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Good's Buffer Ionic Liquids as Relevant Phase-Forming Components of Self-Buffered Aqueous Biphasic Systems

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Abstract

A series of new self-buffering ionic liquids (ILs) based on Good's buffers (GBs) anions and the tetrabutylphosphonium cation ($[P_{4444}]^+$) was here synthesized and characterized. The self-buffering behaviour of the GB-ILs was confirmed by measuring their protonation constants by potentiometry. Further, their ability to form aqueous biphasic systems with the biodegradable potassium citrate salt was evaluated, and further investigated for the extraction of proteins, using bovine serum albumin (BSA) as a model protein. If these ionic structures display self-buffering characteristics as well as a low toxicity towards the luminescent bacteria *Vibrio fischeri*, they were additionally found to be highly effective in the formation of ABS and in the extraction of BSA - extraction efficiencies of 100% to the IL-rich phase obtained in a single-step. The BSA secondary structure in the aqueous IL-rich solutions was evaluated through infrared spectroscopic studies revealing the protein-friendly nature of the synthesized ILs. Dynamic light scattering (DLS), "Conductor-like Screening Model for Real Solvents" (COSMO-RS), and molecular docking studies were finally carried out to better understand the main driving forces of the extraction process. The results suggest that van der Waals and hydrogen-bonding interactions are important driving forces of the protein migration towards the GB-IL-rich phase, while the molecular docking investigations demonstrated a stabilizing effect of the studied ILs over the protein.

Keywords

Good's buffer ionic liquids; aqueous biphasic systems; Microtox; *Vibrio fischeri*; Bovine serum albumin; extraction; stability

Introduction

Aqueous biphasic systems (ABS) are more benign alternatives to replace the conventional liquid-liquid extractions using organic volatile solvents aiming the efficient separation of biomolecules, in which proteins and enzymes are included (Asenjo and Andrews, 2011; Benavides and Rito-Palomares, 2008). ABS are claimed as greener extraction processes, minimizing the consumption of volatile organic solvents which could be not only harmful to

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the environment and human resources, but also for proteins since they could cause their denaturation (Krishna et al., 2002). Several techniques such as electrophoresis, membrane separation, gel filtration, and affinity chromatography, were adopted in protein separation; however, they are some of the most expensive processes and consequently, are associated to several limitations, namely concerning their scale-up (Pei et al., 2009). Traditional ABS consist of aqueous solutions of two polymers (e.g. polyethylene glycol and dextran) or a polymer and a salt. They have been established as an economical method making part of downstream processes exhibiting low-energy consumption, high performance, high biocompatibility and suitability for large scale production. Consequently, the ABS processes have been extensively studied for the separation and purification of different biological products.

As promising substitutes for conventional organic solvents, ionic liquids (ILs) have become ubiquitous in the recent literature, due to their diverse properties, which are responsible for them to be useful in a surprising large number of applications (Freire et al., 2012a; Welton, 1999). ILs are salts composed of a bulky, unsymmetrical organic cation and an inorganic or organic anion, which have a melting point below 100 °C. The wide structural diversity of ILs allows the adjustment of their properties to improve the protein stability and activity (Cantone et al., 2007; Debeljuh et al., 2011; Kumar and Venkatesu, 2012; Lin Huang et al., 2011). On the other hand, a variety of hydrophilic ILs were found to form ABS when mixed with aqueous solutions of inorganic or organic salts (Cláudio et al., 2012; Freire et al., 2012a; Passos et al., 2012b; Pereira et al., 2013) and proved to be efficient in the extraction of proteins and enzymes (Cao et al., 2008; Deive et al., 2011; Deive et al., 2012; Desai et al., 2014a; Ding et al., 2014; Dreyer and Kragl, 2008; Dreyer et al., 2009; Du et al., 2007; Lin et al., 2013; Lu et al., 2011; Novak et al., 2012; Pei et al., 2010; Pei et al., 2009; Ruiz-Angel et al., 2007; Ventura et al., 2012a; Ventura et al., 2011; Zeng et al., 2013). Moreover, and behind the efficiency of IL-based ABS as separation techniques, they have many advantages as compared to the conventional polymer-based systems, such as their lower viscosity and a higher difference in the phases' polarity (Cao et al., 2008; Deive et al., 2011; Deive et al., 2012; Desai et al., 2014a; Ding et al., 2014; Dreyer and Kragl, 2008; Dreyer et al., 2009; Du et al., 2007; Lin et al., 2013; Lu et al., 2011; Novak et al., 2012; Pei et al., 2010; Pei et al., 2009; Ruiz-Angel et al., 2007; Ventura et al., 2012a; Ventura et al., 2011; Zeng et al., 2013). IL-based ABS systems have been applied as extraction techniques of a large range of macromolecules, these including enzymes like horseradish peroxidase (HRP), *Thermomyces lanuginosus* lipase, *Candida antarctica* lipase A, and *Candida antarctica* lipase B (Deive et al., 2011; Ventura et al., 2012a; Ventura et al., 2011), and proteins, namely BSA, cytochrome c, myoglobin, ovalbumin, hemoglobin, trypsin, cytochrome c, γ -globulins, among others (Cao et al., 2008; Dreyer and Kragl, 2008; Dreyer et al., 2009; Du et al., 2007; Lu et al., 2011; Pei et al., 2010; Pei et al., 2009; Ruiz-Angel et al., 2007).

When considering the previously reported works, it is clear that IL-based ABS provide a higher extraction efficiency regarding proteins if compared with the most conventional systems based on polymers. Moreover, some parameters were already defined as important conditions driving the migration of proteins, namely the potential formation of aggregates, or some particular interactions, like electrostatic, dispersive and hydrogen-bonding interactions (Cao et al., 2008; Deive et al., 2011; Deive et al., 2012; Desai et al., 2014a; Ding

et al., 2014; Dreyer and Kragl, 2008; Dreyer et al., 2009; Du et al., 2007; Lin et al., 2013; Lu et al., 2011; Novak et al., 2012; Pei et al., 2010; Pei et al., 2009; Ruiz-Angel et al., 2007; Ventura et al., 2012a; Ventura et al., 2011; Zeng et al., 2013). In the studies reported in the last two decades, phosphate salts are still the most applied as salting-out agents. Included in this condition, some authors used the phosphate buffer to adjust the pH of the system considering some specific proteins/enzymes and ABS (Desai et al., 2014b; Moreira et al., 2013; Souza et al., 2015). However, and contrarily to the pH control by means of the added salt, buffered IL-based ABS can be created with ILs with self-buffering characteristics. For this purpose, we recently developed a series of self-buffering and biocompatible ILs (Good's buffer ionic liquids, GB-ILs) in which five Good's buffers (GBs, namely Tricine, TES, MES, HEPES, and CHES) acted as anions and 1-ethyl-3-methylimidazolium ($[C_2mim]^+$), alkylammonium ($[N_{nnnn}]^+$), and cholinium ($[Ch]^+$) acted as cations (Taha et al., 2015a; Taha et al., 2014; Taha et al., 2015c). GBs were considered because they are *N*-substituted glycine or taurine compounds that are frequently used in diverse biological systems. To expand this range of available GB-based ABS and to better understand their potential in separation processes, in this work, five new GB-ILs with self-buffering characteristics were prepared by the conjugation of a wide variety of GB's anions and the tetrabutylphosphonium cation ($[P_{4444}]^+$). These were characterized, including the evaluation of their toxicity against the luminescent bacteria *Vibrio fischeri* (Steinberg et al., 1995), and further used to form IL-based ABS with the biodegradable potassium citrate salt. The phosphonium cation ($[P_{4444}]^+$) was selected because it has an enhanced aptitude to form ABS (Louros et al., 2010), and thus lower amounts of phase-forming components will be required to create two-phase systems towards the development of more benign and cost-effective separations techniques. After the determination of the corresponding phase diagrams, their capacity to extract BSA was evaluated and compared with that of tetrabutylphosphonium chloride ($[P_{4444}]Cl$). The stability of BSA was also addressed and the molecular-level mechanisms which rule the extraction of BSA to the IL-rich phase were ascertained by COSMO-RS and molecular docking studies. The chemical structure of $[P_{4444}]Cl$ and of the novel GB-ILs prepared in this work are depicted in Figure 1 (the synthetic pathway to prepare the latest is shown in Figure S1 in the Supporting Information).

Materials and Methods

Materials

N-[tris(hydroxymethyl)methyl]glycine (Tricine, purity > 99 wt%), 2-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethane sulfonic acid (TES, purity > 99 wt%), 2-(*N*-morpholino) ethanesulfonic acid (MES, purity > 99 wt%), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES, purity > 99.5 wt%), 2-(cyclohexylamino)ethanesulfonic acid (CHES, purity > 99 wt%), and $[P_{4444}][OH]$ (45 wt% in water), were purchased from Sigma–Aldrich. Sodium hydroxide pellets were supplied from Eka Chemicals. BSA (fraction V) was obtained from Acros Organics. Methanol (HPLC grade, purity > 99.9 wt%) was obtained from Fisher Scientific (UK). Acetonitrile (purity > 99.7 wt%) was supplied from Lab-Scan (Ireland). Purified water passed through a reverse osmosis and a Milli-Q plus 185 water purifying system was used in all experiments. Sodium nitrate (purity > 99.5 wt%) was obtained from Himedia Labs. Potassium nitrate

(purity > 98.0), nitric acid (65 wt%), and potassium hydrogen phthalate (purity > 99.8) were purchased from Panreac (Barcelona, Spain).

Synthesis and characterization of GB-ILs

An aqueous solution of $[P_{4444}][OH]$ was added drop-wised to a GB aqueous solution with a slight equimolar excess. The solution was stirred at room temperature during 12 h; then, the reaction mixture obtained was evaporated at 60 °C under vacuum. A mixture of acetonitrile and methanol (1:1) was added to the viscous liquid previously obtained, stirred vigorously at room temperature for 1 h until the precipitation of the unreacted buffer, which was posteriorly removed by filtration. The solvent (acetonitrile + methanol) was evaporated and the GB-ILs were dried under vacuum for 3 days at room temperature. The water content of each GB-IL prepared (less than 0.05 wt %) was measured by Karl–Fischer coulometer (Metrohm Ltd., model 831). The chemical structures of the GB-ILs were confirmed by 1H and ^{13}C NMR spectroscopy (Bruker AMX 300) operating at 300.13 and 75.47 MHz, respectively. The melting points of GB-ILs were measured by differential scanning calorimetry (DSC), with a Perkin Elmer DSC-7 instrument (Norwalk, CT), at a heating rate of 5 °C.min⁻¹ under a N₂ flow of 40 mL.min⁻¹. These data (NMR data and melting points) are presented in Table S1 in the Supporting Information file.

Potentiometric titrations

pH-potentiometric apparatus—The pH-potentiometric measurements were carried out in a 70 cm³ double-walled glass vessel 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) with a precision of ± 0.001. The temperature of the titration cell was controlled at 25.0 ± 0.1 °C by a thermostatic water bath. The titration cell was equipped with a lid with various openings for insertion of the electrode, burette tip, Pt 1000/B/2 (Metrohm 6.1114.010), and gas inlet and outlet. The titroprocessor was coupled to a personal computer and the titration software Tiamo 2.3 was used to control and record the titration process.

pH profiles procedure—10 mL of GB-ILs (0.05 M) were titrated with 0.1 mol.dm⁻³ of NaOH/HCl under continuous magnetic stirring. The pH electrode was calibrated using three standard buffer solutions of pH 4.01, 7.00, and 9.21.

Determination of protonation constants of GB/GB-ILs—The glass electrode was calibrated considering the hydrogen ion concentrations instead of activities, by means of a strong acid-strong base titration. In this titration, 2 cm³ of 0.1 M of HNO₃ and 50 cm³ of 0.1 M of a KNO₃ solution were titrated with a 0.1 M of carbonate-free NaOH solution. The concentration of NaOH was determined by standardization with potassium hydrogen-phthalate. The water purified was degassed under vacuum using a rotary evaporator at 70 °C and cooled under a stream of nitrogen. The titration cell was kept under a small positive pressure of nitrogen gas to eliminate the effect of atmospheric carbon dioxide. The nitrogen gas was purged for 15 min before starting the titration to expel any dissolved oxygen or carbon dioxide present. The values of volume added (cm³) and electrode potential (mV) were analyzed with the GLEE software (Gans and O'Sullivan, 2000). This computer

program uses a (non-linear) least-squares refinement to fit a modified Nernst equation, Eq. (1),

$$E = E^{\circ} + s \log[H^{+}] \quad (1)$$

where E refers to the potential of the glass electrode, E° and s are the standard electrode potential and slope, and $[H^{+}]$ is the hydrogen ion concentration.

In the acid region, the hydrogen ion concentration, T_H , is obtained from Eq. (2), that is, $\log[H^{+}] = \log(T_H)$.

$$T_H = \frac{a_H v_0 + \gamma b_H v}{v_0 + v_1 + v} \quad (2)$$

where a_H is the acid concentration ($\text{mol}\cdot\text{dm}^{-3}$) and v_0 (cm^3) is the initial volume added to the titration vessel. b_H is the base concentration ($\text{mol}\cdot\text{dm}^{-3}$) in the burette (with negative sign), and γ is a correction factor for the base concentration. v is the volume (cm^3) of base added from the burette, and v_1 (cm^3) is the volume of the electrolyte solution.

In the alkaline region, the hydrogen ion concentration, T_H , is given by Eq. (3), where $\log[H^{+}] = -\text{p}K_w - \log(-T_H)$, v_e^a is the volume (cm^3) at the acid equivalence point, and v_e^b is the volume (cm^3) at the alkali equivalence point.

$$T_H = \frac{a_H v_0 + \gamma \frac{v_e^a}{v_e^b} b_H v}{v_0 + v_1 + v} \quad (3)$$

The Gran plot was used to estimate the carbonate contamination in the titrant and a typical Gran plot is shown in Figure S2. The product of water $\text{p}K_w = 13.778$ at 25°C and ionic strength $I = 0.1 \text{ mol}\cdot\text{dm}^{-3}$ KNO_3 were maintained constant during the refinements (Jameson and Wilson, 1972).

Titration for protonation constant calculations were performed by combining 0.003 M of HNO_3 , 0.1 M of NaNO_3 , and 0.001 M of GB/GB-ILs (total volume of 50 cm^3) and titrated with the NaOH solution under carefully controlled experimental conditions, as described in the glass electrode calibration procedure. Each titration was repeated at least three times (more than 100 data points for each one). The determination of the protonation constants were computed using the HYPERQUAD program (Version 2008) (Gans et al., 1996). This computer program treats the protonation equilibria of ligand as overall association constants; e.g. the protonation equilibria of H_2A which can be expressed as,

The stepwise acid dissociation constants of H_2A is given by

Since $pK_a = -\log(1/K_a)$, the pK_a 's values are given by $pK_{a2} = \log\beta_1$ and $pK_{a1} = \log\beta_2 - \log\beta_1$.

Dynamic light scattering (DLS) measurements

The hydrodynamic radius (R_H) values of the protein were measured at different temperatures using a Zetasizer Nano ZS equipment (Malvern Instruments Ltd., UK). The average R_H was calculated from the scattering intensity data using the instrument software. The light source of the instrument is He-Ne laser light (4mW) with a wavelength (λ) = 633 nm, and the scattering angle were fixed at 173° . The instrument is provided with a thermostatic sampling chamber which controls the temperature in the range from 0°C to 90°C . The samples were prepared with $20\text{ mg}\cdot\text{cm}^{-3}$ of BSA in (0.05 and 0.5) M of GB/GB-ILs/[P₄₄₄₄]Cl, and at pH= 7.4. The samples were incubated at 25°C for 4 h to achieve the equilibrium. Around 1.3 cm^{-3} bubble free sample in a square glass cuvette (PCS8501) was used for the DLS measurements.

Infrared measurements

Attenuated total reflectance (ATR) Fourier transform infrared (FTIR) spectra of 30 mg BSA in (0.05 and 0.5) M of GB/GB-ILs/[P₄₄₄₄]Cl solutions were measured using an ABB MB3000 FTIR spectrometer equipped with PIKE MIRacle™ and a single reflection diamond/ZnSe crystal plate. The spectral region was $400\text{--}4000\text{ cm}^{-1}$ with a resolution of 4 cm^{-1} and 100 scans. At least 5 measurements were performed for each sample. Second-derivative spectra of the amide I ($\sim 1652\text{ cm}^{-1}$) region were used as peak position guides for the Gaussian curve-fitting analysis. The second-derivative and the curve fitting were done using PeakFit v4.0 (AISN software Inc.). The relative amount of α -helices, β -sheets, and turns was estimated by calculating the areas of the bands assigned to a particular substructure.

Phase diagrams and tie-lines

The binodal curve of each phase diagram was determined through the cloud point titration method at $25 (\pm 1) ^\circ\text{C}$ and at atmospheric pressure, as detailed elsewhere (Lee et al., 2015). The tie-lines (TLs) of each phase diagram, including the mixtures compositions for which the extraction of BSA was carried out, were determined by a gravimetric method originally described by Merchuk et al (Merchuk et al., 1998). In this section, the same procedures described by (Lee et al., 2015) were adopted.

Extraction of BSA

The biphasic systems used for the extraction of BSA were gravimetrically prepared at a fixed common mixture composition: (39.4 ± 0.7) wt % of GB-IL/IL + (15.1 ± 0.9) wt % of salt + water. The aqueous solution added to complete the mixture composition contained BSA at a concentration of *circa* $0.5 \text{ g}\cdot\text{L}^{-1}$. BSA was quantified by SE-HPLC and further details can be found elsewhere (Taha et al., 2015b). The wavelength used to quantify the BSA was set at 280 nm whereas the retention time of BSA in the HPLC chromatograms was found to be 9.31 min within an analysis time of 24 min, using the external standard calibration method in the range of 0.001 to $1 \text{ g}\cdot\text{L}^{-1}$ of BSA. In this work, three ABS of each IL were prepared and three samples of each phase were properly quantified. Blank controls were always used to ascertain on the rotation times of the ABS phase-forming components.

The extraction efficiency of BSA, $EE_{\text{BSA}} (\%)$, was calculated to evaluate the success of each ABS to the extraction of BSA. This parameter describes the ratio between the amount of protein quantified in the IL-GB-rich phase and the sum of the amount of the protein in both phases, as defined in Eq. (8):

$$EE_{\text{BSA}} (\%) = \frac{[\text{BSA}]_{\text{IL}} \times w_{\text{IL}}}{[\text{BSA}]_{\text{IL}} \times w_{\text{IL}} + [\text{BSA}]_{\text{Salt}} \times w_{\text{Salt}}} \times 100 \quad (8)$$

where [BSA] is the concentration of protein quantified, w is the weight of each phase, and the subscripts IL and salt represent the IL- and potassium-citrate-rich phases, respectively.

Computational details

COSMO-RS modelling—The detailed theory on “Conductor-like Screening Model for Real Solvents” (COSMO-RS) was described in detail by Klamt (Klamt, 2005). This model integrates concepts from quantum chemistry, continuum solvation model (COSMO), electrostatic interaction and extends to statistical thermodynamics, which are recurrently used for predicting the thermodynamic properties of fluids and liquid mixtures. COSMO-RS is used to analyse the electrostatic interaction ($E_{\text{misfit}}(\sigma \text{ and } \sigma')$), H-bonding (E_{HB}) and van der Waals (E_{vdW}) interaction energies of interacting species in terms of the screening charge densities (σ, σ') of the respective surface segments - Eqs (9-11) (Klamt and Eckert, 2000),

$$E_{\text{MF}}(\sigma, \sigma') = a_{\text{eff}} \frac{\alpha'}{2} (\sigma + \sigma')^2 \quad (9)$$

$$E_{HB} = a_{\text{eff}} c_{hb} \min(0, \sigma \sigma' + \sigma_{hb}^2) \quad (10)$$

$$E_{vdW} = a_{\text{eff}} (\tau_{vdW} + \tau'_{vdW}) \quad (11)$$

where a_{eff} refers to the effective contact area, α' is a general constant, c_{hb} is the strength coefficient, σ_{hb} represent the polarization charge density threshold for hydrogen bond, and τ_{vdW} and τ'_{vdW} are the element-specific parameter for dispersion parameters. The 3D distribution of the polarization charges, σ , of each molecule is converted into a surface composition function, named as sigma profile (σ -profile), by the COSMOtherm program version C30_1401 (Klamt, 1995; Klamt et al., 1998). The σ -profile depicts the surface charge density distribution on the molecular surface, which provides quantitative information about the polarity of the molecules (Klamt, 1995; Klamt and Eckert, 2000). The σ -profiles of [P₄₄₄₄][GB]/[P₄₄₄₄]Cl were computed at the RI-DFT BP/SVP level, as implemented in the TURBOMOLE 6.1 program package (Schafer et al., 2000), and the interaction energies were calculated using the COSMOtherm software version C30_1401.

Octanol-water partition coefficient calculation (log K_{OW})—The DFT/COSMO calculations of GBs were computed using the TURBOMOLE 6.1 program with RI-BP/TZVP method (Schafer et al., 2000). The log K_{OW} (octanol-water partition coefficients) values of all GBs were determined by the COSMOtherm software, version C30_1401 (Klamt, 1995; Klamt et al., 1998). The K_{OW} is obtained through the computation of the ratio of the activity coefficient of the buffer at infinite dilution (γ_i^∞) in the water- and octanol-rich phases, as indicated in Eq. (12).

$$K_{OW, i} = 0.151 K_{OW, i} = 0.1505 \frac{\gamma_i^{\infty, W}}{\gamma_i^{\infty, O}} \quad (12)$$

where the superscripts “W” and “O” refer to the water-rich and the octanol-rich (0.726 mole fraction of octanol) phases, respectively.

QSAR-serum albumin binding model, logK(HSA)—The molecular geometries of [P₄₄₄₄][GB]/[P₄₄₄₄]Cl were first computed with the AM1 semi-empirical method with Polak-Rebiere algorithm to reach a 0.01 root mean square gradient as implemented in the HyperChem (Version 8.0.7, Hypercube, Inc, USA, <http://www.hyper.com>) program. The COSMO files of these ILs were then obtained by single point calculations at the RI-DFT BP/SVP level using the TURBOMOLE 6.1 program package (Schafer et al., 2000). The QSAR-logK(HSA) values were computed using these COSMO files with the COSMOtherm software, version C30_1401 (Klamt, 1995; Klamt et al., 1998).

Molecular docking—The molecular docking between BSA and the diverse [P₄₄₄₄][GB] ions was performed using the Auto-dock Tools vina 1.5.4 program (Trott and Olson, 2010) and the three-dimensional structures of BSA (PDB, 3v03) (Majorek et al., 2012). Further details can be found elsewhere (Taha et al., 2015b). The lowest binding model was searched out from 9 different conformers for each ligand based on the one with the lowest objective function, which includes electrostatic interactions, hydrogen bonding, short range vdW, and solvation energies terms.

Standard Microtox[®] liquid-phase assays

The luminescence inhibition of the marine bacteria *Vibrio fischeri* (strain NRRL B-11177) was measured after contacting with each [P₄₄₄₄][GB] at 15 °C. The protocol followed in this work (81.9% standard test) is described elsewhere in detail (Taha et al., 2015b). Also in this work the effective concentration yielding 50% of inhibition of the bacteria luminescence (EC₅₀) along with the corresponding 95% confidence limits were determined through the Microtox[®] Omni[™] Software version 4.1 (Environmental, 1998), to evaluate the toxicity of each GB-IL.

Results and Discussion

The acid-base behavior of the novel GB-ILs

Good's buffers are zwitterionic compounds with two protonation states, the carboxylic/sulfonic (pK_{a1}) and the amino (pK_{a2}) groups. The first acidic sites of Good's buffers are released below *ca.* pH 3.0, while the second protonation constants are included in the physiological pH region of pH 6 to 9. The pH-profiles of these GB-ILs were measured in aqueous solution (Figure 2a) to identify their buffering action, through which is possible to show their buffering-like region described by the moderate slope length appearing before the inflection point at high pH. Their buffer capacity is a measure of a buffer's ability to resist changes in pH upon the addition of an acid or base. Mathematically, the buffer capacity (β) is normally defined according to Eq. (13),

$$\beta = \frac{dC_b}{d(pH)} = -\frac{dC_a}{d(pH)}, \quad (13)$$

where C_b and C_a , are the number of moles of strong base or acid added *per* liter. The buffer capacity of the GB-ILs in aqueous solution is shown in Figure 2b. From this figure, we can see that the buffer capacities of GB-ILs are relatively high and offer a wide range of pH values. The buffering regions of [P₄₄₄₄][MES], [P₄₄₄₄][TES], [P₄₄₄₄][HEPES], [P₄₄₄₄][Tricine], and [P₄₄₄₄][CHES], are 4.7–7.3, 6.2–8.7, 6.2–8.8, 6.6–9.7 and 7.7–10.7, respectively. The pH at the middle of the buffering region is equal to the pK_{a2}, and thus, the buffering capacity is highest at this point.

The (acid + base) equilibria of GBs and GB-ILs (e.g., MES and [P₄₄₄₄][MES], respectively) are represented in Figure 3. The pK_{a1} and pK_{a2} values of the investigated Good's buffers at 25 °C and the ionic strength of 0.1 M of NaNO₃, as well as the corresponding GB-ILs, are reported in Table 1. The pK_{a1} values of MES, TES, and CHES, are here reported for the first

time. The pK_{a2} values obtained in this work are in good agreement with those found in the literature (Goldberg et al., 2002). Representative potentiometric fitted profiles of MES and $[P_{4444}][MES]$ using the HYPERQUAD 2008 program (Gans et al., 1996) are shown in Figure 2a. The distribution diagrams of species computed with the HySS program (Alderighi et al., 1999) for MES and $[P_{4444}][MES]$ as a function of pH, are presented in Figure 2b. The potentiometric profiles and distribution diagrams of the species for the other investigated GB/GB-ILs are given in Figure S3 in the Supporting Information. From the obtained results (potentiometric profiles and species distribution diagrams) it can be gauged that GB-ILs are more easily deprotonated than the corresponding GBs, as confirmed by the lower pK_a values of the former (Table 1). It should be remarked that it was not possible to determine the pK_{a1} value of $[P_{4444}][TES]$ and $[P_{4444}][CHES]$. The observed decrease in the pK_{a1} and pK_{a2} values of GB-ILs can be attributed to a higher stabilization of their free conjugated bases by electrostatic interactions with the tetrabutylphosphonium cation.

To determine the optimum pH of a protein function, different buffers are used to maintain the solution buffer capacity. However, the available universal buffers are scarce and have drawbacks since their anions interact or chelate metal-ions. The biological buffers MES, HEPES, and CHES are compatible with systems containing metal ions due to their negligible affinity to metal ions, and thus they are used to formulate biocompatible universal buffers in aqueous solution (Figure 5). The universal Good's buffer composed of ' $[P_{4444}][MES] + [P_{4444}][HEPES] + [P_{4444}][CHES]$ ' presents a linear behavior in the pH range from 5.5 to 10.5 and can be seen as a promising option.

ABS phase diagrams

The formation of an aqueous biphasic system depends on the type of IL and its concentration, type of salt and its concentration, temperature and other features such as pH of the aqueous medium. The intensity of the phase-forming ability in each IL-based system relays on the basis of the complex and competing nature of the interactions occurring between the solutes (*i.e.*, ions from the inorganic salt and IL) and water or between the phase-forming components (Freire et al., 2012b). The phase diagrams provide information about the concentration of phase forming components needed for the design of ABS extraction processes. The experimental data corresponding to the ternary phase diagrams determined in this work, as well as the respective correlations, are presented in Tables S2 and S3 in the Supporting Information. For all ABS studied, the top phase corresponds to the IL-rich aqueous layer while the bottom phase is rich in salt and water. Figure 6A depicts the ABS phase diagrams obtained for several $[P_{4444}][GB]/[P_{4444}][Cl]$ and $K_3C_6H_5O_7$. Table S4 in the Supporting Information presents additional data regarding the phase diagrams description, namely tie-lines (TLs) and respective tie-line lengths (TLL). The $[P_{4444}]$ cation is already known for its high ability to form ABS with different salts, which easily justifies the easy formation of ABS of all the GB-ILs prepared in this work. The formation of ABS using these GB-ILs is driven by the low affinity of the phosphonium cation for water, since it has a highly positive shielded charge that is surrounded by four hydrophobic alkyl chains. The smaller the affinity for water and/or the higher hydrophobic nature of the IL, the more prone it is to be salted-out (Marques et al., 2013) by the other phase former, in this case, the potassium citrate salt. Since potassium citrate is a strong salting-out salt (Pegram and

Record, 2007), it has a higher affinity for water and thus the GB-ILs are strongly salted-out from the aqueous solution and create a two-phase system. In Figure 6B, the binodal curves are plotted on a molality scale, together with the data for the ABS composed of $[P_{4444}]Cl + K_3C_6H_5O_7$ obtained from literature (Passos et al., 2012a) for comparison purposes. It can be seen from Figure 6B that the phase-forming ability of all GB-ILs is higher than $[P_{4444}]Cl$, meaning that lower amounts of GB-IL and salt are required to form ABS if compared to more traditional and commercially available ILs, with the benefits of reducing the cost and increasing the biocompatible nature of these systems when applied as purification/fractionation platforms. The GB-IL anions ability to form ABS, for instance at $1.0 \text{ mol}\cdot\text{kg}^{-1}$ of $K_3C_6H_5O_7$ follows the order: $[CHES]^- > [MES]^- > [HEPES]^- > [TES]^- > [Tricine]^-$. At a first glance, this trend can be interpreted in the light of each anion chemical structure (*cf.* Figure 1): *i)* the [Tricine] and [TES] anions' weakest ABS formation ability is a consequence of the presence of three hydroxyl groups that enhance their hydrogen-bonding capacity with water, turning the salting-out process by the citrate-based salt a more difficult task; *ii)* [HEPES]⁻, with one hydroxyl group, is ranked in an intermediate position; *iii)* and finally [MES]⁻ and [CHES]⁻, with no hydroxyl groups, are the more hydrophobic GB-ILs investigated and this with a stronger phase split ability. Meanwhile, the hydrophobic character of the [CHES] anion arises from the cyclohexyl group present in its structure (Figure 1), which allows the easiest phase separation.

The hydrophobic character of GBs can be evaluated through their water-octanol partition coefficients, $\log(K_{ow})$. The higher the value of $\log(K_{ow})$ the higher their solubility in the octanol-rich phase and, consequently, the lower the polarity of the GB. Therefore, higher $\log(K_{ow})$ values correspond to anions that are more easily salted-out and form two-phase systems (ABS). The $\log(K_{ow})$ values of CHES, MES, HEPES, TES, and Tricine are, respectively, 2.04, 0.27, 0.12, -0.51, and -0.69, which is consistent with the phase-forming ability pattern aforementioned.

Figure S4 in the Supporting Information shows a comparison between the effect of $[P_{4444}]^+$ and $[N_{4444}]^+$ (Taha et al., 2014) on the ABS formation with potassium citrate. It can be seen that $[P_{4444}]^+$ is a better phase forming agent than $[N_{4444}]^+$ for all cation-anion combinations. Although both types of compounds are composed of four alkyl chains of similar length, there are also some contributions derived from the central atom. Similar results were obtained in ABS constituted by more conventional ILs and potassium phosphate (Louros et al., 2010), sodium carbonate (Marques et al., 2013) and potassium citrate (Passos et al., 2012a) as salting-out agents. In these studies, the results suggested that phosphonium-based ILs are also more effective in promoting ABS when compared with their ammonium-based congeners. Recently, the influence of the cation's central atom of some ammonium- and phosphonium-based ILs were investigated through experimental and theoretical studies (Carvalho et al., 2014). The authors observed that the ammonium cations are more polar than the corresponding phosphonium ILs, again consistent with the higher ABS phase-forming ability observed for phosphonium-based GB-ILs.

Extraction efficiencies of BSA

Table 2 presents the extraction efficiencies of BSA for the IL-GB-rich phase at a fixed composition and for which the respective phases' compositions are given in Table S4 in the Supporting Information. From the results depicted in Table 2, it is observed that the extraction of BSA is complete ($EE_{BSA} = 100\%$) for the (GB-)IL-rich phase, without significant losses of protein, either by precipitation or denaturation (conclusion obtained by the mass balance results), meaning that these ILs can be seen as protein-friendly and outstanding extraction solvents of proteins. The presence of the potassium citrate salt, a strong salting-out, seems to be the main driving force for the migration of the protein to the top (IL-rich) phase.

The stability of the protein conformation when in contact with the $[P_{4444}][GB]/[P_{4444}]Cl$ was studied in this work through ATR-FTIR, in particular to determine the protein conformation integrity regarding the secondary structure of proteins (Seshadri et al., 1999; Shivu et al., 2013). The IR spectra of the amide I group of BSA in presence of (0.05 and 0.5) M of $[P_{4444}][Tricine]/[P_{4444}][TES]/[P_{4444}][HEPES]/[P_{4444}]Cl$ were measured at pH 7.4, since at this pH, these three GB-ILs present good buffer capacity. The presence of the amide I band is due to carbonyl stretching vibration which appears at $\sim 1653\text{ cm}^{-1}$. In order to analyze the BSA secondary structure, curve fitting was used for the amide I. The bands at (1615, 1631, 1653, 1675, and 1697) cm^{-1} correspond to intermolecular β -sheets, intramolecular β -sheets, α -helix, turns, and antiparallel β -sheets, respectively (Seshadri et al., 1999; Shivu et al., 2013). The α -helix value of BSA in a buffer solution is consistent with those previously reported (Ghosh et al., 2011). The secondary structure of BSA in the studied GB-ILs as well as in the conventional $[P_{4444}]Cl$, is listed in Table 3. The Gaussian curve-fitting analysis of the amide I spectra of BSA in 0.05 M $[P_{4444}]Cl$ and $[P_{4444}][TES]$ are shown in Figure 7, as examples. The α -helices of BSA in GB-ILs are higher than the corresponding GBs. The α -helix contents of BSA in the studied ILs follow the order: $[P_{4444}][TES] > [P_{4444}][HEPES] > [P_{4444}]Cl > [P_{4444}][Tricine]$. It is important to mention that no buffer is used to adjust the pH of the protein solution in presence of the GB-ILs, while 0.05 M of HEPES buffer was used for the analysis with the $[P_{4444}]Cl$.

The evaluation of the BSA aggregation in aqueous solutions of ILs is important to understand the stabilizing/destabilizing effect of the synthesized ILs through proteins. The effect of increasing the concentration of $[P_{4444}][GB]/[P_{4444}]Cl$ on BSA using DLS at pH 7.4 at 25 °C is presented in Figure 8. The size distribution curves of the studied ILs show that BSA in 0.05 M of ILs exhibited two peaks at $R_H = 3.7\text{ nm}$ and $R_H = 100\text{ nm}$. The peak of small particles which appears with a major population is due to the native state of BSA. Meanwhile, the other peak with lower intensity is showing that BSA forms aggregates in these conditions. The size of BSA was increased while increasing the IL concentration from 0.05 M to 0.5 M (Figure 8). This indicates that with the increase of the IL concentration, the BSA molecules aggregate forming oligomeric species. However, these oligomeric species are formed without unfolding as observed from the increase of the α -helices of BSA in the (GB-)ILs.

In order to calculate the binding between $[P_{4444}]Cl/[P_{4444}][GB]$ and BSA, the binding model 'quantitative structure-activity relationship (QSAR)-human serum albumin',

$\log K(\text{HSA})$, was used to predict the binding affinity of $[\text{P}_{4444}]\text{Cl}/[\text{P}_{4444}][\text{GB}]$ to HSA (Xue et al., 2004). This model can be used also to predict the binding of the investigated ILs to BSA because both proteins (HSA and BSA) are similar in terms of amino acid sequence (Benyamini et al., 2006). The $\log K(\text{HSA})$ values of $[\text{P}_{4444}]\text{Cl}$, $[\text{P}_{4444}][\text{TES}]$, $[\text{P}_{4444}][\text{Tricine}]$, and $[\text{P}_{4444}][\text{HEPES}]$ are, respectively, 0.4438, 0.3112, 0.5754, and 0.8292. These phosphonium-based ILs show a binding affinity to BSA, thus confirming the complete extraction of BSA with no losses discussed before.

It is well known that BSA has different binding sites for a variety of biomaterials (e.g. fatty acids) with thermal stabilizing effect, (Michnik et al., 2005) where the hydrophobicity and polarity play a significant role in the binding. The polarity of the synthesized ILs can be qualitatively evaluated from the σ -profiles of their ions. The σ -profile is divided into three regions (Figure 9): H-bond donor, H-bond acceptor, and a non-polar region between them. The H-bond cut-off is $0.079 \text{ e}\cdot\text{\AA}^{-2}$, and the hydrogen bonding is weakened up to $0.01 \text{ e}\cdot\text{\AA}^{-2}$. It is clear from Figure 9 that GB-ILs show strong negative polar peaks over $(0.01 \text{ to } 0.025) \text{ e}\cdot\text{\AA}^{-2}$ (deep red) arising from the oxygen's sulfonic/carboxylic/morpholine groups and hydroxyl groups as well as the nitrogen's amine groups, in which they can provide hydrogen bonds with H-bond donor groups such as protein or water. On the other side, only $[\text{P}_{4444}][\text{TES}]$ shows positive polar peaks at $-0.015 \text{ e}\cdot\text{\AA}^{-2}$ (deep blue) arising from one hydroxyl's hydrogen of $[\text{TES}]^-$, while the other two hydroxyl's hydrogens formed intramolecular hydrogen bonds with the sulfonic group. The hydroxyl's hydrogens of $[\text{Tricine}]^-$ do not show any positive peaks because they form intramolecular hydrogen bonds with themselves and the oxygen's carboxylic group. The hydroxyl's hydrogen of $[\text{HEPES}]^-$ forms intramolecular hydrogen bonds with the nearby tertiary amine group. Furthermore, the amine's hydrogens of $[\text{TES}]^-$, $[\text{Tricine}]^-$, and $[\text{CHES}]^-$ form intramolecular hydrogen bonds with oxygen's sulfonic and carboxylic groups. Therefore, GBs' anions show weak hydrogen bond donor fragments due to the intramolecular hydrogen bond formations. The sharp negative peak of $[\text{P}_{4444}]\text{Cl}$ is a result of the chloride ion while the broad peak in the non-polar area arises from the aliphatic groups of $[\text{P}_{4444}]^+$. This non-polar peak becomes broader in case of GB-ILs due to the methylene groups of the GB's anion, further supporting the higher ability of GB-ILs to form ABS.

Table 4 reports the total mean interaction energy (E_i), electrostatic interaction energy ($E_{i,\text{misfit}}$), hydrogen bond interaction energy ($E_{i,\text{HB}}$), and van der Waals interaction energy ($E_{i,\text{vdW}}$) of the pure ILs derived from COSMO-RS computations. The electrostatic-misfit interaction energy arises from the dissimilarity and mismatching between the hydrogen bond donors and acceptors among ILs ions, as it is evident from the results of the σ -profiles. The data in Table 4 show strongly negative values of van der Waals interaction energy, indicative of their expected strong dispersive interactions with protein. The hydrogen bond energy values are also found to be negative. The electrostatic-misfit contributions are positive due to their lack of H-bond donors which in turn shows the expected attraction towards the H-donors sites of the protein. The total mean interaction energies of the investigated ILs were found to be negative. Thus, the van der Waals and hydrogen bond interactions between BSA and these ILs play an important role in protein partitioning in $[\text{P}_{4444}][\text{GB}]/[\text{P}_{4444}]\text{Cl}$ ABS.

We have previously identified the hydrogen bond interactions between Tricine (Taha et al., 2014), TES (Taha et al., 2015c), and HEPES (Taha et al., 2015c) anions with BSA using molecular docking. Tricine anion has found to form five hydrogen bonds with Leu346, Glu353, and Arg208 amino acid residues of BSA (Taha et al., 2014). TES anion forms two hydrogen bonds with Arg198, while HEPES anion forms only one hydrogen bond with Ser 428 (Taha et al., 2015c). Herein, we also carried out molecular docking to predict the binding sites of MES^- , CHES^- , and $[\text{P}_{4444}]^+$ to BSA as shown in Figure 10. MES anion forms three hydrogen bonds with Ser 428, Arg 458, and His 145. CHES anion forms two hydrogen bonds with Arg458 and His145. The residues found next to the $[\text{P}_{4444}]^+$ are Thr 190, Ser 428, and Leu 454. The binding free energies of the $[\text{MES}]^-$, and $[\text{P}_{4444}]^+$ are (-5.0, -5.6, and -5.0) kcal.mol⁻¹, respectively.

Toxicities of $[\text{P}_{4444}][\text{GB}]$ against the marine bacteria *Vibrio fischeri*—To evaluate the ecotoxicity of the five $[\text{P}_{4444}][\text{GB}]$ ILs, the Microtox[®] test (Corporation, 1992; Steinberg et al., 1995) was used. Table 5 reports the EC₅₀ values determined along with the 95% limits of confidence. The toxic character of $[\text{P}_{4444}][\text{GB}]$ as well as that of $[\text{P}_{4444}]\text{Cl}$ was assessed after 30 minutes of the bacteria exposure, ensuring enough time to verify the effect in the luminescence inhibition, considered in this work the principal output (Ventura et al., 2012b). The results show that the $[\text{P}_{4444}][\text{GB}]$ species present a toxicity that is similar or lower than the toxicity obtained for the $[\text{P}_{4444}]\text{Cl}$. In this sense, the EC₅₀ values can be ranked as follows: $[\text{P}_{4444}][\text{MES}] > [\text{P}_{4444}][\text{HEPES}] > [\text{P}_{4444}][\text{CHES}] > [\text{P}_{4444}]\text{Cl}$ (the commercial IL) $> [\text{P}_{4444}][\text{Tricine}] > [\text{P}_{4444}][\text{TES}]$. This tendency indicates that $[\text{P}_{4444}][\text{MES}]$ is the less toxic compound, while $[\text{P}_{4444}][\text{TES}]$ is the one with the highest toxicity. The $[\text{P}_{4444}][\text{GB}]$ (with the exception of $[\text{P}_{4444}][\text{TES}]$ which belongs to the category “acute 3” – 10 mg.L⁻¹ < EC₅₀ 100 mg.L⁻¹) can be classified as non-hazardous substances (E₅₀ > 100 mg.L⁻¹), according to the limits imposed by the European Legislation for the aquatic environment (Li et al., 2012). The trend for the effect of $[\text{GB}]^-$ anions on toxicity here observed is in general distinct from that observed before, and for other types of $[\text{GB}]$ -ILs (Taha et al., 2014), suggesting a stronger impact of the $[\text{P}_{4444}]^+$ cation. Nevertheless, by finely-tuning the $[\text{GB}]^-$ anion, it is possible to design self-buffering $[\text{P}_{4444}][\text{GB}]$ ILs with similar or lower toxicity than the non-buffering $[\text{P}_{4444}]\text{Cl}$.

Conclusions

The synthesis and characterization of new self-buffering ILs, based on the $[\text{P}_{4444}]^+$ cation and GB's anions (TES, HEPES, CHES, MES, and Tricine), was here addressed. These ILs demonstrated to be able to form ABS with organic salts and are remarkable platforms for the extraction of proteins, using BSA as a model protein. Moreover, these ILs display an improved environmental benignity (as gauged from the EC₅₀ results for *Vibrio fischeri*). The effect of the studied ILs on the BSA structure was investigated using IR absorption spectroscopy, and their binding affinity towards the protein was determined by the QSAR-logK(HSA) model. The $[\text{P}_{4444}][\text{GB}]$ ILs were found to exhibit a stabilizing effect over the BSA structure as compared with the conventional IL, $[\text{P}_{4444}]\text{Cl}$. COSMO-RS and molecular docking were also used to address the polarity of the ILs and to better interpret the extraction

process. Based on these studies, van der Waals and hydrogen-bonding interactions show to play an important role in the protein partitioning.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Chemical structure of the studied [P₄₄₄₄]Cl/[P₄₄₄₄][GB].

Figure 2.

(a) Plots of pH vs. mL of 0.1 mol·dm⁻³ of NaOH/HCl added to 10.0 mL of 0.05 mol·dm⁻³ of GB-IL ([P₄₄₄₄][MES] (blue line), [P₄₄₄₄][TES] (olive line), [P₄₄₄₄][HEPES] (red line), [P₄₄₄₄][CHES] (magenta line), and [P₄₄₄₄][Tricine] (black line)) in water at (25.0 ± 0.1) °C; the reverse titration region is for mL of 0.1 mol·dm⁻³ of HCl added to 10.0 mL of 0.05 mol·dm⁻³ of GB-IL. (b) Buffer capacity as a function of pH for 0.05 M of GB-IL titrated with 0.1 mol·dm⁻³ of HCl/NaOH at (25.0 ± 0.1) °C.

Figure 3.
Protonation equilibria of MES and [P₄₄₄₄][MES].

Figure 4.

(a) pH titration curves of $1 \cdot 10^{-3}$ M of MES and $[\text{P}_{4444}][\text{MES}]$ at 25°C and $I = 0.1$ M of NaNO_3 . The dashed lines are the calculated pH from the refinement operations. (b) Species-distribution diagrams of $1 \cdot 10^{-3}$ M of MES and $[\text{P}_{4444}][\text{MES}]$ at 25°C and $I = 0.1$ M of NaNO_3 .

Figure 5.
Plots of pH vs. mL of $0.1 \text{ mol}\cdot\text{dm}^{-3}$ of NaOH/HCl added to a mixture of 10.0 mL of $0.05 \text{ mol}\cdot\text{dm}^{-3}$ '[P₄₄₄₄][MES]+[P₄₄₄₄][HEPES]+ [P₄₄₄₄][CHES]' at $(25.0 \pm 0.1) ^\circ\text{C}$.

Figure 6.

Ternary phase diagrams for the systems composed of IL + $\text{K}_3\text{C}_6\text{H}_5\text{O}_7$ + water at 25 °C and atmospheric pressure in wt% (left) and in mol.kg⁻¹ (right): (●) [P₄₄₄₄][Tricine], (●) [P₄₄₄₄][MES], (●) [P₄₄₄₄][HEPES], (●) [P₄₄₄₄][TES], (●) [P₄₄₄₄][CHES], and (●) [P₄₄₄₄]Cl (Passos et al., 2012a).

Figure 7.

Gaussian curve-fitting analysis of amide I spectra of BSA in 0.05 M of [P₄₄₄₄]Cl (a) and in 0.05 M of [P₄₄₄₄][TES] at pH 7.4.

Figure 8.

The intensity distribution graph of BSA in (0.05 and 0.5) M of $[P_{4444}]\text{Cl}/[P_{4444}][\text{TES}]/[P_{4444}][\text{Tricine}]/[P_{4444}][\text{HEPES}]$, at pH 7.4 and 25 °C.

Figure 9. σ -profiles of $[P_{4444}]\text{Cl}$ (black line), $[P_{4444}][\text{HEPES}]$ (red line), $[P_{4444}][\text{TES}]$ (blue line), $[P_{4444}][\text{Tricine}]$ (olive line), $[P_{4444}][\text{CHES}]$ (purple line), and $[P_{4444}][\text{MES}]$ (green line).

Figure 10.
Molecular docking of BSA with $[\text{MES}]^-$ (a), $[\text{CHES}]^-$ (b), and $[\text{P}_{4444}]^+$ (c).

Table 1
Protonation constants of GB/GB-ILs in water at 25 °C and $I = 0.1$ M of NaNO_3

GB/GB-ILs	pK _{a1}	SD [a]	pK _{a2}	SD [a]
MES	2.28	0.07	6.12 (6.07) [b]	0.02
[P ₄₄₄₄][MES]	2.21	0.10	5.91	0.06
TES	3.05	0.02	7.30 (7.42) [b]	0.01
[P ₄₄₄₄][TES]	—	—	7.21	0.03
Tricine	2.68 (2.40) [b]	0.03	8.08 (8.00) [b]	0.02
[P ₄₄₄₄][Tricine]	2.39	0.05	7.82	0.01
HEPES	3.17 (3.0) [b]	0.04	7.35 (7.45) [b]	0.04
[P ₄₄₄₄][HEPES]	2.77	0.10	7.23	0.02
CHES	1.76	0.03	9.13	0.01
[P ₄₄₄₄][CHES]	—	—	8.93	0.05

[a] Standard deviation.

[b] Reference (Goldberg et al., 2002).

Table 2
Extraction efficiencies of serum bovine albumin, EE_{BSA} (%), in the ABS composed of $[P_{4444}][GB] + K_3C_6H_5O_7$ at 25 °C, and at the described mixture compositions.

GB-ILs/ILs	<u>weigh fraction composition (wt %)</u>		EE_{BSA} (%)
	IL	$K_3C_6H_5O_7$	
$[P_{4444}][Tricine]$	38.76	15.02	100
$[P_{4444}][HEPES]$	39.45	13.74	100
$[P_{4444}][CHES]$	38.08	13.55	100
$[P_{4444}][MES]$	38.98	16.30	100
$[P_{4444}][TES]$	39.52	14.43	100
$[P_{4444}][Cl]$	38.91	13.99	100

Table 3
Secondary structure analysis (infrared spectra) of BSA in conventional [P₄₄₄₄][GBs]/[P₄₄₄₄][Cl at pH 7.4.

Amide I components	TES 0.05 M	HEPES 0.05 M	Tricine 0.05 M	[P ₄₄₄][Cl		[P ₄₄₄][TES]		[P ₄₄₄][HEPES]		[P ₄₄₄][Tricine]	
				0.05 M	0.5 M	0.05 M	0.5 M	0.05 M	0.5 M	0.05 M	0.5 M
Inter β-sheet	—	—	2.4 ^b	—	—	—	—	—	—	—	—
Intra β-sheet	24.3 ^a	26.4 ^a	25.0 ^b	28.8	29.7	25.7	24.3	25.4	25.8	24.4	23.6
α-helix	59.8 ^a	58.3 ^a	57.6 ^b	60.6	61.2	64.9	65.9	61.2	62.9	59.9	60.5
turn	15.9 ^a	25.3 ^a	14.2 ^b	10.6	9.1	9.4	9.8	13.4	11.3	15.7	15.9
antiparallel β-sheet	—	—	0.8 ^b	—	—	—	—	—	—	—	—

^aReference (Taha et al., 2015c)

^bReference (Taha et al., 2014)

Table 4
Interaction energies of [P₄₄₄₄][GB]/[P₄₄₄₄]Cl at 25 °C.

[P ₄₄₄₄][GB]/[P ₄₄₄₄]Cl	E_i	$E_{i,MF}$	$E_{i,HB}$	$E_{i,vdW}$
	kcal·mol ⁻¹			
[P ₄₄₄₄]Cl	-6.75	16.25	-2.38	-20.62
[P ₄₄₄₄] ⁺	-8.20	10.75	-1.20	-17.75
Cl ⁻	1.45	5.50	-1.18	-2.87
[P ₄₄₄₄][Tricine]	-12.78	14.11	-2.22	-24.67
[P ₄₄₄₄] ⁺	-9.65	8.04	-1.07	-16.62
[Tricine] ⁻	-3.13	6.07	-1.15	-8.05
[P ₄₄₄₄][TES]	-18.75	12.23	-5.31	-25.68
[P ₄₄₄₄] ⁺	-10.26	7.04	-0.72	-16.58
[TES] ⁻	-8.49	5.19	-4.59	-9.10
[P ₄₄₄₄][HEPES]	-13.25	16.43	-2.04	-27.64
[P ₄₄₄₄] ⁺	-9.49	8.27	-1.00	-16.76
[HEPES] ⁻	-3.76	8.16	-1.04	-10.88
[P ₄₄₄₄][MES]	-11.51	16.08	-1.95	-25.64
[P ₄₄₄₄] ⁺	-9.19	8.55	-0.98	-16.77
[MES] ⁻	-2.32	7.53	-0.97	-8.873
[P ₄₄₄₄][CHES]	-14.71	14.39	-1.95	-27.16
[P ₄₄₄₄] ⁺	-10.05	7.77	-0.98	-16.84
[CHES] ⁻	-4.66	6.62	-0.97	-10.32

Table 5
EC₅₀ values after 30 minutes of exposure time with the 95% confidence limits (within brackets) for [P₄₄₄₄][GB] and [P₄₄₄₄]Cl.

Compound	EC ₅₀ (mg L ⁻¹) at 30 min (lower limit; upper limit)
[P ₄₄₄₄]Cl	110.26 (96.57; 123.95)
[P ₄₄₄₄][MES]	231.25 (204.72; 257.78)
[P ₄₄₄₄][TES]	85.98 (72.48; 99.48)
[P ₄₄₄₄][CHES]	154.31 (137.56; 171.07)
[P ₄₄₄₄][Tricine]	107.82 (86.08; 129.55)
[P ₄₄₄₄][HEPES]	185.58 (174.07; 197.10)