ChemPubSoc Europe

DOI: 10.1002/chem.201405693

CHEMISTRY
A European Journal
Full Paper

Ionic Liquids

Novel Biocompatible and Self-buffering Ionic Liquids for Biopharmaceutical Applications

Mohamed Taha,[a] Mafalda R. Almeida,[a] Francisca A. e. Silva,[a] Pedro Domingues,[b] Sónia P. M. Ventura,[a] João A. P. Coutinho,[a] and Mara G. Freire*[a]

Abstract: Antibodies obtained from egg yolk of immunized hens, immunoglobulin Y (IgY), are an alternative to the most focused mammal antibodies, because they can be obtained in higher titers by less invasive approaches. However, the production cost of high-quality IgY for large-scale applications remains higher than that of other drug therapies due to the lack of efficient purification methods. The search for new purification platforms is thus vital. The solution could be liquid–liquid extraction by using aqueous biphasic systems (ABS). Herein, we report the extraction and attempted purification of IgY from chicken egg yolk by using a new ABS composed of polymers and Good's buffer ionic liquids (GB-ILs). New self-buffering and biocompatible ILs based on the cholinium cation and anions derived from Good's buffers were synthesized and the self-buffering characteristics and toxicity were characterized. Moreover, when these GB-ILs are combined with PPG 400 (poly(propylene) glycol with a molecular weight of 400 g mol^{-1}) to form ABS, extraction efficiencies, of the water-soluble fraction of proteins, ranging between 79 and 94% were achieved in a single step. Based on computational investigations, we also demonstrate that the preferential partitioning of IgY for the GB-IL-rich phase is dominated by hydrogen-bonding and van der Waals interactions.

Introduction

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 it is of essential relevance 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 of the animals, which frequently results in distress or even death of the animals.[3] An alternative approach consists of the use of antibodies that exist 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 from 4.3 rabbits over the same time period;[5] therefore, IgY can be obtained in higher titers by non-invasive methodologies.[6] It has already been demonstrated that IgY plays a similar biological role as mammal IgG.[7] IgY can thus be used as an effective replacement for the common mammal antibodies that are of paramount importance in passive immunotherapy. Nevertheless, the production cost of IgY 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 have been 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 proven to be unable to provide high purification factors and yields. Furthermore, the use of hazardous solvents, such as chloroform, should be avoided. Some of these purification strategies also led to the destabilization and loss of specific activity of IgY and cannot be easily scaled to industrial production.[9] Aqueous biphasic systems (ABS), a type of liquid–liquid extraction process, are foreseen as viable alternatives 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] As 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

[a] Dr. M. Taha, M. R. Almeida, F. A. e. Silva, Dr. S. P. M. Ventura, Prof. Dr. J. A. P. Coutinho, Dr. M. G. Freire
CICECO, Departamento de Química
Universidade de Aveiro
3810-193 Aveiro (Portugal)
Fax: (+351) 234370084
E-mail: maragfreire@ua.pt

[b] Dr. P. Domingues
Mass Spectrometry Centre, UI-QOPNA
Department of Chemistry, University of Aveiro
3810-193 Aveiro (Portugal)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201405693.

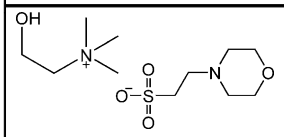
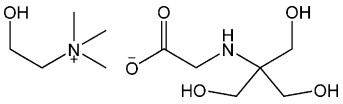
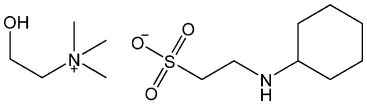
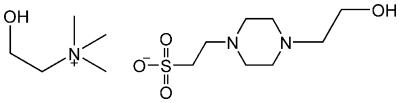
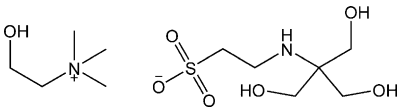
the recovery and purification of (bio)molecules.[10–11] In addition to the more conventional ABS, which already eliminate the use of hazardous and toxic volatile organic compounds (VOCs), the emergence of ionic liquid (IL)-based ABS has led to outstanding extraction performances.[12] Ionic liquids are organic salts with melting points below 100 °C, that present unique characteristics such as negligible volatility, non-flammability, good thermal and chemical stabilities, and have 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 has allowed researchers 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 that have 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 such as calcium, zinc or magnesium, metals 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-based Good's buffer ILs only form ABS with high-charge-density salts.[18] The presence 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 buffer-type ILs based on the cholinium cation.

Cholinium chloride is a water-soluble essential nutrient that is important for cell membrane structure and for the synthesis of folic acid and vitamin B12.[20] Cholinium-based ILs have outstanding biodegradability, low toxicity, and are able to maintain some protein structures and enzyme functions.[21] Yet, none of these cholinium-based ILs have previously been paired with Good's buffers as anions and hence were not able to maintain the pH of aqueous solutions.[21] Furthermore, the use of cholinium as the cation allowed us 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 buffer-type 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) through a simple neutralization reaction (see the Supporting Information for details). Their chemical structures, as well as their abbreviations, are provided in Table 1.

Table 1. Chemical structures of the cholinium-based Good's buffers ILs.

Structure	Name	Abbreviation
	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]

Results and Discussion

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 while 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 group (pK_{a1}) and at the amino (pK_{a2}) group. 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 $NaNO_3$ 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 GB-ILs, possibly by electrostatic interactions that 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.

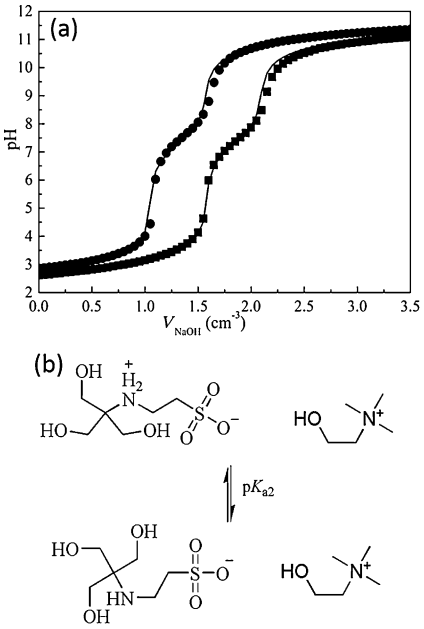


Figure 1. (a) pH titration curves of 1×10^{-3} M TES (■) and [Ch][TES] (●) at 25.0 °C and $I=0.1$ M of $NaNO_3$. (b) Protonation equilibrium of [Ch][TES].

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] with 30 min of exposure time. The final output of this test is the EC_{50} parameter, which represents the effective concentration of a given compound that produces 50% of inhibition of light emission. The EC_{50} 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 g dm^{-3} (181.28; 236.02), respectively.

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

After confirming 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 to *Vibrio fischeri* as it had an EC_{50} value of 6735 mg dm^{-3} (4623; 8847) at 30 min of exposure, a value that agrees 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. Formation of ABS was

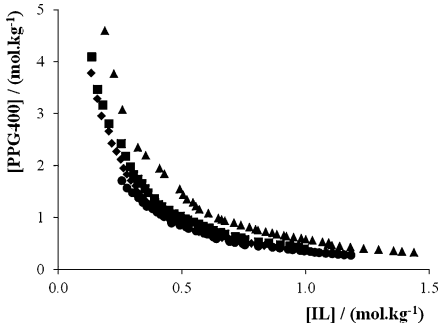


Figure 2. Ternary phase diagrams for the systems composed of PPG 400 + GB-IL + water at 25 °C and atmospheric pressure: [Ch][MES] (triangles), [Ch][Tricine] (diamonds), [Ch[TES] (squares) and [Ch][HEPES] (circles).

not found for the [Ch][CHES]/PPG 400 mixture due to the higher hydrophobicity of this anion (this is discussed in more detail below). 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 (see the 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 polymer 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 compositions above the solubility curve, formation of two aqueous phases was seen; below the solubility curve, the concentration of each component is not sufficient to induce liquid–liquid demixing, thus, falling within the monophasic regime.

Figure 2, thus, also depicts the ability of each GB-IL to induce the phase separation. As all of the GB-ILs share a common cation, the differences in the solubility curves are a result of the anion nature. The GB-IL anions aptitude to form ABS follows the order: $[HEPES]^- \approx [Tricine]^- > [TES]^- > [MES]^-$. As PPG 400 is a moderately hydrophobic polymer, the higher the affinity that each GB has for water, the greater the ability of the IL to promote two phase 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 hydrogen-bonding features of the investigated compounds. The σ-profile is divided into three main regions: hydrogen-bond donor, non-polar, and hydrogen-bond acceptor regions, which are separated by two vertical lines located at the hydrogen-bond cut-off of ± 0.01 e Å^{-2} (Figure 3). The negatively charged sulfonic and carboxylic groups of the ILs display a peak localized at the strongly negative polar region (0.01–0.025 e Å^{-2}) and, therefore, they act as strong hydrogen-bond acceptors. These ILs also show hydrogen-bond donor fragments (located at -0.017 e Å^{-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, for use in the extraction and purification of proteins.

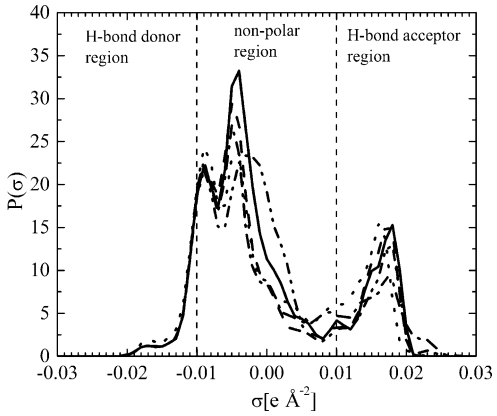


Figure 3. σ-profiles of [Ch][HEPES] (-----), [Ch][MES] (– – –), [Ch][TES] (•••••), [Ch][Tricine] (–•–•), and [Ch][CHES] (——).

Finally, the novel ABS proposed herein were 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), 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 four 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 (see the Supporting Information for more detailed data). For all the investigated systems,

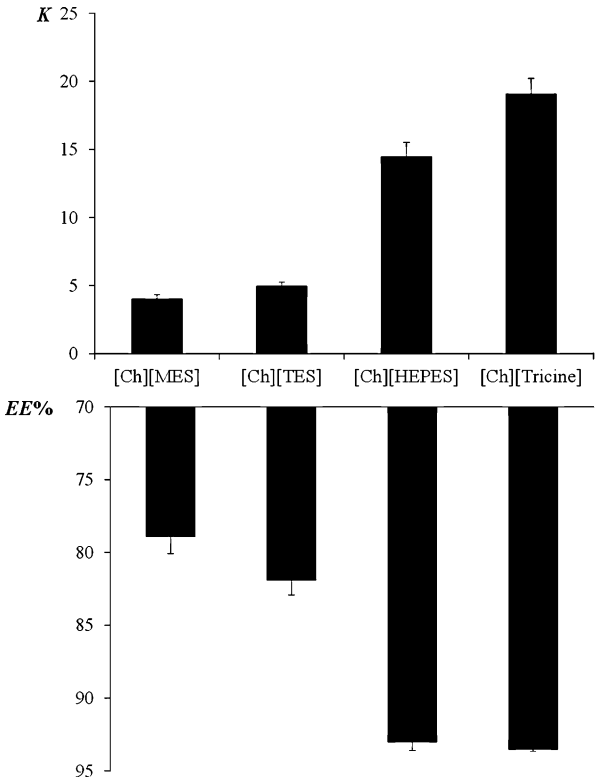


Figure 4. Partition coefficients (*K*) and extraction efficiencies (*EE*%) of total protein content in the water-soluble fraction of egg yolk by using ABS composed of PPG 400 + GB-IL + water at 25 °C. Mixture compositions: 50 wt% of PPG 400 + 7–10 wt% of each GB-IL + 40–43 wt% of an aqueous solution containing the water-soluble proteins from egg yolk.

the partition coefficient is higher than 1.0, confirming preferential partitioning of the proteins into the IL-rich phase (bottom phase). The extraction efficiencies for the IL-rich phase range from 79 to 94%. The ABS composed of PPG 400 and [Ch][Tricine] or [Ch][HEPES] led to the highest extraction efficiencies, above 90%, in a single step. These GB-ILs also present a higher ability to form ABS with PPG 400.

The coexisting phases used in the extraction of the water-soluble fraction of egg yolk were also analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine the proteins present in the coexisting phases, and thus the efficiency of the purification of IgY (Figure 5). Two major proteins were identified in the IL-rich phase, namely IgY and β-livetin (a contaminant protein of the water-soluble fraction). In some systems, a reduction in the intensity of the band corresponding to β-livetin was 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, providing evidence for the lack of antibodies' aggregation or fragmentation in 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 the 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-

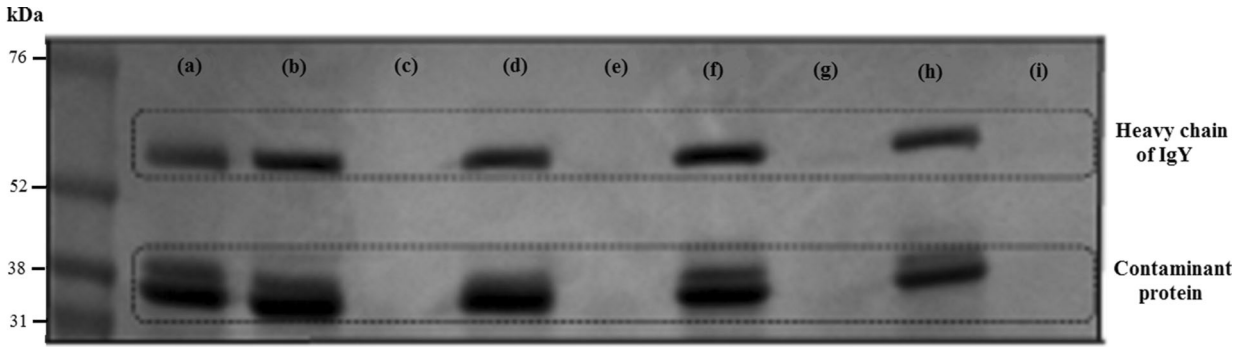


Figure 5. SDS-PAGE of a gel loaded with 0.5 μg of protein per well, stained with Coomassie blue. Lane 1 (Std): standard molecular weights. Lane (a): water-soluble fraction of proteins from egg yolk. Lanes (b) and (c): bottom and top phases, respectively, of the ABS constituted by PPG 400 + [Ch][HEPES]. 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].

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,

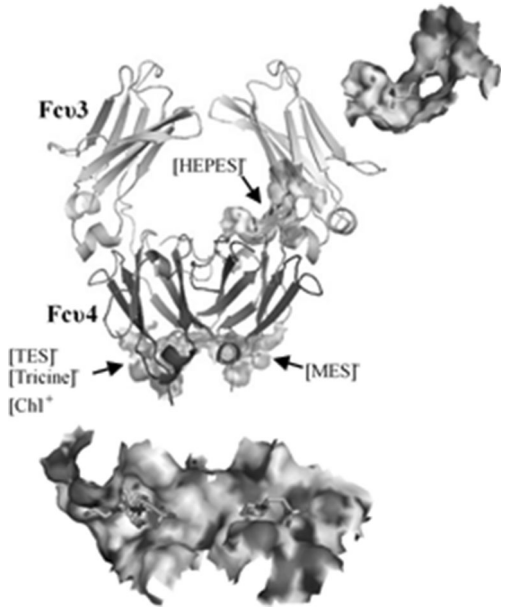


Figure 6. Interactions between IgY and GB-IL ions based on computational docking.

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, THR 561, 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, whereas cholinium forms three hydrogen bonds with GLN 565 and GLN 563. Further details and data are provided in the Supporting Information.

Conclusion

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 these "self-buffering" and "non-toxic" compounds. Based on computational investigations, it was also demonstrated that the partitioning of IgY is dominated by hydrogen-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 through neutralization of choline hydroxide with Good's buffers. A slight excess of equimolar buffer aqueous solution was added drop-wise 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 by 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 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 ^{1}H 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 D_2O as the deuterated solvent. The melting points of the synthesized GB-ILs were measured by differential scanning calorimetry (DSC), on a PerkinElmer DSC-7 instrument (Norwalk, CT), at a heating rate of 5 °C min^{-1} under N_2 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 to accommodate 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 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 mol dm^{-3} $NaNO_3$, were maintained constant during the refinements.[31]

GB/GB-ILs titrations were carried out by titrating a mixture of 0.003 M HNO_3, 0.1 M $NaNO_3$ and 0.001 M GBs/GB-ILs (total volume of 50 cm^3) with 0.1 M NaOH. The titration cell was bubbled with nitrogen gas for 15 min before starting each titration, and then kept under a small positive pressure of N_2 to remove carbon dioxide. All titrations were repeated at least three times. The protonation constants for GBs and GB-ILs were computed by 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 exposure to either GB-IL or PPG 400 aqueous solutions at different concentrations. After 30 min 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 that yielded 50% inhibition of the luminescence (described by the parameter EC_{50}), which was computed by 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. This was 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 ± 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 equilibrium by separation into the two phases over at least 12 h at 25 °C. After the separation of the coexisting phases, the phases 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 as 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. As the phase diagrams are very similar to each other, the similar tie-line lengths (TLL = 38–42 wt%) also correspond to similar mixture compositions.

Each mixture was prepared gravimetrically within ± 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):

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

where $[\text{Protein}]_{IL}$ and $[\text{Protein}]_{PPG}$ 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):

$$EE\% = \frac{W^{IL}_{Protein}}{W^{PPG}_{Protein} + W^{IL}_{Protein}} \times 100 \quad (2)$$

where $W^{PPG}_{Protein}$ and $W^{IL}_{Protein}$ 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 by 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), whereas 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 distained 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/TZVP level, and as implemented in the TURBOMOLE 6.1 program package.[36] The σ-profiles were visualized by the COSMO*therm* 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 by 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×−8.262×16.727) Å, and the grid dimension was (78×70×84) Å.

Acknowledgements

The authors are grateful for financial support from FEDER funds through the program COMPETE and from the national fund through the Portuguese Foundation for Science and Technology (FCT) under the scope of the projects Pest-C/CTM/LA0011/2013 and PTDC/AAC-AMB/119172/2010. The authors are also thankful to FCT for financial support through the doctoral and post-doctoral grants of SFRH/BD/94901/2013, SFRH/BPD/78441/2011 and SFRH/BPD/79263/2011 of F.A.eS., M.T. and S.P.M.V., respectively. M.G.F. acknowledges the European Research Council (ERC) for the Grant ERC-2013-StG-337753.

Keywords: antibodies · aqueous two-phase systems · extraction · Good's buffer · immunoglobulin Y · ionic liquids · purification

[1] C. T. P. Janeway, *Immunobiology: the immune system in health and disease*, Current Biology, Garland Pub., London, **1996**.
[2] L. Zeitlin, R. A. Cone, T. R. Moench, K. J. Whaley, *Microbes Infect.* **2000**, *2*, 701–708.
[3] L. S. Munhoz, G. D. Á. Vargas, G. Fischer, M. d. Lima, P. A. Esteves, S. d. O. Hübner, *Ciênc. Rural* **2014**, *44*, 153–160.
[4] J. C. Jensenius, I. Andersen, J. Hau, M. Crone, C. Koch, *J. Immunol. Methods* **1981**, *46*, 63–68.
[5] Y. Xu, X. Li, L. Jin, Y. Zhen, Y. Lu, S. Li, J. You, L. Wang, *Biotechnol. Adv.* **2011**, *29*, 860–868.
[6] P. Hansen, J. A. Scoble, B. Hanson, N. J. Hoogenraad, *J. Immunol. Methods* **1998**, *215*, 1–7.
[7] J. Kovacs-Nolan, Y. Mine, *Annu. Rev. Food Sci. Technol.* **2012**, *3*, 163–182.
[8] B. De Meulenaer, A. Huyghebaert, *Food Agric. Immunol.* **2001**, *13*, 275–288.
[9] a) F. Javier Hernandez-Campos, E. Brito-De la Fuente, B. Torrestiana-Sanchez, *J. Agric. Food Chem.* **2010**, *58*, 187–193; b) D. Dong, H. Liu, Q. Xiao, R. Li, *J. Chromatogr. B* **2008**, *870*, 51–54.
[10] P.-Å. Albertsson, *Partition of cell particles and macromolecules, Vol. 346*, 3 ed., Wiley, New York, **1986**.
[11] B. Y. Zaslavsky, *Aqueous two-phase partitioning*, Marcel Dekker, New York, **1994**.
[12] M. G. Freire, A. F. M. Claudio, J. M. M. Araujo, J. A. P. Coutinho, I. M. Marrucho, J. N. C. Lopes, L. P. N. Rebelo, *Chem. Soc. Rev.* **2012**, *41*, 4966–4995.
[13] a) C. Wu, J. Wang, Z. Li, J. Jing, H. Wang, *J. Chromatogr. A* **2013**, *1305*, 1–6; b) Z. P. Visak, *J. Solution Chem.* **2012**, *41*, 1673–1695; c) L. C. Branco, J. N. Rosa, J. J. Moura Ramos, C. A. M. Afonso, *Chem. Eur. J.* **2002**, *8*, 3671–3677; d) S. T. Handy, *Chem. Eur. J.* **2003**, *9*, 2938–2944.
[14] J. F. Pereira, L. P. N. Rebelo, R. D. Rogers, J. A. Coutinho, M. G. Freire, *Phys. Chem. Chem. Phys.* **2013**, *15*, 19580–19583.
[15] M. G. Freire, J. F. B. Pereira, M. Francisco, H. Rodriguez, L. P. N. Rebelo, R. D. Rogers, J. A. P. Coutinho, *Chem. Eur. J.* **2012**, *18*, 1831–1839.
[16] a) Z. Du, Y.-L. Yu, J.-H. Wang, *Chem. Eur. J.* **2007**, *13*, 2130–2137; b) M. J. Ruiz-Angel, V. Pino, S. Carda-Broch, A. Berthod, *J. Chromatogr. A* **2007**, *1151*, 65–73; c) Q. Cao, L. Quan, C. He, N. Li, K. Li, F. Liu, *Talanta* **2008**, *77*, 160–165; d) S. Dreyer, U. Kragl, *Biotechnol. Bioeng.* **2008**, *99*, 1416–1424; e) S. Dreyer, P. Salim, U. Kragl, *Biochem. Eng. J.* **2009**, *46*, 176–185; f) Y. Pei, J. Wang, K. Wu, X. Xuan, X. Lu, *Sep. Purif. Technol.* **2009**, *64*, 288–295; g) Y. Pei, Z. Li, L. Liu, J. Wang, H. Wang, *Sci. China Chem.* **2010**, *53*, 1554–1560; h) Y. Lu, W. Lu, W. Wang, Q. Guo, Y. Yang, *Talanta* **2011**, *85*, 1621–1626; i) F. J. Deive, A. Rodriguez, A. B. Pereiro, J. M. M. Araujo, M. A. Longo, M. A. Z. Coelho, J. N. C. Lopes, J. M. S. S. Esperanca, L. P. N. Rebelo, I. M. Marrucho, *Green Chem.* **2011**, *13*, 390–396; j) S. P. M. Ventura, S. G. Sousa, M. G. Freire, L. S. Serafim, Á. S. Lima, J. A. P. Coutinho, *J. Chromatogr. B* **2011**, *879*, 2679–2687; k) S. P. M. Ventura, R. L. F. de Barros, J. M. de Pinho Barbosa, C. M. F. Soares, A. S. Lima, J. A. P. Coutinho, *Green Chem.* **2012**, *14*, 734–740; l) F. J. Deive, A. Rodríguez, L. P. N. Rebelo, I. M. Marrucho, *Sep. Purif. Technol.* **2012**, *97*, 205–210; m) U.

Novak, A. Pohar, I. Plazl, P. Žnidaršič-Plazl, *Sep. Purif. Technol.* **2012**, *97*, 172 – 178; n) X. Lin, Y. Wang, Q. Zeng, X. Ding, J. Chen, *Analyst* **2013**, *138*, 6445 – 6453; o) Q. Zeng, Y. Wang, N. Li, X. Huang, X. Ding, X. Lin, S. Huang, X. Liu, *Talanta* **2013**, *116*, 409 – 416; p) Q. Zeng, Y. Wang, Y. Huang, X. Ding, J. Chen, K. Xu, *Analyst* **2014**, *139*, 2565 – 2573; q) R. K. Desai, M. Streefland, R. H. Wijffels, M. H. M. Eppink, *Green Chem.* **2014**, *16*, 2670 – 2679; r) X. Ding, Y. Wang, Q. Zeng, J. Chen, Y. Huang, K. Xu, *Anal. Chim. Acta* **2014**, *815*, 22 – 32.

[17] N. E. Good, G. D. Winget, W. Winter, T. N. Connolly, S. Izawa, R. M. M. Singh, *Biochemistry* **1966**, *5*, 467 – 477.

[18] M. Taha, F. A. e Silva, M. V. Quental, S. P. M. Ventura, M. G. Freire, J. A. P. Coutinho, *Green Chem.* **2014**, 16, 3149 – 3159.

[19] W. J. Ferguson, K. I. Braunschweiger, W. R. Braunschweiger, J. R. Smith, J. J. McCormick, C. C. Wasmann, N. P. Jarvis, D. H. Bell, N. E. Good, *Anal. Biochem.* **1980**, *104*, 300 – 310.

[20] a) P. Detopoulou, D. B. Panagiotakos, S. Antonopoulou, C. Pitsavos, C. Stefanadis, *Am. J. Clin. Nutr.* **2008**, *87*, 424 – 430; b) C. Signore, P. M. Ueland, J. Troendle, J. L. Mills, *Am. J. Clin. Nutr.* **2008**, *87*, 896 – 902.

[21] a) J. Pernak, A. Syguda, I. Mirska, A. Pernak, J. Nawrot, A. Prądzyńska, S. T. Griffin, R. D. Rogers, *Chem. Eur. J.* **2007**, *13*, 6817 – 6827; b) Y. Fukaya, Y. Iizuka, K. Sekikawa, H. Ohno, *Green Chem.* **2007**, *9*, 1155 – 1157; c) K. D. Weaver, H. J. Kim, J. Sun, D. R. MacFarlane, G. D. Elliott, *Green Chem.* **2010**, *12*, 507 – 513; d) S. P. Ventura, A. M. Gonçalves, J. L. Pereira, F. Gonçalves, J. A. Coutinho, *Ecotoxicol. Environ. Saf.* **2014**, *102*, 48 – 54.

[22] P. Gans, A. Sabatini, A. Vacca, *Talanta* **1996**, *43*, 1739 – 1753.

[23] R. N. Goldberg, N. Kishore, R. M. Lennen, *J. Phys. Chem. Ref. Data* **2002**, *31*, 231 – 370.

[24] a) S. M. Steinberg, E. J. Poziomek, W. H. Engelmann, K. R. Rogers, *Chemosphere* **1995**, *30*, 2155 – 2197; b) L. Li, J. Xie, S. Yu, Z. Su, S. Liu, F. Liu, C. Xie, B. Zhang, *RSC Adv.* **2012**, *2*, 11712 – 11718.

[25] R. West, M. Banton, J. Hu, J. Klapacz, *Rev. Environ. Contam. Toxicol.* **2014**, *232*, 107 – 138.

[26] J. C. Merchuk, B. A. Andrews, J. A. Asenjo, *J. Chromatogr. B* **1998**, *711*, 285 – 293.

[27] A. Klamt, *COSMO-RS from Quantum Chemistry to Fluid Phase Thermodynamics and Drug Design*, Elsevier, Amsterdam, **2005**.

[28] A. I. Taylor, S. M. Fabiane, B. J. Sutton, R. A. Calvert, *Biochemistry* **2009**, *48*, 558 – 562.

[29] O. Trott, A. J. Olson, *J. Comput. Chem.* **2010**, *31*, 455 – 461.

[30] P. Gans, B. O'Sullivan, *Talanta* **2000**, *51*, 33 – 37.

[31] R. F. Jameson, M. F. Wilson, *J. Chem. Soc. Dalton Trans.* **1972**, 2607 – 2610.

[32] *Azur Environmental – Microtox manual*, Carlbad CA, USA **1998**.

[33] M. G. Freire, C. L. Louros, L. P. N. Rebelo, J. A. Coutinho, *Green Chem.* **2011**, *13*, 1536 – 1545.

[34] A. Polson, T. Coetzer, J. Kruger, E. Von Maltzahn, K. Van der Merwe, *Immunol. Invest.* **1985**, *14*, 323 – 327.

[35] Gaussian 09, Revision D.01, M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, B. Mennucci, G. A. Petersson, H. Nakatsuji, M. Caricato, X. Li, H. P. Hratchian, A. F. Izmaylov, J. Bloino, G. Zheng, J. L. Sonnenberg, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, J. A. Montgomery, Jr., J. E. Peralta, F. Ogliaro, M. Bearpark, J. J. Heyd, E. Brothers, K. N. Kudin, V. N. Staroverov, R. Kobayashi, J. Normand, K. Raghavachari, A. Rendell, J. C. Burant, S. S. Iyengar, J. Tomasi, M. Cossi, N. Rega, J. M. Millam, M. Klene, J. E. Knox, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, R. L. Martin, K. Morokuma, V. G. Zakrzewski, G. A. Voth, P. Salvador, J. J. Dannenberg, S. Dapprich, A. D. Daniels, O. Farkas, J. B. Foresman, J. V. Ortiz, J. Cioslowski, D. J. Fox, Gaussian, Inc., Wallingford CT, **2009**.

[36] A. Schäfer, A. Klamt, D. Sattel, J. C. W. Lohrenz, F. Eckert, *Phys. Chem. Chem. Phys.* **2000**, *2*, 2187 – 2193.

[37] A. K. F. Eckert, COSMOtherm Version C2.1 Release 01.08, COSMOlogic GmbH & Co. KG, Leverkusen, Germany, **2006**.

Received: October 17, 2014
Published online on February 4, 2015

