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# Long-term protein packaging in cholinium-based ionic liquids: improved catalytic activity and enhanced stability of cytochrome c against multiple stresses†

Meena Bisht,[a,b] Dibyendu Mondal,[b,c] Matheus M. Pereira,[b] Mara G. Freire,[b] P. Venkatesu *[a] and J. A. P. Coutinho *[b]

There is considerable interest in the use of structurally stable and catalytically active enzymes, such as cytochrome c (Cyt c), in the pharmaceutical and fine chemicals industries. However, harsh process conditions, such as temperature, pH, and the presence of organic solvents, are the major barrier to the effective use of enzymes in biocatalysis. We demonstrate the suitability of cholinium-based ionic liquids (ILs) formed by the cholinium cation and dicarboxylate-based anions as potential media for enzymes, in which remarkable enhanced activity and improved stability of Cyt c against multiple stresses were obtained. Among the several ILs studied, an exceptionally high catalytic activity (>50-fold) of Cyt c was observed in the aqueous solutions of cholinium glutarate ([Ch][Glu]; at 1 : 1 weight ratio of IL : $H_2O$) compared to the commonly used phosphate buffer solutions (pH 7.2), and >25-fold compared to the aqueous solutions of cholinium dihydrogen phosphate ([Ch][Dhp]; 1 : 2 weight ratio of IL : water)—the best known IL for long term stability of Cyt c. The catalytic activity of the enzyme in the presence of ILs was retained against several external stimuli, such as chemical denaturants ($H_2O_2$ and GuHCl) and temperatures of up to 120 °C. The observed enzyme activity is in agreement with its structural stability, as confirmed by UV–vis, circular dichroism (CD), and Fourier transform infrared (FT-IR) spectroscopy. Taking advantage of the multi-ionization states of di/tri-carboxylic acids, the pH was switched from acidic to basic by the addition of the corresponding carboxylic acid and choline hydroxide, respectively. The activity was found to be maximum at a 1 : 1 molar ratio of [Ch][carboxylate], with a pH in the range from 3 to 5.5. Moreover, it was found that the cholinium-based ILs studied herein protect the enzyme against protease digestion and allow long-term storage (at least for 21 weeks) at room temperature. An attempt by molecular docking was also made to better understand the efficacy of the investigated cholinium-based ILs towards the enhanced activity and long term stability of Cyt c. The results showed that dicarboxylate anions interact with the active site's amino acids of the enzyme through H-bonding and electrostatic interactions, which are responsible for the observed enhancement of the catalytic activity. Finally, it is demonstrated that Cyt c can be successfully recovered from the aqueous solution of ILs and reused without compromising its yield, structural integrity and catalytic activity, thereby overcoming the major limitations in the use of IL–protein systems in biocatalysis.

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## Introduction

Enzymatic biocatalysis has been recognized as a key process in diverse fields of applications, including the synthesis of valuable pharmaceutical intermediates and biofuels from renewable resources.[1–3] Nowadays, there is considerable interest in the use of structurally stable and catalytically active enzymes in the food, pharmaceutical, and fine chemicals industries.[4] However, enzymes have evolved to work in cellular environments,[1–4] and are usually unstable under harsh process conditions, such as high temperature, high pressure,

[a]*Department of Chemistry, University of Delhi, Delhi – 110 007, India. E-mail: venkatesup@hotmail.com, pvenkatesu@chemistry.du.ac.in; Fax: +91-11-27666605; Tel: +91-11-27666646-142*
[b]*Departamento de Química, CICECO, Universidade de Aveiro, 3810-193, Aveiro, Portugal. E-mail: jcoutinho@ua.pt; Fax: +351234 370 084; Tel: +351 234 401 507*
[c]*Sustainable Energy Materials and Processes group, Center for Nano and Material Science, Jain University, Jain Global Campus, Kanakapura, Ramanagaram, Bangalore-562112, Karnataka, India*

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and the presence of organic solvents. These are often the major limitations behind a more extended use of enzymes in industrial processes.[5] Organic solvents usually have deleterious effects over enzymes, for instance by leading to the unfolding of the enzyme conformations, loss of bound water from the protein surface, and damage of the protein structure.[6] In order to overcome these problems, some strategies have been proposed: (i) modification of the enzyme surface, thereby increasing its resistance to harsh conditions; and (ii) manipulation of the solvent environment to improve the enzyme stability and activity.[7] Among these, the entrapment of enzymes has shown great promise toward the improvement of their catalytic activity and stability.[8] However, such a strategy carries the risk of enzyme deactivation, particularly by shrinking and confinement of the modified enzyme during condensation and purification processes, thereby limiting its bioavailability.[9] Interestingly, enzyme deactivation might be overcome by employing ionic liquids (ILs) as protective media.[10]

ILs are molten salts with unique properties, such as high ionic conductivity, high electrochemical stability, and low volatility, and are becoming popular as promising solvents for many industrial processes.[11–13] A large number of reports are available for the processing of biomacromolecules, such as enzymes/proteins and DNA in ILs.[14–19] Nevertheless, the IL–protein systems reported to date have mainly comprised imidazolium-based ILs, which may display low biocompatibility features.[20] Alternatively, cholinium-based ILs have been proposed in more recent years due to their enhanced biocompatible[21] and biodegradable[22] nature. Various studies have shown that cholinium-based ILs are remarkable solvents to maintain the activity and stability of several proteins, including cytochrome c (Cyt c), which is an industrially important and ubiquitous peroxidase enzyme.[23–26] Cyt c is primarily known for its function in the mitochondria as a key participant in the life-supporting function of ATP synthesis and electron-transfer processes in the respiratory system.[27] Cyt c has many functions beyond the respiration.[28] The release of Cyt c into the cytoplasm also activates caspase-dependent apoptosis.[29] Very recently, the Arnold research group discovered that cytochrome c can catalyze the carbon–silicon bond formation which was unknown in nature. This carbon–silicon bond-forming biocatalyst offers an environmentally friendly and highly efficient route for producing enantiopure organosilicon molecules.[30] Cyt c has a lot of advantages as a biocatalyst when compared with peroxidases for its application.[31] Due to the presence of the heme prosthetic group, it became more important for catalysis in the presence of organic solvents, since Cyt c does not lose its heme catalytic group in these systems, however the secondary structure of the protein is affected by these organic solvents.[32] Cyt c can also be used as an electron transfer element between electrodes and surface bound enzymes.[33] Friebe *et al.* also showed that cyt C can act as an electron-funneling antenna for efficient photocurrent generation in a reaction center biophotocathode.[34] Therefore, it is very important to improve the catalytic activity and stability of Cyt c for many applications. Amongst the series of cholinium-based ILs investigated, cholinium-dihydrogen phosphate ([Ch][Dhp]) has been highlighted as the most biocompatible medium for Cyt c.[35–40] Fujita *et al.*[35,39,40] studied the structural stability of Cyt c in [Ch][Dhp] and showed that the secondary structure is retained at a high temperature (80 °C), and that the activity of the enzyme is kept intact even after storage at room temperature for long periods. However, no significant improvement in the catalytic activity of Cyt c was recorded by the authors.[35,39,40] On the other hand, it has been suggested that the IL anion exerts a dominating effect on the catalytic activity and stability of enzymes.[41] Recently, ILs based on anions derived from carboxylic acids and dicarboxylic acids with high hydrogen-bonding characteristics have been introduced in order to design protein friendly solvent systems.[42–47] Towards this endeavor, cholinium-based ILs comprising anions derived from monocarboxylic acids have shown to be able to increase the activity of proteases, even after storage at room temperature for 13 months.[44] More recently, pH switchable cholinium ILs based on dicarboxylic acids have been explored as protein friendly media for simultaneous biomass saccharification and fermentation to produce biofuels.[42]

The present work explores the widening properties of cholinium-based ILs through their combination with anions derived from natural compounds such as carboxylic acids due to the abundance and the reasonable cost of synthesizing ILs based on these anions. Our results indicate the potential application of these ILs as "green" media in diverse applications such as protein purification, process engineering, nanotechnology, biotechnology, and green biocatalysis. Particular applications can be found in the extraction and recovery of different biomolecules from various sources. Nowadays, IL-based aqueous biphasic systems (ABSs) are a promising protein purification technique used in the biotechnology industry due to their efficiency and simplicity of operation.[10] However, a high concentration of ILs is used for the preparation of ABSs and it is thus important to know the impact of high concentration IL solution on the structural stability of proteins. Most of the existing reports either deal with IL–protein interactions, and propose the suitability of the IL–protein systems for biotechnological applications,[48] or demonstrate a higher catalytic activity of enzymes in IL media without any systematic mechanistic understanding.[49,50] Moreover, studies undertaken concerning both the thermodynamic stability and catalytic activity of enzymes in the presence of ILs are scarce.[51,52] The back extraction of structurally stable and catalytically active enzymes from the IL solution has also not yet been studied. Therefore, based on this lacuna, a comprehensive study towards the finding of efficient solvents for long-term protein packaging, while evaluating the enzyme activity and stability against multiple stresses, such as high temperature, a broad pH range, the presence of chemical and biological denaturants, and the presence of organic solvents, was carried out in this work.

The present study reports the potential of cholinium-based ILs for long-term protein packaging, for which an improved activity and stability of Cyt c are shown. For this purpose, a series of ILs comprising the cholinium ion and di/tri-carboxylate

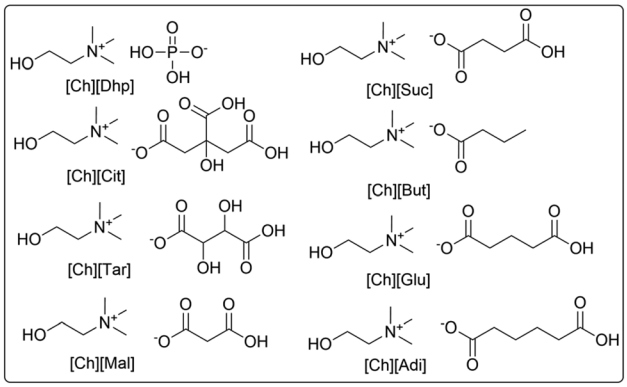


Fig. 1 Chemical structure of the investigated cholinium-based ILs.

anions (Fig. 1) have been investigated. [Ch][Dhp] was also included for comparison since it is the most well documented IL regarding its compatibility with Cyt c.[35–40] Efforts were made to assess the structural stability of the enzyme by various techniques, such as UV-vis, circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopy. Furthermore, molecular docking was carried out to better understand the reasons behind the enhanced activity and stability. To further confirm the long-term activity and stability of the enzyme under ambient conditions, Cyt c was dissolved in aqueous solutions of ILs and stored for several months. Finally, the recovery of Cyt from the aqueous solutions of ILs was carried out allowing the enzyme reuse, with no significant losses in activity and stability. Therefore, the present study discloses the potential of novel cholinium-based ILs as biocompatible media for long-term packaging of proteins/enzymes, thereby overcoming the common obstacles faced in biocatalysis.

# Experimental section

### Materials

Cytochrome c (Cyt c) from equine heart with a purity of >95%, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) with a purity of >98%, hydrogen peroxide solution ($H_2O_2$) 30% (w/w) in $H_2O$, choline bicarbonate ~80% pure in $H_2O$, cholinium dihydrogencitrate with a purity of >98%, cholinium bitartrate with a purity of 99%, glutaric acid with a purity of 99%, adipic acid with a purity of 99%, α-chymotrypsin from bovine pancreas, guanidine hydrochloride with a purity of >99%, absolute ethanol for HPLC ≥99.8%, and phosphate buffered saline (PBS) tablets were purchased from Sigma-Aldrich. Succinic acid, propanoic acid, butanoic acid, and malonic acid with a purity of >98% were acquired from Fluka, while [Ch][Dhp] was purchased from IoliTec. All other chemicals used were of analytical grade. A sodium phosphate buffer solution (10 mM) of pH 7.0 was used as a reference solvent. The water employed was double distilled, and it was passed across a reverse osmosis system and further treated with a Milli-Q plus 185 water purification apparatus.

### Synthesis of cholinium-based ILs

Cholinium-based ILs were synthesized using a standard procedure through a simple acid base reaction.[17] Equimolar solutions (1 : 1) of aqueous cholinium bicarbonate (80 wt% in water) and the corresponding carboxylic acids were slowly mixed and stirred for 12 h at 75 °C. The mixture was then kept under stirring for 12 h. The ILs were collected and dried for 24 h in a vacuum line under a nitrogen atmosphere for moisture removal. The chemical structure and purity (>98%) of the ILs were confirmed by $^1$H-NMR and elemental analysis. The structure of the ILs employed in the present study is shown in Fig. 1.

### Peroxidase activity of Cyt c in the presence of cholinium-based ILs

The peroxidase activity of Cyt c was measured using ABTS as a substrate in the presence of $H_2O_2$. Cyt c catalyzes the oxidation of ABTS in the presence of $H_2O_2$ and produces the green-colored $ABTS^+$ radical. The formation of the $ABTS^+$ radical was monitored by the changes in the absorption spectra at 420 nm. The absorption spectra were acquired for mixtures containing 2 μM of Cyt c, 3 mM of ABTS, 1 mM of $H_2O_2$ and different concentrations of ILs (1 : 4, 1 : 2, 3 : 4 and 1 : 1 weight ratio). The reaction was initiated with the addition of $H_2O_2$. The reaction was subjected to 1 min of incubation with continuous measurements of absorbance changes at 420 nm. The percentage of relative activity was calculated considering 100% of enzymatic activity in the presence of PBS at pH 7.2.

### Peroxidase activity of Cyt c in the presence of cholinium-based ILs under multiple stresses

The peroxidase activity of Cyt c was measured using ABTS as a substrate in the presence of PBS (pH 7.2), a 1 : 2 weight ratio of [Ch][Dhp], and a 1 : 1 weight ratio of the remaining ILs against multiple stresses, namely temperature, pH, and the presence of chemical and biological denaturants. An enzyme-free reference was taken in all cases to rule out the interference of the ILs and other chemicals in the enzymatic reaction assay (Fig. S1†). The activity of Cyt c was determined at 20, 40, 60, 80, 100 and 120 °C, after incubation from 30 min to 4 h. In order to investigate the activity of Cyt c against chemical denaturants, Cyt c was incubated in the presence of 1 mM $H_2O_2$ for 15 and 30 min at 25 °C. The activity of Cyt c was also determined in the presence of 2, 4, and 6 M of the commonly used chemical denaturant guanidium hydrochloride (GuHCl), after the incubation for 15 min. To study the long storage effect, Cyt c was stored at room temperature (25 ± 2) °C in PBS and in IL aqueous solutions, and its activity was determined along regular periods, up to 21 weeks. To address the effect of pH on the enzyme activity, the pH of ILs was switched from acidic to basic by the addition of the corresponding carboxylic acid and cholinium hydroxide to the [Ch][carboxylate] IL, and then the activity was determined using ABTS as a substrate. The remaining activity was calculated by considering the difference of the initial activity and the activity after the

 

completion of the reaction under various stress conditions. Relative activity was calculated considering 100% of enzyme activity in PBS.

### Peroxidase activity of Cyt c and protection afforded by cholinium-based ILs against protease digestion

To appraise the effect of cholinium-based ILs on the biological stability of Cyt c, chymotrypsin treatment was employed to hydrolyze the enzyme, thereby causing deactivation. For this purpose, Cyt c was incubated for 24 h with α-chymotrypsin (1 μM) in the presence of both PBS and ILs at 37 °C. The activity was measured before and after incubation using ABTS substrate. The formation of the $ABTS^{+}$ radical was monitored by changes in the absorption spectra at 420 nm. The concentrations of 1 : 2 of [Ch][Dhp] and a 1 : 1 weight ratio of the remaining ILs were used. The remaining activity was calculated from the difference of the activity before and after the incubation of Cyt c with α-chymotrypsin.

### Stability of Cyt c in the presence of cholinium-based ILs

The stability of Cyt c in the presence of ILs and under multiple stresses was studied by UV-vis, FTIR and CD spectroscopy. UV-vis spectra and the activity of Cyt c in the absence and presence of various concentrations of ILs were recorded using a Shimadzu UV-1800 spectrophotometer. The spectra of Cyt c were acquired with quartz cuvettes of path length 0.1 cm. The concentration of Cyt c was 0.5 mg $mL^{-1}$. CD spectroscopy studies were performed using a Jasco-1500 spectrophotometer, equipped with a Peltier system for temperature control. CD calibration was performed using (1*S*)-(+)-10-camphorsulphonic acid (Aldrich, Milwaukee, WI), which exhibits a 34.5 M $cm^{-1}$ molar extinction coefficient at 285 nm and 2.36 M $cm^{-1}$ molar ellipticity ($\Theta$) at 295 nm. The sample was pre-equilibrated at the desired temperature for 15 min and 100 scan speed was fixed for adaptive sampling (accuracy of ±0.01) with a response time of 1s and 1 nm bandwidth. Each sample spectrum was obtained by subtracting the appropriate blank media from the experimental spectrum and was collected by averaging three spectra. All samples were pre-equilibrated at 25 °C for 25–30 min. Far-UV and near-UV CD spectra were monitored in a cuvette with a path length of 0.1 cm, with 0.5 mg $mL^{-1}$ concentration of Cyt c. Unfortunately, due to the intrinsic absorbance of the ILs in far and near-UV regions, only the region from 350 to 450 nm was investigated. However, the CD spectra in the region between 200 and 300 nm was recorded for the Cyt c extracted from the aqueous solutions of ILs, redissolved in PBS buffer at pH 7.2. FT-IR spectra were recorded using a PerkinElmer Spectrum Bx spectrophotometer in the wavelength range from 1800 to 1300 $cm^{-1}$. For each spectrum, 64 scans were made at a resolution of 2 $cm^{-1}$. The concentrations of Cyt c and ILs were 25 mg $mL^{-1}$, and 1 : 2 and 1 : 1 weight ratios, respectively.

### Molecular docking

The interaction sites of Cyt c with the IL ions were identified using the Auto-dock vina 1.1.2 program.[53] The crystal structure of Cyt c (PDB:1hrc) was used. AutoDockTools (ADT)[54] was used to prepare the protein input files by merging non-polar hydrogen atoms, and adding partial charges and atom types. Ligand (IL cation and anions) 3D atomic coordinates were computed by Gaussian 03w and the ligand rigid root was generated using AutoDockTools (ADT), setting all possible rotatable bonds defined as active by torsions. The grid center at the center of mass (*x*-, *y*-, and *z*-axes, respectively) to cover the whole interaction surface of Cyt c was 40 Å × 42 Å × 58 Å. The binding model that has the lowest binding free energy was searched out from 10 different conformers for each ligand.

### Recovery of Cyt c from the aqueous solution of ILs

Cyt c was precipitated from the aqueous solution of ILs using ice cold ethanol. The precipitated fraction was carefully separated using centrifugation (3 min at 500 rpm) and dried under an inert atmosphere. After that, Cyt c was redissolved in PBS buffer of pH 7.2, and UV-vis, FTIR, and CD spectra were acquired as described before. The yield and concentration of Cyt c were determined by spectroscopy at 280 nm, using a calibration curve previously established with Cyt c. The peroxidase activity of the recovered Cyt c was determined using ABTS as a substrate at 420 nm.

## Results and discussion

### Effect of cholinium-based ILs and their concentration on the activity of Cyt c

The present study demonstrates the potential of cholinium-based ILs comprising anions from carboxylic acids to enhance the activity and structural stability of Cyt c under several external stresses. To know the effect of different ILs having monocarboxylates, dicarboxylates and tricarboxylates as anionic constituents on the enzyme activity, the activity of Cyt c was determined using ABTS as a substrate in the presence of different ILs at a concentration of 0.5 g $mL^{-1}$. For comparison purposes, the activity was also studied in phosphate buffer solutions (PBS, 10 mM) at pH 7.2 and a 1 : 2 weight ratio of [Ch][Dhp] (the best IL reported to date for Cyt c).[35,38–40] Fig. 2a shows the relative activity of Cyt c in PBS and different ILs of 1 : 2 weight ratio. A ~2.5-fold increase in the enzyme activity was recorded in the presence of [Ch][Dhp] compared to its activity in PBS, which is in agreement with earlier reports.[39,40] There is no improvement in the enzyme activity in the presence of monocarboxylate-based ILs, namely [Ch][But] and [Ch][Prop], compared to PBS. Nevertheless, a remarkable increased activity ranging from ~3 to 25-fold compared to PBS and from ~1.5 to 12-fold compared to [Ch][Dhp], was recorded in the presence of di- and tri-carboxylate-based ILs. These results indicate that the presence of more than one –COOH group is beneficial towards the improvement of the enzyme activity.

To address the effect of the alkyl chain length of the dicarboxylate-based ILs, the ILs with the formula $[Ch][HO_2C\text{-}(CH_2)_n\text{-}COO^{-}]$, with $n$ = 1 to 4, were studied. Interestingly, Cyt c activity was enhanced with the increase of the alkyl chain length, and

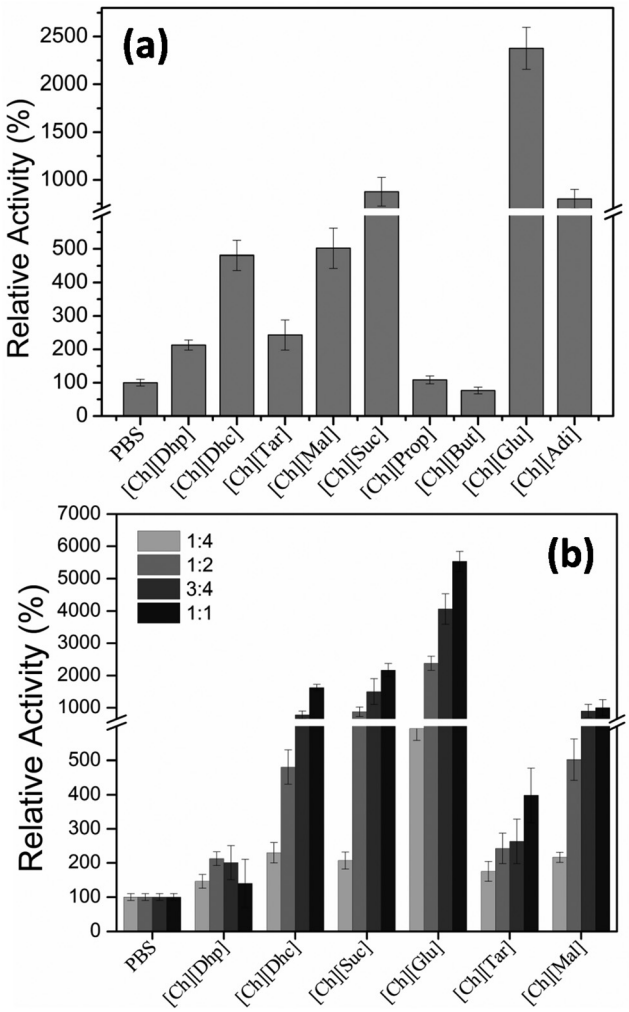


Fig. 2 (a) Relative activity of Cyt c in aqueous solutions containing a 1 : 2 weight ratio of different cholinium-based ILs. (b) Relative activity of Cyt c in aqueous solutions of cholinium-based ILs at different concentrations (a weight ratio of IL/water).

found to be the highest when $n$ = 3, *i.e.* with [Ch][Glu]. For a further increase in the chain length, when $n$ = 4 corresponding to [Ch][Adi], a deleterious effect on the activity was observed. This decline in the enzyme activity with the increase in the anion alkyl chain length of the ILs, from propanoate to butanoate and from glutarate to adipate, can be due to stronger dispersive-type interactions occurring between the ILs and the enzyme polypeptide backbone, and that may destroy the structural integrity of the enzyme. Overall, [Ch][Dhp], [Ch][Dhc], [Ch][Tar], [Ch][Mal], [Ch][Suc] and [Ch][Glu] were identified as the most promising media and thus chosen for further studies.

From the previous study, it is clear that the activity of Cyt c strongly depends on the nature of the IL anion; however, it is also pertinent to know the effect of the IL concentration on the activity of Cyt c. Fig. 2b shows the relative activity of Cyt c at different concentrations of cholinium-based ILs. In general, the activity of Cyt c decreases in the order: [Ch][Glu] > [Ch][Suc] > [Ch][Dhc] > [Ch][Mal] > [Ch][Tar] > [Ch][Dhp]. The activity of Cyt c increased till 1 : 2 weight ratio of [Ch][Dhp], and started to decrease with a further increase in the concentration. On the other hand, the activity of Cyt c drastically increased with the increase in the concentration of other dicarboxylate-based ILs, up to 1 : 1 weight ratio, with [Ch][Glu] identified as the best IL (>50-fold activity increase compared to that observed in PBS).

### Effect of cholinium-based ILs and their concentration on the stability of Cyt c

The three-dimensional structure and Met80–Fe bond are essential for the Cyt c activity. The upper portion of the heme pocket, including the Met-80 ligand, is the most labile ligand, which can easily dissociate from the heme iron. Any structural alterations, namely unfolding or denaturation of proteins, are mainly responsible for the loss of activity. In contrast, the catalytic activity of Cyt c is reported to be enhanced significantly by the partial unfolding of the protein tertiary structure, as monitored by Trp59 and Fe–S (Met80).[55,56] As discussed above, the catalytic activity of Cyt c improves substantially in the presence of different cholinium-based ILs, but whether such an enhancement causes any irreversible structural changes in the protein structure is still unknown. Towards this endeavor, the stability of Cyt c in the presence of different ILs at different concentrations was evaluated by UV-vis, CD and FTIR spectroscopy (Fig. 3 and 4, and ESI, Fig S2–S6†).

UV-vis absorption allows one to infer the conformational changes of proteins in solvent media. Due to the presence of the heme prosthetic group, Cyt c shows some characteristic absorption bands. As shown in Fig. 3a and b, the oxidized (Fe(III)) form of Cyt c in PBS (pH 7.2) shows characteristic bands at ~280 nm (due to the n–π* transition of aromatic amino acids), an intense Soret band at ~409 nm, and a

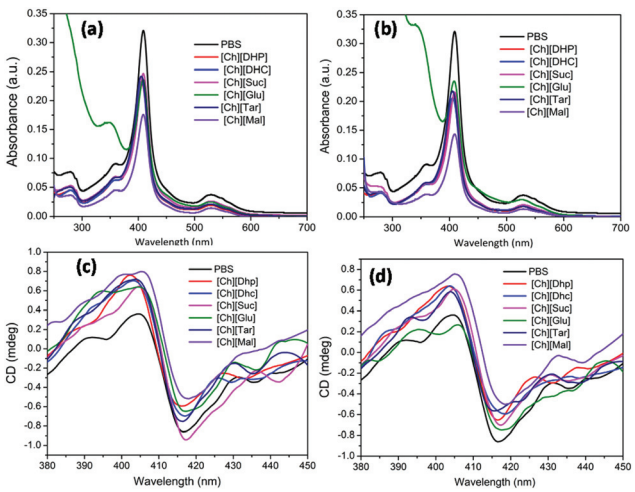


Fig. 3 (a) & (b) UV-vis absorption spectra of Cyt c in the presence of various IL aqueous solutions at 25 °C. (c) & (d) Near-UV CD spectra of Cyt c in the presence of various IL aqueous solutions at 1 : 2 and 1 : 1 weight ratios of all ILs except for 3 : 4 of [Ch][Dhp] at 25 °C.

Q band at 528 and 550 nm (which reflect π–π* transitions due to the porphyrin chromophore). These are in good agreement with previous reports.[57,58] From Fig. 3a and b, it is evident that there is no significant shift in the wavelength maxima of Cyt c in the presence of different ILs at a concentration of 1 : 1 weight ratio, which indicates that the aqueous solutions of all ILs did not affect the polypeptide environment around the heme group. However, the decrease in the absorbance of the 409 nm peak indicates that there are some interactions occurring between the enzyme and ILs. Similar results were observed in the presence of lower concentrations of ILs (Fig. S2 in the ESI†). The UV-vis spectrum of Cyt c in [Ch][Glu] changed significantly below 400 nm wavelength due to the absorption of the pure IL in the UV region (Fig. S3 in the ESI†).

To gain more insights into the possible structural changes of Cyt c, CD spectroscopy studies were carried out in the presence of different cholinium-based ILs, at different concentrations. Fig. 3c & d show the CD spectra of Cyt c in PBS and in the presence of different ILs at a concentration of 1 : 1 weight ratio, while Fig. S3 in the ESI† shows the CD spectra of Cyt c in the presence of different ILs at variable concentrations. Due to the strong absorbance of the cholinium-based ILs below 250 nm, only the region from 250 to 450 nm was investigated, which can yet provide insight into the tertiary structural changes in the heme vicinity. The weak spectral features in the Soret region (350–450 nm) have been assigned to the charge transfer transitions between the porphyrin and heme iron, thus sensitive to the changes in the axial ligands.[59,60] As can be seen from Fig. 3b, native Cyt c exhibits an intense positive band at ~406 nm and a strong negative band around ~416 nm in PBS solutions. The negative CD band around 416 nm is related to the presence of Met80 at the sixth coordination position of Cyt c.[40,60] After the addition of all ILs, there is no shift in the positive and negative bands, only the intensities of the positive and negative bands are different. These non-significant changes in the CD spectra suggest that the incubation of Cyt c in the aqueous solutions of ILs does not affect the heme iron microenvironment and the conformation of Cyt c.[57] Possible changes in the tertiary structure of Cyt c in the presence of different cholinium-based ILs were also investigated from the near-UV CD spectra (250–300 nm), data provided in the ESI, Fig S5.† The tertiary structure of Cyt c is predominantly preserved by dispersive-type interactions by the side chain amino acids (tyrosine (Tyr), tryptophan (Trp) and phenylalanine (Phe)), which show characteristic bands in the mid-UV-CD spectra.[61] As can be seen in Fig. S5 in the ESI,† Cyt c shows bands at ~263 nm due to Tyr, at ~282 nm due to Phe, and at ~290 nm due to Trp residues in the mid-UV CD spectra in the presence of PBS buffer (pH 7.2). These bands are kept intact in the presence of 1 : 2 weight ratio of [Ch][Dhp], and may be responsible for the improvement in the catalytic activity compared to PBS. Although there was no alteration in the Soret band in the presence of other ILs as confirmed by the UV-vis and far UV-CD spectra, a significant shift of the amino acid bands in the mid-UV CD spectra was observed (Fig. S5 in the ESI†). This may be the reason for the improved catalytic activity in the presence of dicarboxylate-based ILs, as changes in the side chain amino acid residues near the active center of the enzyme may enhance the enzyme activity.[62] This observation was further proved by molecular docking studies, as discussed below (*vide infra*).

The changes in the secondary structure of Cyt c in the presence of cholinium-based ILs were further investigated by FTIR analysis. In FTIR spectra, the amide I band (1600–1700 $cm^{-1}$) of Cyt c is primarily related to the C=O stretching vibration of the peptide backbone conformation, and amide II mainly corresponds to the N–H bending vibration and the C–N stretching vibration of the peptide backbone. Therefore, any conformational changes in the secondary structure of Cyt c cause disturbance in the amide I and amide II regions in the FTIR spectra of the enzyme.[63] Fig. S6 in the ESI† shows the FTIR spectra of Cyt c in PBS and in the presence of cholinium-based ILs. In PBS, amide I and II peaks are at 1652 and 1546 $cm^{-1}$, respectively. In aqueous solutions containing 1 : 1 weight ratio of ILs, there is no change in the amide I band, except in the case of [Ch][Suc], where a shift from 1652 $cm^{-1}$ to 1632 $cm^{-1}$ was observed, although with negligible changes in the amide II band (Fig. S6a in the ESI†). Similarities in the amide I and II peak positions of Cyt c in the presence of ILs demonstrate that the secondary structure remains almost intact in all ILs at 1 : 2 weight ratio, whereas at 1 : 1 weight ratio of ILs there is a substantial shift of both amide I and amide II bands, showing that the secondary structures have undergone some structural changes (Fig. S6b in the ESI†). Therefore, from FTIR results, it is evident that at lower concentrations (1 : 2 weight ratio) of ILs, Cyt c retains its native structure, whereas a perturbed secondary structure starts to prevail at higher concentrations (1 : 1 weight ratio) of ILs. Nevertheless, such perturbation in the secondary structure did not lead to the complete unfolding or irreversible damage of Cyt c since a substantially higher catalytic activity was still observed at this higher concentration of ILs.

### Activity of Cyt c against $H_2O_2$ as an oxidant, GuHCl as a chemical denaturant and protease digestion in cholinium-based ILs

The usefulness of heme peroxidases in biosensors and in immunoassay reactions is limited due to their poor stability in the presence of $H_2O_2$.[64] Cyt c is degraded by $H_2O_2$ because it leads to the opening of the heme porphyrin ring, rapidly inactivating Cyt c.[64,65] The *in vivo* degradation of Cyt c by $H_2O_2$ can interfere with respiration, accelerate aging, and enhance the metabolism of carcinogens. Therefore, it is important to protect Cyt c from the adverse effects of $H_2O_2$. In order to investigate the stability of Cyt c against $H_2O_2$ in the presence of ILs, allowing thus to infer the potential of ILs to act as protective solvents over chemical denaturants, Cyt c was incubated in 1 mM $H_2O_2$ for 15 and 30 min at 25 °C, both in PBS and in the presence of various IL aqueous solutions. Fig. 4a shows the remaining relative activity of the enzyme in these aqueous solutions after being incubated with $H_2O_2$. The peroxidase activity of Cyt c in PBS retained only 70% and 48% of the

initial activity after 15 min and 30 min of incubation, respectively. Similar results were obtained in the presence of [Ch][Dhp]. Remarkably, the remaining activity of Cyt c in most IL aqueous solutions was higher than that observed in PBS, especially in the presence of [Ch][Dhc] and [Ch][Glu], indicating the potential of these to protect the protein from $H_2O_2$ deactivation, however the possibility of the ILs' role as a sacrificial reagent in the presence of $H_2O_2$ can't be ruled out.

Besides $H_2O_2$, enzyme deactivation is also affected by chemical denaturants such as guanidinium chloride (GuHCl), a well-known denaturant for most of the proteins *in vitro*, as well as *in vivo*.[66,67] Its deleterious action on Cyt c is quite prominent which results in a decrease in Cyt c activity and structure stability.[68] In this regard, ILs have been reported to allow its refolding by displaying a counteracting effect against the deleterious action of chaotropic agents, such as urea and GuHCl.[69,70] Fig. 4b shows the changes in the activity of Cyt c at different concentrations of GuHCl in aqueous solutions containing 1 : 2 weight ratio of ILs. In the presence of 2 M GuHCl, Cyt c still shows a higher activity, and only at higher concentrations the activity decreases. In the presence of aqueous solutions of [Ch][Dhp], [Ch][Dhc], [Ch][Tar], and [Ch][Mal], Cyt c shows a marginal loss in activity, up to 6 M of GuHCl. Therefore, these cholinium-based ILs seem suitable to protect Cyt c against the GuHCl-induced denaturation. However, ILs such as [Ch][Suc] and [Ch][Glu] are not able to counteract the denatured action of GuHCl, since Cyt c was precipitated as soon as GuHCl was added. This counteracting effect was further ascertained by CD spectra analysis. Fig. S7 in the ESI† shows that Cyt c exhibits a strong positive band at ~401 nm and a strong negative band at ~416 nm in PBS. When buffer-dissolved Cyt c was treated with GuHCl, the negative band consequently vanishes. These results confirm the disruption of the coordination between Met80 and heme iron.[71] Upon the addition of GuHCl, only a broader positive band centered at 406 nm was observed (Fig. S7 in the ESI†), indicating the conformational changes around the heme crevice. In the presence of ILs, ellipticities for negative bands decrease, but are not completely destroyed as in the case of PBS. This is an indication of the counteracting ability of all ILs, with the only exceptions of [Ch][Glu] and [Ch][Suc], against the adverse effect of GuHCl on the activity and native structure of Cyt c.

To further validate the potential of ILs employed against the degradation of Cyt c by biological denaturants, α-chymotrypsin was used. As shown in Fig. 7b, in the presence of [Ch][Tar], 60% of the initial activity was retained after 24 h digestion with α-chymotrypsin, whereas only 5% of the activity remained in the presence of PBS and [Ch][Mal]. The effect of ILs was also monitored on the enzymatic activity of α-chymotrypsin. Proteolytic activity results indicate that enzyme activity was maintained in all ILs and the used ILs did not inactivate the protease (The enzymatic activity was recorded in the presence of ILs (1 : 4 weight ratio of IL : $H_2O$) using casein as the substrate after a incubation of 15 min). These results indicate that [Ch][Tar] has potential utility for protecting the enzyme against biological degradation.

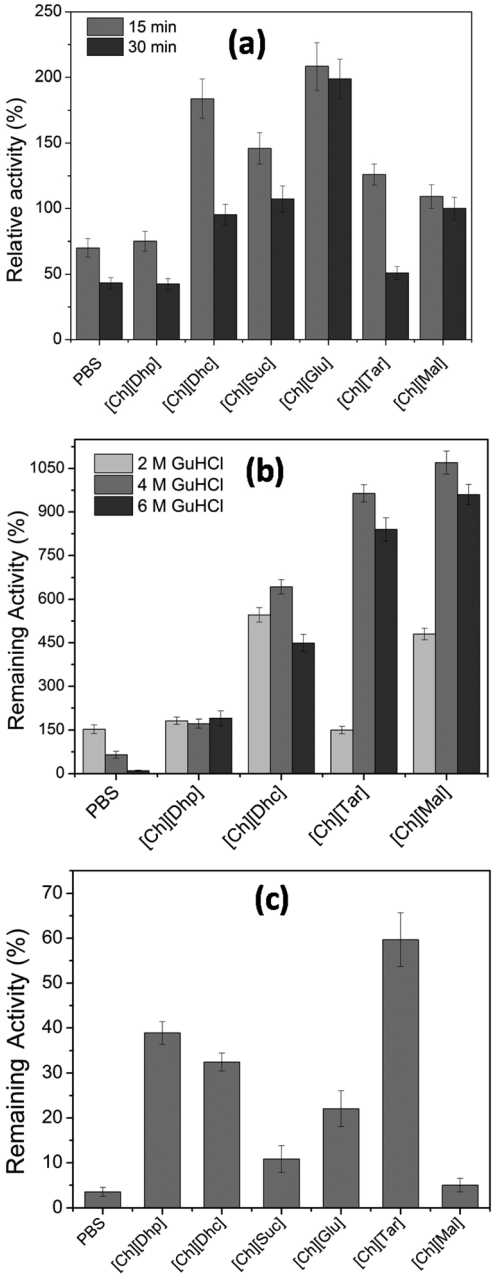


**Fig. 4** (a) Activity of Cyt c in PBS and aqueous cholinium-based IL solutions (1 : 2 of [Ch][Dhp] and a 1 : 1 weight ratio of other ILs), after incubation for 15 min and 30 min with 1 mM $H_2O_2$ at room temperature. (b) Activity of Cyt c in buffer and IL aqueous solutions at different concentrations of GuHCl. (c) Remaining activity of Cyt c after incubation in CT in the presence of ILs 1 : 4 weight ratio of ILs : $H_2O$.

### Effect of temperature and pH on the stability and activity of Cyt c in cholinium-based ILs

Protein stability and activity vary significantly with temperature. Therefore, we also investigated the thermal stability and activity of Cyt c in the presence of cholinium-based ILs. The activity of Cyt c was determined in the presence and absence of ILs at 1 : 2 weight ratio and at various temperatures ranging from 20 to 120 °C (Fig. 5a). The enzyme activity increases up to

 

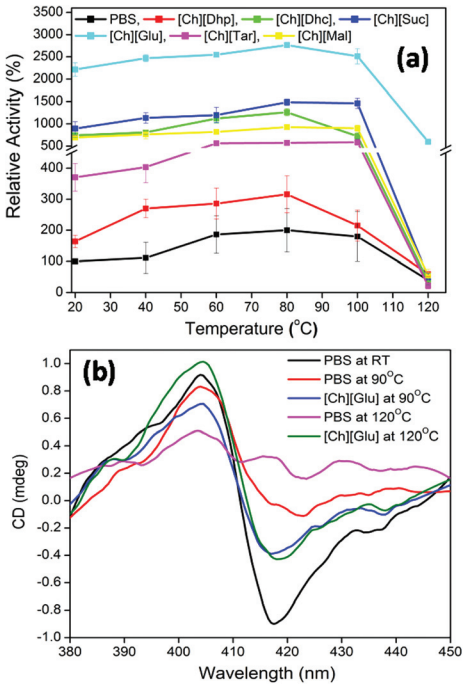


**Fig. 5** Effect of temperature on the conformation of Cyt c: (a) relative activity of Cyt c as a function of temperature; (b) CD spectra of Cyt c at different temperatures.

80 °C, and then decreases. In the presence of PBS, Cyt c is almost completely inactivated at 80 °C, while peroxidase activity was retained in the presence of all ILs when exposed at 100 °C for 30 min. There was a dramatic decrease in peroxidase activity at 120 °C, even in the presence of the ILs. The only exception was verified with [Ch][Glu] due to a thermal-induced conformational change in the enzyme. $T_{50}$, the temperature of half-inactivation of the enzyme, was also calculated with and without ILs using Fig. 5(a) and the data are provided in Table S1.† $T_{50}$ is the temperature at which the activity is reduced by half after a defined time period.[7]

Fig. 5(b) shows the CD spectra of Cyt c at different temperatures. From the CD results, it is clear that the Soret bands completely disappear at 120 °C in the presence of PBS, while in the presence of [Ch][Glu], Cyt c exhibits both positive and negative bands. These results suggest that the incubation of Cyt c in aqueous solutions of [Ch][Glu] is able to protect Cyt c against thermal denaturation. The increased thermal stability of Cyt c up to 80 °C was observed in [Ch][Dhp] by Ohno and co-workers[35,39] whereas, in our case, and up to 120 °C, Cyt c shows a ~7-fold higher activity than in the presence of PBS at 20 °C. These results reveal that both the biocatalytic activity and stability of Cyt c are enhanced in [Ch][Glu], demonstrating the suitability of these cholinium-based ILs as high temperature bio-catalytic media.

Besides temperature, pH induced denaturation of enzymes may also be a major problem in biocatalysis when envisaging a broad range utility of IL–protein systems. Therefore, an attempt was made to investigate the effect of pH on the catalytic activity of Cyt c in the presence of different ILs. It can be noticed that all ILs employed in this work have more than one exchangeable acidic proton and thus the pH can be modified by the addition of a base. Instead of adding mineral acids or bases which would add extra foreign ions to the IL solutions, [Ch][OH] and carboxylic acids were used to adjust the pH. Fig. 6 shows the effect of pH on the activity of Cyt c in the presence of cholinium-based ILs. The activity of Cyt c in the presence of different ILs is maximum in the pH range 3.2–5.4, whereas below this pH range the activity markedly decreases. A strong decrease of the activity was recorded in the pH range 6.2–12.9, in agreement with the previous studies that demonstrated that the activity of Cyt c decreases substantially at alkaline pH.[65]

## Reasons behind the improved catalytic activity and enhanced stability of Cyt c in the presence of cholinium-based ILs

In order to evaluate the enhanced activity and stability of Cyt c afforded by ILs at the molecular level, a molecular docking study was carried out. The binding sites of each IL ion to Cyt c at its lowest binding energy structure were analyzed with 9 conformers. Cyt c is constituted by 104 amino acids in a small single domain with a heme group coordinated between HIS18 and MET80.[53,54] The lowest absolute value of affinity (kcal $mol^{-1}$) for each IL ion to Cyt c, as well as the molecular interaction diagrams, are shown in the ESI (Fig. S8– S24†). Moreover, the best binding pose and docking affinities, the type of interaction, and the geometry distance (Å) are shown in the ESI (Table S2†). Besides the energy scoring function, molecular docking analysis allows the identification of the hydrogen bonding, electrostatic interactions and dispersive-type interactions between the IL ions and Cyt c. All ILs share a common cation ($[Ch]^+$), and the docking affinity values of IL anions to Cyt c follow the rank: $[Dhc]^-$ > $[Glu]^-$ > $[Tar]^-$ >

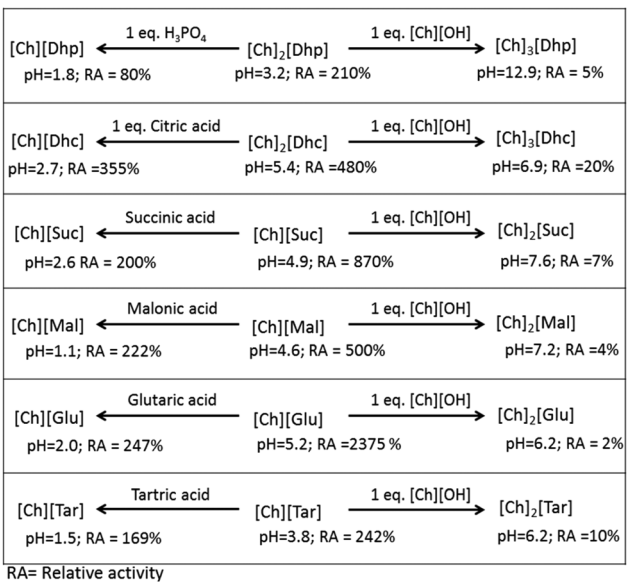


**Fig. 6** Effect of different cholinium-based ILs at various pH values in the activity of Cyt c.

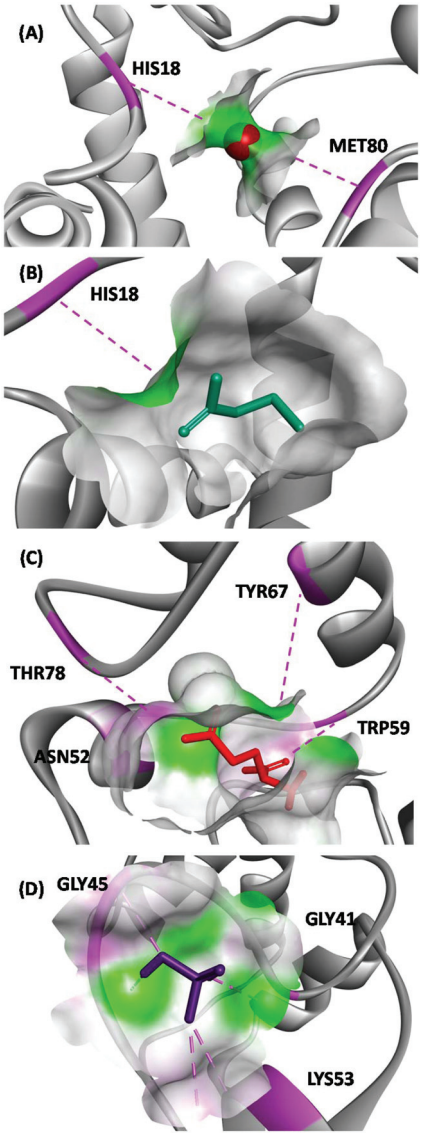


Fig. 7 Cyt c docking pose with the lowest absolute value of affinity: (A) Cyt c heme group, (B) with $[But]^-$, (C) with $[Glu]^-$, and (D) with $[Dhp]^-$.

$[Suc]^-$ > $[Adi]^-$ > $[Mal]^-$ > $[But]^-$ > $[Prop]^-$ > $[Dhp]^-$ (ESI, Table S1†). According to Fig. 7a, the heme group in Cyt c is adjacent to the residues HIS18 and MET80. The binding of the IL anions to HIS18 appears to be the major driving force in the catalytic behaviour of Cyt c. Fig. 7b and c show the positions displayed by $[But]^-$ and $[Glu]^-$. $[But]^-$ is only adjacent to the residue HIS18 of Cyt c. On the other hand, $[Glu]^-$, which leads to higher Cyt c activity (Fig. 2), is surrounded by ASN52, TRP59, TYR67 and THR78. All other anions studied show the same behaviour, binding preferentially to residues that do not lead to a decrease in the Cyt c activity (Fig. S8–S14, in the ESI†). In contrast, $[Dhp]^-$ does not interact with the active site of Cyt c (Fig. 8D); rather, it interacts with the side chain amino acids of the enzyme, and thus there is no significant improvement in the enzyme catalytic activity in the presence of the corresponding IL. In short, the capabilities of dicarboxylate

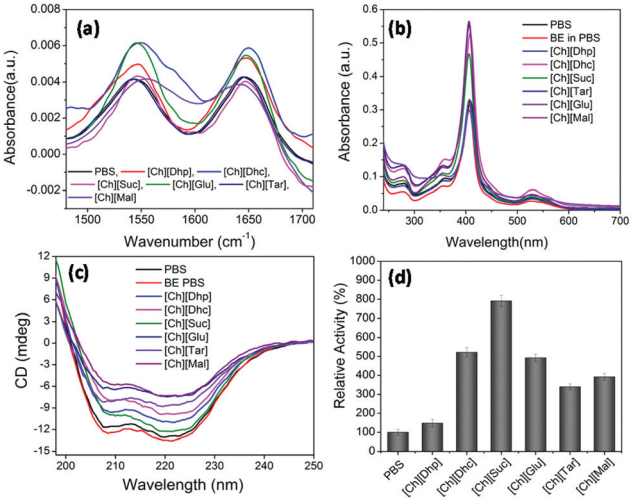


Fig. 8 Structural stability and enzymatic activity of Cyt c after its recovery from aqueous solutions containing 3 : 4 of [Ch][Dhp] and a 1 : 1 weight ratio of the remaining ILs: (a) FTIR (b) UV-vis and (c) CD spectra; and (d) relative activity.

anions to interact with the adjacent amino acid peptide chain at the catalytically active center of Cyt c play a major role towards the substantial improvement in the catalytic activity.

### Recovery of Cyt c from aqueous solutions of cholinium-based ILs

From FTIR results (Fig. S6 in the ESI†), it is clear that at high concentrations of ILs (1 : 1 weight ratio) there are strong interactions between Cyt c and ILs. The question is whether such interactions may affect the enzyme's structure. For this purpose, Cyt c was precipitated from IL solutions using ice cold ethanol, with a >90% recovery yield of the enzyme. Cyt c was then resuspended in PBS aqueous solutions. The extracted Cyt c was characterized by UV-vis, CD and FTIR spectroscopy. Fig. 8 shows that both the structural stability and enzymatic activity are retained after the recovery of Cyt c from the IL solutions. From FTIR spectra (Fig. 8a), it can be seen that there are no shifts in the amide I and amide II bands of Cyt c, which indicate no changes in the enzyme secondary structure. Fig. 8b shows the UV-vis absorption spectra of Cyt c in the presence and absence of different ILs; the presence of the characteristic bands discussed before indicates no significant structural changes in the enzyme structure after the recovery step.

Since ILs were removed in the Cyt c recovery step, they do not interfere in far UV regions, thus allowing one to obtain the far-UV CD spectrum of Cyt c. Native Cyt c exhibits two negative maxima at 220 and 208 nm, as shown in Fig. 8c, which are similar in all samples of the recovered enzyme. With the aim of evaluating whether enzymatic activity was affected during the recovery process, the activity of Cyt c was further studied using ABTS as a substrate in the aqueous solutions of novel ILs. The results (Fig. 8) show that the potential of ILs for the enhancement of the enzyme activity is maintained for the

 

recovered Cyt c, further demonstrating the storage ability of ILs for the enzyme.

### Effect of long-term storage on the activity of Cyt c in cholinium-based ILs

It is well known that the structural integrity of proteins is disturbed when stored at room temperature for long periods of time in aqueous buffer solutions, whereas the potential of [Ch][DhP] as potential storage media of Cyt c was demonstrated by Ohno and co-workers.[35] The authors showed that Cyt c retained more than 60% of its activity after storage for 18 months in the aqueous solutions of [Ch][Dhp]; however, this IL does not exhibit significant improvements in the initial enzyme activity, as shown before. As discussed earlier, all other ILs in the present study not only lead to an improved activity of Cyt c compared to [Ch][Dhp], but also are able to preserve the enzyme against structural damage when stored at room temperature for 20 weeks. As can be seen from Fig. 9, Cyt c is deactivated after 1 week of storage in buffer, whereas >70% of the initial activity is retained after 21 weeks of storage under ambient conditions, showing therefore the high potential of ILs as storage media for Cyt c.

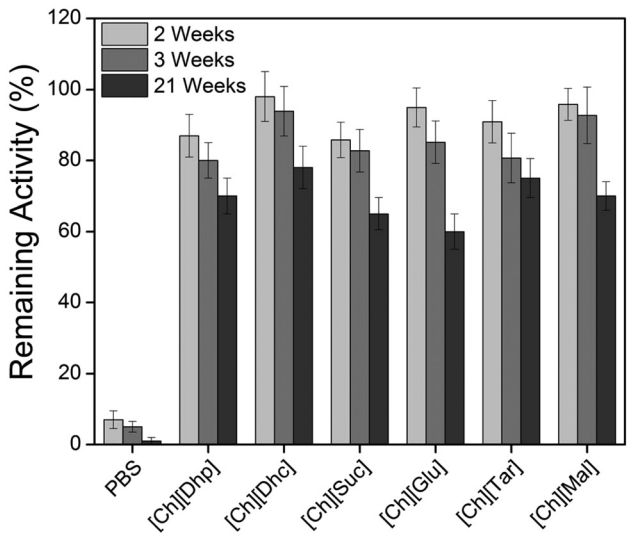


**Fig. 9** Activity of Cyt c in buffer, a 1 : 2 weight ratio of [Ch][Dhp] and a 1 : 1 weight ratio of the remaining IL aqueous solutions after incubation for 2, 3 and 21 weeks at room temperature.

## Conclusions

The novel class of ILs studied here, formed by cholinium and anions derived from di-carboxylic acids, when in aqueous media, are remarkable solvents for the packaging of Cyt c, while being able to provide exceptionally high catalytic activity and enhanced stability against multiple stresses. Under the optimized conditions, an outstanding high catalytic activity (>50 fold) of Cyt c was observed in the aqueous solution of [Ch][Glu] (1 : 1 weight ratio), compared to phosphate buffer solutions (pH 7.2), and ~25-fold compared to the most suited IL ([Ch][Dhp]) reported to date. This study shows that the use of dicarboxylate-based ILs can improve not only the enzymatic activity but also the stability of Cyt c, by protecting it against chemical and biological denaturants. Some ILs have an incredible tendency to offset the denaturing effects of $H_2O_2$, GuHCl and protease on the activity and stability of Cyt c, thereby broadening the widespread utility of this enzyme in biocatalysis. The catalytic activity of the enzyme in the presence of ILs was retained against other external interferences, such as high temperature and pH. The enzyme activity in the presence of different ILs was found to be maximum in the pH range from 3 to 5.5, and in the presence of [Ch][Glu] (1 : 1 molar ratio) it is preserved up to 120 °C. The observed enzyme activity correlates well with the structural stability of the enzyme, as confirmed by UV–vis, circular dichroism (CD), and Fourier transform infrared (FT-IR) spectroscopy. As confirmed by molecular docking, the capability of the dicarboxylate anions to interact with the catalytically active center of Cyt c plays a major role towards the remarkable improvement observed in the catalytic activity. The successful recovery and reuse of the enzyme from the aqueous solutions of ILs was also achieved, without compromising its structural integrity and catalytic activity. Moreover, it was found that the ILs studied herein allow long-term storage (~5 months) of the enzyme at room temperature, without a significant decrease of its initial activity, valuable to overcome the most commonly encountered problems in the therapeutic applications of Cyt c.

In summary, novel cholinium-based ILs capable of enhancing both the stability and activity of Cyt c, and able to preserve the enzyme for at least up to 5 months under ambient conditions, have been found. These ILs are of high interest for protein packaging aiming at overcoming some of the major limitations found in the development of robust biocatalytic processes.

## Conflicts of interest

There are no conflicts to declare.

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