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Aqueous biphasic systems: a benign route using cholinium-based ionic liquids†

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Ionic liquid based aqueous biphasic systems (ABS) have been the focus of a significant amount of research in the last decade. However, only (moderately) toxic and poorly biodegradable ionic liquids have been explored hitherto. Focusing on the development of more benign and sustainable approaches, a novel class of ABS using cholinium based ionic liquids is proposed. For the first time, it is shown that a large assortment of cholinium based ionic liquids is capable of undergoing liquid liquid demixing in the presence of aqueous solutions with strong salting out species. In order to assess the applicability of these systems for separation purposes, the partitioning of two antibiotics and/or their hydrochloride forms was also investigated. Cholinium based ABS are shown to be improved routes for the extraction of pharmaceuticals, achieving complete extractions in a single step by way of the proper tailoring of the phase forming components and their concentrations in the aqueous media.

Introduction

The pioneering work of Albertsson launched aqueous biphasic systems (ABS) as alternative and biocompatible liquid–liquid extraction techniques for the recovery and purification of distinct (bio)molecules, namely proteins and nucleic acids.[1] Conventional ABS are formed by two water-soluble polymer–polymer, polymer–salt or salt–salt combinations that phase separate above given concentrations.[2]

In the last decade a large amount of work has been devoted to ABS composed of a novel class of compounds—ionic liquids. After the proof of principle reported by Rogers and co-workers in 2003,[3] several researchers have been working with ionic liquid-based ABS, either by providing their phase diagrams or by exploring their ability to extract value-added compounds.[4] Most research has dealt with ABS made up of ionic liquids and typical organic/inorganic salts as feasible alternatives to polymer–salt systems[5] while, more recently, ionic liquids have also been shown to sucessfully replace traditional salts and to be strong enough to induce the salting-out of a polymeric-rich phase.[6] In addition, aqueous solutions of imidazolium-based ionic liquids also undergo liquid–liquid demixing in the presence of more benign species such as amino acids[7] and carbohydrates.[8]

Ionic liquids (ILs) are low-temperature molten salts that can be designed for specific purposes by preselecting diverse combinations of cations and anions. Therefore, a main advantage of using ionic liquids in the formation of ABS rests on the possibility of tailoring their polarities and affinities by the proper organization of the ions which constitute a given fluid. This feature is a major benefit of ionic-liquid-based ABS given the difficulty of overcoming the limited polarity range of polymer-based ABS.[9] Polymer–polymer-based systems usually display two hydrophobic phases whereas polymer–salt ABS have both an extreme hydrophobic phase and a highly hydrophilic one. This limited difference in polarities between the coexisting phases prevents the widespread use of polymer-based ABS for extraction purposes since the tailoring and selective partitioning are difficult to achieve. Usually, either the functionalization of the polymer or the addition of specific salts is used to overcome this drawback.[10] On the other hand, by virtue of their tunability, ionic liquids can ''ideally'' cover the full hydrophilicity–hydrophobicity range and specific (and effective) extractions can be directly envisaged.

Despite fascinating findings regarding the extraction efficiencies of ionic liquid-based ABS for amino acids, proteins, pharmaceuticals and phenolic compounds amongst others,[4] the large-scale application of these systems is still

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† Electronic supplementary information (ESI) available: Weight fraction solubility data, comparison of the data gathered in this work with literature results, ternary phase diagrams in weight fraction, density and viscosity of the coexisting phases in the temperature range between 298.15 and 328.15 K, partition coefficients of antibiotics and respective standard deviations and NMR spectra of the synthesized ionic liquids. See DOI: 10.1039/c2ra22972b

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hampered by the price and hazardous toxicities of some ionic liquids. Even though a large number of ionic liquids have been used in the formulation of ABS, they have been limited to imidazolium-, pyridinium-, piperidinium-, pyrrolidinium-, and phosphonium-based cations, combined either with halogens, fluorinated anions, sulfates, sulfonates, or with more complex anions such as tosylate.[4] Nevertheless, several of these ions possess some toxicity and are poorly biodegradable, although side chain modifications may enhance their primary biodegradability potential and decrease their toxicity.[11] In addition, the feedstock commonly used to produce these ionic liquids is of nonrenewable origin. Thus, the search for safer and cheaper ionic liquids for ABS while taking advantage of their benefits, is still an imperative issue. The ample versatility of ionic liquids should permit the synthesis of new fluids with both an acceptable environmental footprint and enhanced biocompatibility.

Choline chloride (also known as 2-hydroxyethyltrimethylammonium chloride) is a water soluble essential nutrient which supports several biological functions.[12] Choline chloride is a salt with a high melting point (302 °C)[13] and recent advances have demonstrated the synthesis of novel cholinium-based ionic liquids combined with different anions.[14–16] Indeed, these ionic liquids have shown excellent biodegradability[17] and low toxicity to filamentous fungi and the freshwater crustacean *Daphnia magna*.[15] While both biocompatible and biodegradable, cholinium-based ionic liquids share the most relevant ionic liquid properties: a wide liquidus temperature range, non-flammability and negligible vapour pressure at ambient conditions. Due to all the outstanding properties of cholinium-based ionic liquids, scientific interest in them has increased in the past few years, with applications as diverse as crosslinking agents for collagen-based materials,[18] in the pretreatment and dissolution of biomass,[16,19] in improving the visualization of hydrous samples by scanning electron microscopy,[20] and use as cosubstrates for microorganisms in the degradation of azo dyes.[21] Moreover, a number of works have described novel cholinium-based ionic liquids in which the protein structure and enzyme function can be maintained or even increased.[14,22] The work of Deive *et al.*[23] has shown that fungi are more able to grow (compared to bacteria) during their exposure to these biocompatible ionic liquids and evaluated the ionic liquid degradation during cultivation.

Taking into account all the benefits of cholinium-based ionic liquids, we proposed the first use of these fluids and inorganic salts as the main constituents of ABS. Novel phase diagrams, tie-lines, and tie-line lengths, as well as pH values, densities and viscosities of the coexisting phases were determined at 298 K. In order to establish the extractive potential of the studied ABS, two antibiotics and/or their hydrochloride forms, were screened *via* their partitioning between the two aqueous phases. Tetracycline, tetracycline·HCl and ciprofloxacin·HCl are produced by fermentative processes and by synthetic routes, respectively.[24] The study of different antibiotics allows us to gauge the potential of these novel ABS as alternative purification platforms.

Results and discussion

Phase diagrams and tie-lines

At 298 K and atmospheric pressure, groundbreaking ternary phase diagrams were determined for the common inorganic salt K_3PO_4, water and the following cholinium-based ionic liquids: cholinium chloride ([Ch]Cl), cholinium acetate ([Ch][Ac]), cholinium levulinate ([Ch][Lev]), cholinium glutarate ([Ch][Glu]), cholinium salicylate ([Ch][Sal]), cholinium succinate ([Ch][Suc]) and benzyldimethyl(2-hydroxyethyl)ammonium chloride ([BCh]Cl). It should be mentioned that cholinium L-malate, cholinium bicarbonate, cholinium bitartarate and cholinium dihydrogencitrate were also tested albeit without liquid–liquid demixing. Fig. 1 depicts the chemical structures of the cholinium-based fluids that form aqueous two-phase systems in the presence of a K_3PO_4 aqueous solution.

The experimental phase diagrams of the various ternary systems are graphically presented in Fig. 2. To remove the influence of divergences which could be the mere result of the different molecular weights of the dissimilar ionic liquids, the saturation curves are compared in molality units (mol of solute *per* kg of solvent). The experimental weight fraction data of the liquid–liquid ternary systems are detailed in the ESI†.

Although there are several reports in the literature of various ionic liquids undergoing liquid–liquid demixing in the presence of inorganic salts, for instance imidazolium-,

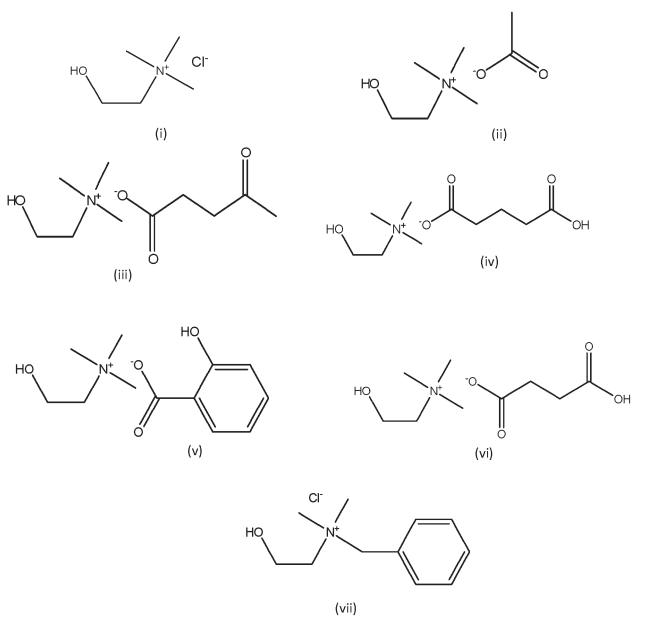

Fig. 1 Chemical structures of the cholinium based ionic liquids studied: (i) cholinium chloride ([Ch]Cl); (ii) cholinium acetate ([Ch][Ac]); (iii) cholinium levulinate ([Ch][Lev]); (iv) cholinium glutarate ([Ch][Glu]); (v) cholinium salicylate ([Ch][Sal]); (vi) cholinium succinate ([Ch][Suc]); (vii) benzyldimethyl(2 hydro xyethyl)ammonium chloride ([BCh]Cl).

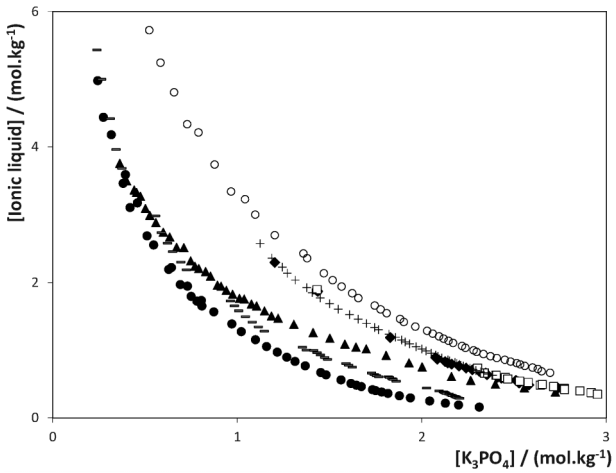

Fig. 2 Phase diagrams of the ternary systems composed of K_3PO_4 + cholinium based ionic liquid + H_2O at 298 K: (○) [Ch]Cl; (+) [Ch]Ac; (□) [Ch][Suc]; (◆) [Ch][Glu]; (▲) [Ch][Lev]; (▬) [BCh]Cl; (●) [Ch][Sal].

pyridinium-, pyrrolidinium- and piperidinium-based cations,[4] this work constitutes the first evidence that ionic liquids based on the non-toxic and biodegradable cholinium cation and sustainable organic acid-based anions also form ABS in the presence of strong salting-out species. Obviously, new screenings of cholinium-based ionic liquids and other inorganic or organic salts should be explored to enlarge the collection of more benign ABS.

When a high charge density salt such as K_3PO_4 is dissolved in an aqueous solution, the isolated ions are surrounded by a layer of water molecules, a phenomenon known as ionic hydration. Thus, when K_3PO_4 is added to an aqueous medium containing an ionic liquid, the two solutes compete for the solvent molecules. Typically, the competition is won by the inorganic ions which are more able to form hydration complexes and, therefore, a ''migration'' of solvent molecules away from the ions of the ionic liquid towards those of the inorganic salt takes place, which in turn leads to liquid–liquid demixing.[25] As a result, a phase rich in ionic liquid separates from the rest of the inorganic salt solution. Nevertheless, our data indicate that some cholinium-based ionic liquids are salted-out by the inorganic salt, whereas other cholinium-based salts are not. These results suggest that, in order to form ABS, the ionic liquid ions must present a delocalized charge and, thus, be poorly hydrated in solution. Only the choliniums with an aliphatic moiety at the anion, or completely hydrogenated carbons, can undergo phase separation with the addition of the phosphate salt solution. Even though bitartarate, dihydrogencitrate and malate are organic anions, they present a large number of hydroxyl groups with improved hydrogen-bonding. Therefore, these anions compete with the inorganic salt ions for the formation of hydration complexes and are not sufficiently ''hydrophobic'' to undergo liquid–liquid demixing.

Fig. 2 reports all the ternary phase diagrams for the cholinium-based fluids and allows us to assess the capacity of the various ionic liquids to form two macroscopic aqueous phases. The larger the two-phase region, the easier it is for the ionic liquid to undergo liquid–liquid demixing. The overall likelihood of these ionic liquids forming ABS occurs in the following order: [Ch][Sal] > [BCh]Cl > [Ch][Lev] > [Ch][Glu] ≈ [Ch][Suc] ≈ [Ch][Ac] > [Ch]Cl.

The ionic liquid most easily separated from aqueous solution is [Ch][Sal]. Although it has an anion with an hydroxyl group which could enhance hydrogen-bonding with water, it also boasts an aromatic ring with a high number of carbon atoms and is thus very effective in promoting ABS formation. An increase in the number of carbons leads to a decrease in the hydrophilic nature (weaker affinity for water).[26] Indeed, this trend agrees with our previously reported findings regarding imidazolium-based ionic liquids and inorganic salts in which the tosylate anion is more prone to undergoing liquid–liquid demixing than a wide variety of other anions.[27] The same argument is valid for the inclusion of a benzyl group at the cholinium cation, [BCh]Cl, as compared to the phase diagram of [Ch]Cl. Taking into account the log K_{ow} values (octanol–water partition coefficients) as a measure of the differential solubility of a given substance between octanol and water and, thereby, as a descriptor of hydrophobicity of the three cholinium-based ionic liquids, we obtain the following rank: $\log K_{ow}$([Ch][Sal]) = 2.06 > $\log K_{ow}$([BCh]Cl) = 2.54 > $\log K_{ow}$([Ch]Cl) = 3.70.[28] As expected, the log K_{ow} trend agrees well with the observed capacity for ABS formation, *i.e.*, the higher the hydrophobicity of the ionic liquid the more easily it is salted-out by K_3PO_4.

Comparing the anions with carboxyl groups, but not with hydroxyl groups, it appears that [Ch][Lev], with its longer aliphatic chain, is more able to form two phases than [Ch][Ac]. In addition, when evaluating both [Ch][Lev] and [Ch][Glu], with a similar number of carbons at the anion, the presence of additional hydroxyl groups enhances the affinity of the anion for water and consequently decreases the capacity for phase separation. In the case of cholinium malate, with its two hydroxyl terminal groups, no phase separation was in fact observed, as previously mentioned. In general, based on these findings, it can be postulated that the ionic liquids' affinity for water is the driving force behind their ability to be salted-out by the high charge density salt, and accordingly, to form ABS.

These findings reveal that the structural characteristics of the ionic liquid ions have a large impact on phase diagram behaviour. In summary, two major factors were found to govern their salting-out/-in capacity: (i) the number of –OH groups, and (ii) the size of the hydrocarbon moiety.

Recently, the use of cholinium-based ionic liquids (with conjugate bases of organic acids as anions) was proposed for the formation of ABS when combined with a hydrophobic polymer (poly(prolylene glycol) 400, PPG 400).[29] Wang and co-workers[29] reported the preferential hydration of the ionic liquids and thus their salting-out capability through the polymer aqueous phase. The authors showed that the salting-out aptitude of an anion is directly related to its hydration capacity, and that citrate- and oxalate-based ionic liquids are the stronger salting-out species and are more prone to inducing the liquid–liquid demixing of PPG 400 from aqueous media.[29] In the current work, more hydrophobic cholinium-based ionic liquids were selected so that they could be salted-

out by a high charge density salt (K_3PO_4). In contrast to the previous work,[29] the most hydrophobic cholinium-based ionic liquids, *i.e.*, those that are less prone to hydration, are better phase separated and more able to form ABS.

We have shown that cholinium-based ionic liquids are able to form ABS in the presence of salting-out species. Even so, it should be stressed that the ability of these cholinium-based fluids for phase separation is lower than that of ionic liquids made up of other cation families. For instance, chloride-based ionic liquids composed of the cations 1-butyl-3-methylimidazolium, 1-butyl-1-methylpyrrolidinium, tetrabutylphosphonium or tetrabutylammonium are more easily salted-out than [Ch]Cl (ESI†).[30] This is an inherent consequence of the two short chain methyl moieties and an hydroxyl group at the cholinium cation which enhance its affinity for water and impedes its phase separation.

The experimental binodal curves were fitted by least-squares regression using the non-linear eqn (1):[31]

$$[\mathrm{IL}] = A \exp[(B \times [\mathrm{K_3PO_4}]^{0.5}) \quad (C \times [\mathrm{K_3PO_4}]^3)] \qquad (1)$$

where [IL] and $[K_3PO_4]$ are the ionic liquid and the salt weight fraction percentages, and A, B and C are constants obtained by the regression of the experimental binodal data.

The correlation parameters A, B and C, along with their standard error of estimate (σ) and correlation coefficients (R^2), are listed, for each system, in Table 1.

On the basis of the standard deviations obtained, it is safe to assume that eqn (1) provides a good description of the experimental data, as previously observed for other families of ionic liquids,[5] and can be used to determine a particular composition for which no experimental data are available.

The experimental results for the tie-lines (TLs), tie-line lengths (TLLs) and the percentage weight fraction of salt and ionic liquid in the top and bottom phases are reported in Table 2. It should be noted that "*T*", "*B*", and "*M*" designate the top phase, the bottom phase and the initial mixture, respectively; $[K_3PO_4]$ and [IL] represent the concentration in weight fraction percentage of salt and ionic liquid. In every system studied the bottom phase corresponds to the K_3PO_4-rich one whereas the cholinium-rich phase is on top.

An example of the TLs representation along with the correlation of the binodal data using eqn (1) is shown in Fig. 3.

Table 1 Correlation parameters of eqn (1) adjusted to the binodal experimental data (also a standard error of estimate, σ, and correlation coefficients, R^2) at 298 K

Ionic liquid	$A \pm \sigma$	$B \pm \sigma$	$10^5\ (C \pm \sigma)$	R^2
[Ch]Cl	102 ± 1	−0.254 ± 0.004	1.92 ± 0.03	0.9993
[Ch][Ac]	94 ± 11	−0.223 ± 0.027	2.62 ± 0.13	0.9982
[Ch][Lev]	88 ± 1	−0.246 ± 0.005	1.89 ± 0.06	0.9989
[Ch][Glu]	171 ± 37	−0.294 ± 0.050	2.34 ± 0.18	0.9985
[Ch][Sal]	119 ± 2	−0.339 ± 0.006	4.50 ± 0.15	0.9983
[Ch][Suc]	210 ± 60	−0.352 ± 0.065	2.10 ± 0.22	0.9987
[BCh]Cl	97 ± 1	−0.274 ± 0.004	3.42 ± 0.07	0.9987

Table 2 Experimental weight fraction compositions (wt%) for the TLs and TLLs, and compositions of ionic liquid and K_3PO_4 at the top phase (*T*), initial mixture (*M*) and bottom phase (*B*) at 298 K

Ionic Liquid	$[IL]_T$	$[K_3PO_4]_T$	$[IL]_M$	$[K_3PO_4]_M$	$[IL]_B$	$[K_3PO_4]_B$	TLL
[Ch]Cl	36.51	14.51	20.46	27.51	7.30	38.16	37.59
	36.51	14.51	29.70	19.99	7.30	38.16	37.59
	42.37	11.20	20.82	29.80	3.24	44.97	51.69
	51.61	7.02	24.95	30.24	1.35	50.80	66.66
	55.12	5.77	20.65	34.33	1.13	51.83	70.97
[Ch][Ac]	62.94	3.28	30.90	19.88	19.25	25.91	49.20
	64.96	2.79	30.04	23.00	7.04	36.30	66.92
[Ch][Lev]	53.54	4.09	28.05	20.56	14.47	29.33	46.51
	59.13	2.63	30.15	20.22	13.38	30.40	53.52
[Ch][Glu]	78.53	6.83	30.32	26.90	11.18	34.86	72.94
	81.42	6.24	29.64	30.03	4.91	41.39	84.19
[Ch][Sal]	60.78	3.90	30.39	19.91	3.35	34.15	64.91
	62.77	3.58	29.53	22.84	1.29	39.21	71.05
[Ch][Suc]	89.47	5.82	30.09	26.93	13.45	32.85	80.68
	92.45	5.39	30.28	30.00	6.54	39.40	92.39
[BCh]Cl	56.16	3.96	30.10	17.84	10.35	28.35	51.90
	61.93	2.68	29.64	20.30	5.54	33.46	64.25

pH, density and viscosity

The physicochemical properties of ABS, such as pH, density and viscosity are key features when the intent is to use these systems as extractive approaches in biotechnological applications, and particularly, when envisaging their design and scale-up. Therefore, aiming at further evaluating the potential of the ABS herein suggested, the pH values, densities and viscosities of the coexisting phases were determined for the following ternary mixture compositions: 30 wt% of [Ch]Cl, [Ch][Ac], [Ch][Lev], [Ch][Sal] or [BCh]Cl + 20 wt% of K_3PO_4 and 30 wt% of [Ch][Suc] or [Ch][Glu] + 27 wt% of K_3PO_4. The mixture compositions of the last two ionic liquids are different than those of the remaining fluids due to the different phase diagrams obtained when analyzed in weight fraction compositions (ESI†). Mixture compositions were chosen within the liquid–liquid region, but not within the solid–liquid regime. The results appear in Table 3. The weight fraction compositions of each phase at the selected points are presented in

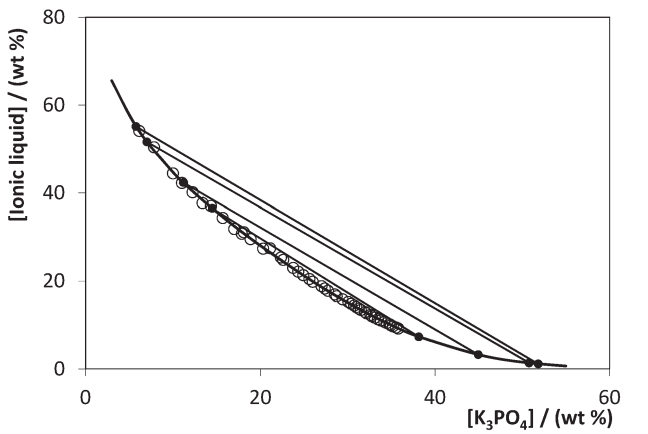

Fig. 3 Phase diagram for the ternary system composed of [Ch]Cl + K_3PO_4 + H_2O at 298 K: (○) binodal curve data; (●) TL data; (▬) adjusted data through eqn (1).

Table 3 pH values, viscosity (η) and density (ρ) of the coexisting phases in ABS composed of cholinium based ionic liquids + K_3PO_4 + H_2O at 298 K

	K_3PO_4 rich phase			Cholinium rich phase		
Ionic liquid	pH	η (mPa s)	ρ (g cm ^{3})	pH	η (mPa s)	ρ (g cm^3)
[Ch]Cl	13.06	8.616	1.493	13.23	5.859	1.126
[Ch][Ac]	12.11	9.271	1.487	12.76	6.012	1.142
[Ch][Lev]	13.47	10.233	1.522	13.71	8.219	1.123
[Ch][Glu]	8.81	28.342	1.620	9.62	14.929	1.223
[Ch][Sal]	13.08	4.866	1.382	13.06	7.497	1.148
[Ch][Suc]	9.40	20.874	1.538	9.27	14.308	1.289
[BCh]Cl	12.71	4.019	1.390	12.69	11.472	1.122

Table 2. The dependence of density and viscosity on temperature (from 298 to 328 K) is reported in the ESI.†

We tested the potential of several hydrophilic cholinium-based ionic liquids combined with K_3PO_4 (a strong salting-out species) to form ABS. Therefore, all systems present alkaline phases due to the presence of the basic PO_4^{3-} anion. The pH of the coexisting phases ranges between 8.81 and 13.71 which further depends on the ionic liquid present in the system. Glutarate- and succinate-based systems present lower pH values (pH $<$ 10) than the other systems—a fact related to the ionic liquids' nature.

Both density and viscosity for ionic-liquid- and inorganic-salt-rich phases were found to decrease as the temperature increased (data shown in ESI†). In all equilibrated systems, the density of the K_3PO_4-rich phase is higher than that of the cholinium-rich one. However, larger differences between the densities of the two phases in cholinium-based systems are observed than with ABS composed of imidazolium- or phosphonium-based ionic liquids and the same inorganic salt.[32] This, as experimentally observed, enables a faster and easier phase separation of the cholinium systems and can be regarded as a main advantage when envisaging large-scale applications.

At 298 K, the K_3PO_4-rich phase is generally more viscous than the corresponding ionic-liquid-rich phase for the systems with [Ch]Cl, [Ch][Ac], [Ch][Lev], [Ch][Glu] and [Ch][Suc]. Two exceptions to this pattern arise in the systems including [Ch][Sal] and [BCh]Cl, where the ionic-liquid-rich phases are more viscous than the corresponding salt-rich ones. The viscosity of the ionic-liquid-rich phase ranges from 6 to 15 mPa s whereas the viscosity of the salt-rich one lies between 4 and 28 mPa s. Compared to analogous systems composed of K_3PO_4 with other ionic liquids,[32] the viscosities of cholinium-based ionic liquids are within the same range. On the other hand, the viscosity values of cholinium-based systems are substantially lower than those of typical PEG-salt ABS.[33] Thus, the low viscosity of cholinium-based ABS will favour both the mass transfer in extraction processes as well as the industrial handling of the phases.

Partitioning of antibiotics

Due to their therapeutic and prophylatic qualities both for humans and animals, antibiotics have received widespread use all around the world. Nowadays, most antibiotics such as tetracyclines are produced by staged fermentation in which strains of microorganisms producing high yields are grown under optimum conditions. The mold is strained out of the fermentation broth, and the antibiotic is separated from the broth by filtration, precipitation, liquid–liquid extraction and other separation methods.[34] Other antibiotics, such as ciprofloxacin, are laboratory synthesized, or produced by chemical modification of natural substances. For an antibiotic to be economically viable, manufacturers must be able to maximize the yield of the drug in the fermentation process or in the reaction media, whilst minimizing purification costs. Therefore, we tested the use of cholinium-based ABS as an alternative purification platform for antibiotics.

Three different antibiotic forms (tetracycline, tetracycline·HCl and ciprofloxacin·HCl) were studied by way of their partitioning behaviour between the coexisting phases of ABS. The molecular structures of the antibiotics are shown in Fig. 4.

The partition coefficients (K) of tetracycline, tetracycline·HCl and ciprofloxacin·HCl were determined at 298 K in several systems and at different compositions according to the following equation:

$$K = \frac{[\text{Antiobiotic}]_{\text{IL}}}{[\text{Antibiotic}]_{\text{K3PO4}}} \quad (2)$$

where $[\text{Antibiotic}]_{\text{IL}}$ is the concentration of each antibiotic in the ionic-liquid-rich phase and $[\text{Antibiotic}]_{\text{K3PO4}}$ is the respective concentration in the K_3PO_4-rich phase.

The success of the extractive potential of ABS largely depends on the main components of the medium and on the coexisting phase properties (aiming at obtaining selective extractions and high extraction yields). Thus, the first approach was to compare the partition coefficients of the 3 antibiotic forms using several systems at fixed compositions. The results appear in Fig. 5. The selected overall biphasic mixtures contain 30 wt% of [Ch]Cl, [Ch][Ac] or [Ch][Lev] + 20 wt% of K_3PO_4 and 30 wt% of [Ch][Glu] or [Ch][Suc] + 27 wt% of K_3PO_4. The composition of the coexisting phases, as well as their physicochemical properties, are presented in Tables 2 and 3, respectively. The partition coefficients are the result of at least three independent determinations. The detailed partition coefficients, respective standard deviations and

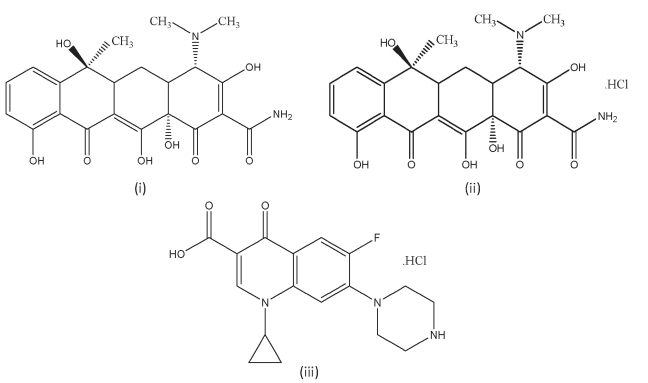

Fig. 4 Molecular structures of the antibiotics: (i) tetracycline; (ii) tetracycline·HCl; (iii) ciprofloxacin·HCl.

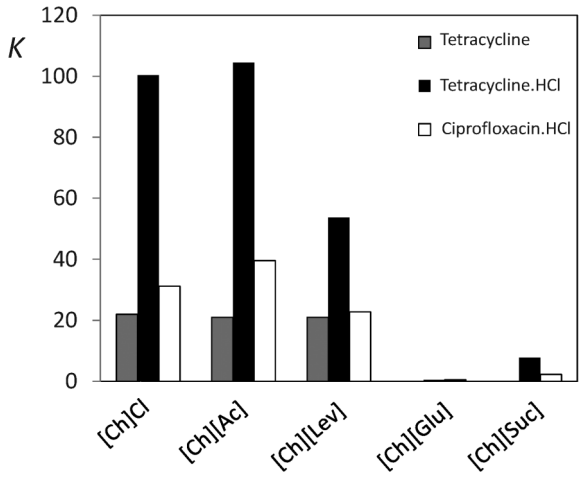

Fig. 5 Partition coefficients of the three antibiotics forms at 298 K. The overall biphasic region mixture contains 30 wt% of [Ch]Cl, [Ch][Ac] or [Ch][Lev] + 20 wt% of K_3PO_4 and 30 wt% of [Ch][Glu] or [Ch][Suc] + 27 wt% of K_3PO_4.

weight fraction compositions of each constituent are provided in the ESI.†

For the antibiotics tetracycline·HCl and ciprofloxacin·HCl, the partition coefficients follow the rank: [Ch][Ac] > [Ch]Cl > [Ch][Lev] ≫ [Ch][Suc] > [Ch][Glu]. For tetracycicline, the partition coefficients are similar in all systems studied. Particularly high partition coefficients were attained for systems composed of the most hydrophilic ionic liquids, namely [Ch][Ac] and [Ch]Cl. On the other hand, the lowest partition coefficients are observed in the systems constituted by [Ch][Suc] and [Ch][Glu], even though these systems have a greater amount of K_3PO_4 which should promote the preferential migration of the antibiotic to the ionic liquid-rich phase—see discussion below on the influence of the inorganic salt content. Indeed, whilst the partition coefficients of tetracycline·HCl and ciprofloxacin·HCl are always higher than unity, *i.e.*, the antibiotics preferentially partition for the cholinium-rich phase, with [Ch][Glu] the opposite occurs—the antibiotics migrate towards the salt-rich phase. These results suggest that the ionic liquid, or at least the properties that it confers to the medium, has a major impact on the partitioning of the antibiotics studied.

The partition behaviour of ionizable (bio)molecules is also related to the different chemical species present in solution. Most antibiotics have acidic and/or basic functionalities and their ionization state is ruled by both the solution pH and acidic dissociation constants. The various chemical species (cationic, neutral/zwiterionic or anionic) may therefore display different partitioning behaviour. Tetracycline shows three dissociation constants (pK_a = 3.32, 7.78, 9.58) assigned to the tricarbonyl group, and dimethylamine and β-diketone groups, whilst cyprofloxacin exhibits four dissociation constants (pK_a = 3.01, 6.14, 8.70, 10.58) attributed to the carboxyl group and three basic nitrogen sites.[35] Based on these dissociation constants, the speciation curves of each antibiotic as a function of pH are well described in the literature.[35] In summary, for systems with a pH higher than 12 (Table 3), tetracycline takes the form of a divalent anion and ciprofloxacin a monovalent anion. Only for the systems composed of [Ch][Glu] or [Ch][Suc], where the pH of the phases is lower (pH ≈ 9), is tetracycline mostly present as a monovalent anion whereas ciprofloxacin has equivalent amounts of a non-charged and a positively charged species. Taking into account the results for [Ch]Cl, [Ch][Ac] and [Ch][Lev] (pH > 12), it seems that the electrostatic interactions between the inorganic salt ions and the charged antibiotics are of low importance since they preferentially migrate towards the ionic liquid-rich phase. On the other hand, it appears that the salting-out effect exerted by PO_4^{3-} (main species present at high pH values) is a dominant effect. This high charge density anion, with an improved ability to create hydration complexes, leads to the ''exclusion'' of each antibiotic from the inorganic salt-rich phase in favour of the more ''organic'' ionic liquid-rich phase. However, in the systems with lower pH values, and taking into account the basicity constant of PO_4^{3-},[27] the species HPO_4^{2-} and $H_2PO_4^-$ are also present and are weaker salting-out agents, and may thus lead to the lower partition coefficients observed.

In view of the molecular structures of the antibiotics, as well as the wide variety of ionic liquids employed, hydrogen-bonding interactions and dispersive-type interactions can also be anticipated. However, dispersive-type interactions seem to be non-favourable since the systems composed of the ionic liquids with longer aliphatic chains, [Ch][Glu] or [Ch][Suc], are those where particularly low partition coefficients were observed. On the contrary, hydrogen-bonding interactions are of high relevance due to the preferential partitioning of antibiotics for ionic-liquid-rich phases composed of more hydrophilic fluids. In fact, it has already been well-documented that the tetracycline partitioning for an organic phase increases with the hydrogen bonding tendency of the solvent.[36]

When comparing the data obtained for the 3 antibiotic forms, the partition coefficients for the ionic-liquid-rich phase follow the order: tetracycline·HCl > ciprofloxacin·HCl > tetracycline. The trend is supported by the octanol–water partition coefficients of both antibiotics ($\log K_{ow}$(tetracycline) = 1.19 and $\log K_{ow}$(ciprofloxacin) = 0.4).[37] Ciprofloxacin favourably migrates to more ''organic'' phases than tetracycline. Nevertheless, the cholinium-based systems studied are composed of highly hydrophilic ionic liquids with a large amount of water, and therefore antibiotics with a higher affinity for hydrophilic phases are better extracted. The same explanation can be extrapolated to the higher partition coefficients observed with tetracycline·HCl. The addition of the hydrochloride group in tetracycline leads to the enhancement of its affinity for water or aqueous solubility.[38]

Fig. 6 depicts the effect of the systems' composition, whilst changing both the ionic liquid and inorganic salt weight fraction towards the partitioning of tetracycline·HCl and ciprofloxacin·HCl. [Ch]Cl was chosen to illustrate this study since it is one of the ionic compounds leading to the highest partition coefficients. The detailed partition coefficients and respective standard deviations are provided in the ESI.† The compositions of each phase according to the initial mixture point are presented in Table 2.

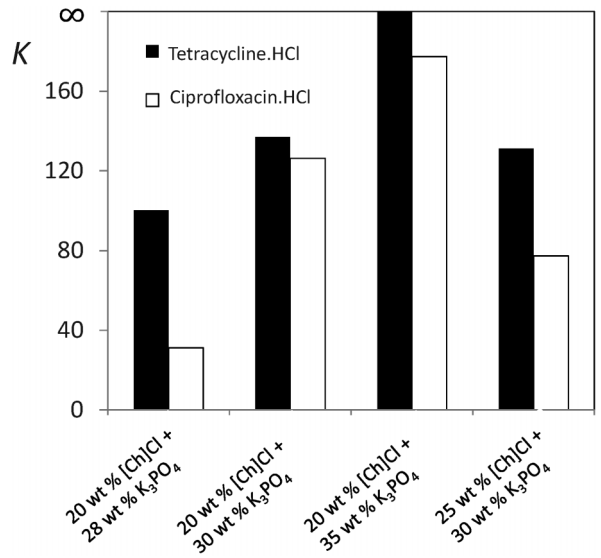

Fig. 6 Partition coefficients of tetracycline·HCl and ciprofloxacin·HCl in the system composed of [Ch]Cl + K_3PO_4 at 298 K and at different mixture compositions; $K = \infty$ represents complete extraction.

From the results in Fig. 6 it can be inferred that the amount of K_3PO_4 has a significant influence. However, the impact of the ionic liquid is more moderate. The continual increase of the salt content generally leads to an increase in the partition coefficients due to the strong salting-out effect exerted by the high charge density salt. Exceptionally, at the composition of 20 wt% of [Ch]Cl + 35 wt% of K_3PO_4, the complete extraction of tetracycline.HCl into the ionic-liquid-rich phase in one single-step was observed (*i.e.* no antibiotic was detected at the salt-rich phase). On the other hand, and to a milder degree, the increase on [Ch]Cl content leads to a decrease in the partition coefficients of both antibiotics. The reason for such a fall may be attributed to a decrease in the amount of ''free'' water available and to the salting-out effect exerted by the ionic liquid itself.

Partion coefficients of antibiotics in conventional polymer-based systems fall within 0–6 and complete extractions remain far from being achieved.[39] Moreover, previous results for the extraction of ciprofloxacin and ciprofloxacin·HCl using imidazolium-based ionic liquid ABS and several amino acids as the salting-out agents confirmed low extraction efficiencies into the ionic-liquid-phase.[7] Therefore, the high partition coefficients obtained with cholinium-based ABS reveal that they are actually improved alternatives to traditional extraction methods and certainly deserve further attention from the biotechnology field.

Conclusions

This work shows, for the first time, that cholinium-based ionic liquids are able to form ABS in the presence of aqueous solutions of inorganic salts. In addition to their benign character, cholinium-based ABS are also enhanced purification platforms for pharmaceutical compounds. The data obtained with the extraction of 3 antibiotics forms confirmed that cholinium-based ABS provide real and improved alternatives to traditional extraction methods, offering simpler, quicker, greener, and highly more efficient procedures. Compared to the low partition coefficients observed in typical polymer-based ABS, cholinium-based systems led to the complete extraction of antibiotics for the ionic liquid-rich phase in a single-step, by way of the targeted manipulation of the phase-forming components and their concentration.

The use of cholinium-based ionic liquids combined with other inorganic/organic salts, as well as with gentler and more biodegradable species such as carbohydrates or polymers, is obviously envisaged and clearly recommended for biotechnological purposes.

Experimental

Materials

The inorganic salt, K_3PO_4, with a stated purity > 98 wt%, came from Sigma as did the ionic liquid (2-hydroxyethyl)trimethylammonium chloride ([Ch]Cl), ⩾99 wt% pure. Fluka provided the (2-hydroxyethyl)trimethylammonium salicylate ([Ch][Sal]), > 95 wt% pure. The benzyl(2-hydroxyethyl)dimethylammonium chloride ([BCh]Cl), ⩾ 97.0 wt%, was from Aldrich. The (2-hydroxyethyl)trimethylammonium acetate ([Ch][Ac]), > 98 wt% pure, was purchased from Iolitec. The ionic liquids (2-hydroxyethyl)trimethylammonium levulinate ([Ch][Lev]), (2-hydroxyethyl)trimethylammonium glutarate ([Ch][Glu]) and (2-hydroxyethyl)trimethylammonium succinate ([Ch][Suc]) were synthesized by the reaction of the cholinium bicarbonate with the corresponding carboxylic acid according to published protocols.[17] All ionic liquid samples were dried for at least 24 h at a moderate temperature (≈323 K) before use. The purities of the synthesized ionic liquids were confirmed by ^1H and ^{13}C NMR spectra (ESI†) and shown to be ⩾ 98 wt%. The water content of all ionic liquids was ⩽1000 ppm as determined by Karl Fischer titration.

Double distilled water, passed through a reverse osmosis system and further treated with a Milli-Q plus 185 water purification equipment, was used in all experiments.

The antibiotics used as partitioning solutes were tetracycline ⩾ 98.0 wt% from Fluka, tetracycline·HCl > 95 wt% from Sigma-Aldrich, and ciprofloxacin·HCl > 99.8 wt% that was generously provided by Bayer HealthCare AG.

Methods

Phase diagrams and tie-lines. Aqueous solutions of K_3PO_4 at *circa* 55 wt% and aqueous solutions of the different ionic liquids at variable concentrations (ranging from 60 wt% to pure ionic liquid) were prepared and used to determine the phase diagrams. The ternary phase diagrams were determined at 298 K (± 1 K) and at atmospheric pressure using cloud point titration.[5] Repetitive drop-wise addition of the aqueous salt solution to the ionic liquid aqueous solution was carried out until the appearance of a cloudy solution (biphasic regime), followed by the drop-wise addition of water until

the formation of a clear and limpid solution (monophasic regime). To complete and extend the phase diagrams, the opposing addition of the ionic liquid aqueous solution to the salt solution was also carried out. All the additions occurred under constant stirring. The ternary system compositions were determined by weight quantification of all components within $\pm$ 10 ^{4} g.

The experimental binodal curves were correlated according to eqn (1), and the tie-lines were further determined by a gravimetric method originally described by Merchuck *et al.*[30] for polymer-based ABS, and later applied to ionic-liquid-based ABS.[3,5] For the determination of each tie-line, a ternary mixture was prepared by mixing water, ionic liquid and salt with specified concentrations within the biphasic region and vigorously agitating. The mixtures were then allowed to settle for *circa* 12 h at 298 K ($\pm$ 1 K). Then, the top and bottom phases were carefully separated and individually weighed within $\pm$ 10 ^{4} g. The tie-lines were determined by mass balance using the relationship between the weight of the top phase and the weight and composition of the overall mixture as detailed elsewhere.[4,5]

The tie-line lengths (TLL) were determined according to eqn (3),

$$\mathrm{TLL} = \sqrt{([\mathrm{K_3PO_4}]_T \quad [\mathrm{K_3PO_4}]_B)^2 + ([\mathrm{IL}]_T \quad [\mathrm{IL}]_B)^2} \qquad (3)$$

where [IL] and [K_3PO_4] are the ionic liquid and the salt weight fraction percentages, and the subscripts "*T*" and "*B*" designate the top phase and the bottom phases, respectively.

pH determination. The pH values ($\pm$ 0.02) of both the ionic-liquid-rich and salt-rich phases were measured at 298 K ($\pm$ 1 K) using a HI 9321 Microprocessor pH meter from HANNA Instruments. The pH meter was calibrated with two buffers (pH values of 4.00 and 7.00). All the biphasic mixtures were gravimetrically prepared within $\pm$ 10 ^{4} g. After vigorous stirring and a subsequent period of settling (at least 12 h), the phase separation was carried out, and the pH values measured.

Density and viscosity determination. Measurements of viscosity and density were performed in the temperature range between (298.15 and 328.15) K at atmospheric pressure using an automated SVM 3000 Anton Paar rotational Stabinger viscometer-densimeter. The dynamic viscosity has a relative uncertainty of 0.35% while the absolute uncertainty on density is within 0.0005 g cm ^{3}. Further details on the equipment can be found elsewhere.[40]

Density and viscosity measurements were carried out at selected biphasic regions. Individual mixtures at the biphasic region were prepared by weight, vigorously shaken and allowed to reach equilibrium by the separation of both phases for at least 12 h and at 298 K ($\pm$ 1 K). After the separation step, viscosity and density measurements were performed for both aqueous phases.

Partitioning of antibiotics. Specific mixtures in the two-phases regime were selected. For each experiment the aqueous two-phase systems were prepared by mixing the exact amount of each ionic liquid, inorganic salt and an aqueous solution containing the antibiotic. Aqueous solutions, with a concentration of approximately 0.1 g dm ^{3} for tetracycline, 1.0 g dm ^{3} for tetracycline·HCl and 0.4 g dm ^{3} for ciprofloxacin·HCl were used. After the complete dissolution of all components in the mixture by stirring, the mixture was allowed to settle (without stirring and in an environment with no light) for *circa* 12 h at 298 K ($\pm$ 1 K). After the phase separation, each antibiotic was measured by spectroscopy using a UV-vis spectrophotometer, SHIMADZU UV-1700, at the wavelengths of 276 and 275 nm, corresponding to the tetracycline and tetracycline·HCl, and ciprofloxacin·HCl maximum absorption wavelengths, respectively. Calibration curves for each solute had previously been established.

Possible interferences of both the salt and the ionic liquid with the analytical method were taken into account and found to be of no significance at the dilutions carried out and for the ionic liquids used. Nevertheless, the partition coefficients in systems composed of [BCh]Cl and [Ch][Sal] were not determined due to the interference of the aromatic ring of the ionic liquid within the quantification of the antibiotic.

At least three individual vials were prepared, and three samples of each aqueous phase were quantified, allowing us to determine the average partition coefficient (eqn (2)) and corresponding standard deviation.

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