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Extraction of an active enzyme by self-buffering ionic liquids: a green medium for enzymatic research†

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In this work, the extraction of a model enzyme α-chymotrypsin in its active conformation from an aqueous solution by using new biocompatible and self-buffering Good's buffer ionic liquids (GBILs)-based aqueous biphasic systems (ABSs) is demonstrated. The experimental liquid–liquid phase boundary data for these new GBIL-based ABSs were measured at 298.2 K. These new ionic liquids are derived from biological Good's buffers as anions and tetra-alkylammonium as cations. The buffering potential of these GBILs was confirmed by measuring their pH profiles and protonation constants (pK_a) in aqueous solution by a potentiometric method at various temperatures. Moreover, these GBILs provide a greater stabilizing effect on the activity of α-chymotrypsin, as compared with the corresponding Good's buffers. Therefore, the new self-buffering GBILs may not only serve as a biocompatible medium for enzymatic research, but also avoid the use of an external buffering agent, which is often needed for pH control in the case of using a conventional ILs-based medium. Therefore, these new biocompatible GBILs are more advantageous over the conventional ILs-based media for enzymatic research.

1. Introduction

The use of enzymes has been proven to achieve higher selectivity and better yields of the final products at comparatively milder conditions of temperature and pressure. However, their high sensitivity towards external factors, such as biocompatibility and pH value, still limits their industrial applications. Recently, ionic liquids (ILs), a new class of environmentally friendly solvents, have been identified as a favorable biocompatible medium to maintain the active conformation for enzymes or proteins.[1–7] Basically, ionic liquids are entirely composed of ions, but exist as liquids below the boiling point of water (100 °C).[8–14] ILs have special physical and chemical properties, such as negligible or very low vapor pressure, low flammability, high inherent conductivities, high thermal stability, existing as liquid state over a wide temperature range, easy recycling, and being a good solvent for a wide range of organic and inorganic chemical compounds.[15–19] At present, ILs are widely used in many research fields, including electrochemistry,[20–22] organic chemistry,[23,24] catalysis,[25–27] engineering process,[28–32] and pharmaceutical applications.[33] Thus, they are regarded as future solvents and emerging as a potential replacement for the conventional volatile organic solvents that are frequently used in various industries.[34–36] In the last decades, number of reports[37–43] mentioned that ILs, either in pure or in aqueous state, can be served as biocompatible media to biomolecules such as deoxyribonucleic acid (DNA), protein, and enzyme and also can stabilize biomolecules' structures against external or internal impetus. In addition, the applications of ILs as a potential biocatalyst were also extensively studied.[44,45]

Biomolecules are highly sensitive system and can be functionally active only in their optimum pH range. External buffering compound is often needed to maintain the pH values of media in a suitable range. In a current practice, phosphate-based buffer is recommended to maintain the required pH of biological media due to its protein stabilizing tendency.[46] However, the complexation of phosphate ions with metal ions such as magnesium, zinc, and calcium was reported in literature.[47] Since these metal ions are commonly found in various proteins and therefore, phosphate-based buffer solutions possess the possibility to interfere with the proteins.

Many efforts have been made on designing the task specific ionic liquids for biological research, but very few studies[48–53] focused on designing ILs with self-buffering potential. Ou *et al.*[48] reported three imidazolium based ionic liquids, 1-butyl-3-methylimidazolium hydrogen phosphate [BMIM][HP], 1-octyl-3-methylimidazolium hydrogen phosphate [OMIM][HP], and 1-butyl-3-methylimidazolium hydrogen tartrate [BMIM][HT] with self-buffering potential. These ionic liquids are suitable to maintain pH values in highly acidic region (pH ∼ 3–4). Since

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most of the biological researches are conducted in the physiological pH range of 7 to 9, therefore those ionic liquid buffers cannot be used in the biological researches. MacFarlane *et al.*[49] investigated three ionic liquid buffers, choline dihydrogenphosphate/choline hydrogen phosphate, choline tartrate, and hydrated lysine, which can help to maintain pH values in a variety of applications of ionic liquids. However they did not systematically determined pK_a values of these ionic liquid buffers experimentally. Matias *et al.*[50] synthesized seven choline derivatives by using of Good's buffers (BES, DIPSO, CAPS, EPPS, MES, TAPSO, and MOPSO) as anions. It was found that the pH can be maintained from 5.33 to 11.82 in aqueous solution by varying the buffer anions. Since the synthesized choline derivatives appear as salts instead of ionic liquids, the application of those buffers in enzymatic research may be limited to the only buffering compounds. Moreover, neither the ATPS forming tendency nor the extraction efficiency for biomolecules by those choline salts were studied. Recently, Taha *et al.*[51,52] synthesized similar self-buffering ionic liquids derived from the biological buffers TES, MES, HEPES, CHES, and tricine; however, their GBILs did not cover the whole pH range of enzymatic reactions. The buffering range of our new GBILs will cover pH from 6 to 12, thus these GBILs can be used in enzymatic reactions although higher pH values are needed.

In the present study, we attempt to synthesize some new self-buffering ILs, derived from the commonly used Good's buffers (GB) as anions and the ammonium ions with various chain lengths as cations. The selected Good's buffers, *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid (TAPS), 3-(*N*-morpholino)propanesulfonic acid (MOPS), 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (EPPS), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), and *N*,*N*-bis(2-hydroxyethyl)-glycine (BICINE) are amino acid derivatives and are widely used in biological field. Each of these compounds exists as a zwitterion in aqueous solution and thus can be used as a cation or anion. In our previous researches, we have found that GB behave as a kosmotropic salt in nature and suitable to stabilize the native structure of the protein.[54–57] Since the GB themselves serve as a protein stabilizer, the new self-buffering ionic liquids derived from these buffers are expected to be highly biocompatible.

The properties of these new GBILs are fundamentally important for practical uses. In the present study, we measured the thermal stability temperature (T_d) and melting point (T_m) of the GBILs with thermal gravimetric analysis (TGA) and differential scanning calorimetry (DSC), respectively. Their structures were analyzed with NMR spectroscopy and water contents were measured with Karl Fischer titration. To analyze their buffering potential in aqueous solution, we measured their pH profiles and then determined the acid dissociation constant (pK_a) values for each synthesized GBIL at various temperatures.

Moreover, these GBILs are found to be formed two-liquid phase with sodium sulfate solution. Aqueous bi-phasic system (ABS) has been widely applied to the separation of cell membrane, organelles, and enzyme from the cell lysates or extracts.[58–60] To characterize these new GBILs-based ABS, we measured the phase boundaries data for each GBIL-based ABS at 25 °C and checked their extraction efficiency for a model enzyme α-chymotrypsin (α-CT). We chose α-CT as a model enzyme because of its unique structural and functional importance. α-CT is commonly used for exploring the mechanism of protein folding or unfolding in the presence of cosolvents.[61,62] Overall, converting Good's buffers into ionic liquids not only provides a suitable biocompatible medium with comparable self-buffering capability, avoiding the use of external buffering compound, but also may help in the extraction and purification of the enzyme.

2. Experimental section

2.1 Materials

All the materials used in this study were analytical grade and obtained from the reliable commercial sources. TAPS (mass fraction purity ≥ 0.995), MOPS (mass fraction purity ≥ 0.995), EPPS (mass fraction purity ≥ 0.995), CAPS (mass fraction purity ≥ 0.99), BICINE (mass fraction purity ≥ 0.99), sodium sulfate salt (mass fraction purity ≥ 0.99), α-chymotrypsin (α-CT) from bovine pancreas type II, essentially salt free and tetra-methylammonium hydroxide [TMA][OH] (25 wt% in H_2O) were obtained from Sigma Chemical Co. (USA). Tetra-ethylammonium hydroxide [TEA][OH] (35 wt% in H_2O), and tetra-butylammonium hydroxide [TBA][OH] (40 wt% in H_2O) were purchased from Alfa Aesar (USA). The solvents acetonitrile (mass fraction purity ≥ 0.998), ethanol (mass fraction purity ≥ 0.998), deuterium oxide (mass fraction purity = 0.990 atom D), and dimethyl sulfoxide-d_6 (DMSO-d_6, mass fraction purity = 0.999 atom D) were purchased from the Sigma Aldrich (USA). All the materials were used as they received, without further purification. De-ionized water used in the synthesis process was obtained from NANO pure-Ultra pure water system with resistivity of 18.3 MΩ cm. All the synthesis samples were prepared gravimetrically by using an electronic balance (R&D, Model GR-200) with a precision of ±0.1 mg.

2.2 Synthesis and characterization of the GBILs

In the synthesis process, aqueous ammonium-hydroxide solution of tetramethylammonium (TMA), tetraethylammonium (TEA) and tetrabutylammonium (TBA) were added drop-wise to a slightly excess of an equimolar aqueous buffer solution in a round bottom flask, fitted with a reflux condenser. The reaction mixture was stirred uniformly with magnetic stirrer for about 12 hours under ambient conditions to ensure the completion of reaction. The mixture was then evaporated at 50–60 °C under reduced pressure, by using a rotary evaporator (Panchum Evap., Chering Huei Co., Ltd). To remove the unreacted materials or any other impurities presented in the obtained viscous liquid, a mixture of ethanol and acetonitrile (1 : 1) was added to the viscous liquid and stirred uniformly at room temperature for 1 hour. The solution was then filtered and the filtrate solution was dried under reduced pressure for about 8 to 12 hours to obtain the GBIL with lower water content. The water content in each of these synthesized GBIL samples was analyzed by Karl Fischer titration. The measured values of the water content in the GBILs are compiled in Table S1 of the ESI.† The structure of these new synthesized GBILs have

characterized by measuring ^{1}H NMR spectra with a Bruker advance 500 spectrometer using tetramethylsilane (TMS) as an internal reference. The elemental analysis for the synthesized GBILs was made with Elementar Vario EL cube (Germany) to further ensure the structures of these GBILs.

2.3 Thermal gravimetric analysis (TGA)

The TGA profile were measured by using Pyris Diamond TG-DTA instrument. The samples were weighted (~10 mg) and heated from 50 °C to 600 °C under a dry nitrogen atmosphere at a heating rate of 10 °C min^{-1}.

2.4 Differential scanning calorimeter (DSC)

The DSC measurements performed under inert nitrogen atmosphere with heating rate of 10 °C min^{-1}. The samples were weighted (10–15 mg) and loaded into aluminum pans and then heated to the desired temperature.

2.5 pH profiles

The pH profile for each synthesized IL in aqueous solution was measured by the potentiometric titration method, using an automatic titrate (Metrohm 888 Titrando, USA) at 25 °C. The measurement was performed in the closed, jacketed equilibrium cell of volume 50 cm^3, equipped with a magnetic stirrer for proper mixing. The temperature of the equilibrium cell was controlled by water circulating thermostatic bath (FIRSTEK, B402L, Taiwan). The temperature of the titration solution inside the cell was measured with a precise digital thermometer (Model-1560, Hart Scientific Co., USA) with an uncertainty of ±0.1 °C. The concentration of the stock solution of the ILs, hydrochloric acid (HCl), and sodium hydroxide (NaOH) was fixed to 0.05 M. A constant volume of 30 cm^3 from the stocked solution of IL was introduced into the equilibrium cell. This titration solution was titrated with the acid or base in the inert nitrogen atmosphere to obtain the respective pH profile for the pH range of 2 to 12.

2.6 Protonation constant (pK_a)

The protonation constants of all the GBILs were determined by direct potentiometric titration using standardized NaOH titrant. The detail procedure has been given in our previous articles.[63] For each titration, a constant volume of 50 cm^{-3} with GBIL concentration of 0.01 mol dm^{-3} were used. The ionic strength of the titration solution was maintained to 0.1 mol dm^{-3} by adding a calculated amount of sodium nitrate ($NaNO_3$). Based on the obtained potentiometric data, the calculation of protonation constants of all the investigated GBILs were performed by using the HYPERQUAD program (Version 2008).[64] The refinement of the equilibrium constants by this program required the inputs such as the concentration of each species in solution and the potentiometric data. The quality of the obtained results decide on the basis of parameters such as possible low value of the standard deviation (σ) and the curve fitting between calculated and measured pH.

2.7 Activities measurements

The detailed procedure for measuring the activity of α-CT in aqueous solutions of GBILs has been described in the literature.[65] For the sake of better understanding, the brief procedure of measuring activity is given here. The progress of the hydrolysis reaction in presence or absence of the α-CT is monitored by estimating the appearance of the *p*-nitrophenoxide (PNP^-) at 400 nm, at 25 °C, and at pH = 8.0, for 3 minutes by using UV-visible spectrophotometer (JASCO, V-550). The activity is expressed as millimoles (mM) of PNP^- formed per minute, per g of α-CT.

2.8 Phase boundaries data

The binodal curve of each GBIL based ABS system was measured by using the cloud point titration method at 25 (±1)° C and atmospheric pressure. To determine the binodal curve, similar experimental procedure was used as described in the previous reports.[66,67] The freshly prepared solution of sodium sulfate was added drop-wise to the aqueous solution of the GBIL until the cloud point appear and then drop-wise addition of water carried out to get monophasic region. The procedure was repeated under the constant stirring as far as the appearance of the cloud point not observed on addition of any amount of salt solution. The composition of GBIL, salt, and water in the mixture determine by weight evaluation with an analytical balance, with measured uncertainty of ±5 mg.

2.9 Extraction of chymotrypsin

The ternary mixture of desired composition of GBIL (30 ± 1.5 wt%) + salt (13 ± 1.5 wt%) + water (57 ± 1.5 wt%), used in the partitioning of α-CT, were prepared gravimetrically by using an electronic balance at room temperature and pressure, and at pH of 8.0. The calculated amount of α-CT (0.5 g dm^{-3}) were added to the extraction medium and vortexed for 10 minutes, centrifuged for 15 minutes, and then left for about 30 minutes to ensure the complete partitioning of enzyme between two phases. Afterwards, the two phases were separated carefully and the amount of α-CT in each phase was determined by using UV-visible spectrophotometer. For the UV-spectra measurement each phase was diluted at 1 : 50 by volume in MOPS buffer solution. Prior to the analysis, the UV-visible spectrophotometer was calibrated by using series of standard solution of α-CT in the range of 0.001 g dm^{-3} to 1.0 g dm^{-3} at pH 8.0, at 25 °C, and at atmospheric pressure. At least three separate biphasic mixture vials were prepared for each GBIL based ABS and the amount of α-CT were determined from minimum 3 repeated measurements. To avoid the interference from the salt, and GBIL in the calculation of α-CT, the absorption of blank solutions containing IL and salt were measured prior to each sample of α-CT. The extraction efficiency (EE) of α-CT by using GBIL based ABS system is ascertain by the following equation:

$$\mathrm{EE}(\%) = \frac{[\mathrm{CT}]_{\mathrm{GBIL}} \times w_{\mathrm{GBIL}}}{[\mathrm{CT}]_{\mathrm{GBIL}} \times w_{\mathrm{GBIL}} + [\mathrm{CT}]_{\mathrm{salt}} \times w_{\mathrm{salt}}}$$

where [CT], represents the concentration of α-chymotrypsin in g dm^{-3}, the subscripts, GBIL and salt, represents the Good's

buffer ionic liquid rich-phase and salt-rich phase, respectively. The symbol w is the weight of each phase. Each experimental procedure is repeated at least three times in order to get the good quality of result with minimum error and the provided value is the average of the repeated measurements.

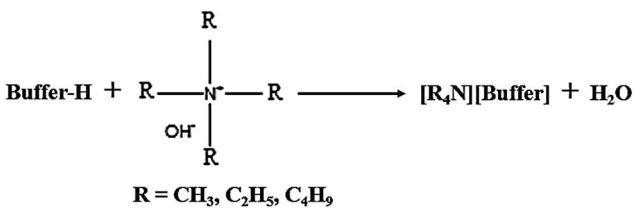
2.10 Fluorescence spectra measurements

The emission spectra were recorded at room temperature for the sample of α-chymotrypsin (about 2.5 g per liter) in 30% aqueous solution of GBILs and for the samples of α-chymotrypsin from the upper and the lower phase of GBILs-based ABS at pH = 8.0 using an RF-5301PC Spectro-fluorophotometer (SHIMADZU), with a 1.0 cm quartz cell. The spectra were recorded at excitation wavelengths of 295 nm with an excitation and emission slit width of 5 nm for the emission range of 300 nm to 450 nm. Prior to the analysis of the sample, fluorescence spectrum for the blank solution was measured.

3. Results and discussion

Here we are introducing fifteen new, buffer and ammonium based, Good's buffer ionic liquids (GBILs). These GBILs have been synthesized by using different combination of Good's buffer as an anion and ammonium as a cation. The synthesis of these GBILs is based on the fact that the structure of the selected biological buffers (TAPS, MOPS, EPPS, CAPS, and BICINE) contains acidic sulfonic or carboxylic group that can be easily neutralize by a suitable base. Therefore, all the reported new GBILs are synthesized by the simple acid–base neutralization reaction. The process of synthesis is shown in Scheme 1. All the TMA-based GB-ILs appeared as solid, while TEA-based GBILs appear as highly viscous liquids at room temperature. [TBA][TAPS] appears as solid at room temperature, while [TBA][MOPS], [TBA][EPPS], [TBA][CAPS], and [TBA][BICINE] exist as viscous liquids at room temperature. Since the melting point of [TMA][BICINE] is found to be higher than the normal boiling point of water (as seen from Table 1), it would be better named as Good's buffer ionic salt. However, to keep the discussion general for all the synthesized ionic liquids or ionic salts, we are using the same abbreviations "GBILs" for all the synthesized Good's buffer derivatives.

To characterize the exact structure of these new buffers based GBILs, we have measured the NMR spectra of each synthesized IL or ionic salt. The measured ^{1}H NMR spectra for TAPS, MOPS, EPPS, CAPS, and BICINE-based GBILs are given in Fig. S1 to S5, respectively, of the ESI.† The outcomes of NMR analysis and the corresponding possible molecular structure of each synthesized GBILs are given in Table S1 of the ESI.†



Scheme 1 Synthesis pathways of the new GBILs.

Table 1 Thermal decomposition temperature (T_d) and melting point (T_m) of the synthesized GBILs

GBILs	T_d (°C)	GBILs	T_m (°C)
[TMA][TAPS]	218	[TMA][TAPS]	88.4
[TEA][TAPS]	224	[TMA][MOPS]	36.4
[TBA][TAPS]	271	[TMA][EPPS]	80.0
[TMA][MOPS]	228	[TMA][CAPS]	50.9
[TEA][MOPS]	239	[TMA][BICINE]	112.2
[TBA][MOPS]	215		
[TMA][EPPS]	195		
[TEA][EPPS]	228		
[TBA][EPPS]	221		
[TMA][CAPS]	205		
[TEA][CAPS]	212		
[TBA][CAPS]	216		
[TMA][BICINE]	173		
[TEA][BICINE]	162		
[TBA][BICINE]	163		

To further confirm the structure of the synthesized GBILs, we have selected [TMA][TAPS], [TEA][TAPS], and [TBA][TAPS] as the representative systems and measured elemental analysis for these selected GBILs. The results of the analysis are given in Table S1 of the ESI.† As can be seen from the tabulated values (Table S1†), the experimentally obtained individual percentages of the elements (carbon, hydrogen, and nitrogen) in each GBIL are found very close to the analytically calculated values for the corresponding GBIL.

The thermal degradation of the synthesized GBILs buffers was examined by thermal gravimetric analysis (TGA), by using Pyris Diamond TG-DTA instrument. The thermal profiles for TAPS based GBILs ([TMA][TAPS], [TEA][TAPS], and [TBA][TAPS]) are presented in Fig. 1 and the thermal profiles for the rest of the synthesized GBILs are given in Fig. S6(a–d) of the ESI.†

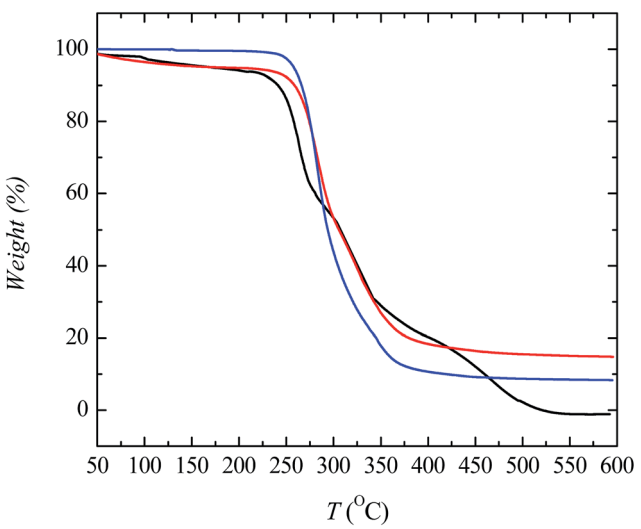


Fig. 1 Thermal profiles of the TAPS-based GBILs: (–), [TMA][TAPS]; (–), [TEA][TAPS]; and (–), [TBA][TAPS].

The decomposition temperature (T_d) of each GBIL corresponding to the beginning of decomposition (estimated from the respective decomposition curve) is listed in Table 1. It can be seen clearly from the decomposition curves (Fig. 1 and S6†) and the T_d values (Table 1). Each GBIL possesses a unique characteristic stability behavior against thermal response. In general, all these new GBILs are found to be thermally stable up to 200 °C to 300 °C. At temperatures slightly higher than 100 °C, a small loss in weight was observed (2 to 7 wt%). Erdemi *et al.*[68] have mentioned that weight loss of samples above the normal boiling point of water represents the removal of the physically bounded water. The degradation of most of these investigated GBILs were completed in two steps. In the first degradation step, an about 75 to 95 percent mass loss occurs immediately after the respective decomposition temperature (Table 1). Whereas, the rest mass depletion takes places in a slow fashion during a second degradation step. The results of TGA analysis show that the thermal stability behavior of synthesized GBILs is unique and depends on the combination of cation or anion.

Those GBILs, appeared solid at room temperature, were also analyzed with the dynamic scanning calorimeter (DSC). The results of DSC measurements are presented in Fig. S7 of the ESI.† The determined melting points (T_m) are listed in Table 1, indicating that the melting points of these GBILs are higher than room temperature but lower than 100 °C, except for [TMA][BICINE]. However we would like to bring into attention that the aqueous solution of IL is generally used as a biological medium in various studies instead the viscous IL, therefore these TMA based ionic salts are of equally important. As can be seen from Table 1, the melting point of TMA-based GBILs follows the order of BICINE > TAPS > EPPS > CAPS > MOPS.

To ascertain the buffering action of these new GBILs, we measured their pH profiles in the aqueous media in the pH range of 2 to 12. The measured pH profiles for [TMA][MOPS], [TEA][MOPS], and [TBA][MOPS] are shown in Fig. 2. The pH profiles for the other investigated GBILs are compiled in Fig. S8 of the ESI.† The region of moderate slope in the pH profile, before the inflection point, represents the buffering region. In this region, the pH is controlled by the equilibrium between the deprotonated and protonated forms of the GBILs. Therefore, from the pH profile curves (Fig. 2 and S8†), it can be seen that these newly synthesized GBILs are able to maintain their buffering potential in aqueous medium.

To further characterize the buffering nature of these GBILs, the protonation constants (pK_{a_2}) of each GBIL were determined at temperatures of 20 °C, 25 °C, and 35 °C with ionic strength of 0.1 mol dm^{-3} $NaNO_3$. The experimentally determined pK_{a_2}

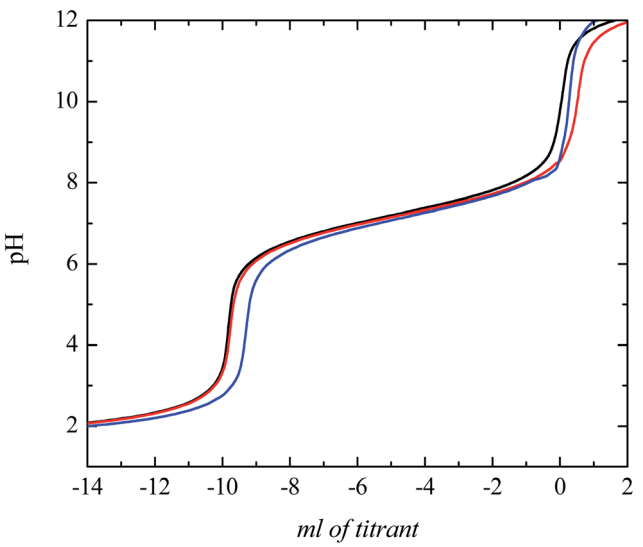


Fig. 2 pH profiles of the MOPS-based GBILs: (–), [TMA][MOPS]; (–), [TEA][MOPS]; and (–), [TBA][MOPS].

Table 2 Protonation constants (pK_{a_2}) of the GBILs in water at 20 °C, 25 °C, and 35 °C and ionic strength I = 0.1 mol dm^{-3} $NaNO_3$, with corresponding standard deviation (σ) and pH range used in pK_{a_2} determination. The pK_{a_2} values of the Good's buffer (GB) at 25 °C and I = 0.1 mol dm^{-3}

GB	pK_{a_2}	GBILs	pK_{a_2}	σ	pK_{a_2}	σ	pK_{a_2}	σ
25 °C			20 °C		25 °C		35 °C	
TAPS	8.33[a]	[TMA][TAPS]	8.53	0.02	8.36	0.01	8.24	0.02
MOPS	7.17[a]	[TEA][TAPS]	8.58	0.02	8.48	0.02	8.19	0.02
EPPS	7.87[b]	[TBA][TAPS]	8.53	0.02	8.33	0.01	8.27	0.02
CAPS	10.39[b]	[TMA][MOPS]	7.24	0.02	7.18	0.02	7.07	0.02
BICINE	8.22[b]	[TEA][MOPS]	7.30	0.05	7.21	0.02	7.11	0.03
		[TBA][MOPS]	7.23	0.09	7.16	0.02	7.03	0.06
		[TMA][EPPS]	8.08	0.04	7.90	0.05	7.76	0.05
		[TEA][EPPS]	7.96	0.08	7.87	0.09	7.75	0.08
		[TBA][EPPS]	8.08	0.03	7.75	0.03	7.71	0.05
		[TMA][CAPS]	11.04	0.04	10.56	0.02	9.96	0.02
		[TEA][CAPS]	10.96	0.04	10.45	0.02	9.85	0.02
		[TBA][CAPS]	11.00	0.04	10.53	0.01	9.96	0.02
		[TMA][BICINE]	8.33	0.03	8.26	0.02	8.08	0.02
		[TEA][BICINE]	8.34	0.02	8.26	0.02	8.13	0.02
		[TBA][BICINE]	8.33	0.05	8.23	0.02	8.12	0.02

[a] Ref. 69. [b] Ref. 70.

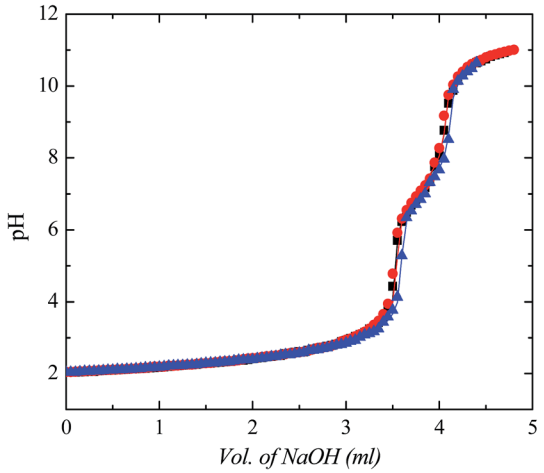


Fig. 3 Representative titration curves for [TMA][MOPS] (■), [TEA][MOPS] (●), and [TBA][MOPS] (▲) at 25 °C and at 0.1 mol dm^{-3} of $NaNO_3$. The smooth lines represent the corresponding best-fit from the Hyperquad 2008.

values of all the GBILs from pH-metric data by using HYPERQUAD 2008 are reported in Table 2. Fig. 3 shows a representative titration curves and the corresponding best fit for [TMA][MOPS], [TEA][MOPS], and [TBA][MOPS] at 25 °C with ionic strength of 0.1 mol dm^{-3} of $NaNO_3$.

From the Fig. 3, it can be seen that the agreement between the experimental and correlated values is excellent. Similar curves were obtained for the rest of the studied GBILs. For the sake of clarity, we are not presenting all the titration curves. The zwitterionic Good's buffers TAPS, MOPS, EPPS, MOPS, CAPS, and BICINE known to have two protonation constants. The first protonation constant (pK_{a_1}) corresponds to the dissociation of sulphonic or carboxylic group. The second protonation constant (pK_{a_2}) corresponds to the dissociation of the amino group, which is responsible for their observed buffering action.

As can be seen from Table 2, the pK_{a_2} values of the each investigated GBILs decreasing with the increase of temperature. This trend was expected because with the increase of temperature extent of dissociation increases. For the comparison purpose, the pK_{a_2} values of the Good's buffer (GB), TAPS, MOPS, EPPS, CAPS, and BICINE in aqueous solutions under the similar experimental condition at 25 °C taken from the literature[69,70] and presented in the Table 2. The order of pK_{a_2} for these buffers follows the order of CAPS > TAPS > BICINE > EPPS > MOPS. The same trend in the pK_a's of GBILs has been observed for the respective GB (Table 2). Moreover, the presence of ammonium cations (TMA, TEA, and TBA) in the solutions increase the pK_a's of GBILs in comparison with the corresponding GBs (CAPS, TAPS, BICINE, EPPS, and MOPS).

The selected biological buffers (TAPS, MOPS, EPPS, CAPS, and BICINE) are considered as biocompatible, nontoxic, and green compounds. Especially, TAPS, MOPS, and EPPS buffers were found to protect the native structure of the protein BSA against thermal denaturation, in aqueous solutions containing high concentration of the respective buffer.[54,55,71] Thus, the synthesized GBILs could be biocompatible and green compounds. However,

Table 3 Catalytic activities of α-CT in 0.05 M, MOPS, [TMA][MOPS], [TEA][MOPS], and [TBA][MOPS], at 400 nm, 25 °C, and pH 7.8

Compound	Activity
MOPS	5.689
[TMA][MOPS]	8.684
[TEA][MOPS]	8.965
[TBA][MOPS]	10.918

to make it certain that the addition of these ammonium cations (TMA, TEA, and TBA) to the GBs does not change its inherent biocompatible nature, we selected MOPS based ionic liquids, [TMA][MOPS], [TEA][MOPS], and [TBA][MOPS], as representative compounds and measured the biological activities of the enzyme α-chymotrypsin (α-CT) in the presence of these selected GBILs. The basic idea is, if the GBILs are non-biocompatible for biological system, enzyme α-CT will be denatured and lose its biological activity. The enzyme α-CT is highly reckoned protease enzyme and known for its capability to catalyze the hydrolysis of *p*-nitrophenylacetate (PNPA).[72,73] Thus, this enzyme catalyzed hydrolysis reaction serves as the basis for measuring the biological activity of the enzyme α-CT because the enzyme can work as a catalyst only in its active or re-nature form. Any deviation from the native or active conformation induced by co-solvent can be directly detected by monitoring the progress of the hydrolysis reaction. Therefore, we measured the activity of α-CT in 0.05 mol md^{-3} MOPS, [TMA][MOPS], [TEA][MOPS], and [TBA][MOPS] to catalyze the hydrolysis of PNPA at 25 °C and pH = 7.8. The results of activity measurements are shown in Table 3. From the tabulated values of the enzyme activity, it can be seen that the activities of α-CT in the aqueous solutions of [TMA][MOPS], [TEA][MOPS], and [TBA][MOPS] is greater than that in the aqueous buffer solution of MOPS.

It indicates that the synthesized GBILs are biocompatible in nature and provide more active conformation to the α-CT in comparison with the conventional buffer. These results of activity measurement show that it is advantageous to convert Good's buffers into ionic liquids. Moreover, the activity of α-CT in aqueous solutions of GBILs follows the order of [TBA][MOPS] > [TEA][MOPS] > [TMA][MOPS]. It indicates that the activity of α-CT or biocompatibility of GBILs increases with the increase in alkyl chain length of the counter cation part in the GBILs. Some conventional ILs were also found to be biocompatible for the enzyme α-CT, but the activity testing always required an additional buffer compounds to maintain the pH value. However, in the present case, GBILs themselves can also be used as a buffer to maintain the pH of the system. Thus these new GBILs are more useful in comparison with the conventional non-buffering biocompatible ILs used in biological or biochemical research because they are not only biocompatible but also can avoid using an extra buffering agent to the biological or enzymatic system.

The separation or extraction of novel compounds by using aqueous biphasic systems (ABSs) is not only highly valuable but also very economical because the process usually occurs at ambient condition. Many reports are available in the literature[74–76] emphasizing the scope of IL-based ABS for the recovery

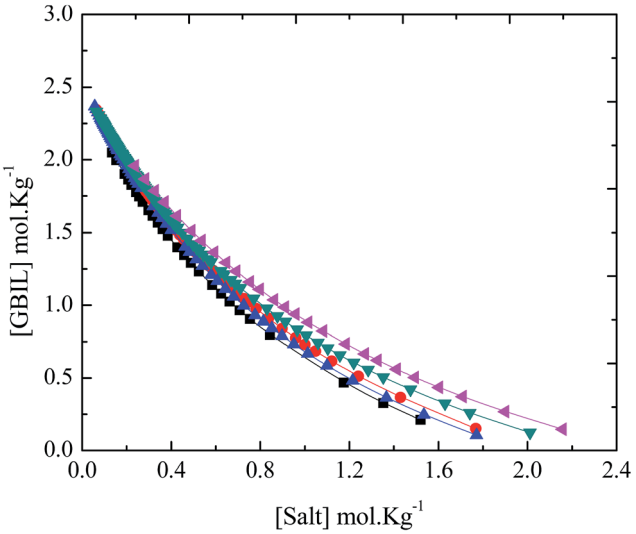


Fig. 4 Liquid–liquid phase boundaries of the systems composed of GBIL + Na_2SO_4 + H_2O at 25 °C and at atmospheric pressure: (-■-), [TBA][TAPS]; (-●-), [TBA][MOPS]; (-▲-), [TBA][EPPS]; (-▼-), [TBA][CAPS]; (-◀-), [TBA][BICINE].

of a broad range of valuable materials including precious drugs, metal ions, alkaloids, amino acids, proteins, and enzymes. Therefore, we attempt to test the possibility of inducing the liquid–liquid phase splitting of the aqueous solution of sodium sulfate by adding these new GBILs. Interestingly, all the TBA-based GBILs can induce ABS for the aqueous solution of sodium sulfate, while GBILs with short alkyl chain (TMA and TEA) fail to form ABS. In these new GBIL-based ABSs, the top is GBIL-rich phase and the lower is aqueous phase containing electrolytic compound.

To further characterize the new GBIL-based ABSs, we measured the phase boundaries data (binodal curve) for each reported ABS system by using well-reckoned cloud point titration method under ambient condition. The results of measurements are presented in Table S3 of the ESI.† The phase diagram for all the TBA-based GBILs are graphically shown in Fig. 4. The region just above the curve represents the two phase regions.

In addition, to check the extraction efficiency of these GBIL-based ABSs especially for the biomolecule, we have selected enzyme α-chymotrypsin (α-CT) as a model enzyme. The ternary mixture composed of GBIL (30 ± 1.5 wt%) + salt (13 ± 1.5 wt%) + water (57 ± 1.5 wt%), used in the partitioning of α-CT, were prepared gravimetrically with an electronic balance at pH of 8.0. Here, we would like to bring into notice that the pH (about 8.0) of the extraction medium in each case maintain by the self-buffering action of these GBILs, without using any external buffering agent. Since the required experimental pH is away from the suitable pH range of the [TBA][CAPS], we did not test the extraction of α-CT in the [TBA][CAPS] based ABS system. The results of measurement including, initial mixture compositions and the percentage extraction efficiency (EE) of α-CT are given in Table 4. From the tabulated values of the percentage of EE in Table 4, it can be seen that GBIL completely extract enzyme α-CT from the aqueous

Table 4 Initial mixture compositions in weight percentage (*w*), extraction efficiency (EE) of the α-chymotrypsin in the GBIL based ABS systems and activities of α-chymotrypsin in GBIL phase after extraction at 25 °C, at atmospheric pressure and pH 8.0

GBIL	w_{IL} (%)	w_{salt} (%)	w_{water} (%)	EE (%)	Activity
[TBA][TAPS]	29.67	13.47	56.86	100	12.842
[TBA][MOPS]	30.24	13.66	56.10	100	17.095
[TBA][EPPS]	29.95	13.63	56.42	100	12.026
[TBA][BICINE]	30.35	13.70	55.95	100	17.382

solution. These results indicate toward the excellent capability of these GBIL based ABS systems for completely recovering the precious biomolecule through a single step.

To ascertain the complete extraction of the tested enzyme in the GBIL phase and to analyze the structure of enzyme after extraction, we have relied on the fluorescence technique. The fluorescence method is highly recognized for the exact analysis of the structural conformation of the enzymes in solvent.[77,78] Since the fluorescence intensity of the fluorophore molecule is very sensitive to the molecular environment, it serves as a basis to analyze the structural and conformational change in the molecule brought by the surrounding solvent.[79] The aromatic amino acid residues like tryptophan (Trp), phenylalanine (Phe), and tyrosine (Tyr) are commonly found fluorophores in the enzyme or proteins and are responsible for their respective fluorescence behavior in polar or non-polar solvent. Moreover, it is recognized that, at the excitation wavelength of the 295 nm, the emission spectra of enzyme appears mainly due to the Trp residue and the contribution of Tyr or Phe became minor.[80] On the basis of these facts, Park *et al.*[81] suggested that the fluorescence spectra of protein using Trp as a fluorescence probe can provide an exact idea of protein conformation. Fortunately, the crystal structure of enzyme α-CT was noticed to have eight

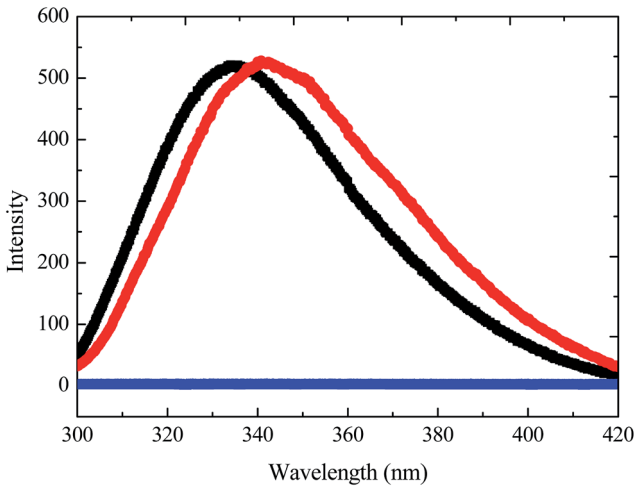


Fig. 5 The fluorescence spectra of α-chymotrypsin in [TBA][TAPS] before and after extraction: (■); α-chymotrypsin in aqueous [TBA][TAPS] (before extraction), (●); α-chymotrypsin in upper phase after extraction, (■); α-chymotrypsin in lower phase after extraction.

tryptophan residues (Trp27, Trp29, Trp51, Trp141, Trp172, Trp 207, Trp215, and Trp 237).[82]

Therefore, to analyze the structural changes in the enzyme α-CT in GBIL-rich phase, we have measured emission fluorescence spectra for the α-CT samples taken from upper and lower phases of the GBIL-based ABS system at an excitation wavelength of 295 nm. The obtained emission spectra of α-CT in aqueous solution of [TBA][TAPS] (before extraction), α-CT in upper phase after extraction, and α-CT in lower phase after extraction are presented in Fig. 5 and the results for the rest of GBILs are given in Fig. S9 of the ESI.† From these figures, it can be seen that no fluorescence peak observed for α-CT in the lower phase of the ABS, which further confirms the complete extraction of α-CT from the aqueous solution to the upper phase of the ABS. In addition, the fluorescence intensity of the peak of α-CT after extraction is almost comparable with that of α-CT before extraction (Fig. 5 and S9†), indicating that enzyme remains in its native conformation after extraction. However, the peak of α-CT after extraction was noticed to shift slightly toward a higher wavelength. From the study of the crystal structure of α-CT,[83] it reveals that two tryptophan amino acid residues (Trp 207 and Trp 237) of the α-CT are easily available for the external solvents. Moreover, it is observed that[80] the increase in the hydrophobic environment around Trp residues may cause the shifting in the emission peak towards lower wavelength and the increase in hydrophillic environment around Trp residues results in the shifting of the emission peak towards the higher wavelength. Probably the observed slightly blue shift in the peak of α-CT in upper phase is the result of comparatively hydrophillic microenvironment around Trp residue provided by the GBIL and the phase forming sodium sulphate in the upper phase. The outcomes of the fluorescence measurements suggest that α-CT remains in its native conformation after extraction. This expectation is in accordance with our previous reports,[54,55,71] in which we found that the selected GBs (TAPS, MOPS, and EPPS) are highly biocompatible and are suitable for protecting the native structure of protein against thermal denaturation and maintain the stable and compact native conformation to the protein. However, to further confirm our expectation that enzyme remains in its active conformation in GBIL-rich phase, we have performed the activities measurement for the sample of α-CT in GBIL-rich upper phase at 25 °C and pH 8.0. The results of the measurement are compiled in Table 4. The tabulated values of the activities in Table 4 suggest that the GBILs assist the enzyme maintains in its active conformation and are suitable for extracting the enzyme in active conformation from aqueous solutions. Since the complete extraction of α-CT in its active form was attained in a single step and the self-buffering nature of these GBILs avoids using external buffer compounds in the extractive medium, it suggest that these new GBIL-based biological media can be more promising and economical for the enzymatic research. The conventional ionic liquids have a tendency to interfere with external buffering compounds, but the use of our synthesized self-buffering GBILs can avoid such a situation from happening and can be more effective over the conventional phosphate buffer systems, which possess strong complexation tendency with various metallo-proteins.

4. Conclusion

The main objective of this study is to synthesize and characterize the new ionic liquids that solve the most of problems encountered in enzymatic research, including biocompatibility and pH control. In the present study, fifteen new self-buffering GBILs have been produced from Good's biological buffers as anions and ammonium as cations. The structure and purity of these new IL buffers have been checked with NMR technique. The thermal decomposition and melting point of these new ionic liquids analyzed by the thermal gravimetric analysis (TGA) and dynamic scanning calorimetry (DSC) techniques, respectively. These new compounds are found to be thermally stable over wide range of temperature. The reported GBILs not only provide suitable biocompatible medium for conducting enzymatic or biological research but also can avoid using an external buffering agent. The buffering potential of these new ILs confirmed by measuring their pH profile and protonation constants in aqueous solutions, at different temperatures (20 °C, 25 °C, and 35 °C). The results indicate that these new GBILs are promising towards maintaining the required pH of the medium. Moreover, the enhanced enzyme activity and the excellent extraction efficiency of α-chymotrypsin in the presence of these new GBILs indicate towards the suitability of using GBILs based medium for the enzymatic research. Thus, in comparison with the conventional ionic liquids or buffer itself, these new ionic liquid buffers can serve as a better biological medium as well as a buffering agent and thus can help in conducting various biological processes in more effective, and green way.

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