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Recent advances in the use of ionic liquids as solvents for protein-based materials and chemistry

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Abstract

Proteins are a diverse class of molecules that can act as catalysts and structural components. Interest in their interactions with ionic solvents is on the increase due to the tuneable possibilities for non-aqueous biocatalysis, improved thermostability of biomaterials, and possible roles in medicine, such as drug delivery and use as cell-growth scaffolds. We summarise here the recent examples of these exciting new aspects of protein-ionic solvent interactions, highlighting future directions.

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Ionic liquids, proteins, stability, catalysis, separations, enzymes.

Introduction

In the last few years, the tunability of ionic liquids has been recognised as a solution to many challenges in bioscience, including issues with contrasting solubility of enzymes and substrates that impact catalyst accessibility; challenges in separations; problems with protein–drug aggregation, stability, and delivery [1]; and as an opportunity for improving the green credentials and economics of protein-based processes. We address here a selection of studies from the past three years, building on our previous review [2] and highlighting the increasing scope of protein-based chemistry using ionic liquids (ILs) as solvents. We further provide a perspective on where this combination of biomaterials and ionic solvents may be deployed for greater sustainability impact, with the chemical structures of selected IL cations and anions appearing in this review highlighted in Figure 1. While imidazolium-based (general formula $[C_nC_m\text{Im}]^+$, as seen for cations **1**–**3**) cations are still a popular feature of many of the processes studied in the last few years, there is an increasing shift towards bio-based and biocompatible ILs [1], including those based on the cholinium $[\text{Ch}]^+$ cation. This shift has been driven both by a strong awareness of the need for more sustainable ILs, paired with the increasing understanding of the properties of ions and how they relate to function [3]. Much recent work in this latter aspect is directed to going beyond the early Hofmeister series-based observations [4,5] to encompass a more nuanced 'specific-ion' model that is more relevant for complex biological and IL structures [5,6]. Such foundational models will not be discussed in detail in this review, which concentrates more on recent application highlights, but have been comprehensively covered by others [7].

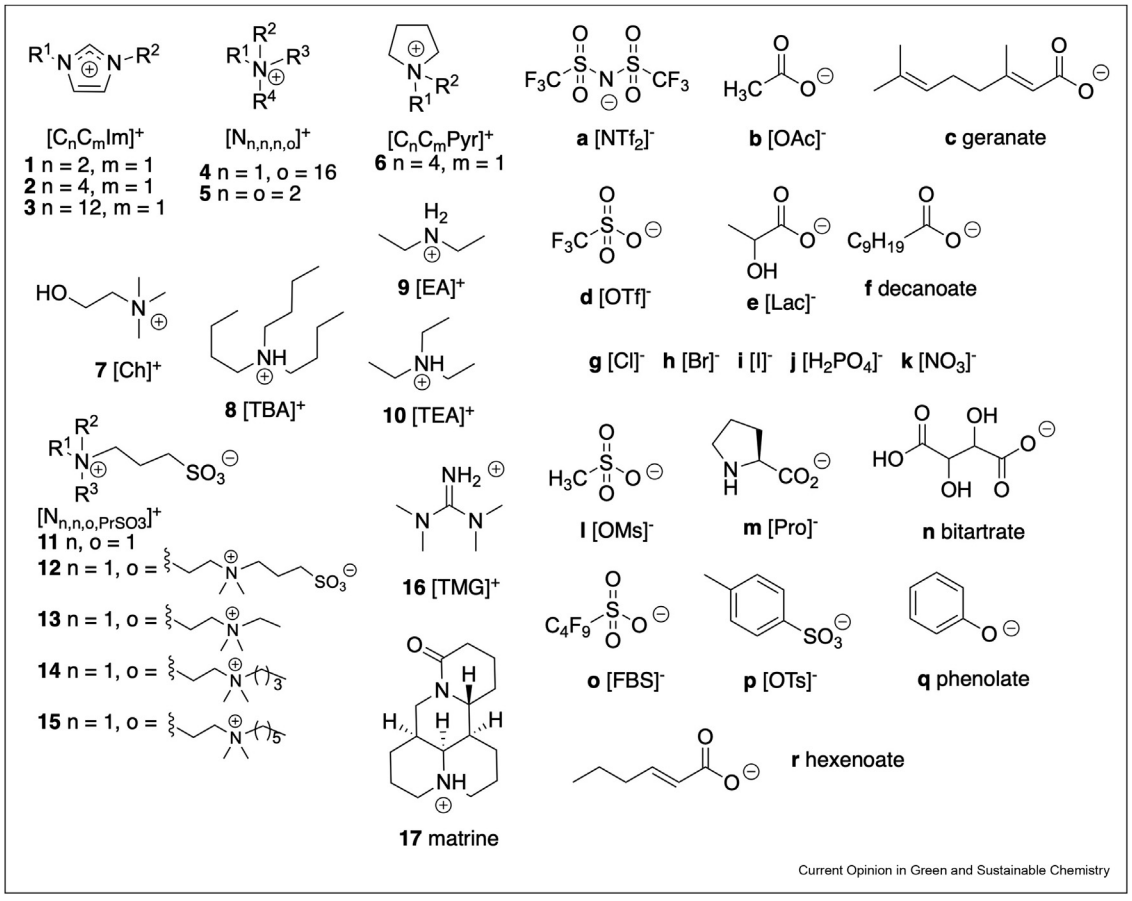
Dissolving proteins – catalyst accessibility, resistance to the detrimental impacts of ionic liquid, and processing

Biocatalysis with ionic liquids and their mixtures has continued to attract significant interest in recent years [8–10]. The solubilisation of proteins in ionic liquids has provided an important route to addressing challenges of substrate access to enzyme active sites, particularly in the processing of recalcitrant biomaterials, such as lignocellulose.

Cellulases are extremely important in industrial biomass conversions, with limitations brought about by the inactivation of these enzymes by residual ionic liquids used in pretreatment [11]. Recently, a rational understanding of the impact of $[C_4C_1\text{Im}][\text{Cl}]$ **2g** using a combination of experimental and computational approaches provided design routes to a more stable cellulase. In particular, minimisation of ionic liquid-induced enzyme aggregation by mediating protein dynamics through engineered structural changes led to a significant improvement in observed activity (Figure 2(a)). This work provides a basis for the importance of protein dynamics, and mediation of this dynamic behaviour through IL-protein interactions, in controlling reaction outcomes.

Laccases are also industrially important enzymes utilised in waste-water treatment and lignin degradation [12]. ILs can enhance substrate availability, and imidazolium-based ILs have been utilised as a surface modifier for chitosan to create magnetic immobilised nanoparticles (Figure 2(b)) [13]. The presence of the

Figure 1



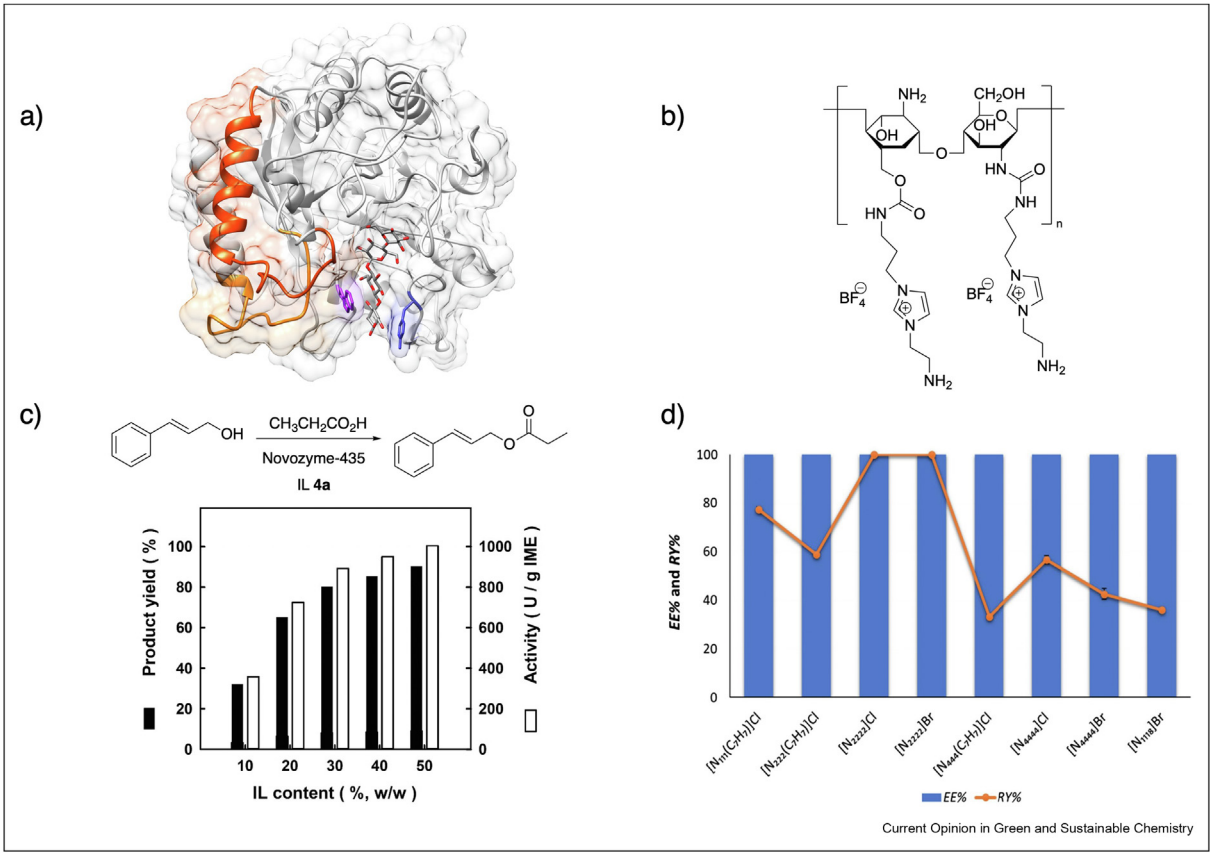
Cations and anions utilised in some recent protein-IL studies.

IL-based linker enhances laccase loading onto the nanoparticles by over 25%, with an increased expressed activity of 1.6-fold. In this environment, with added Cu^{2+}, the laccase could achieve a specific activity of more than 65%, relative to free laccase, with the added benefits of being recyclable, having an improved substrate removal efficiency, being less temperature sensitive, and being more storage stable.

Although different ionic liquids can be selected to match against enzyme activity, an alternative approach involves either evolution or design of enzymes that are more tolerant to specific ILs and mixtures containing them. Rational design rules have been difficult to achieve due to the complexity of possible interactions, with recent progress being made in this area. Structure-based modifications of proteins to enable resistance to the damaging impacts of ionic liquids in aqueous solutions have been systematically evaluated for *Bacillus subtilis* lipase A [14]. Here, data-driven approaches, directed by an experimental site-saturation mutagenesis library, have been used to reveal stabilising mutations towards aqueous $[C_4C_1Im]^+$-based ILs (**2d**, **2g**, **2h** and **2i**). By analysing a number of previously used approaches, five key calculable properties of a protein (solvent accessibility, relative volume, hydropathy, unfolding energy, and residue conservation) were identified that, when combined, contributed to the most effective identification of relevant single-point mutants conferring stability of the enzyme (and hence function) in mixtures containing each of the four ILs. This approach was improved further by integration with molecular dynamics (MD) predictions of structural stability (or structural weak spots) in the protein, where previous work had shown multi-μs simulations of this type predict IL interactions with the surface protein residues that induce long-range perturbations of the non-covalent forces within the structure [15]. This study brings computational screening for functional outcomes one step closer.

Separation processes are extremely important in bioprocessing, and optimising these is essential for industrially efficient processes. Temperature-switchable, sponge-like ionic liquids have been used as matrices for

Figure 2



Ionic liquids and their interactions with ILs when dissolving proteins. (a) Cellulase (1 ECE) shows highly dynamic regions (marked in red and orange) in **2g** that can be stabilised through protein engineering. The key residue W213 (purple) is thought to interact specifically with the $[C_4C_1Im]^+$ cation **2**. (b) An ionic liquid modified chitosan increases laccase loading on a nanoparticle, along with its specific activity. (c) The effects of different amounts of the ionic liquid **4a** on the biocatalytic efficiency of lipase in the preparation of cinnamoyl propionate. (Adapted with permission from E. Alvarez, J. Rodriguez, R. Villa, C. Gomez, S. Nieto, A. Donaire, P. Lozano, Clean Enzymatic Production of Flavor Esters in Spongelike Ionic Liquids, ACS Sustainable Chem. Eng. 7(15) (2019) 13,307−13314 [16]. Copyright 2019 American Chemical Society). (d) Extraction efficiencies (EE%) and recovery yields (RY%) of ovalbumin from different IL mixtures. Reprinted in part from *Purification and Separation Technology Science*, **233**, D.C.V. Belchior, M.V. Quental, M.M. Pereira, C.M.N. Mendonça, I.F. Duarte, M.G. Freire, Performance of tetraalkylammonium-based ionic liquids as constituents of aqueous biphasic systems in the extraction of ovalbumin and lysozyme, 116,019 (2020) [18], with permission from Elsevier. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

bioproduction, including the synthesis of 16 different flavour esters using lipase B [16], and chemo-enzymatic synthesis of glycerol carbonate (meth)acrylate using carbon dioxide [17]. The hydrophobic nature of these water-immiscible ILs with long alkyl side chains in the cations (*e.g.* $[C_{12}C_1Im][NTf_2]$ **3a**, $[N_{1,1,1,16}][NTf_2]$ **4a**) could be exploited to solubilise the reagents and the sponge-like properties enhanced clean separation, either utilising a membrane [16], or with a combination of cooling and centrifugation [17]. They additionally afforded good recyclability (>99% yield for up to six reaction cycles) of the IL/biocatalyst system affording a potentially attractive and scalable process for industry, with excellent yields maximised by a longer alkyl chain length of the cation in combination with the $[NTf_2]^-$ anion **a**. High IL: H_2O weight ratios of 50% w/w (χ_{IL} = 0.031) appeared to maximise the activity of lipase through presumed protection against deactivation of the enzyme (Figure 2(c)) [16].

Protein fractionation and separation with ionic liquids are exemplified in a study separating ovalbumin from lysozyme, two of the major protein constituents of egg white [18]. Tetraalkylammonium chlorides and bromides were utilised for aqueous biphasic separations, with the aqueous component varying in pH. Increased aggregation of ovalbumin was seen at higher cation hydrophobicities, with best recovery for $[N_{2,2,2,2}]Cl$ **5g** for both lysozyme (99%) and ovalbumin (100%). Regeneration of lysozyme from the IL-rich phase was achieved with cold ethanol precipitation. A similar strategy, but utilising glycine−betaine analogue ionic liquids, was

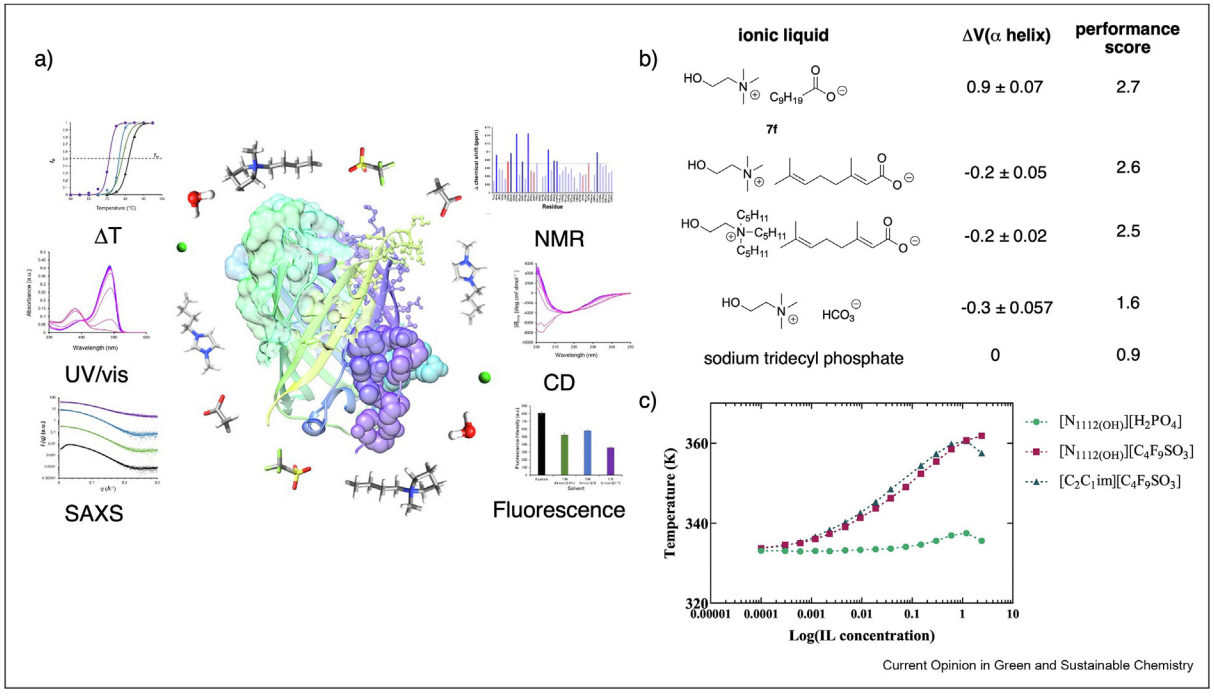
able to recover anti-interleukin-8 monoclonal antibodies and IgG from Chinese hamster ovary cell culture [19]. The IL was able to be recycled without loss in separation performance, and the process shows good potential for industrial purification of these high-value biopharmaceuticals (Figure 2(d)).

How does protein structure change in mixtures containing an ionic liquid?

The changing electrostatic and intermolecular interactions induced by ionic liquids necessarily have an impact on protein structure and dynamics, which themselves relate to changes in the function of the catalyst — both good and bad.

Elegant work by Hallett and coworkers has shown the importance of addressing protein-ionic liquid interactions by using a broad range of complementary techniques, with the emphasis on multi-technique approaches being required as a gold standard to analyse changes in protein structure [20]. A combination of UV—vis, circular dichroism (CD), fluorescence and NMR spectroscopies, and SAXS provide a holistic approach that avoids the pitfalls of using single methods. These methods were utilised to provide a comprehensive view of green fluorescent protein (GFP) interactions and structure changes in mixtures containing imidazolium (**1b**, **1d** and **1g**) and pyrrolidinium (**6b**, **6d** and **6g**) salts (Figure 3(a)). Native and modified avidin in mixtures containing cholinium-based ILs, studied using a combination of differential scanning calorimetry (DSC), differential scanning fluorimetry (DSF), dynamic light scattering (DLS), variable temperature CD, thermogravimetric analysis (TGA), and SAXS, indicated that rare conformations could be accessed and stabilised through changes in the protein energy landscape, and are dependent on the specific IL used [21]. A similar suite of analytical approaches was used to probe the structure of surface-modified avidin that was stabilised with a surfactant to produce a thermally stable biofluid [22]. Antibody formulations were also able to show similar exploration of their conformational landscape accelerated by the presence of IL, with variable-temperature CD and MD methods additionally combining to provide insight into the controlling thermodynamics [23]. The key take-home messages from these studies were that ionic liquid—protein interactions are highly complex, with specific knowledge of the binding location of individual ions with the protein needed to understand underpinning structural changes.

Figure 3



Changing protein structure with ionic liquids. (a) A range of techniques, including temperature dependence studies, UV/vis, SAXS, NMR, CD and fluorescence spectroscopy, are needed to fully understand IL-protein interactions (b) Change in proportion of α-helix in keratin and proposed performance score for transdermal delivery for a series of ionic liquids. (c) Melting temperature data for Bovine Serum Albumin in the presence of increasing concentrations of the ionic liquids shown. Reprinted from *Journal of Molecular Liquids*, **322**, M.M.S. Alves, J.M.M. Araújo, I.C. Martins, A.B. Pereiro, M. Archer, Insights into the interaction of Bovine Serum Albumin with Surface-Active Ionic Liquids in aqueous solution, 114,537 (2021) [30], with permission from Elsevier.

This molecular-level representation can be accessed as seen already through techniques such as NMR spectroscopy [11,24] and computation [11]. In particular, complementary interactions, which vary depending on protein-IL combinations, strengthened observed molecular confinement, impacting dynamics and conformational access of the proteins. Overall though, anion composition plays a significant role in dictating the nature of interactions, as has been seen in other studies.

Fourier Transform Infrared (FTIR) spectroscopy has been a powerful tool to understand changes in protein secondary structure, particularly keratin, within a biomatrix when assessing 31 ammonium-based ionic liquids in studies towards transdermal drug delivery [25]. A loss of α-helical content was identified as resulting in destabilisation and denaturation of the protein and was mitigated by unbranched anions such as decanoate **f**. A properties-derived 'performance score' for each ionic liquid, correlating well with Hansen solubility parameters for that ionic liquid, identified cholinium decanoate **7f** and the deep-eutectic variant cholinium geranate **7c** as preferred vectors for transdermal delivery (Figure 3(b)). The minimal impact on ovalbumin α-helices was also noted as a key factor in the development of adjuvants for SARS Cov-2 spike-protein vaccines with cholinium lactate **7e** solutions [26]. Similarly, cholinium-based ionic liquids have shown enhanced intestinal absorption of drugs without substantial mucosal layer disruption [27]. Sheer-thinning and reduced viscosity of the glycoprotein-rich mucus was observed in the presence of ionic liquids while maintaining the viscoelastic gel behaviour seen naturally. The topical application of nucleic acids was tested with six choline-based ILs, and the best transdermal transport was found with choline octanoate, with excellent stability of the nucleic acids [28]. A proposed mechanism from 200 ns MD simulations, supporting the use of ionic liquid **7c** in insulin delivery, indicated nanostructure formation with strong interaction of the geranate anion with surrounding waters, minimising disruption to insulin structure [29]. In combination, these works highlight the potential of ionic liquids to act as carriers of nanoparticles and other therapeutics across protein-containing barriers with minimal structural disruption.

Clear stabilisation of protein structure was also shown in the surface association of fluorinated ionic liquids **1o** and **7o** with bovine serum albumin [30], relative to other previously studied systems involving lysozyme. Assessment of key properties was achieved through a combination of DSF, nano-differential scanning fluorimetry (nanoDSF), DSC, CD, conductometry, and isothermal titration calorimetry (ITC) and is clearly seen in the melting temperature data shown in Figure 3(c). The maximum impact on thermostability was around 20–30 °C, relative to when cholinium dihydrogen phosphate was used. CD showed a relative increase in α-helical content (8%) on IL addition, with a concomitant decrease in β-sheet and random coil structure.

With a focus on membrane proteins, bacteriorhodopsin and its plasma-treated counterpart (to simulate oxidative damage) were used as models to investigate the impact of three tetrabutylammonium ILs **8k**, **8l** and **8o** using a combined experimental (UV–vis) and computational (MD) approach [31]. An absence of effect on the UV–vis spectrum in the presence of ionic liquid **8l**, compared with quenching seen on the addition of either salt **8k** or **8o** was interpreted as mesylate **8l** maintaining the structural stability of the native protein. This observation was supported through slightly lower average RMSD values, C_α-atomic positional fluctuations, and solvent accessible surface area values after 100 ns of MD simulation. None of the ILs prevented plasma-induced degradation of the protein. These studies, thus, point to the protein context being important in assessing anion effects on structure.

Changes in protein structure can induce aggregation, which is critical in disease processes and to be avoided in the preparation of protein-based medicines but can also be exploited. Cholinium tosylate **7p** generates fibrils of egg-white proteins, upon which cytochrome *c* could be immobilised with enhanced activity [32]. CD, FTIR and UV–vis spectroscopies confirmed a transition of α-secondary structure to increased β-sheet content, consistent with the changes expected to form fibrils and the involvement of hydrophobic and π-π interactions presumably through the interaction of the tosylate anion **p** with phenylalanine and tyrosine residues.

Related fibrillation has been extensively studied for the amyloid-β peptides implicated in Alzheimer's disease. Recent MD simulations in aqueous ethylammonium- **9** [33] and triethylammonium-based **10** [33,34] ILs supported experimental observations that amyloid-β fibrils can be destabilised with [TEA][H_2PO_4] **10j** and especially [TEA][OMs] **10l** by driving a reduction in β-sheet content mediated through the increased van der Waals interactions of the TEA cation **10** and affinity for mesylate anions **l**.

Avoiding protein–protein and protein-surface interactions

Tanner and coworkers exploited the tunability of ionic liquids to reduce protein interactions and absorption onto biomedical surfaces [35]. Here choline hexenoate acted as a biocompatible surface modifier that resisted protein adhesion under *in vitro* conditions, and indicated opportunities for selective drug delivery *in vivo*.

To investigate anti-fouling surfaces, Level *et al.* [36] utilised myoglobin as a probe to understand protein

interactions with 10–50 mM solutions of zwitterionic [$N_{n,n,n,PrSO3}$] bromides in detail. They surmised that no interaction was present with these species from the lack of change in the UV–vis spectrum at 409 nm, indicating these IL functionalities could be integrated into surfaces to prevent protein adhesion (Figure 4(a)).

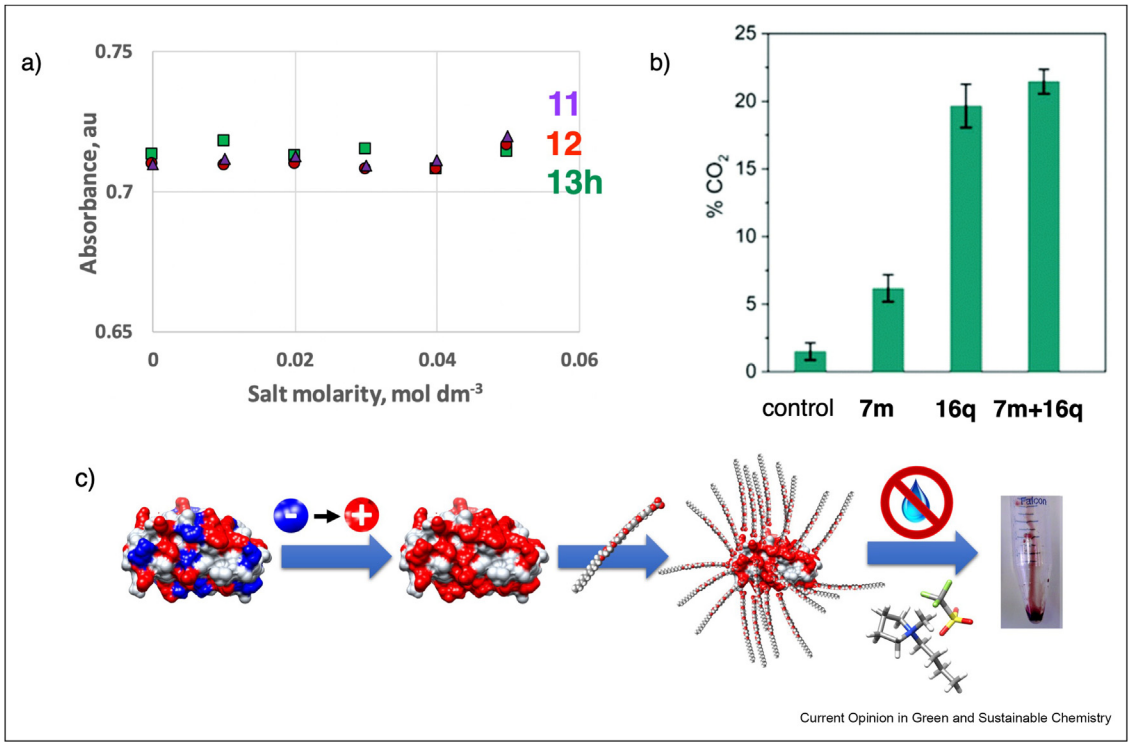
Reduction in protein–protein aggregation could also be achieved through forming tuned mixtures of ionic liquids [37]. Monitoring with UV–vis, CD, DLS and fluorescence spectroscopies established that the detrimental impact of cholinium iodide inducing aggregation of β-lactoglobulin was attenuated through binary mixing with cholinium salts of acetate, bitartrate, chloride and dihydrogen phosphate, enhancing protein stability. This tuning of protein interactions with ILs demonstrates how structural/functional behaviour might be systematically controlled for different outcomes.

Creating stable materials

Gels and nanocomposites comprising protein/peptide scaffolds and containing ionic liquids are being increasingly developed for a range of applications, including drug delivery [39,40], functional scaffolds [41,42], membranes [43], and bioprocess [38,44,45]. A focus on renewable sources has meant that materials such as packaging films for food with antibiotic and antioxidant properties could be produced from gelatin, the alkaloid and Chinese medicine matrine, and coconut acids (*e.g.* lauric acid, capric acid, caprylic acid, caproic acid, and other fatty acids) [46].

Chemical modification of the surface protein coat of filamentous bacteriophage M13 enabled it to be converted into thermally stabilised nanoconjugates compatible with ionic liquids [47]. Here, α-helicity, monitored by synchrotron radiation circular dichroism spectroscopy, increases significantly (25%–49%) on

Figure 4



Protein surface interactions and forming stable materials. (a) Stability of myoglobin in increasing concentration of zwitterionic liquids **11** and **12** and the bromide **13h**. Reprinted from *Journal of Colloids and Interfacial Science*, **562**, G. Level, J. Zhang, J. Brown, O. Hammond, B. Hannigan, L. Stella, P. Nockemann, M. Blesic, Multicharge zwitterionic molecules: Hydration, kosmotropicity and anti-fouling potential, 391–399 (2020) [36], with permission from Elsevier. (b) The effect of two ionic liquids on the absorption of carbon dioxide. Reproduce in part from X. Ji, Y. Xue, Z. Li, Y. Liu, L. Liu, P.K. Busk, L. Lange, Y. Huang, S. Zhang, Inozyme: ionic liquids as solvent and stabiliser for efficient bioactivation of CO_2, Green Chem. 23(18) (2021) 6990–7000 with permission from the Royal Society of Chemistry [38]. (c) A schema for the formation of stable liquid proteins. Protein is reacted at anionic centres to convert these to cationic centres; the cationised protein is then encased in anionic surfactant, water removed and material suspended in ionic liquid to generate the stable liquid form. Adapted from A.P.S. Brogan: Preparation and application of solvent-free liquid proteins with enhanced thermal and anhydrous stabilities, New J. Chem. 45(15) (2021) 6577–6585 [48]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

nanoconjugate formation; this level was maintained both once solvent-free (42%) and in the presence of selected pyrrolidinium-based ionic liquids (39–44%). These IL-stabilised materials exhibited thermal stabilities ~60–120 °C higher than the corresponding aqueous nanoconjugates. The role of ILs in protein nanoconjugate stabilisation was further indicated by the preparation of a surface-modified α-chymotrypsin conjugate, which could be solubilised in neat imidazolium-based IL [45]. The overall process has been generalised, as illustrated in Figure 4(c) [48]. Chemical modification of the outer-surface protein affords consistent electrostatic anchor points for a complementarily charged ionic surfactant. The resultant surfactant coat provides the main stabilising interaction, also limiting protein dynamics and unfolding, consistent with the improved stability against heat. A further advantage of the liquid form is that this can be induced with either a hydrophobic or hydrophilic ionic liquid, offering significant scope in mix-and-match systems. This approach shows good promise for similar modification strategies to avoid the cold chain and exploit anhydrous environments [48].

Of practical significance in potential climate change mitigation, an ionic liquid-stable formate dehydrogenase afforded efficient CO_2 conversion to formate when a nanoconjugate of this enzyme was created with a mixture of choline prolinate **7m** and tetramethylguanidinium phenolate **16q** [38]. Here synergistic intensification (Figure 4(b)) and activation of CO_2 were attributed to reaction at the anions to provide an enriched substrate environment for the enzyme. MD simulation suggested further activation of the enzyme from enhanced action of the NADH cofactor, affected by IL stabilisation of the protein structure.

Future perspectives

The rational understanding of protein-ionic liquid interactions, including in aqueous media, is improving, pushing forward the boundaries on using these systems for biocatalysis, and leading to a broader range of applications in the medical sector. This is supported by an increasing number of complementary techniques to examine complex, and particularly protic, systems (see, for example, the use of Far IR spectroscopy to understand hydrogen bonding [49]; new computational methods for protic ILs [50,51]). Upcoming advances in machine learning applied to ionic liquid properties will enable even more rapid *in silico* screening, optimising these goals further [52–55]. In addition, there is an increasing exploration of ionic liquids that are not just biocompatible but also able to be sourced from renewables, providing a trajectory towards fully bio-sourced materials and processes as needed to tackle both climate change and circular environments needed for off-planet living.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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