibiotics, recovery of saponins and polyphenols, and separation of antioxidants). The next two subsections will discuss these applications in the same order, starting with the extraction of proteins.

11.4.1 Protein Extraction and Purification

The list of biotechnological applications of proteins and enzymes (therapeutic biopharmaceuticals, diagnostic biomarkers, etc.) is continuously growing. The traditional methods to extract and purify proteins are too complex, thus too expensive, time-consuming, and hard to scale-up. Moreover, most organic solvents usually applied in these separation processes are harmful to these sensitive and extremely unstable molecules and can cause their denaturation and consequently the loss of their biological activity. Thus, there is an urgent need for more benign processes, and the use of benign and biocompatible ILs in stable, water-rich, liquid-liquid extraction systems seems to be the ideal approach for a more "natural" step in protein purification.

In the seven studies summarized in Table 11.4, the applicability of Bio-ABS for protein extraction was frequently proved with model proteins/enzymes (cytochrome-c, BSA, OVA, BHb, lysozyme, papain, trypsin). Most of these studies were tested to examine the partitioning mechanisms, the interaction phenomena governing protein migration between the phases, and the effect of the ABS on protein stability. The high purification efficiency of Ch-ILs-based ABS was observed even in the earliest work of the Wang group in 2012 [35]. After one single-step extraction of four model proteins, yields in the range of 86.4–99.9 % were achieved with a trend in extraction efficiency decreasing in the following order: lysozyme > papain > trypsin > BSA [35]. The authors [35] justified that the size of the protein has a major effect on its extractability; the transfer of a protein molecule into the IL-rich phase depends on the interactions of the phase-forming components that have to be broken in order to create a cavity where the protein will be entrapped. Thus, larger proteins require larger cavities that need more energy to break solvent interactions [35].

The influence of two other parameters, tie line length (TLL) and pH, was also examined [35]. It was found that the IL will be more concentrated in the bottom, IL-rich phase with increased TLL, and consequently the energy required to disrupt the IL-water network will be higher, decreasing the partitioning coefficient of the protein [35]. The effect of pH change, however, could not be explored reliably as it was observed that, when the pH was adjusted between pH 5 and 11, more than 86 % of the protein molecules were already transferred into the IL-rich phase and thus not sensitive to any change in the pH administered in the other phase [35].

This extensive study [35] also incorporated the evaluation of the change in enzymatic activity accountable to Ch-ILs/PPG-400 ABS and the recyclability of the PPG polymer. According to their results [35], the addition of some ILs (especially [Ch][Lac]) to water mixtures can apparently enhance the activity and thermal stability of certain enzymes, trypsin in particular. On the other hand, the PPG-400 phase-forming polymer could easily be recovered and recycled by convenient heating [35]. All these interesting data obtained by the Wang group [35] are fundamental to show how to increase the more benign and biocompatible character of ABS, combining cholinium-based ILs and polymers, which suggests that their application can be a viable alternative to the common protein separation processes.

Following this work, Huang et al. [101] compared the extraction efficiency of a cholinium-like IL-based ABS to that of imidazolium-based IL-ABS, all formed with aqueous solutions of K_2HPO_4, using BSA as a model protein. The authors [101] found the cholinium-like IL-ABS to be the most efficient extractive system, and, after optimizing the most crucial parameters (temperature, extraction time, IL-salt content), yields of 92–100 % were achieved in single-step extractions [101]. They also determined that intermolecular processes, micelle formation, and salting-out effects were the major driving forces in the protein transfer process, while aggregation phenomena were responsible for protein separation [101]. Analytical results (including UV-Vis, FLS, FT-IR, and TEM) also proved that the conformation of the model protein BSA was not altered by this IL extraction process, indicating that this system has a potential future in biotechnological separations [101].

Wu et al. [98, 99] reported similar observations with AA-ILs. These ILs were synthesized with imidazolium cations [98] or ammonium or phosphonium cations [99] and then ABS were prepared with K_3PO_4 [98] or K_2HPO_4 [99] solutions, and their ability to extract cytochrome-c was tested. The determination of cytochrome-c partition coefficients and the polarity of the corresponding ionic-liquid-rich phase suggested that hydrophobic interactions between the IL and cytochrome-c are mainly responsible for achieving the higher partition coefficients [98, 99]. The authors also elucidated, however, that protein separation can be effected by other types of interactions as well, like specific electrostatic interactions between the ions of the AA-ILs and the protein or salting out of the protein by the inorganic salt [98, 99].

The occurrence of liquid-liquid demixing in aqueous mixtures containing 1,1,3,3-tetramethylguanidinium-based ILs was also proved [37, 100]. Zeng et al. [100] examined the extractive potential of the obtained ABS with three model proteins, namely, BSA, BHb, and OVA. The authors [100] maximized the extraction efficiency for every system using single factor experiments by optimizing time, temperature, and the concentration of the components. However, the efficiency and the mechanism of protein partitioning were observed to be dependent on the type of the protein and the IL as well. In the case of BSA, yields up to 99.62 % were achieved, while no change in the conformation of the protein was observed. The authors also demonstrated that the mechanism of protein partitioning was mainly controlled by the aggregation and embrace phenomena, which depends on the interactions of the phase-forming components that have to be broken in order to create a cavity where the protein will be entrapped [100].

Similar experiments with another set of model proteins (lysozyme, trypsin, OVA, and BSA) and guanidinium-IL-based ABS also proved that protein separation using these systems can be more effective than with conventional IL-ABS [37]. It was further concluded that the trend in the observed extraction efficiencies (in decreasing order: lysozyme > trypsin > OVA > BSA) was governed by the size effect of proteins, that is, larger proteins require a larger inclusion cavity to fit in, necessitating the separation of more interacting solvent components [37], a mechanism similar to the one discussed earlier with regard to Ch-ILs-based ABS [35]. These systems were also found to be very effective in protein separation without causing any change in the structural features and biological activity of the model subjects [37].

Taha et al. [36] performed similar experimental studies with GB-ILs. The authors [36] explored if these ILs can be employed as self-buffering compounds, protein stabilizers, and, ultimately, extracting agents in ABS formulations. GB-ILs with buffering capacity at pH 7 were combined with a biodegradable and nontoxic organic salt, potassium citrate, and then the extraction of the model protein BSA was examined. In every experiment, extraction efficiencies close to 100 % were observed, with no apparent signs of BSA loss in the mass balances, either by precipitation or denaturation [36].

Thus, as we saw in the examples mentioned in this subsection, these ABS, formulated with more benign and/or biocompatible ILs, have the ability to extract proteins with high efficiency without altering their conformation or biological activity. It has to be highlighted for future ABS design, however, that energy is required to transport the protein into the IL-rich phase, and this energy is apparently proportional to the size of the protein molecule and probably to the strength of the solvent-solvent interactions.

11.4.2 Approaches for Extraction of Other Biomolecules

The examples in Table 11.4 show that the extractive potential of Bio-ABS can probably be extended toward other solutes, such as antibiotics, antioxidants, saponins, and polyphenols. Antibiotics are high-added value pharmaceutical agents that can be naturally produced by some microorganisms, however, in relatively low concentrations. The separation of antibiotics is usually a complex and consequently costly process (processes), including extraction with organic solvents, precipitation or crystallization techniques, and/or even more expensive methods, such as ultrafiltration or chromatography. The extensive production and usage of pharmaceuticals, including antibiotics, on the other hand, have raised several concerns about their potential adverse effects on human health and the environment. Accidental release of pharmaceuticals/antibiotics, even by passing through the human body with no chemical change into the environment, combined with their usually low degradation rate, hence their accumulation and migration among water systems, have already generated severe issues [12]. To address these issues, the design and widespread application of more benign and environmentally friendly, and hopefully more economic, separation/concentration processes are necessary. ABS incorporating the new and biocompatible IL families are certainly viable alternatives for traditional methods and hold great future potential.

It was proposed in 2013 that ABS composed of cholinium salts and PEG-600 can be cheaper, more sustainable, and biocompatible methods for the extraction of tetracycline (used as a model) from the fermented broth of *Streptomyces aureofaciens* [102]. ABS, composed of aqueous solutions of PEG-600 and five different cholinium-based salts ([Ch]Cl, [Ch][OAc], [Ch][DHcit], [ChDHph], and [Ch][Bic]), were tested and optimized first with commercially available tetracycline, in order to gain information about the partitioning coefficients of tetracycline. The composition of the IL and the pH had the major influence on the partitioning coefficient of tetracycline; the highest values of partitioning were obtained for the most alkaline ABS, the [Ch][OAc]- and [Ch][Bic]-based ones, while the ABS composed of PEG-600 and [Ch]Cl were not able to induce a preferential partitioning [102]. The most fascinating observation was that, depending on the cholinium salt employed, tetracycline can be concentrated either in the polymer-rich or in the salt-rich phase, suggesting further options for future tailored applications [102]. When the two systems showing the highest and lowest partitioning, [Ch][Bic] and [Ch]Cl, respectively, were used to extract tetracycline directly from fermented broth, extraction efficiencies over 80 % were obtained [102]. These promising results further support the idea of applying these biocompatible Ch-ILs-based ABS as an alternative technique in a pre-purification stage of downstream processes designed to recover antibiotics from complex and natural matrices.

Shahriari et al. [89] also examined the applicability of ABS composed of Ch-ILs and K_3PO_4 for the separation of antibiotics using two model compounds, tetracycline and ciprofloxacin, and their hydrochloride forms [89]. In general, the observed partitioning coefficients decreased with the following rank: [Ch][OAc] > [Ch]Cl > [Ch][Lev] > > [Ch][Suc] > [Ch][Glu]. On the other hand, the IL-rich phases appeared to be more successful in extracting the hydrochloride forms in the following order: tetracycline· HCl > ciprofloxacin·HCl > tetracycline [89]. The mechanisms behind the migration of these antibiotics seem to be far more complex than the phenomenon observed for proteins, where the dominant factor was the size of the protein molecule. The authors supposed, however, that partitioning of these antibiotics may be influenced by specific ionization effects and hydrogen bonding interactions (since the portioning was favorable toward the IL-rich phase) and that the hydrochloride forms have higher affinity for water [89]. Although the extraction mechanisms are not well understood yet, this study provided another example of the potential applicability of the Ch-ILs-based ABS as a real alternative to the traditional extraction methods.

Attempts to extract other biomolecules with the aid of Ch-ILs-based ABS were published as well. The antioxidant *tert*-butylhydroquinone (TBQH) was extracted using four different IL/PEG-600 ABS, where the ILs of choice were [Ch][Mal], [Ch][Suc], [Ch][Fum], and [Ch][L-Ma] [2]. The obtained partition coefficients for the PEG-rich phase varied between 3.55 and 11.79, while the extraction efficiencies ranged from 83 to 93 % [2]. Interestingly, the results are in accordance to those measured when the partitioning of tetracycline in certain ABS, composed of cholinium-based salts and PEG-600, also favored the polymer-rich phase [102].

Ribeiro et al. [103] examined if [Ch]Cl/K_3PO_4 ABS were suitable to extract saponins and polyphenols from complex biological extracts of tea and mate. The high partitioning coefficients obtained suggest that the application of Ch-ILs-based ABS can be a viable method for the extraction of these metabolites even from complex natural matrices [103].

The examples in this section clearly illustrated that Bio-ABS have the potential to be used as biocompatible and stable methods for purification and separation of various biomolecules with high efficiency, even at the industrial level. Proper selection of phase-forming agents and optimized conditions can further improve selective partitioning of the desired compounds.

11.5 Conclusions and Future Prospects

Environmental concerns and the growing market for bio-products with added value have initiated new research activities to find and design more benign and biocompatible methods and processes to produce them. ABS incorporating ILs may one day replace conventional organic solvent-based extraction processes assuming that their environmental and economic impacts are reduced below a required level. This chapter tried to collect and explain the outcomes of the emerging research focusing on biocompatible ILs and ABS thereof. Various aspects and combinations of the three components required to form IL-based ABS, cation and anion of the IL complemented with a phase-forming agent, were discussed. The most promising ABS variants were those composed of cholinium- and/or amino-acid-based ILs, especially the ones that employ a cholinium cation and an amino acid anion. Both compound classes are abundant in nature, thus highly biocompatible, and therefore can be obtained from renewable natural sources, vehemently sought after and highly appreciated properties. The study of these ILs, however, is far from being complete leaving plenty of room available for further research.

Besides the research on more variations of Ch- and/or AA-ILs, it is also important to identify and thoroughly test possible candidates for the role of benign and biocompatible phase-forming agents. They can most likely be found among (bio)polymers, (bio)surfactants, and biodegradable salts. The cholinium-based ILs/PEG ABS can be considered as early examples since they exhibit strong potential toward the environmentally friendly extraction of various bio-products at low cost.

Although the research of these environmentally friendly IL-ABS has already produced promising results, the mechanisms governing the liquid-liquid demixing at the molecular level or the phenomena behind the migration of biomolecules between the phases are still not fully understood. The theoretical understanding of these processes can lead to further developments and, combined with necessary improvements in the modeling and simulation of the scale-up processes, will be highly beneficial at a future industrial level. The full picture obliges us, however, to continuously study ways of recovering phase-forming agents, as well as ILs, in order to minimize the environmental impact of the entire industrial production system. In summary, it is evident, even from the few studies reporting the use of Bio-ABS so far, that these systems can become an effective, environmentally friendly sustainable, and biocompatible alternative to the more traditional extraction systems, including IL-ABS, in the near future to obtain high-value proteins, such as biopharmaceuticals.

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