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Novel biocompatible and self-buffering ionic liquids for biopharmaceutical applications

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Keywords

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Antibodies, or immunoglobulins, are Y-shape proteins produced by the body's immune system to identify and neutralize harmful substances, such as bacteria, viruses, fungi, parasites and toxins.[1] Passive immunization is a new therapy which acts through the administration of specific antibodies. It is an emerging alternative to antimicrobial chemotherapy, conventional vaccines, and use of antibiotics, and essentially relevant in an era where we are facing the emergence of antibiotic-resistant microorganisms.[2] Traditionally, the antibodies investigated for such a purpose are produced by small mammals. These antibodies (IgG) are usually collected from repeated bleeding or heart puncture which may frequently result in distress or even death of the animals.[3] An alternative approach consists on the use of antibodies existent in egg yolk (immunoglobulin Y, IgY). Egg yolk contains immunoglobulins in large quantities which are transferred from the hen plasma through the egg follicle.[4] The amount of IgY produced by a single hen over a year is equivalent to the production of 4.3 rabbits,[5] and therefore, IgY can be obtained in higher titres by non-invasive methodologies.[6] It was already demonstrated that IgY plays a similar biological role as mammal IgG.[7] IgY can thus be used as an effective replacement to the well-known IgG mammal antibodies with paramount importance in passive immunotherapy. Nevertheless, the production cost of IgY still remains higher than other drug therapies due to the lack of effective purification techniques. Egg yolk is a very complex matrix, rich in lipoproteins and other water-soluble proteins, and the proper isolation of IgY remains a major challenge.

Several methodologies were investigated with the goal of purifying IgY from egg yolk, including multiple precipitation stages with polymers or salts, ultrafiltration or extraction with chloroform.[8] However, these lengthy and cumbersome techniques have shown to be unable to provide high purification factors. Furthermore, the use of solvents, such as

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chloroform, should be avoided. Some of these purification strategies also lead to the destabilization and loss of specific activity of IgY and cannot be easily scaled to industrial production.[9] Aqueous biphasic systems (ABS), which belong to the liquid-liquid extraction processes, can be foreseen as a viable alternative for the IgY extraction from egg yolk and further purification. Traditional ABS consist of two immiscible aqueous-rich phases based on polymer-polymer, polymer-salt or salt-salt combinations.[10] Since ABS are mainly composed of water they are accepted as biocompatible media for cells, cell organelles and biologically active substances, and have been widely used for the recovery and purification of (bio)molecules.[10–11] In addition to the more conventional ABS, which eliminate the use of hazardous and toxic volatile organic compounds, the emergence of ionic liquid (IL)-based ABS has led to outstanding extraction performances.[12] Ionic liquids are organic salts with melting points below 100°C, that present unique characteristics, such as negligible volatility, non-flammability, good thermal and chemical stabilities, and an improved ability for the dissolution of a wide variety of biomaterials.[12–13] One of the most important features of ILs as phase-forming components of ABS results from their tunability by the proper arrangement of the chemical structures of their constituting ions. Consequently, the introduction of ILs into ABS allowed to overcome the restricted range of polarities of the coexisting phases of the polymer-based ABS, which have been limiting the purifications achievable with these systems.[14] IL-based ABS are nowadays seen as a novel class of liquid-liquid partitioning systems with tunable extraction efficiencies and selectivities.[12,15]

IL-based ABS have been successfully used in the extraction of proteins without denaturation.[16] However, most of the ILs investigated for ABS formation affect the pH of the aqueous solution – a major drawback when the goal is the extraction of proteins such as antibodies. Previous reports focused mainly on the use of imidazolium-based ILs with anions with a strong alkaline or acidic character.[12] Hence, phosphate-based buffered solutions were used to maintain the pH of the aqueous medium aiming at avoiding the denaturation of proteins.[12] Nevertheless, phosphate ions can bind with metal ions like calcium, zinc or magnesium that are essential to maintain the integrity of some proteins/enzymes.[17] Recently, a novel class of ILs with buffering characteristics was proposed.[18] It is believed that Good's buffers are currently the most inert and non-toxic buffers for use in protein studies.[17,19] However, these tetraalkylammonium- and imidazolium-based Good's buffers ILs only form ABS with high-charge density salts.[18] The prevalence of two aqueous phases of high ionic strength are not favorable for the purification of high-value proteins such as antibodies. Aiming at overcoming these drawbacks, herein we report the synthesis of more biocompatible Good's buffers ILs based on the cholinium cation.

Cholinium chloride is a water soluble essential nutrient important for cell membrane structure and for the synthesis of folic acid and vitamin B12.[20] Cholinium-based ILs present outstanding biodegradability, low toxicity, and are able to maintain some protein structures and enzyme functions.[21] Yet, none of these cholinium-based ILs were paired with Good's buffers as anions and hence are not able to maintain the pH of aqueous solutions.[21] Furthermore, the use of cholinium as the cation allowed to create ABS with biodegradable and biocompatible polymers as phase-forming substituents of salts.

In this work, we report the synthesis of a new class of biocompatible ABS composed of cholinium-based Good's buffers ILs (GB-ILs) and their use in the formation of ABS combined with a polymer, namely poly(propylene) glycol, and their further application in the extraction/purification of IgY from egg yolk. These novel ILs were synthesized with anions derived from Good's buffer anions (MES, Tricine, TES, HEPES, and CHES) *via* a simple neutralization reaction (*cf.* Supporting Information). Their chemical structures, as well as their abbreviation, are provided in Table 1.

After the synthesis of the GB-ILs, their self-buffering characteristics were firstly ascertained and proved to be within the physiological pH region (between 6 and 8). Their protonation constants have been potentiometrically determined using the HYPERQUAD 2008 program. [22] Good's buffers are zwitterionic amino acids, either *N*-substituted taurine or glycine derivatives, with two protonation sites at the carboxylic/sulfonic (pK_{a1}) and the amino (pK_{a2}) groups. The latter protonation is responsible for their buffering ability near the physiological pH region. The pK_{a2} values of MES, TES, HEPES, Tricine, and CHES in aqueous solution at 25.0 °C and ionic strength of 0.1 M of NaNO₃ are, respectively, 6.12, 7.30, 7.35, 8.08, and 9.12, which agree well with literature values.[23] On the other hand, the pK_{a2} values of [Ch][MES], [Ch][TES], [Ch][Tricine], [Ch][HEPES] and [Ch][CHES] are, respectively, 6.01, 7.26, 7.17, 7.87 and 8.96, meaning that the cholinium cation reduces the pK_{a2} values of IL-GBs possibly by electrostatic interactions which stabilize the negatively charged GBs. Figure 1 shows a representative pH-metric titration profile of TES and [Ch][TES], as well as the protonation (pK_{a2}) equilibrium of the latter - the remaining pH profiles are shown in the Supporting Information.

To evaluate the toxicity of the synthesized GB-ILs, we measured their toxicity towards the bioluminescent bacteria *Vibrio fischeri*, by the Microtox[®] standard assay[24] at 30 minutes of exposure time. The final output of this test is the EC₅₀ parameter, which represents the effective concentration of a given compound that produces 50% of inhibition of light emission. The EC₅₀ values, with the respective 95 % confidence limits shown in brackets, of [Ch][HEPES], [Ch][MES], [Ch][Tricine], and [Ch][CHES] are 19584 (12207; 26962), 9789 (3953; 15626), 4588 (2266; 9289), and 208.65 (181.28; 236.02) g·dm⁻³, respectively.

These results reveal that the GB-ILs investigated present a non-toxic character as indicated by their high EC₅₀ values - compared with the limits imposed by the European Classification.[24] Furthermore, the [Ch][TES] may also be considered as non-toxic, since in the range of concentrations studied, it was not possible to achieve 50% of luminescence inhibition.

After attesting the buffering capacity of the new GB-ILs and their low toxicity towards the gram-positive bacteria *Vibrio fischeri*, their ability to form ABS with PPG 400 (poly(propylene) glycol with a molecular weight of 400 g·mol⁻¹) was investigated. It should be remarked that the ecotoxicity of PPG 400 was also determined and shown to be non-toxic over *Vibrio fischeri* since it revealed an EC₅₀ value of 6735 (4623; 8847) mg·dm⁻³ at 30 minutes of exposure, agreeing with the available data regarding the non-toxic nature of propylene glycol.[25]

The ternary phase diagrams of the ABS composed of [Ch][HEPES], [Ch][Tricine], [Ch][TES] or [Ch][MES] + PPG 400 + water at 25 °C are depicted in Figure 2. For the [Ch][CHES]/PPG 400 mixture it was not found the formation of ABS due to the higher hydrophobicity of this anion - *cf.* discussion below on the phase diagrams trend. The experimental weight fraction data are reported in the Supporting Information. All the experimental binodal curves were also fitted by an empirical correlation[26] and the corresponding regression parameters were further estimated – *cf.* Supporting Information. From these, tie-lines (TLs), along with their respective lengths (TLLs), were also measured and are reported in the Supporting Information. These parameters are important to define the monophasic/biphasic regimes for which no experimental data are available as well as to ascertain on the phases' compositions at the mixture point where the extractions are carried out. In all studied ABS, the top phase corresponds to the PPG-rich phase while the bottom phase is mainly composed of IL and water (confirmed by conductivity measurements).

Figure 2 depicts the solubility curves displayed in molality of polymer (mole of IL *per* kg of solvent) *versus* molality of IL (mole of IL *per* kg of solvent). Molality was chosen in order to avoid distortions in the comparisons that could be a consequence of the different molecular weights of the GB-ILs involved. For mixtures with composition above the solubility curve there is the formation of two aqueous phases, whereas below the solubility curve the concentration of each component is not enough to induce the liquid–liquid demixing, falling thus within the monophasic regime.

Figure 2 depicts the ability of each GB-IL to induce the phase separation. Since all the GB-ILs share a common cation, the differences on the solubility curves are a result of the IL anion nature. The GB-IL anions aptitude to form ABS follows the rank: [HEPES][−] ≈ [Tricine][−] > [TES][−] > [MES][−]. Since PPG 400 is a moderately hydrophobic polymer, the higher the affinity of each GB affinity for water, the greater the ability of each IL to promote the two phases formation. Indeed, this trend can be rationalized based on the polarity of the GB anions through their dipole moment values (Debye), namely 21.02, 19.97, 18.23, and 16.74 for [HEPES][−], [Tricine][−], [TES][−], and [MES][−], respectively, and as determined in this work – see computational details below.

Figure 3 presents the σ -profiles of the several [Ch][GB]-ILs computed by the Conductor-like Screening Model for Real Solvents (COSMO-RS).[27] The σ -profiles provide a detailed description of the polarity distribution and H-bonding features of the investigated compounds. The σ -profile is divided in three main regions, namely hydrogen bond donor, non-polar, and hydrogen bond acceptor regions, which are separated by two vertical lines located at the H-bond cut-off of $\pm 0.01 \text{ e} \cdot \text{\AA}^{-2}$ (Figure 3). The negatively charged sulfonic and carboxylic groups of the ILs display a peak localized at the strongly negative polar region (0.01 to $0.025 \text{ e} \cdot \text{\AA}^{-2}$) and, therefore, they act as strong hydrogen bond acceptors. These ILs also show hydrogen bond donor fragments (located at $-0.017 \text{ e} \cdot \text{\AA}^{-2}$) due to the hydroxyl groups present in the cholinium cation and the different GB anions. Furthermore, [Ch][GB]-ILs also present an electronic charge located in the non-polar region mainly derived from the aliphatic groups. According to the COSMO-RS theory, these GB-ILs can interact strongly with water or proteins through hydrogen-bonding and van der Waals forces. Thus, [Ch]

[BG]-ILs are viable candidates, as phase-forming components of ABS, envisaging their use in the extraction and purification of proteins.

The novel ABS here proposed were finally investigated for the extraction of IgY from aqueous solutions containing the water-soluble fraction of proteins existent in egg yolk. The mixture compositions (50 wt% of PPG 400 + 7-10 wt% of each IL + 40-43 wt% of an aqueous solution containing the water soluble proteins), and which fall within the biphasic region, were chosen according to a fixed tie-line length to avoid differences in the compositions of the coexisting phases amongst the 4 IL-based ABS. The partition coefficients (K) and extraction efficiencies ($EE\%$) of IgY and contaminant water-soluble proteins, at 25 °C, are depicted in Figure 4 - *cf.* the Supporting Information with the detailed data. For all the investigated systems, the partition coefficient is higher than 1.0, confirming the proteins preferential partitioning for the IL-rich phase (bottom phase). The extraction efficiencies for the IL-rich phase range between 79 and 94 %. The ABS composed of PPG 400 and [Ch][Tricine] or [Ch][HEPES] lead to the highest extraction efficiencies, above 90%, in a single-step. These GB-ILs are also those that present a higher ability to form ABS with PPG 400, and that for a given mixture composition, display higher amounts of water at the IL-rich phase – *cf.* the Supporting Information with the tie-lines data.

The coexisting phases used in the extraction of the water soluble fraction of egg yolk were also analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to conclude on the proteins present at the coexisting phases, and thus on the purification of IgY – Figure 5. Two major proteins are identified at the IL-rich phase, namely IgY and β -livetin (a contaminant protein of the water-soluble fraction). In some systems, a reduction on the intensity of the band corresponding to β -livetin is observed, indicating that these systems deserve to be further explored for the purification of IgY. Moreover, the SDS-PAGE results demonstrate that the corresponding band of the IgY heavy chain does not significantly change, precluding the antibodies stability at the IL-rich phase. Nevertheless, it should be emphasized, that the values presented in Figure 4 correspond to the combined extraction efficiencies and partition coefficients of two proteins, namely IgY and β -livetin.

The crystal structure of the fragment crystallizable region (Fc region or tail region) of IgY[28] was used to identify hydrogen-bonding interactions and binding sites between the IL ions and the protein in order to gather additional insight on the enhanced affinity and stability of IgY in IL-rich aqueous phases. For this purpose, the Auto-dock Tools vina 1.5.4 program was used.[29] The binding sites of the cholinium cation, and [MES]⁻, [TES]⁻, [Tricine]⁻ and [HEPES]⁻ anions are shown in Figure 6, with binding free energies of -3.1, -4.1, -4.4, -4.4, and -4.1 kcal·mol⁻¹, respectively. Cholinium, [MES]⁻, [TES]⁻ and [Tricine]⁻ are located next to the GLN 563, GLN 565, THR561, HIS 464, PRO 460, and ALA 462 residues. On the other hand, for [HEPES]⁻, ASN 449, TYR 447 and ARG 485 are the adjacent residues. The GB anions, namely [Tricine]⁻, [TES]⁻, [MES]⁻ and [HEPES]⁻ form 9, 5, 5, and 4 hydrogen-bonds, respectively, while cholinium forms three hydrogen-bonds with GLN 565 and GLN 563. Further details and data are provided in the Supporting Information.

In summary, novel biodegradable and biocompatible ABS, composed of a polymer and a novel class of ILs with buffering characteristics, were investigated for the extraction and purification of IgY from the water soluble fraction of proteins of egg yolk. Outstanding extraction efficiencies for the IL-rich phase ranging between 79 and 94 % were attained in a single step, while using “self-buffering” and “non-toxic” compounds. Based on computational investigations, it was also demonstrated that the partitioning of IgY is dominated by H-bonding and van der Waals interactions. Although it was not possible to completely separate IgY from the major contaminant (β -livetin) in this process, these novel systems represent an adequate strategy for future investigations due to their benign character and ability to maintain the integrity of the proteins. Further investigations envisaging the purification of IgY are ongoing.

Experimental Section

Materials

PPG 400, choline hydroxide solution (46 wt% in methanol), 2-(*N*-morpholino)ethanesulfonic acid (MES, purity > 99 wt%), 2-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethane sulfonic acid (TES, purity > 99 wt%), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES, purity > 99.5 wt%), *N*-[tris(hydroxymethyl)methyl]glycine (Tricine, purity > 99 wt%), and 2-(cyclohexylamino)ethanesulfonic acid (CHES, purity > 99 wt%), were supplied by Sigma–Aldrich Chemical Co. Sodium hydroxide was purchased from Eka Chemicals. Methanol (HPLC grade, purity > 99.9%) was supplied from Fisher Scientific and acetonitrile (purity > 99.7%) was from Lab-Scan. Sodium nitrate (purity > 99.5%) was acquired from Himedia Labs. Nitric acid (65 %), potassium nitrate (purity > 98.0%), and potassium hydrogen phthalate (purity > 99.8%) were obtained from Panreac. All solutions were prepared using ultra-pure water (passed previously through a Milli-Q plus 185 system). Fresh eggs were purchased in a local market. The molecular weight standards for SDS-PAGE, namely marker molecular weight full-range, were acquired from VWR.

Synthesis and characterization of GB-ILs

The GB-ILs were synthesized *via* neutralization of choline hydroxide with Good’s buffers. A slightly excess of equimolar buffer aqueous solution was added drop-wised to choline hydroxide solution. The mixture was stirred continuously for at least 12 h at ambient conditions. The mixture was then evaporated at 60 °C under reduced pressure using a rotary evaporator yielding a viscous liquid. A mixture of acetonitrile and methanol (1:1, v:v) was added to this liquid and then vigorously stirred at room temperature for 1 h to precipitate any excess of buffer. The solution was then filtrated to remove the precipitated solid, and the filtrate was evaporated up to dryness under vacuum (10 Pa) for 3 days at room temperature to yield each GB-IL. The water content in each GB-IL was measured by Karl–Fischer (KF) titration, using a KF coulometer (Metrohm Ltd., model 831) with the Hydranal Coulomat AG reagent (Riedel-de Haën). The water content in each GB-IL was found to be less than 0.05 wt%. The chemical structures of the synthesized compounds were confirmed by ^1H and ^{13}C NMR spectroscopy (Bruker AMX 300) operating at 300.13 and 75.47 MHz, respectively. Chemical shifts are expressed in δ (ppm) relative to tetramethylsilane (TMS) as

the internal reference and D₂O as the deuterated solvent. The melting points of the synthesized GB-ILs were measured by differential scanning calorimetry (DSC), a Perkin Elmer DSC-7 instrument (Norwalk, CT), at a heating rate of 5 °C/min under N₂ flow of 40 mL·min⁻¹. The NMR data and melting points are reported in the Supporting Information.

Determination of protonation constants of GB/GB-ILs

The pH-metric titrations were carried out in a double-walled titration vessel of about 70 cm⁻³ at (25.0 ± 0.1) °C (the temperature was maintained by means of a thermostatic refrigerated water bath). The titration vessel was sealed by a lid with holes accommodating the electrode, Pt 1000/B/2 (Metrohm 6.1114.010), burette tip, and inlet and outlet gas. The titrations were performed automatically by using an automatic titrator (Metrohm 904) equipped with a 801 magnetic stirrer, Dosino buret model 683, and a pH glass electrode (Metrohm 6.0262.100). The whole titration system was controlled by means of a computer using the software Tiamo 2.3 (Metrohm), which was also used to record the titration process.

The calibration of the pH electrode was performed in terms of hydrogen ion concentrations instead of activities, by titrating a standardized strong acid with a strong base, by the Gran method, and using the GLEE software.[30] The ionic product of water, $pK_w = 13.778$ at 25.0 °C, and ionic strength, $I = 0.1 \text{ mol} \cdot \text{dm}^{-3} \text{ KNO}_3$, were maintained constant during the refinements.[31]

GB/GB-ILs titrations were carried out by titrating a mixture of 0.003 M of HNO₃, 0.1 M of NaNO₃, and 0.001 M of GBs/GB-ILs (total volume of 50 cm³) with 0.1 M of NaOH. The titration cell was bubbled with nitrogen gas for 15 min before starting each the titration, and then kept under a small positive pressure of N₂ to remove carbon dioxide. All titrations were repeated at least three times. The protonation constants for GBs and GB-ILs were computed using the HYPERQUAD program (Version 2008).[22]

Standard Microtox® assays

The Microtox® test[24] was used to evaluate the inhibition of the luminescence of *Vibrio fischeri* after the exposure to either each GB-IL or PPG 400 aqueous solutions, at different concentrations. After 30 minutes of incubation, the light output of the luminescence bacteria was measured and compared with the light output of a blank control. The toxicity is represented by the effective concentration yielding 50% of inhibition of the luminescence (described by the parameter EC₅₀) which was computed using the Microtox® Omni™ Software, version 4.3.0.1.[32]

Phase diagrams (ABS)

The binodal curve of each phase diagram was determined through the cloud point titration method at 25 ± 1 °C and atmospheric pressure.[33] Aqueous solutions of ILs at 70 wt % and pure PPG 400 were used in the determination of the PPG 400-IL-water phase diagrams. Repetitive drop-wise addition of the aqueous solution of each GB-IL to the PPG 400 was carried out until the detection of a cloudy biphasic solution, followed by the drop-wise addition of water until the detection of a monophasic region. This procedure was carried out

under constant stirring and at 25 °C. The systems compositions were determined by the weight quantification of all components added within $\pm 10^{-4}$ g.

The tie-lines (TLs) of each phase diagram were determined by a gravimetric method originally described by Merchuk et al.[26] A mixture at the biphasic region was gravimetrically prepared with PPG 400 + GB-IL + water, vigorously stirred, and allowed to reach the equilibrium by the separation of the two phases for at least 12 h at 25 °C. After the separation of the coexisting phases they were further weighted. Finally, each individual TL was determined by the application of the lever-arm rule to the relationship between the weight of the top and bottom phases and the overall system composition. Further details can be found elsewhere.[26]

Extraction of IgY/Proteins

To obtain the proteins water soluble fraction from egg yolk the following protocol was adopted: (i) the egg yolk was manually separated from egg white and transferred to a filter paper to carefully remove remaining egg white; (ii) the yolk skin was cut with a lancet and the yolk was poured into a tube and diluted in water in a proportion of 1:3 (v:v, yolk: water); (iii) the solution was supplemented with 3.5% (w/v) of PEG 6000 and mixed until the polymer was completely dissolved; (iv) the aqueous solution was centrifuged at 4 °C for 60 min at 4600 g. The solid phase, achieved by the addition of PEG 6000, and which consists of “yolk solids and fatty substances”, was then separated from the watery phase containing the IgY and other water-soluble proteins (WSP). This procedure was adapted from that described previously by Polson et al. [34]. This step is required since the use of ABS, majorly composed of water, are not adequate to dissolve and to maintain the integrity of lipoproteins. The water-phase was then recovered and used in the formation of each ABS. A common tie-line length (TLL = 38-41 wt %) for given mixture compositions of the IL-GB-based ABS (≈ 50 wt% PPG 400 + ≈ 7 -10 wt% IL + ≈ 42 wt% aqueous solution containing the water soluble proteins) was chosen based on the phase diagrams determined in advance. Since the phase diagrams are very close to each other, the similar tie-line lengths (TLL = 38-42 wt %) also correspond to similar mixture compositions.

Each mixture was prepared gravimetrically within $\pm 10^{-4}$ g, vigorously stirred and left to equilibrate for at least 12 h (a time period established in previous optimizing experiments) and at 25 °C, to achieve the complete partitioning of IgY and other contaminant proteins between the two phases. After the careful separation of the phases, using small glass ampoules designed for the purpose, the amount of protein was quantified in each phase. At least three individual experiments were carried out for each ABS allowing the determination of the average partition coefficients and extraction efficiencies and respective standard deviations. The protein content was quantified through UV-spectroscopy, using a SHIMADZU UV-1800, UV Spectrometer, at a wavelength of 280 nm.

The partition coefficients of the proteins, K were determined according to Equation 1,

$$K = \frac{[Protein]_{IL}}{[Protein]_{PPG}} \quad (1)$$

where $[Protein]_{IL}$ and $[Protein]_{PPG}$ are the concentration of proteins in the IL-rich and in the PPG-rich aqueous phases, respectively.

The percentage extraction efficiencies of the proteins, $EE\%$ are defined as the percentage ratio between the amount of protein in the IL-rich aqueous phase and that in the total mixture, according to Equation 2,

$$EE\% = \frac{w_{Protein}^{IL}}{w_{Protein}^{PPG} + w_{Protein}^{IL}} \times$$

where $w_{Protein}^{PPG}$ and $w_{Protein}^{IL}$ are the weight of protein in the PPG-rich and in the IL-rich aqueous phases, respectively.

Control or “blank” solutions at the same mixture point used for the extraction studies (with no proteins added) were used in all systems.

SDS-PAGE

The protein profile of the coexisting phases was investigated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using an Amersham ECLTM Gel from GE Healthcare Life Sciences. The proteins concentration was determined using the Bio-Rad protein assay (Bio-Rad). The top phases (lower protein content) were directly mixed with the Laemmli buffer (1:1, v:v) while the bottom phases were initially diluted, in order to achieve a total protein content of 0.005 mg, and further mixed with the Laemmli buffer. Both phases were then subjected to SDS-PAGE in 20% polyacrylamide gels. The proteins were stained with Coomassie Brilliant Blue G-250 for 2-3 h and then destained at room temperature. All gels were analyzed using the Image Lab 3.0 (BIO-RAD) analysis tool.

Computational details

Density functional theory (DFT) calculations—The molecular dipole moments of the GB anions have been computed in water with a polarizable continuum model (IEF-PCM) using the DFT/B3LYP/6-311++G(d,p) method and using the natural bond orbital (NBO) by Gaussian 09 package.[35]

COSMO-RS modelling—The quantum chemical basis of “Conductor-like Screening Model for Real Solvents” (COSMO-RS) has been described in detail by Klamt.[27] The σ -profiles of the GB-ILs were estimated at the RI-DFT BP/SVP level, and as implemented in the TURBOMOLE 6.1 program package.[36] The σ -profiles were visualized by the COSMOtherm software, version C30_1401 (COSMOlogic GmbH & Co KG, Leverkusen, Germany). [37]

Molecular docking—The molecular docking between IgY-Fc and the GB-IL ions was performed using the Auto-dock Tools vina 1.5.4 program.[29]The crystal structure of IgY-Fc (2W59)[28] was used in the docking. The natural bond orbital (NBO) charges for the GB anions and cholinium in water phase were used. The center of the grid in the x-, y-, z-axes was $(-17.141 \times -8.262 \times 16.727) \text{ \AA}$, and the grid dimension was $(78 \times 70 \times 84) \text{ \AA}$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) pH titration curves of 1×10^{-3} M of TES (■) and [Ch][TES] (●) at 25.0°C and $I = 0.1$ M of NaNO_3 . (b) Protonation equilibrium of [Ch][TES].

Figure 2.

Ternary phase diagrams for the systems composed of PPG 400 + GB-IL + water at 25 °C and atmospheric pressure: (▲) [Ch][MES], (◆) [Ch][Tricine], (■) [Ch][TES] and (●) [Ch][HEPES].

Figure 3.
 σ -profiles of [Ch][HEPES] (black line,—), [Ch][MES] (red line, ---), [Ch][TES] (blue line,), [Ch][Tricine] (dark green line, -.-), and [Ch][CHES] (light green line, -.-.-).

Figure 4.

Partition coefficients (K) and extraction efficiencies ($EE\%$) of total proteins in the water soluble fraction of egg yolk using ABS composed of PPG 400 + GB-IL + water at 25 °C. Mixture compositions: 50 wt% of PPG 400 + 7-10 wt% of each GB-IL + 40-43 wt% of an aqueous solution containing the water soluble proteins from egg yolk.

Figure 5.

SDS-PAGE of a gel loaded with 0.5 μg of protein/well, stained with Coomassie blue. Lane 1 (Std): Standard molecular weights; Lane (a): water soluble fraction of proteins from egg yolk; Lanes (b) and (c): Bottom and top phases, respectively, of the ABS constituted by PPG 400 + [Ch][HEPES]; Lanes (d) and (e): Bottom and top phases, respectively, of the ABS constituted by PPG 400 + [Ch][TES]; Lanes (f) and (g): Bottom and top phases, respectively, of the ABS constituted by PPG 400 + [Ch][Tricine]; Lanes (h) and (i): Bottom and top phases, respectively, of the ABS constituted by PPG 400 + [Ch][MES].

Figure 6.
Interactions between IgY and GB-IL ions based on computational docking.

Table 1

Chemical structure of the cholinium-based Good's buffers ILs

Cholinium 2-(N-morpholino)ethanesulfonate ([Ch][MES])	Cholinium N-[tris(hydroxymethyl)methyl]glycinate ([Ch][Tricine])	Cholinium 2-cyclohexylamino)ethanesulfonate ([Ch][CHES])

Cholinium 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonate ([Ch][HEPES])	Cholinium 2-[2-hydroxy-1,1-[bis(hydroxy methyl)ethyl]amino]ethane sulfonate ([Ch][TES])	