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Biomolecular structure, behavior, interactions and utilization within deep eutectic solvents

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REVIEW

The study and application of biomolecules in deep eutectic solvents

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Biomolecules have been thoroughly investigated in a multitude of solvents historically in order to accentuate or modulate their superlative properties in an array of applications. Ionic liquids have been extensively explored over the last two decades as potential replacements for tradition organic solvents, however, they are sometimes associated with a number of limitations primarily related to cost, convenience, accessibility, and/or sustainability. One potential solvent which is gaining considerable traction in recent years is the so-called *deep eutectic solvent* which holds a number of striking advantages, including biodegradability, inherently low toxicity, and a facile, low-cost, and solventless preparation from widely available natural feedstocks. In this review, we highlight recent progress and insights into biomolecular behavior within deep eutectic solvent-containing systems, including discussions of their demonstrated utility and prospects for the biostabilization of proteins and nucleic acids, free enzyme and whole-cell biocatalysis, various extraction processes (*e.g.*, aqueous biphasic systems, nanosupported separations), drug solubilization, lignocellulose biomass treatment, and targeted therapeutic drug delivery. All indications point to the likelihood that these emerging solvents have the capacity to satisfy the requirements of environmental responsibility while unlocking biomolecular proficiency in established biomedical and biotechnological pursuits as well as a number of academic and industrial ventures not yet explored.

1. Introduction

1.1 Introduction of DESs

The solvent environment of a biomolecule is critical to its optimal performance in various scenarios, including biocatalysis, drug delivery, therapeutics, and biosensing. Many researchers have implemented ionic liquids (ILs) as alternative solvent systems for biomolecules due to a range of advantages such as low vapor pressure, stability (*e.g.*, thermal, chemical, redox), non-flammability, and tailorability. While this review is not directly concerned with conventional ILs, it appears sensible, to briefly distinguish between an ionic liquid and its cousin, the deep eutectic solvent. Over the past two decades, ILs have become one of the most widely studied topics in science and engineering.[1, 2] A commonly accepted general definition of an IL is that of a fluid solely comprising ions that remains molten at temperatures below 100 °C. Besides their potential environmental impact, the cost associated with the synthesis, purification, recycle, and disposal of ILs has presented a barrier to more widespread use. Although ILs certainly display many fine qualities, it has been shown in certain settings that ILs can be prohibitively costly to synthesize and can also be toxic to cells and enzymes.[3, 4] In the light of these limitations, the recent emergence of deep eutectic solvents (DESs) is both significant and timely. Briefly, DESs are low-melting mixtures of Lewis or Brønsted acids and bases. Notably, these melts are related to ILs and exhibit many of their favorable features but are frequently based on nontoxic, inexpensive, readily available, and completely biodegradable natural compounds. Additionally, DESs can generally be prepared by simple mixing of the desired components in specific molar ratios,[5-14] usually with gentle heating in 50-100 °C range to encourage mixing (other methods of synthesis include the freeze-drying method[15, 16] or solventless mortar-and-pestle grinding of components, although these are less common). The purity of the starting materials defines the final DES purity and, in contrast to ILs (which produce stoichiometric waste during metathesis), DES preparation is essentially 100% atom efficient. With these decisive benefits, DESs have emerged as a highly promising class of green solvent, which retains many of the versatile properties displayed by ILs, while offering additional advantages in terms of cost and sustainability.

The DESs most pertinent to biomolecular studies are “type III” DESs consisting of a hydrogen bond acceptor (HBA; typically, a quaternary ammonium salt like choline chloride (ChCl), choline acetate, or ethylammonium chloride) and a hydrogen bond donor (HBD; such as an amine, amide, carboxylic acid, sugar or other polyol). The molar ratio of the individual components is very important, and, at a particular eutectic ratio, a number of molecules of the HBD (typically, 1–4) binds per salt (ion pair) to form a liquid mixture at a temperature well

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below the melting points of the individual constituents. DESs can be prepared that are more hydrophilic than conventional ILs and most are water miscible. The conception of DESs was first introduced by Abbott *et al.* in 2003 where ChCl (melting point 302 °C) was mixed with urea (melting point 133 °C) to form a DES with a reported melting point of 12 °C.[12] The charge delocalization arising from hydrogen bonding between the halide anion and the HBD moiety (in this case, urea) was assigned as responsible for the decreased melting point observed for the mixture relative to the melting points of the individual components. In terms of nomenclature, DESs are typically classified into four groups, as summarized in Table 1. DESs can be described by the basic formula Cat^+X^-zY, in which Cat^+ represents the cation of various sulfonium, ammonium, or phosphonium salts, X^- is the halide (typically) anion of the salt, Y is a Lewis or Brønsted acid, and z is the number of molecules of Y. Among the four types of DESs listed, type III is the most encountered in the literature because of their simple preparation, low cost, nonreactivity with water, and biocompatibility. To date, the most widely used HBA is choline chloride (ChCl) because it is cheap, extensively available, biodegradable, and presents low toxicity (*e.g.*, it is used in animal feed; choline itself is present in foodstuffs as phosphatidylcholine). Currently, the most popularly studied DESs comprise choline chloride mixed with a two-fold molar equivalent of the HBD species urea, ethylene glycol, or glycerol to produce reline, ethaline, or glyceline, respectively, as shown in Fig. 1A. Examples of the depressed melting points observed for representative binary mixtures between choline chloride and HBD species are provided in Fig. 1B using reported DES melting points taken from the literature.[6]

Table 1. General formulas for the classification of DESs adapted with permission from reference[17].

Type	General formula	Terms
Type I	$Cat^+X^-zMCl_x$	M = Zn, Sn, Fe, Al, Ga, In
Type II	$Cat^+X^-zMCl_x \cdot yH_2O$	M = Cr, Co, Cu, Ni, Fe
Type III	Cat^+X^-zRZ	Z = $CONH_2$, COOH, OH
Type IV	$MCl_x + RZ = MCl_{x-1}^+ \cdot RZ + MCl_{x+1}^-$	M = Al, Zn, and Z = $CONH_2$, OH

Due to their environmentally-responsible qualities as well as their tailorable physicochemical properties, there is considerable interest in applying DESs in a number of important areas. Not surprisingly, the number of publications focused on DES synthesis, properties, and applications has grown exponentially since their emergence.[17-19] DESs have already been explored as media or co-solvents for organic synthesis, electrochemistry (*e.g.*, electrodeposition of metal films), polymers, chemical analysis (*e.g.*, detection, chromatography), extractions, separations (*e.g.*, CO_2 and SO_2 capture) and nanotechnology, in addition to biotechnological utilization in biocatalysis, lignocellulose processing, and pharmaceutical and biomedical application examples, a number of which will be summarized herein.

Natural DESs (NADESs) were distinguished for the first time by Choi *et al.* in 2011 as eutectic solvents being composed of primary plant metabolites (*e.g.*, sugars, carboxylic acids, amino acids) that appear in living tissues and play an important role in cellular processes.[20] NADESs were introduced into analytical practice as an alternative type of extraction solvent. Besides the many advantages of DESs, some have argued that NADESs are even more environmentally friendly due to their natural origin. The genesis of this notion is that NADESs may occur in all organisms and are thought to be involved in the biosynthesis, solubilization, and storage of hydrophobic metabolites and unstable compounds in living cells. Given the growing interest in NADESs for extractive processes, biotransformations, nucleic acid manipulation, biocatalysis, pharmaceuticals and therapeutics, it is important to include them in this review, however, it should be noted that they fall well within the purview of conventional DESs in all respects.

1.2 Scope of Review

Numerous reviews on DESs, summarizing their synthesis, toxicity profiles, physicochemical properties, and various applications,[17, 18] have appeared elsewhere and the interested reader is referred to such reviews throughout, as these topic will not be covered explicitly here. Instead, this review is focused on the implementation of DESs as an emerging and alternate solvent system for biomolecular applications, including aspects of protein thermostabilization and extraction (*e.g.*, aqueous biphasic systems) and the role of DESs in biocatalytic systems, such as those involving lipases or whole cells. Including in the current review is a discussion of DESs for the modulation of nucleic acid systems, drug solubilization and delivery, and emerging biomedical applications (*e.g.*, therapeutic deep eutectic solvents). Overall, the intent of this review is to elucidate some of the advantages of implementing DESs in a multitude of biomolecule-driven purposes and illustrate the versatility and potential of these unique, environmentally-responsible solvents for next-generation biotechnological and biomedical applications.

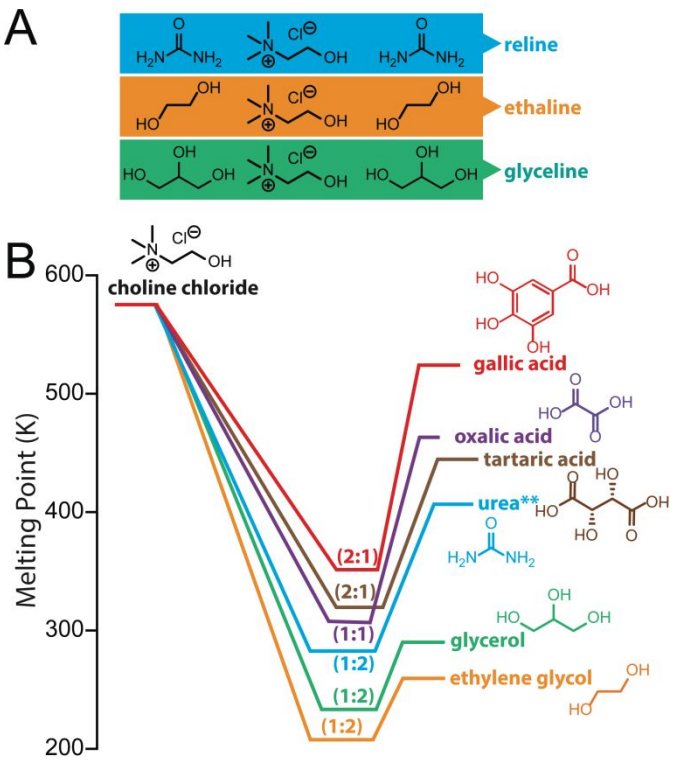

Fig. 1 (A) The "notorious three" DESs (*i.e.*, reline, ethaline, glyceline) currently responsible for the bulk of the research activity in the area. (B) Diagram of select binary combinations of choline chloride with various hydrogen bond donor species illustrating the melting points of the constituents compared with the eutectic formed at the stoichiometry indicated. **It is noteworthy that the trend shown for 1:2 choline chloride:urea coincides nearly exactly with that of the 1:1 choline chloride:malonic acid system.

2. Proteins in deep eutectic solvents

2.1 Model protein folding studies and thermostability

Proteins behave differently depending on the solvent in which they reside, which makes solvent environment of utmost importance. For many years, the use of enzymes was restricted to dilute aqueous solutions; however, the use of organic solvents became increasingly more popular which widened the field to many more applications.[21, 22] Even though organic solvents have played a major role in protein folding studies, there have been numerous disadvantages of these solvents showing toxicity, instability, degradation, and inactivation of some proteins. Therefore, considerable attention has been focused on developing novel solvents, including the use of ILs, and, although some of these ILs show great enhancement in protein thermal stability, some have been proven to be protein denaturants and expensive to synthesize. DESs have become increasingly popular in protein stability studies to further enhance their thermostability, while also providing a more biofriendly solvent environment.

Esquembre *et al.* were among the first groups to study the thermal unfolding and refolding of hen egg white lysozyme in neat DESs consisting of ChCl:urea and ChCl:glycerol, both employing a 1:2 molar ratio.[23] Thermal treatment of the protein to 80 °C resulted in stabilization when ChCl:glycerol was used, while ChCl:urea effectively destabilized lysozyme as confirmed by fluorescence spectroscopy and circular dichroism (CD) measurements, as can be seen in Fig. 2.

Small-angle neutron scattering (SANS) has also been implemented to investigate any conformational changes seen in lysozyme as well as bovine serum albumin (BSA) when neat and hydrated solutions of ChCl:glycerol and ChCl:urea were used as solvents. [24] In neat ChCl:urea, the maximum dimension of the scatterer (D_{max}), an indicator of protein conformation, was similar to the protein in ChCl:glycerol, although it displayed a lower intensity. The D_{max} values for lysozyme in neat ChCl:glycerol and neat ChCl:urea were D_{max} = 57.3 ± 1.1 Å and 58.0 ± 0.8 Å, respectively. This result was interesting because urea has been known to denature proteins, but according to the SANS data, lysozyme retained its structure in a solvent with high urea content. In comparison with data presented from Esquembre *et al.*[23], a decrease in protein activity was seen with increasing DES concentration. Therefore, although the structure of the protein might remain the same in high urea concentrations, the activity could potentially be reduced. Additionally, although the protein structure remained intact, higher ionic strength and slower diffusion (due to increased DES viscosity) could lead to decreased protein activity.

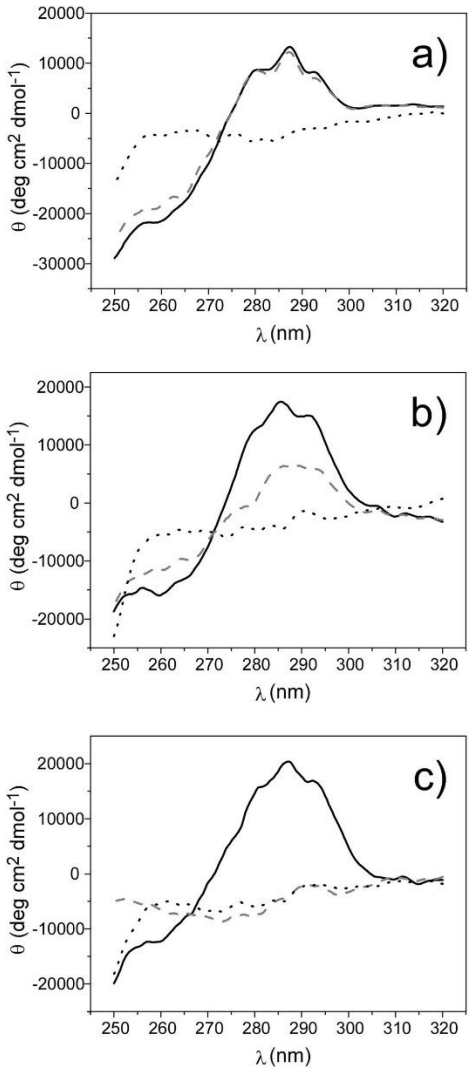

Fig. 2 Near UV CD spectra of lysozyme dissolved in MES buffer solution (a), neat ChCl:glycerol (b), and neat ChCl:urea (c), at 20 °C before thermal treatment (solid line), at 80 °C (dotted line), and at 20 °C after cooling (dashed grey line). Reprinted with permission from reference[23]. Copyright © 2012 Royal Society of Chemistry.

Furthermore, by replacing any water naturally available around the active site of the protein with DES, activity can be further reduced due to specific binding and decreasing any solvophobic effect. CD results showed that lysozyme in the DES/water mixtures (50 and 75 wt% DES) retained its conformation, indicative of protein stabilization.

It was also determined that SANS data for BSA in DES/water mixtures with 50 and 75 wt% DES (D_{max} = 94 ± 5 Å, 98 ± 3 Å, respectively), agreed well with results from BSA in PBS (D_{max} = 93 ± 3 Å). However, for BSA in neat ChCl:glycerol, the D_{max} was larger and slightly red shifted but still shorter than the fully denatured protein (D_{max} = 131 ± 4 Å in neat DES *vs.* 314 ± 12 Å for denatured BSA). As a result of this, it was concluded that the protein in neat DES showed a partially folded structure, whereas the addition of water allowed the protein to fold into a more efficient globular shape. Overall, when water was incorporated alongside a DES, it ultimately formed a shell around the active sites of the protein, most likely due to hydrogen bonding, allowing for better protein folding.

Xin *et al.* have performed differential scanning calorimetry (DSC) measurements from 30 °C-90 °C to determine the thermostability of lysozyme in 25, 50, and 75 wt% ChCl:trehalose (3:1 molar ratio). The transition temperature, T_m, for lysozyme in water was 70.2 °C, while the protein in 25 and 50 wt% DES showed increased transition temperatures of 74.4 °C and 80.6 °C, respectively, indicating that these DESs increased protein stability.[25] The thermal stability of lysozyme was further increased in 75 wt% ChCl:trehalose due to the disappearance of any denaturing peak in the DSC spectrum. Upon thermal treatment to 90 °C, CD measurements displayed an intact tertiary structure and complete folding/unfolding reversibility in 75 wt% DES, and partial reversibility in 25 and 50 wt% DES for the protein. Additionally, the individual DES components were studied alongside lysozyme and agreed with previous literature reports that have stated that the supramolecular complexes that form DESs begin to break down when DES concentrations are less than 50 wt% resulting in DES solutions that have basically become solutions of their individual components, thus losing some of their unique properties.[5, 15, 16, 26] In general, when the concentration of a DES is above 50 wt%, the hydrogen bonding between the hydrogen bond acceptors and donors will remain intact. Since trehalose provides many areas for hydrogen bonding, this DES allowed for increased protein stabilization.

Fibrillar formation in hen egg white lysozyme has been successfully studied by Silva *et al.* who used ChCl:acetic acid (1:1 molar ratio) with lysozyme along with HCl and glycine at 70 °C to create fibrils, which were then indirectly measured through fluorescence spectroscopy.[27] Higher temperatures and low pH (around 2) favoured the unfolding of lysozyme; once cooled back down, it refolded into a β-sheet configuration which led to the formation of protein nanofibers. Thioflavin T is a cationic benzothiazole dye that binds to these β-sheet amyloid structures, therefore, an increase in β-sheets led to an increase in fluorescence intensity. Fluorescence spectra showed that solutions made with 5% (v/v) DES displayed the highest fluorescence intensity (out of 1, 5, and 10% v/v DES) and fibrillar formation did not occur without the aid of the DES. Additionally, the use of ChCl alone produced high fluorescence intensity, similar to aqueous 5% (v/v) DES; however, no increase in fluorescence intensity was seen when acetic acid alone was used. Therefore, it was determined that ChCl played the major role in fibril formation, although an enhanced fluorescence was seen when the DES was used. Overall, after thermal treatment, more than 90% of the native lysozyme misfolded upon cooling and arranged themselves into nanofibers.

DESs have also been employed as solvent environments for proteins other than lysozyme and BSA. The activity and stability of horseradish peroxidase (HRP) have been investigated with various DESs, with a consensus that, although an increase in DES concentration can negatively affect the activity, the stability of the protein will be enhanced. Wu *et al.* showed that the overall activity of HRP was higher in aqueous ChCl-based DESs over DESs that used choline acetate (ChAc) as the HBA.[28] Although the protein activity was favoured in ChCl-based DESs over ChAc-based DESs, the protein stability was increased with ChAc-based

DESs. Similar activity results have been seen with Sanchez-Leija *et al.* who determined that the catalytic activity of HRP was higher in 80% ChCl:glycerol over 80% ChCl:urea (both at 1:2 molar ratio), however, the activity decreased in both systems with increasing DES concentration. [29]

Papadopoulou *et al.* later determined that ethylammonium chloride (EAC) as a HBA, along with the same HBDs of urea, glycerol, and ethylene glycol, showed enhanced HRP activity over ChCl-based DES when used for the oxidation of guaiacol. [30] It was also determined that EAC-based DESs led to higher cytochrome c peroxidase activity over ChCl-based DESs. For instance, 30% (v/v) ChCl:urea (1:2) resulted in an 8-fold peroxidase activity enhancement, while 30% (v/v) EAC:urea (1:1.5 molar ratio) displayed almost 100-fold activity enhancement. The increased peroxidase activity of cytochrome c in EAC-based DESs could be due to different interactions between the protein and salt as well as the changes in viscosities, since ChCl-based DESs tend to be more viscous, potentially increasing mass transfer limitations. Interestingly, the use of urea resulted in a relatively higher activation of cytochrome c as compared to glycerol and ethylene glycol, regardless of which salt was used. This result contradicts previous reports that claim that urea is a protein denaturant. The heme group in this protein is somewhat responsible for catalysing peroxidation reactions and when the protein was in the urea-containing DES, this heme group potentially became exposed, which could allude to the increased activity observed.[31] The CD spectra displayed similar results, showing that the protein in 30% (v/v) EAC- or ChCl-based DES underwent an orientation in the active site, allowing the heme active center of cytochrome c to be more accessible, which led to increased peroxidase activity.

Most recently, Kist *et al.* studied the thermostability of bovine ribonuclease A (RNase A) in DESs using DSC.[32] RNase A, although widely studied with ILs, has not been rigorously investigated with DESs;, therefore, ChCl:urea, ChCl:ethylene glycol, and ChCl:glycerol (all in 1:2 molar ratio) ranging from 5-35 wt% were studied alongside the protein. Using DSC, iterative thermal cycles from 10-80 °C showed that enhanced thermal stability of RNase A was achieved in all concentrations of ChCl:glycerol and ChCl:ethylene glycol as compared to the protein in water. However, RNase A showed complete denaturation in solutions above 5 wt% ChCl:urea after only one thermal cycle. The transition temperatures (T_m), as can be seen in Fig. 3, steadily increased with increasing ChCl:glycerol (glyceline) concentration, yielding a T_m = 63.0 °C at 35 wt% ChCl:glycerol, as compared to a T_m = 59.7 °C for the protein in water. The transition temperature was fairly constant for ChCl:ethylene glycol (ethaline) regardless of concentration; however, the transition temperature for the protein in ChCl:urea (reline) continually decreased from T_m = 57.0 °C at 5 wt% to 51.8 °C in 35 wt%. The IL 1-butyl-3-methylimidazolium chloride ([bmim]Cl) was also investigated with RNase A for comparison purposes, to show the strong denaturing ability of that solvent on the protein.

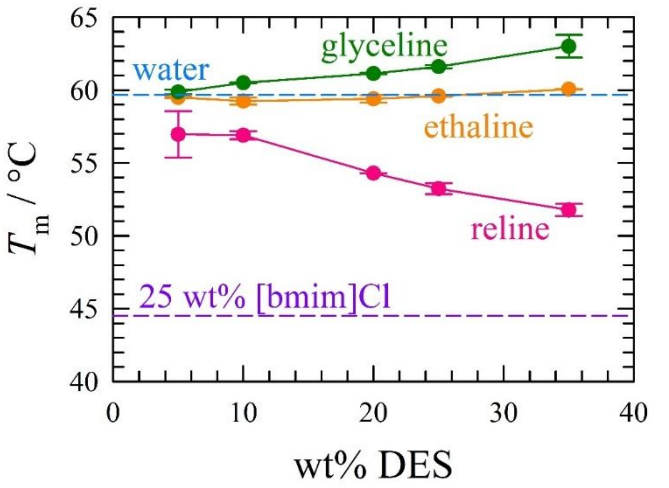

Fig. 3 The transition temperature, T_m, versus wt% DES. The ionic liquid [bmim]Cl shows a much lower T_m, confirming its reputation as a protein denaturant. The T_m for RNAse A in water is shown for reference. Reprinted with permission from reference[32]. Copyright © 2019 American Chemical Society.

DESs have also been used to increase stability of therapeutic proteins. Lee *et al.* evaluated NADES as a stabilizing medium for the therapeutic protein, human interferon-α2 (IFN-α2), which is used clinically to treat hepatitis B and C, leukemia, and AIDS-related Kaposi's sarcoma.[33] DESs composed of ChCl:fructose, ChCl:citric acid, and ChCl:malic acid all in 1:1 molar ratio, were used to evaluate their capability of thermally stabilizing IFN-α2 during short- and long-term storage. Interestingly, the DES-protein formulations were synthesized by the freeze-drying method which involved the weighed DES components mixed together along with deionized water, after which the protein was incorporated. The resulting formulation was then freeze-dried to ensure that the protein integrity remained intact. Among the DESs tested, ChCl:fructose was the only one that showed no detrimental effect on the IFN-α2 activity as compared to the other two DESs, that drastically decreased IFN-α2 activity. Regarding the short-term storage, the activity of IFN-α2 in PBS buffer was greatly reduced after 30 minutes at 70 °C and CD results showed loss of secondary structure and complete unfolding of the protein. However, when IFN-α2 was incorporated with ChCl:fructose, the activity remained consistent after 120 minutes at 70 °C and no structural changes were seen after thermal treatment as confirmed by CD and fluorescence spectroscopy. When 30% or more water was added to the DES-protein system, conformational changes were seen in CD spectra as well as an increase in fluorescence, which was indicative of IFN-α2 inactivation. No conformational changes were seen in the proteins' structure when the protein was stored for 90 days at 37 °C, showing that the DES of ChCl:fructose can be a suitable solvent for the storage of a therapeutic protein.

Overall, DESs can provide excellent thermostability for proteins, depending on the hydrogen bond acceptors and donors implemented while also providing new and more environmentally friendly alternatives to aqueous or organic

solvents. Additionally, these solvents may help to give more insight on protein activity and behaviour in extreme environments (*e.g.* cryogenic temperatures or total absence of water) that can further advance the information gained in this field.

2.2 Protein solubilization and extraction

2.2.1 Aqueous biphasic systems (ABS)

There has been great interest in isolating and extracting pure proteins, however, the main challenge is to maintain the proteins' structure and function after extraction. Traditional methods for protein extraction and purification include ammonium sulfate precipitation, electrophoresis, ion exchange, and affinity chromatography; however, they typically involve organic solvents which have proven to be inefficient due to high costs and low yields.[34, 35] More recently, aqueous biphasic (or two-phase) systems (ABS) have been proposed as an alternative and environmentally friendly extraction method with the use of ILs.[36] Due to the limitations of ILs mentioned in the introduction section, researchers have turned their attention to implementing DESs into these novel and efficient protein extraction systems. Fig. 4 shows a generalized scheme for DES-based ABSs for extracting proteins. In a typical ABS set up, once various parameters have been optimized, an appropriate amount of DES is added to a centrifuge tube. Afterwards, a salt solution (typically K_2HPO_4) is added followed immediately by the protein of interest. The ABS system is then shaken for an appropriate amount of time, resulting in two separate distinguishable phases to form during which the protein of interest preferentially resides in the DES-rich top layer, allowing for facile extraction. Once the protein has been extracted, the concentration is determined by measuring the absorbance and then the extraction efficiency can be calculated using the following equation:

$$E = C_t V_t / (C_t V_t + C_b V_b) \quad (1)$$

Where C_t and C_b are the concentrations of the proteins in the DES-rich top phase and salt-rich bottom phase, respectively, and V_t and V_b are the volumes of the top phase and bottom phase, respectively. Generally, the following parameters are investigated to yield the optimal extraction efficiency per mass of DES used, concentration of salt solution, separation/shaking time, and temperature. Zeng *et al.* were among the first groups to extract proteins using DESs in aqueous biphasic systems.[37] BSA and trypsin were easily extracted with ChCl:urea (1:2), yielding extraction efficiencies of 99.94% and 95.53%, respectively. The extraction efficiencies were also high for BSA in tetramethylammonium chloride (TMACl):urea (1:2) and trypsin in ChCl:methylurea (1:2), yielding 91.69% and 95.27%, respectively. Interestingly, when the protein ovalbumin was studied, the extraction efficiencies were ≤50% in all DESs investigated.

Trypsin and BSA have also shown high extraction efficiencies when ChCl:glycerol (1:2) has been implemented into an ABS, yielding 94.36% and 98.16%, respectively. [38] Additionally, Xu *et al.* have analysed a mixed sample of BSA and bovine hemoglobin (BHb) in ChCl:glycerol resulting in extraction efficiencies of 96.19% and 62.09%, respectively.

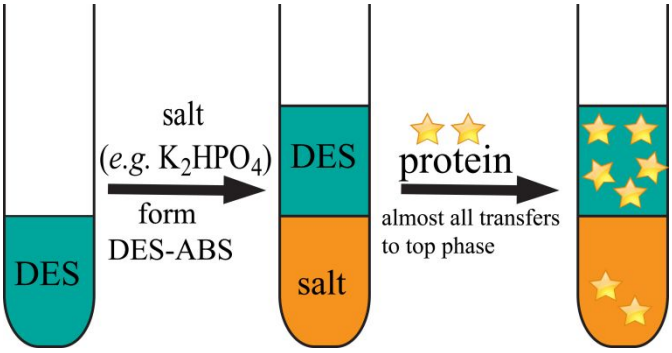

Fig. 4 General schematic of the extraction process of proteins using an aqueous biphasic system implementing deep eutectic solvents. Adapted with permission from references [37,38,39]. Copyright © 2014 Royal Society of Chemistry; 2015, 2016 Elsevier.

When the DES of betaine:urea:H_2O (1:2:1) was used in an aqueous biphasic system, Li *et al.* were able to successfully extract and separate BSA and BHb from a mixed sample with extraction efficiencies of 96.28% and 91.26%, respectively, showing a 30% improvement for BHb extraction, most likely due to increased hydrogen bonding in the DES. [39] Similar results have reported that higher extraction efficiencies can be found with betaine-containing DESs as compared to ChCl-containing DESs for these biphasic systems,[40] although this area of research is still in its infancy. Typically K_2HPO_4 is used as the aqueous salt-rich phase due to its high solubility and strong phase-forming ability in water, however, Pang *et al.* synthesized an ABS using Na_2CO_3 as the aqueous salt-rich phase and ChCl:polyethylene glycol 2000 (ChCl:PEG; 20:1 molar ratio) as the DES-rich phase to extract BSA and papain separately, yielding extraction efficiencies of 95.16% and 90.95%, respectively, after parameter optimization.[41]

Another interesting approach to these aqueous biphasic systems has been to implement ternary DESs. Zhang *et al.* compared binary and ternary DESs in ABSs for the extraction of BSA.[42] The ternary DES composed of tetramethylammonium chloride (TMAC):glycerol:urea (1:1:1) was able to successfully extract BSA with an efficiency of 98.95%, comparing well with the extraction efficiencies of the binary DES of TMAC:urea (1:2), which gave results of 99.31%. Interestingly, the ternary DES was able to achieve a higher back extraction of protein (71.89%) as compared to the binary DES which only yielded 21.02%. Lastly, these biphasic systems have been synthesized with two DESs, specifically, tetrabutylammonium chloride:polypropylene glycol 400 (TBAC:PPG400; 1:2 molar ratio) and L-proline:xylitol (1:1 molar ratio), the latter of which replaced the salt-rich phase, which allowed chymotrypsin to preferentially reside.[43] This DES/DES ABS gave an overall extraction efficiency of 97.30% for chymotrypsin. Overall, the results discussed herein show that DESs can provide a stable environment for proteins in these aqueous biphasic systems.

2.3 Protein extraction using magnetic NPs and DESs

2.3.1 Magnetic composites for solid-phase extraction of proteins

Graphene oxide (GO) has been used in magnetic solid-phase extraction processes over the years due to its large number of carboxyl and hydroxyl groups. These functional groups can form hydrogen bonds or have electrostatic interactions with various adsorbates containing oxygen- and nitrogen-functionalized groups. Yuzhi Wang's research group have loaded magnetic Fe_3O_4 nanoparticles (NPs), onto GO alongside DESs to increase the hydrophilicity and enhance the extraction efficiency of proteins. Three similar yet somewhat different schemes are shown in Fig. 5 to represent the general set up and procedure for protein extraction using this magnetic solid-phase extraction technique. As discussed in the ABS section, various parameters need to be optimized in order to achieve the best extraction capability, including temperature, extraction time, protein concentration, and amount of magnetic GO/DES used.

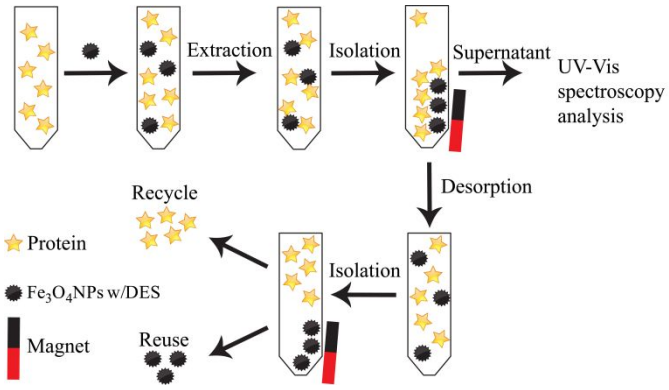

Fig. 5 General protein extraction process using magnetic iron oxide nanoparticles with deep eutectic solvents. Adapted with permission from references [44-46]. Copyright © 2015, 2016 Elsevier.

In the first study, a DES consisting of ChCl:glucose (1:1) was coated onto the surface of Fe_3O_4@GO NPs to aid in extracting BSA.[44] Once the protein was extracted, the Fe_3O_4@GO-DES NPs were recovered using an external magnetic and the concentration of the protein was determined via UV-Vis. The Fe_3O_4@GO-DES NPs were able to be fully recovered and therefore could be used again for subsequent extractions. The extraction amount (Q) was calculated by the following equation:

$$Q = (C_0\text{-}C)V / m \qquad (2)$$

where Q (mg g^{-1}) is the mass of protein adsorbed onto a unit amount of Fe_3O_4@GO-DES NPs, C_0 and C (mg mL^{-1}) are the initial and final concentrations of protein in the solution, V (mL) is the volume of the initial solution and m (mg) is the mass of the magnetic DES-NP substituent.

Interestingly, after the incorporation of DES onto the Fe_3O_4@GO NPs, the surface charge of the material changed from negatively charged (Fe_3O_4@GO NP zeta potential = –10.6 mV) to positively charged (Fe_3O_4@GO-DES NP zeta potential = 19.4 mV), showing that the addition of DES could selectively extract acidic proteins over neutral and basic proteins. Three consecutive extraction-desorption cycles were performed although a significant decline in the amount of protein extracted occurred after each cycle: 73.1 mg g^{-1} for the first cycle, 34.0 mg g^{-1} after the second cycle, and 20.9 mg g^{-1} after the third cycle. When Fe_3O_4@GO NPs were used without any DES, the amount of protein that could be extracted was only 27.4 mg g^{-1}. In a subsequent report, it was determined that the DES of ChCl:glycerol (1:1) could be successfully coated onto a surface of Fe_3O_4-NH_2@GO NPs to yield an extraction capability of 44.59 mg g^{-1} for BSA.[45] Interestingly, when ChCl:glycerol was coated onto the Fe_3O_4@GO NPs, the extraction capability was only 38.0 mg g^{-1}, showing that each system is able to provide varying degrees of extraction capabilities depending on the magnetic NPs implemented.

In a final method of extracting proteins using the magnetic solid-phase extraction technique, Fe_3O_4 NPs have been coated with tetraethyl silicate (TEOS) and further modified with 3-(trimethoxysilyl)-propyl methacrylate (γ-MPS), to make microspheres.[46] The DES ChCl:itaconic acid (1:1) was then coated onto the Fe_3O_4@SiO_2-MPS microspheres, which were then used to test the capability of extracting trypsin. Interestingly, this system could perform the extraction-desorption process with only a slight loss in the extraction capacity over six cycles, which was better than the aforementioned systems. The extraction capability of trypsin in the magnetic microsphere-DES system was 287.5 mg g^{-1}, which was two times higher than that of typical magnetic polymers (75.9-140.65 mg g^{-1}). Overall, the magnetic solid-phase protein extraction technique has been shown to be enhanced when DESs have been coated onto magnetic nanoparticles or microspheres.

2.4 Protein extraction using molecularly imprinted polymers (MIPs)

Molecular imprinting technology is very similar to the previously discussed extraction techniques although template molecules (typically the protein of interest) are incorporated into the system and once they are removed, they create cavities that can specifically adsorb the protein and successfully isolate it. Typically, for these systems, the magnetic provider has been shown to be Fe_3O_4 NPs, alongside a crosslinker and DES. The DESs have been incorporated into these systems as functional monomers that can help increase the adsorption capacity of the protein of interest. The amount of DES is critical because if not enough is added, less than optimal interactions between the DES and protein occur; however, too much DES results in a thick imprinting layer which hinders the adsorption capacity of the template molecule. The adsorption capacity, as can be seen in equation 2, is then calculated and an imprinting factor (IF) can be determined to evaluate the specific recognition ability of the magnetic DES-MIPs system, which is defined as:

$$IF = Q_{MIP} / Q_{NIP} \qquad (3)$$

where Q_{MIP} and Q_{NIP} are the adsorption capacity of the protein on the magnetic DES-MIPs and the protein on the magnetic non-imprinted polymers (DES-NIPs), respectively. BHb has been successfully isolated in MIPs using various DESs. For example, Liu *et al.* used ChCl:methacrylic acid (1:2) as the functional monomer in MIPs to yield an adsorption capacity of 175.44 mg g^{-1} and an IF of 4.77.[47] This adsorption capacity was almost four times higher than the adsorption capacity of the non-imprinted polymers, which was 39.59 mg g^{-1}. Similar results were seen when the DES (3-acrylamidopropyl) trimethylammonium chloride:urea (1:2) was used in MIPs, yielding an adsorption capacity for BHb of 164.20 mg g^{-1} and an IF of 4.93.[48] In another report, the protein transferrin was able to be isolated when ChCl:acrylic acid (1:2) was used, yielding an adsorption capacity of 37.5 mg g^{-1} and an IF of 3.50.[49] Interestingly, this MIPs system was also able to isolate BHb, with an adsorption capacity of ~14 mg g^{-1}. BHb and transferrin have similar configurations and sizes, therefore, BHb could be similar enough to successfully occupy the imprinting cavities. Overall, MIPs including DESs have shown to provide a biocompatible method for isolating and purifying proteins.

3. Nucleic acids in deep eutectic solvents

Known as a genetic information-carrying macromolecule, deoxyribonucleic acid (DNA) consists of repeating units called nucleotides attached to the negatively charged phosphate backbone via sugar molecules. Earlier studies have suggested that ions and ionic solvents (e.g. ionic liquids and DES) have multi-level interactions with DNA molecules including electrostatic attraction between organic cations and the phosphate backbone, hydrophobic and polar interactions of ionic solvents with DNA major and minor grooves, and possible anion interactions with bases through hydrogen bonds.[50, 51] Despite their structural differences, DES have many similar properties with ionic liquids. The stability of duplex DNA in DES has been examined by several groups. The Hud group[52] visualized the secondary structures of DNA and RNA in neat ChCl:urea (1:2) by CD spectroscopy. They made several interesting findings: (a) An A-form duplex can be seen in DES for the 32 bp mixed-sequence DNA while B-form helix was observed in aqueous salt solutions (such as 3.7 *m* NaCl); (b) A left-handed Z-form helix was found in DES and aqueous solutions of ChCl (3.7 *m*) and NaCl (3.7 *m*) for oligonucleotide $[d(CG)_8]_2$; (c) A similar B-form helix was formed in DES as in aqueous solutions for oligonucleotide $[d(AT)_{16}]_2$; (d) $[d(A_4T_4)_4]_2$ yielded an altered B-form helical structure (B* form). However, these DNA molecules exhibited lower duplex stability under dehydrating and high ionic strength conditions as suggested by their lower melting transition midpoints (T_M) in DES. Interestingly, certain triplex and G-quadruplex structures were observed in DES although these structures are different from those in aqueous solutions. The same group[53] further reported that neat ChCl:glycerol (1:4) enabled the folding of a two-dimensional DNA origami at 20 °C in 144 h while the folding completed in 3 h in hydrated ChCl:glycerol (1:4; 90%) (see Fig. 6). Furthermore, the hydrated ionic solvent allows for the folding of a three-dimensional DNA origami and a DNA tail system. More interestingly, folded DNA structures are transferrable between aqueous solvent and ChCl:glycerol (1:4).

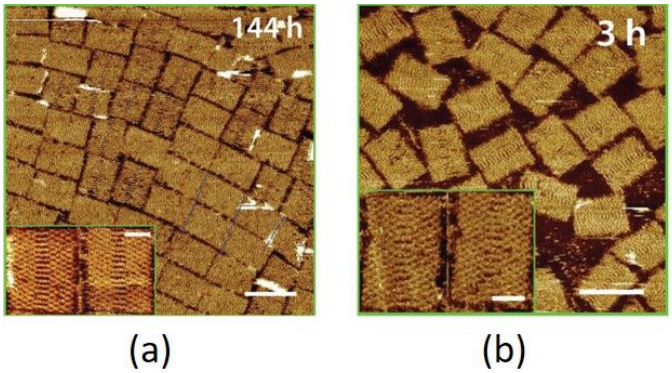

Fig. 6 AFM images illustrating the folding of DNA origami structures: (a) in anhydrous choline:glycerol (1:4) at 20 °C for 144 h; b) in (hydrated) 75% choline:glycerol (1:4) at 20 °C for 3 h. Reprinted with permission from reference[53]. Copyright © 2015 Wiley.

The Prasad group[54] dissolved up to 5.5 wt% and 2.5 wt% salmon testes DNA in two DES (ChCl:ethylene glycol and ChCl:glycerol, both at 1:2 molar ratios) respectively; they confirmed B-form helical structures in DES and suggested a high thermal and pH stability for the regenerated DNA. The Zhao group[55] reported that salmon testes DNA molecules preserve their characteristic B-form helical duplex structures in 1.0 M ChCl:glycerol (1:2) as confirmed by CD spectra; in addition, the use of 0.2 M ChCl:glycerol (1:2) enabled a high activity of DNA-based hybrid catalyst for the Michael addition, resulting in a high enantioselectivity (94.0%) and a high yield (88%) at room temperature for 24 h. The Kohler group[56] evaluated the excited-state deactivation in $d(AT)_9 \cdot d(AT)_9$ duplex DNA strands dissolved in deuterated ChCl:ethylene glycol (1:2) by femtosecond time-resolved IR spectroscopy. Their data confirmed the existence of long-lived exciplex forms exclusively in the solvated duplex, but not in the denatured single strands. In addition, DES enables a longer lifetime of the exciplex state than the aqueous solution, which is attributed to reduced stabilization of the charge transfer state and slower charge recombination due to the Marcus inverted behavior.

Guanine-rich nucleic acids tend to form four-stranded G-quadruplex structures by the stacking of planar quartets composed of four guanines that interact by Hoogsteen hydrogen bonding.[57, 58] G-quadruplex DNA usually form polymorphic structures, e.g. at least five intramolecular G-quadruplexes formed from human telomeric sequences.[59] Typically, G-quadruplex DNA structures are constructed in aqueous solutions, but recent studies reported these structure could be found in DES. G-quadruplex with the parallel-stranded structure was folded from human telomere sequence (HTS) DNA in ChCl:urea (1:2),[60] which is consistent with the observation of the parallel fold under a reduced water activity and alternative folds at a high water activity. On the other hand, after rapid cooling the thermally denatured HTS to room

temperature, refolding DNA back to the parallel structure in DES took much longer time (several months) than that in aqueous solutions (< 2 min) as can be seen in Fig. 7.

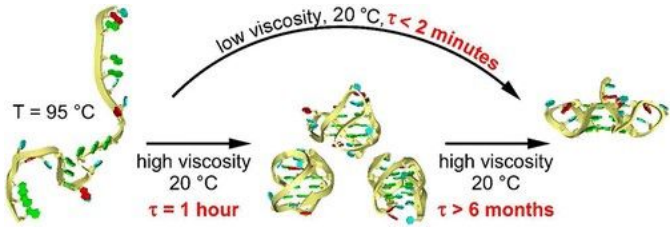

Fig. 7 DNA refolding in viscous choline chloride:urea (1:2 molar ratio) Reprinted with permission from reference[60]. Copyright © 2012 American Chemical Society.

Structures of ten G-quadruplexes were evaluated in neat ChCl:urea (1:2) by UV melting, CD and fluorescence spectroscopy, and various intramolecular, intermolecular, and higher-order G-quadruplex structures (particularly the parallel structure) as shown in Fig. 8. [61] Higher thermal stability of G-quadruplexes (even at over 110 °C) was found in neat DES than in aqueous media. Pal and Paul investigated the interaction between ChCl:urea (1:2) with guanine-rich quadruplex thrombin-binding aptamer (TBA) DNA at 300 K using 10 μs all-atom molecular dynamics simulations.[62] They observed that at a higher DES concentration, the higher density of DES molecules near quadruplex TBA led to more rigid DNA structure and conformation; at low DES concentrations, guanine-8 and thymine-9 nucleoside bases of loop-2 stacked to each other.

Higher compatibility and stability of duplex and quadruplex DNA in DES systems allows for the preservation of these biomolecules in ionic solvents, and enables potential applications in catalysis, biosensors, and DNA-based architectures.

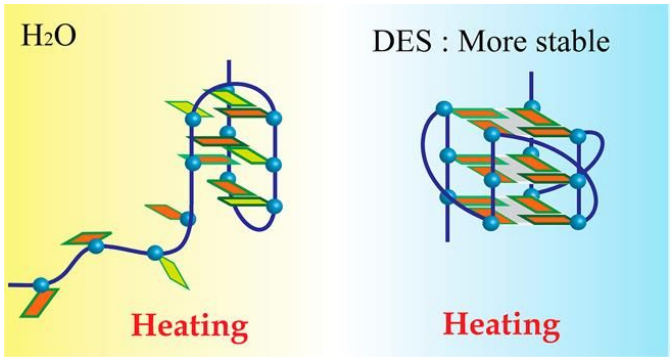

Fig. 8 Human telomeric DNA, Tel_{22}, adopting a more stable parallel G-quadruplex structure in water-free DES (containing K^+). Reprinted with permission from reference[61]. Copyright © 2013 American Chemical Society.

4. Biocatalysts in deep eutectic solvents

Biocatalysis can be defined as a reaction catalyzed by isolated enzymes or whole cells and is considered a green and sustainable technology due to reduced synthetic steps, less energy required, decreased raw material consumption, minimized undesired side product yields, and overall less waste generated. Solvents for biocatalysis reactions have been widely investigated in order to find an environmentally friendly replacement over the traditionally used hazardous organic solvents. Water has been considered as the greenest solvent considering its quality and quantity; however, its high polarity can hinder its application in some biocatalytic reactions due to some substrates' water-immiscibility properties. Therefore, other solvents have been investigated as suitable media for both water-miscible and water-immiscible enzymatic reactions. To this, Gorke *et al.* were among the first to successfully use ILs as solvent media for biocatalysis[63]; however, due to some of the aforementioned disadvantages, researchers have turned their attention to DESs as more suitable solvents for certain biocatalytic reactions.[64], [65] A complete list of the discussed works within this section are presented in Table 2 including the enzyme, reaction, conditions, conversion efficiencies, etc.

4.1 Lipases

Hydrolases are the most commonly used enzymes in biocatalysis because they use water to cleave chemical bonds, dividing large molecules in multiple smaller molecules.[66] Examples of common hydrolases include epoxide hydrolases, esterases, proteases, glycosidases, nucleosidases, and lipases. Lipases, acyl hydrolases that cleave long-chain fatty acids into polar lipids, are typically more active toward transesterification reactions; however, the water by-product that results from direct esterification reactions is more desirable for greener processes. Additionally, even though most lipases maintain good activity in organic solvents and have been shown to be good catalysts in biodiesel production[67], the toxicity and damaging properties of organics have led researchers to lean towards DESs for lipase-catalyzed biotransformations.[68] By using DESs as transesterification media, undesired side reactions, such as hydrolysis, may be avoided, owing to limited amounts of water present in the biocatalytic systems. In some cases, DESs can even play a bifunctional role, that is, substrate and solvent, in lipase-catalyzed reactions.

Gorke *et al.* were among the first to investigate the lipase-catalyzed transesterification of ethyl valerate with 1-butanol and various DESs using *Candida Antarctica* lipase B (CALB).[69] The use of ChCl:glycerol (1:2), ChCl:urea (1:2) and ethylammonium chloride (EAC):glycerol (1:1.5) all resulted in conversions to butyl valerate over 90% for both CALB and immobilized CALB. The immobilized CALB was also able to catalyse the aminolysis reaction of ethyl valerate with 1-butylamine in ChCl:glycerol, ChCl:urea, and ChCl:acetamide, yielding conversions of >90%, >90%, and 39%, respectively, after 4 h. ChCl-based DESs have also been shown to enhance the solubility of certain compounds as well as improve the activity of CALB immobilized on polyacrylate beads (Novozym® 435), which inherently results in increased conversion and product yields for other transesterification reactions. [70, 71]

Papadopoulou *et al.* found that EAC-based DESs also performed well in the transesterification reaction of ethyl

ferulate with 1-octanol catalysed by CALB nanoflowers to produce various esters.[72] When EAC:urea, EAC:glycerol, and EAC:ethylene glycol (molar ratios all 1:1.5) were all used as reaction media, yields were 42.5%, 14.0% and 61.4%, respectively, as compared to the ChCl-based DES, which yielded conversions of 24.6%, 13.0%, and 4.0% for ChCl:urea, ChCl:glycerol, and ChCl:ethylene glycol, respectively (molar ratios 1:2). The increased catalytic activity of CALB-nanoflowers observed in EAC-based DESs could be attributed to lower viscosity which could decrease the mass transfer limitations of the substrates to the active site of the enzyme and/or promote conformational dynamics of the protein.[73] When seven reaction cycles were completed, the residual activity of the immobilized CALB was 50%, which was higher than that reported for immobilized lipases in organic solvents (~40%).[74]

Choline acetate (ChAc)-based DESs have also been explored as suitable solvents in the transesterification reaction of ethyl sorbate with 1-propanol catalyzed by Novozym® 435.[75] Initial lipase activity was significantly low when ChAc:ethylene glycol (1:2) and ChAc:urea (1:2) were used, yielding results of 0.07 μmol min^{-1} g^{-1} and 0.21 μmol min^{-1} g^{-1}, and selectivity's of 12% and 40%, respectively. However, when ChAc:glycerol (1:1.5) was used, the initial activity was greatly enhanced to 1.02 μmol min^{-1} g^{-1}, which was twice as much as the traditional organic solvent (0.57 μmol min^{-1} g^{-1}) and compared well with ChCl-based DESs of ChCl:urea (1:2) and ChCl:glycerol (1:2) that yielded initial activities of 1.00 μmol min^{-1} g^{-1} and 1.12 μmol min^{-1} g^{-1}. Additionally, the selectivity was enhanced from 45% with ChCl:glycerol to 99% with ChAc:glycerol. Lastly, ChAc:glycerol was studied in the enzymatic synthesis of biodiesel from Miglyol® oil 812, which resulted in high conversions between 82-97% within 1-3 h using Novozym® 435. ChAc:glycerol has also been shown to increase the activity and stability of another lipase, specifically *Penicillium expansum* lipase, when the DES molar ratio was 1:2 (instead of 1:1.5), although it displayed poor performance of biodiesel production from *Millettia pinnata* seed oil, yielding a conversion of only 7.6%.[76] In similar fashion to Zhao *et al*.[75], Novozym® 435 displayed the highest conversion of biodiesel production with *Millettia pinnata* seed oil when ChAc:glycerol (1:2) was implemented, although the yield was only 54.8%.

DESs have also been used as solvents for alcoholysis reactions. For instance, Durand *et al.* found that neat ChCl:urea (1:2) and ChCl:glycerol (1:2) were both successful in the alcoholysis reaction of vinyl laurate with varying chain length alcohols (butanol, octanol, and octadecanol), by yielding 100% conversions, regardless of what alcohol was implemented, with >99% selectivity.[77] Additionally, ChCl:oxalic acid (1:1) and ChCl:malonic acid (1:1) showed highest conversions when 1-octanol was used, yielding 43% and 36%, respectively (>99% selectivity for both). Interestingly, ChCl:ethylene glycol (1:2) resulted in conversions of 33% or less in all alcohols investigated while also yielding the lowest selectivity's (<30%). Ethylammonium chloride:urea (1:2) showed an excellent conversion of 94% and >99% selectivity when butanol was the alcohol implemented; however, the conversions were ≤10% when octanol and octadecanol were used. Two other alcoholysis reactions,

Journal Name

REVIEW

Enzyme	Reaction catalyzed	DES	Conditions	Enzyme efficiency/ conversion	Selectivity	Ref.
Lipase						
Candida Antarctica lipase B (CALB)	Transesterification of ethyl valerate with 1-butanol	1:2 ChCl:glycerol; 1:2 ChCl:urea; 1:1.5 EAC:glycerol	60 °C	>90% conversion over 90 min	N.R.	69
Candida Antarctica lipase B; immobilized as Novozyme® 435	Aminolysis reaction of ethyl valerate with 1-butylamine	1:2 ChCl:glycerol; 1:2 ChCl:urea; 1:2 ChCl:acetamide	60 °C	>90%, >90%, 39% conversion, respectively, in 4 h	N.R.	69
Candida antarctica lipase B	Transesterification of ethyl ferulate with 1-octanol	1:1.5 EAC:ethylene glycol	60 °C	61.4% conversion after 72 h	N.R.	72
Candida antarctica lipase B	Transesterification of ethyl ferulate with 1-octanol	1:2 ChCl:urea	60 °C	24.6%, conversion after 72 h	N.R.	72
Candida Antarctica lipase B; immobilized as Novozyme® 435	Transesterification of ethyl sorbate with 1-propanol	1:1.5 ChAc:glycerol	1.0 vol% water, 50 °C	Initial activity of 1.02 µmol min^{-1} g^{-1}	99%	75
Candida Antarctica lipase B; immobilized as Novozyme® 435	Transesterification of Miglyol® oil 812 with methanol	1:1.5 ChAc:glycerol	1 vol% water, 50 °C	97% conversion in 3 h	N.R.	75
Penicillium expansum lipase; Novozyme ® 435	Enzymatic production of biodiesel from *Millettia pinnata* seed oil	1:2 ChAc:glycerol	50 °C	7.6% and 54.8% conversion within 48 h	N.R.	76
Candida Antarctica lipase B;	Alcoholysis reaction of vinyl laurate	1:2 ChCl:urea; 1:2 ChCl:glycerol	<1% water, 60 °C	100% conversion after 16 h	>99%	77

immobilized as Novozyme ® 435	with varying chain length alcohols					
Candida Antarctica lipase B; immobilized as Novozyme ® 435	Lipophilization reaction of methyl *p*-coumarate with 1-octanol	1:2 ChCl:urea; 1:2 ChCl:glycerol	10% water, 60 °C	98% conversion after 72 h	N.R.	[78]
Candida Antarctica lipase B; immobilized as Novozyme ® 435	Esterification of acetic anhydride with 1-butanol	1:2 ChCl:ethylene glycol	5% (w/w) water, 25 °C	80% yield within 3 h	N.R.	[79]
Candida Antarctica lipase B; immobilized as Novozyme ® 435	Enzymatic synthesis of α-monobenzoate glycerol from glycerol and benzoic acid	1:2 ChCl:glycerol	8% (v/v) water, 60 °C	99% conversion after 24 h	N.R.	[80]
Candida Antarctica lipase B; immobilized as Novozyme ® 435	Esterification of glycerol and benzoic acid to synthesize α-monobenzoate	1:2 ChCl:glycerol	10% (v/v) water, 60 °C	>90% conversion within 24 h	N.R.	[81]
Candida rugosa lipase	Lipase catalyzed esterification of DES compounds to synthesize menthol fatty acid esters	65:35 (-)-menthol:decanoic acid	10wt% water	83% conversion after 7 days	N.R.	[82]
Candida Antarctica lipase B; immobilized as Novozyme ® 435	Synthesis of fatty acyl ethanolamines, specifically, DHEA and EPEA	5:2 ChCl:glucose	8.50% water, 60 °C	96.84% conversion for EPEA; 90.06% for DHEA after 1 h	N.R.	[83]
Lipase from *Aspergillus niger*	Enzyme-catalyzed Henry reaction of 4-nitrobenzaldehyde	1:2 ChCl:glycerol	30 vol% Water, 25 °C	92% yield in 4 h	N.R.	[84]
Porcine pancreas lipase	Lipase-catalyzed aldol reaction	1:2 and 1:1.5 ChCl:glycerol	20% (v/v) water, 60 °C	>97% conversion after 24 h	High selectivity toward aldol product	[85]

	between 4-nitrobenzaldehyde and acetone					
Candida Antarctica lipase B; immobilized as Novozyme ® 435	Chemoenzymatic epoxidation reaction of 1-octadecene	1:1 ChCl:sorbitol	40 °C	72.4% conversion after 24 h	N.R.	[86]
Penicillium camemberti lipase	Chemoenzymatic epoxidation of soybean oil	1:1 ChCl:sorbitol	70% DES, 40 °C	95% conversion after 48 h	N.R.	[87]
Candida Antarctica lipase B	Lipase-mediated epoxidation of monoterpenes	1:1 ChCl:xylitol; 1:1 ChCl:sorbitol; 1:2 ChCl:fructose	Room temp (5 °C for ChCl:fructose)	100% conversion after 24 h	N.R.	[88]
Lipase G from *Penicillium camemberti*	Chemoenzymatic epoxidation reaction of hydrogen peroxide and glyceryl trioleate	1:1 ChCl:xylitol	40 °C	>80% conversion after 24 h	N.R.	[89]
Phospholipase D	Transphosphatidylation of phosphatidylcholine with L-serine to synthesize phosphatidylserine	1:2 ChCl:ethylene glycol	0.5% water, 40 °C	90.3% yield after 7 h	N.R.	[90]
Candida rugosa lipase	Lipase-catalyzed hydrolytic reaction	1:1:1 ChCl:urea:glycerol	0.59% water, 25 °C	155% activity	N.R.	[91]
DES as catalyst	Mono N-alkylation of aniline with hexyl bromide	1:2 ChCl:urea	50 °C	78% yield in 4 h	DES recycled 5 times (65% yield after 5th)	[92]
N-substituted aniline or indole	Synthesis of tricyanovinyl substituted aniline and indole derivatives	1:2 ChCl:urea	35 °C	89% yield in 5 min	DES recycled up to 4 runs (73% yield after 4th run)	[93]
hydrolase						

Epoxide hydrolase AD1 from *Agrobacterium radiobacter*	Epoxide hydrolase-catalyzed hydrolysis of styrene oxide	1:2 ChCl:glycerol,	25 vol% DES, 37 °C	92% conversion in 2 h	N.R.	[69]
Solanum tuberosum potato epoxide hydrolase 1	Hydrolysis of (1*S*, 2*S*) enantiomer of (1,2)-*trans*-2-methylstyrene oxide	1:2 ChCl:ethanediol; 1:2 ChCl:glycerol; 1:2 ChCl:urea	40% (v/v) DES, 30 °C, 250 min	Enantiomeric excess yields of 98%, 97%, 96%	Regioselectivity of (1*R*, 2*R*) enantiomer 38% and 40% in ChCl:glycerol and ChCl:ethanediol	[94]
β-glucosidase	Hydrolysis of *p*-nitrophenyl-β-glucopyranoside	1:2 ChCl:propylene glycol	6% water, 60 °C	225% β-glucosidase activity	V_{max} = 18.19 μM min^{-1} in 1 h	[95]
Burkholderia cepacia lipase (BCL)	Hydrolysis reaction of *p*-nitrophenyl palmitate	1:2 ChCl:ethylene glycol	40% (v/v) DES, 37 °C	230% enzyme activity	N.R.	[96]
6-O-α-rhamnosyl-β-glucosidase	Deglycosylation of hesperidin reaction	1:2 ChCl:ethylene glycol	30% (v/v) DES, 60 °C	>100% enzyme activity after 1 h	N.R.	[97]
Other enzymes						
Subtilisin immobilized on chitosan	Transesterification reaction of *N*-acetyl-L-phenylalanine ethyl ester with 1-propanol	1:2 ChCl:glycerol	3% (v/v) water, 50 °C	High activity of 2.9 μmol min^{-1} g^{-1}	98% selectivity	[98]
α-chymotrypsin	Chymotrypsin-catalyzed peptide synthesis	1:2 ChCl:glycerol	10-25% (v/v) water, room temp.	90% yield and no hydrolysis side reaction after 24 h	100%	[99]
Benzaldehyde lyase from *Pseudomonas fluorescens*	Carboligation of aldehydes	1:2 ChCl:glycerol	40% water, room temp	98% conversion of valeraldehyde after 24 h	27% (*R*) enantiomeric excess	[100]
Haloalkane dehalogenases	Hydrolysis of 1-iodo-hexane	1:2 ChCl:ethylene glycol	50% (v/v) DES, 37 °C	>100% activity	N.R.	[101]

ketoreductases	Bioreduction of propiophenone to 1-phenyl-1-propanol	1:1 ChCl:sorbitol; 1:2 ChCl:glycerol	50% (w/w) DES, 30 °C	>99% conversion after 24	>99% enantiomeric excess	102
Alcohol dehydrogenase *Ralstonia* sp. ADH	Stereoselective reduction of ketones	1:2 ChCl:glycerol	60% (v/v) DES; 30 °C	>80% conversion after 24 h	>90% enantiomeric excess	103
Alcohol dehydrogenase isolated from horse liver	Bioreduction of cyclohexanone to cyclohexanol coupled with butane-1,4-diol	1:2 ChCl:glycerol	20% (v/v) water, 25 °C	>40 mM cyclohexanol product in 72 h	N.R.	104
Laccase from *Bacillus* sp. HR03	Stability of laccase	2:1 Glycerol:betaine	20% (v/v) DES, 80 °C	70% activity after 20 min	N.R.	105
Laccase from *Trametes versicolor*	Laccase-catalyzed reactions	1:2 Choline dihydrogen phosphate:xylitol	10 and 25 wt% DES, 25 °C	170% enzyme activity	N.R.	106
Phenolic acid decarboxylase from *Bacillus subtilis* (*Bs*PAD)	Decarboxylation of *p*-hydroxycinnamic acids (**1**) followed by Ru-catalyzed metathesis of olefins (**2**) OR Pd-catalyzed metathesis of styrene-type olefins (**3**)	1:2 ChCl:glycerol	(**1**) 50% water, 30 °C (**2**) 50% water, 50 °C (**3**) 50% water, 100 °C	(**2**) 15% yield after 24 h (**3**) >90% conversion	(**3**) isolated yield of 65% on (*E*)-4-hydroxystilbene after 8 h	107
Phenolic acid decarboxylase from *Bacillus subtilis* (*Bs*PAD)	Decarboxylation of *p*-coumaric acid (**1**) and subsequent Pd-catalyzed Heck cross coupling with an aryl halide (**2**)	1:2 ChCl:glycerol	(**1**) 50% water, 30 °C (**2**)22.5% water, 85 °C	Full conversion of (**1**) and (**2**)	20% yield of (*E*)-4-hydroxystilbene after 16 h	108
Whole cell						
Baker's yeast	Enantioselective reduction	1:2 ChCl:glycerol	50 vol% water,	20-30% conversion after 72 h	95% enantiomeric excess (*R*) in	109

	of ethyl acetoacetate		room temp.		neat DES; racemic mixture at DES with 30 vol% water	
Baker's yeast	Baker's yeast bioreduction of aryl-containing ketones	1:2 ChCl:glycerol	50 wt% water, 37 °C	88% yield after 6 days	(*S*) enantiomer favored; 10% water led to (*R*) enantiomer favored	[110]
Baker's yeast	Reduction of ethyl-3-oxobutanoate	1:2 ChCl:glycerol; 1:2 ChCl:glucose; 3:2 ChCl:fructose	90% (w/w) water, 22 °C	Yields at 24 h: 94.1% 91.9% 94.7%	Enantiomeric excess%: 85S 91S 92S	[111]
Lysinibacillus fusiformis CGMCC1347 cells	Bioconversion of isoeugenol to vanillin	4:1 ChCl:lactose	20% (v/v) DES, 30 °C	132% yield after 72 h	N.R.	[112]
Whole cells of *Trichoderma asperellum* ZJPH0810	Asymmetric reduction of 3,5-bis(trifluoromethyl)acetophenone	1:1 ChCl:glutathione	1% (w/v) DES, 30 °C	90.7% yield after 24 h	>99% enantiomeric excess	[113]
Acetobacter sp. CCTCC M209061 cells	Asymmetric oxidation of 1-(4-methoxyphenyl)ethanol (MOPE)	1:2 ChCl:glycerol	20% (v/v) DES, 30 °C; initial rate: 90.2 µmol min^{-1}	49.4% conversion in 9 h	98.7% enantiomeric excess after 9 h	[114]
Acetobacter sp. CCTCC M209061 cells	Asymmetric reduction of 3-chloropropiophenone	1:2 ChCl:urea	5% (v/v) DES, 30 °C; initial rate: 2.39 mmol h^{-1}	86% yield after 6 h	>99% enantiomeric excess	[115]
Acetobacter pasteurianus GIM1.158 cells	Biocatalytic reduction of 2-octanone	1:2 ChCl:ethylene glycol	10% (v/v) DES, 35 °C; initial rate: 1.61 µmol min^{-1}	85.5% conversion in 2 h	98.8% enantiomeric excess	[116]
Biomass pretreatment						
Biomass	**DES**	**Conditions**	**Amount removed**	**Enzymatic hydrolysis**	**DES recycled?**	**Ref.**

Pretreatment of sugar cane bagasse	1:5 ChCl:lactic acid	80 °C	Removed 50.6% lignin after 12 h	50-60% glucan yields	DES recycled 3 times (69% recovery after 3rd cycle)	117
Pretreatment of corncob	1:10 ChCl:lactic acid; 1:2 ChCl:glycerol	90 °C	86.1% and 71.3% lignin extracted after 24 h	83.2% and 96.4% glucose yield	N.R.	118
Pretreatment of lignocellulosic biomass rice straw	1:5 ChCl:lactic acid	60 °C	>90% lignin extracted lignin after 12 h	Saccharification efficiency 36% in 24 h	DES recycled up to 3 cycles (>60 mg g^{-1} each time)	119
Pretreatment of *Eucalyptus camaldulensis*	1:10 ChCl:lactic acid	110 °C	64.0% lignin removed after 6 h	94.3% glucose yield	DES recycled up to 4 pretreatment cycles (90% yield of DES per cycle)	120
Pretreatment of lignocellulosic oil palm empty fruit punch	1:15 ChCl:lactic acid 1:2 ChCl:formic acid	120 °C	61% lignin removed after 8 h	Phenolic hydroxyl content: 3.33-3.72 mmol g^{-1} and 2.66 mmol g^{-1}	N.R.	121
Furfural production from oil palm fronds	1:1 ChCl:oxalic acid	16.4 wt% water, 100 °C	72.97% cellulose composition	26.34% furfural yield after 135 min	N.R.	122
Delignification of oil palm fronds	1:2 ChCl:urea	30% water, 120 °C	16.31% lignin removed after 4 h	N.R.	N.R.	123
Fractionation of Eucalyptus globulus wood	1:2 ChCl:ethylene glycol	170 °C for 4 h and then 90 °C for 24 h	90.2% lignin removed; 97.4% hemicellulose removal	94.5% cellulose retained	N.R.	124
Pretreat herb residues of *Cortex albiziae* (HRCA)	1:1 ChCl:*p*-coumaric acid	5% water, 160 °C	79.78% hemicellulose removed after 5 h	~90%	N.R.	125
Selective fractionation of beechwood	1:4 ChCl:KOH	80 °C	55-63% dissolution of biomass after 8 h	Successful fractionation and	N.R.	126

				dissolving of cellulose		
Saccharification of rice husks	1:2 ChCl:ethylene glycol; 1:2 ChCl:glycerol	20% (v/v) DES, 35 °C	Halophilic cellulase activity of 40-50% after 48 h	>1 mM glucose yields	N.R.	[127]
Delignification process of unbleacehd beech pulp	1:1:3 Malonic acid:ChCl:propanediol	5.14 wt% water	39.8% lignin removed	Solubilized 2.09 wt% cellulose	N.R.	[128]
Delignification process of unbleacehd beech pulp	1:2:3 ChCl:acetamide:lactic acid	6.36 wt% water	33.8% lignin removed	Solubilized 0.66 wt% cellulose	N.R.	[128]
Pretreatment of switchgrass	1:1.94:0.06 Guanidine hydrochloride: ethylene glycol:p-toluenesulfonic acid	120 °C for pretreatment; 50 °C for enzymatic hydrolysis	79% xylose 82% lignin removed in 6 min	>90% glucose yields	N.R.	[129]
Pretreatment of softwood loblolly pine	1:2 ChCl:formic acid	60 °C	N.R.	68% glucose yield after 70 h	N.R.	[130]

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specifically the lipophilization of methyl *p*-coumarate and methyl ferulate with 1-octanol and CALB alongside DESs as solvents have also been investigated.[78] Differing from the previous reaction, neat ChCl:urea and ChCl:glycerol both resulted in no conversions with methyl *p*-coumarate, even after four days; however, 98% conversion was seen when 10% water was added to ChCl:urea, and the hydrolysis side product remained very low. On the other hand, once 15% water was added, the hydrolysis reaction became prevalent (as well as substrate degradation) and led to an overall decrease in yield to 86%. When the alcoholysis reaction was performed with ChCl:glycerol, similar trends were seen; however, this system was much slower and resulted in lower yields (62%) even at the highest water content of 20% and hydrolysis made up 10% of the total yield. Additionally, water was found to be necessary for the alcoholysis reaction of methyl ferulate as well. In agreement with the previous reaction, ChCl:urea resulted in faster reaction rates and higher yields (>90%) than ChCl:glycerol (~50%) with both having 20% water, and the hydrolysis side reaction was significantly lower (<2%) regardless of DES used and water content.

Esterification reactions have also been investigated with DESs as the solvent media in order to increase conversions and yields. Cvjetko Bubalo *et al.* investigated DESs in the Novozym ® 435-catalyzed synthesis of the short chain esters of butyl acetate by the esterification of 1-butanol and acetic anhydride.[79] Similar to Durand *et al.*, when neat DESs (ChCl:glycerol, ChCl:urea, or ChCl:ethylene glycol, all in 1:2 molar ratio) were used, less than 5% conversions were seen.[78] However, when 0.5-2.5 moles of water were incorporated, the esterification yield gradually increased with the increase of water content, regardless of what DES was implemented. Interestingly, when the water content was too low, 1-butanol was strongly bound to the DES by hydrogen bonding, making it unavailable for the reaction; however, when the water content was over the optimal amount, the excess water molecules near the active site generated a hydrolysis reaction which led to lower yields. At optimal water content, the substrate was released from the hydrogen bonding network and the DES could also bind some water molecules formed during esterification in that same network, thus preventing hydrolysis altogether. The initial rate in ChCl:ethylene glycol:water at a 1:2:2 molar ratio was 1.2 μmol min^{-1} mg^{-1} which was 4-fold higher than when the ratio was 1:2:0.5 (rate = 0.3 μmol min^{-1} mg^{-1}). The highest initial rate occurred with ChCl:glycerol containing 2.5 mol of water (rate = 2.2 μmol min^{-1} mg^{-1}), however, it was still lower than the initial rate of the traditional solvent of *n*-heptane, which was rate = 4.5 μmol min^{-1} mg^{-1}. In terms of HBD, glycerol and ethylene glycol resulted in higher enzyme activity and esterification yield as compared to urea, which is in agreement with previously discussed results.[76,94] Overall, the highest yield (80%) resulted from ChCl:ethylene glycol with the addition of 1.5 mol (5% w/w) water, which was strongly increased from *n*-heptane or the IL [C_5mim][Tf_2N], which resulted in yields of 50% and 41%, respectively. The addition of a small amount of water (5-20%) to DESs has also been shown to increase conversions in other esterification reactions, specifically to increase the product yield of α-monobenzoate glycerol (α -MBG),[80, 81] to aid in the synthesis of menthol fatty acid esters,[82] and to assist in the synthesis of fatty acyl ethanolamines, specifically, docosahexaenoyl ethanolamide (DHEA) and eicosapentaenoyl ethanolamide (EPEA).[83] Additionally, the addition of 30% water to ChCl:glycerol and 50% water to ChCl:ethylene glycol has shown to drastically increase yields in the classic enzyme-catalyzed Henry reaction of 4-nitrobenzaldehye with nitromethane to 92.2% and 82.0%, respectively as compared to that in water, which only produced a yield of 33.4%.[84] Furthermore, the addition of 20% (v/v) water with ChCl:glycerol (molar ratios of 1:2 and 1:1.5) in a similar 4-nitrobenzaldehyde biocatalytic reaction (acetone used as a starting substituent instead of nitromethane) resulted in complete conversions with yields >97%.[85]

It has been noticed that the lipase CALB tends to display decreased stability when tested against the harsh conditions of a chemoenzymatic epoxidation reaction as compared to esterification reactions. Therefore, Zhou *et al.* have investigated the potential benefits of incorporating DESs as the solvents with the chemoenzymatic epoxidation reaction of 1-octadecene.[86] The DESs of ChCl:urea and ChCl:acetamide (both 1:2 molar ratios) resulted in the lowest conversions, yielding only 30.2% and 13.9%, respectively. The conversion was somewhat increased to 37.7% with ChCl:ethylene glycol (1:2); however, sugar-based DESs of ChCl:glycerol, ChCl:xylitol, and ChCl:sorbitol (all with 1:1 molar ratios) all showed even higher conversions yielding 56.7%, 65.1%, and 72.4%, respectively. When this reaction was performed in PBS buffer, the resulting conversion was 62.5%, showing that although most of the DESs do not result in increased conversions, ChCl:xylitol and ChCl:sorbitol show promise as better, more environmentally friendly solvent systems. These two DESs have also been shown to have a positive effect on the chemoenzymatic epoxidation of soybean oil, by increasing conversions 6-fold and 8-fold when 30% ChCl:xylitol (1:1) and 50% ChCl:sorbitol (1:1) were used as compared to 10% conversion in aqueous media.[87] Furthermore, Ranganathan *et al.* successfully used ChClxylitol (1:1), ChCl:sorbitol (1:1), and ChCl:fructose (1:2) as solvents for the

CALB-catalyzed epoxidation of monoterpenes, resulting in 100% conversions after 24 h. [88]

Interestingly, ChCl:xylitol has also been shown to stabilize lipase G from *Penicillium camemberti* in the chemoenzymatic epoxidation reaction of hydrogen peroxide and glyceryl trioleate to produce epoxidized glyceryl trioleate.[89] The product formation increased more than 22-fold in ChCl:xylitol resulting in a rate of 172 μM h^{-1} as compared to the reaction in buffer, which had a rate of 7 μM h^{-1}. Additionally, 60% conversion yield was seen after 6 h, as compared to <10% in buffer. These results were compared with Novozym ® 435 as the catalyst and it was determined that lipase G gave significantly higher yields of the desired epoxide product, as confirmed by ^{1}H NMR while also resulting in no hydrolysis products.

Another interesting example of DESs being incorporated as reaction media involves the phospholipase D (PLD)-catalyzed transphosphatidylation of phosphatidylcholine (PC) with L-serine to synthesize phosphatidylserine (PS) as shown by Yang *et al.*[90] The highest PS yield of 92.1% was achieved in ChCl:glycerol (1:2), although it required 12 h for reaction completion. A slightly decreased yet still great yield of 90.3% was obtained in only 7 h in ChCl:ethylene glycol (1:2), with the lipase displaying excellent stability, maintaining 81% of its original activity after 10 synthesis batches. ChCl-based DESs have also shown promise as cosolvents for lipase-catalyzed hydrolytic reactions by implementing two hydrogen bond donors into one DES.[91] The activity of lipase in buffer was considered to be 100%, but the activity in almost every DES investigated was >100%, with ChCl:urea:glycerol (1:1:1 molar ratio) resulting in activity of 155%. The DESs of ChCl:urea:formamide (1:1:1), and ChCl:glycerol:formamide (1:1:1) both resulted in lipase activity between 80–90%. These results are in agreement with previous reports in which urea and glycerol are efficient HBDs for lipase-catalyzed reactions in DESs.[77, 78] The DESs also greatly improved the thermal stability of lipase at 40 °C from 50% activity in buffer to over 80% activity in DESs. After 12 h, the lipase activity in buffer had diminished to 0%, while the activity in 10% ChCl:ethylene glycol:formamide (1:1:1) and 10% ChCl:glycerol:ethylene glycol (1:1:1) was still 30.6% and 39.1%, respectively.

In addition to solvent media, DESs have also been used as the catalyst in some biocatalytic reactions. Singh *et al.* used ChCl:urea and ChCl:glycerol (both at 1:2) as both a catalyst and a recyclable solvent for the selective mono N-alkylation of aromatic primary amines.[92] The reaction was also performed with a lipase from *Pseudomonas* sp. for comparison purposes in organic solvents of ethanol, chloroform, dichloromethane, and hexane, which resulted in yields of 85%, 81%, 75%, and 50%, respectively, with reaction times anywhere from 4-12 hours. When DESs were used, the reaction times were somewhat shortened (4-8 h) and the yields for ChCl:glycerol and ChCl:urea were 65% and 78%, respectively. The DES-catalyzed reactions were further explored in mono N-alkylation reactions of different aromatic amines. Overall, when the alkyl group was hexyl or butyl, the lipase-catalyzed reaction produced somewhat higher yields (74-85%); however, when benzyl alkyl groups were used, the DES-catalyzed reactions prevailed (79-89%). Even though some of the resulting yields were slightly lower in DES-catalyzed reactions, the reaction times were either the same as or faster in almost all reactions tested, as compared to lipase-catalyzed systems. DESs have also been implemented as catalysts during the synthesis of tricyanovinyl substituted aniline and indole derivatives.[93] Multiple DESs were tested, and although ChCl:malonic acid, ChCl:oxalic acid, and ChCl:urea:dichloromethane (ratios not provided) all showed high product yields of 70%, 70%, and 85%, respectively, the reaction times were relatively long (between 15–60 min). However, ChCl:urea resulted in the highest yield of 89% taking only five minutes for the reaction to proceed. It was also determined that the ChCl:urea could be recycled up to four separate runs with very little loss in activity (89%–73%).

In general, water can play a crucial role in the success or failure of lipase reactions. Excess water could induce the unwanted reaction of hydrolysis, but not enough water might result in solutions with high viscosity, increasing mass transfer limitations and decreasing the overall yield. In DESs, however, even the addition of 5% water to the system has been shown to decrease the viscosity and change the activity and selectivity of the enzyme of interest. Additionally, even though urea is claimed to be a protein denaturant, when it is paired with ChCl in a DES, the hydrogen bonding that results has been shown to stabilize various lipases and increase product yields, which has been further confirmed through molecular dynamic simulations. [131]

4.2 Other hydrolases

Gorke *et al.* were among the first to investigate the effects of ChCl:glycerol (1:2) on epoxide hydrolases (EH).[69] The rate of EH-catalyzed hydrolysis of styrene oxide to styrene glycol by EH AD1 from *Agrobacterium radiobacter* resulted in a conversion of only 4.6% when buffer was used. However, upon addition of 25 vol% ChCl:glycerol to the reaction, the conversion increased 20-fold to 92%. Interestingly, when the solutions contained 50 vol% DES, the conversion of the EH AD1-catalyzed reaction drastically decreased to 2%. Lindberg *et al*. have investigated the stability and activity *Solanum tuberosum* epoxide hydrolase 1 (StEH1), using 40% (v/v) ChCl:ethanediol, ChCl:glycerol and ChCl:urea all in 1:2 molar ratios for the hydrolysis of the (1*S*,2*S*) enantiomer of trans-2-methylstyrene oxide (2-MeSO) substrate, and yielded enantiomeric excesses (*ee*) of 98%, 97%, and 96%, respectively.[94] Additionally, the regioselectivity of the (1*R*,2*R*)-2-MeSO enantiomer was improved from 15% in PBS buffer to 38% and 40% in 40% (v/v) ChCl:glycerol and 40% (v/v) ChCl:ethanediol, respectively.

The activity and stability of glucosidase in ChCl-based DESs has also been investigated. Xu *et al.* incorporated 40% (v/v) ChCl:propylene glycol, ChCl:ethylene glycol, and ChCl:glycerol (all with molar ratios of 1:2) in a β-glucosidase-catalyzed hydrolysis reaction of *p*-nitrophenyl-β-glucopyranoside (*p*NPG) to produce *p*-nitrophenol (*p*NP).[95] β-glucosidase showed strongly enhanced activity of 230%, 200%, and 140% in ChCl:propylene glycol, ChCl:ethylene glycol, and ChCl:glycerol, respectively. Interestingly, the maximum reaction rates (V_{max})

were faster in the DESs containing only 6 vol% water, with V_{max} = 18.19 ± 0.46 μM min^{-1} and 15.37 ± 4.35 μM min^{-1} for the enzyme in ChCl:propylene glycol and ChCl:ethylene glycol, respectively as compared to the neat DESs (V_{max} = 10.56 ± 3.28 μM min^{-1} and 3.79 ± 0.78 μM min^{-1}, respectively). Additionally, these results were much higher than when the enzyme was with methanol (V_{max} = 1.58 ± 0.91 μM min^{-1}), showing that DESs greatly improved β-glucosidase activity and that the addition of a little water can strongly enhance reaction rates, most likely due to decreased viscosity in the system. Similar results have been seen in the hydrolysis reaction of *p*-nitrophenyl palmitate (*p*NPP) using *Burkholderia cepacia* lipase (BCL) and DESs.[96] The DESs of 40% (v/v) ChCl:glycerol and ChCl:ethylene glycol (both 1:2 molar ratio) showed increased enzyme activity of 230% and 180%, respectively, with ChCl:ethylene glycol resulting in the fastest V_{max} regardless if it was implemented as a cosolvent (40% v/v) or main solvent (with 4% water) yielding rates of 21.78 ± 2.3 μM min^{-1} and 25.70 ± 3.1 μM min^{-1}, respectively. Additionally, the lipase activity after 20 minutes in ChCl:ethylene glycol with 4% (v/v) water was 1.5 times and 14 times faster than the IL 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF_4]) and methanol, respectively. Interestingly, in the deglycosylation of hesperidin reaction using 6-O-α-rhamnosyl-β-glucosidase as the biocatalyst, Weiz *et al.* determined that 30% (v/v) ChCl:urea (1:2) resulted in no enzyme activity; however, the activity was greatly enhanced to 95% in 40% (v/v) ChCl:glycerol (1:2) and >100% in 30% (v/v) ChCl:ethylene glycol (1:2).[97]

4.3 Other enzymes

There are many other enzymes that use DESs as the solvent system in biocatalysis reactions other than hydrolases. Zhao *et al.* have investigated protease activation in ChCl:glycerol and ChAc:glycerol (in various molar ratios) with catalysts subtilisin and α-chymotrypsin (both free and immobilized) in the standard transesterification reaction of *N*-acetyl-L-phenylalanine ethyl ester with 1-propanol.[98] When immobilized subtilisin was used with ChAc:glycerol (1:2), the resulting activity was 2.0 μmol min^{-1} g^{-1} (99% selectivity), which was four times higher than the enzyme activity in *t*-butanol (with 2% (v/v) water) which was 0.50 μmol min^{-1} g^{-1} and a selectivity of only 29%. However, when the mole ratio was changed from 1:2 to 1:1.5 and water contents between 2-4% (v/v) were incorporated, the subtilisin activity decreased to 0.42-0.90 μmol min^{-1} g^{-1}, although the selectivity remained at 99%. For ChCl:glycerol (1:2) containing 3% (v/v) water, a high activity of 2.9 μmol min^{-1} g^{-1} was seen with immobilized subtilisin alongside excellent selectivity of 98%. Compared with subtilisin, α-chymotrypsin exhibited far less activity in both free and immobilized forms, resulting in 0.028 μmol min^{-1} g^{-1} and 0.031 μmol min^{-1} g^{-1}, respectively, in ChCl:glycerol (1:2) at 2% (v/v) water.

Building off of these results, Maugeri *et al.* studied α-chymotrypsin in the reaction involving *N*-acetylphenylalanine ethyl ester (APEE, **1**) and glycinamide hydrochloride (GH, **2**) to produce various peptides in ChCl-based DESs, as can be seen in Fig. 9.[99] Typically, APEE (**1**) undergoes dissolution in hydrophobic solvents, whereas (GH, **2**) is usually more soluble in hydrophilic solvents. The resulting dipeptide, *N*-Ac-Phe-Gly-NH_2 (APG, **3**) has been shown to be useful in some therapeutic applications. One of the challenges for peptide synthesis is the solvent system, since hydrophobic and hydrophilic substrates are combined simultaneously. The use of aqueous solutions can lead to hydrolysis, therefore, DESs were used as the reaction media to help alleviate those potential issues. Additionally, it has been shown that the formation of APG **3** could be hampered if hydrolysis occurs to yield N-acetylphenylalanine (AP, **4**), especially if there is a significant amount of water present. Some molecules of water are crucial for the enzymatic activity, therefore, it was necessary to optimize the reaction conditions carefully to minimize the hydrolytic side reactions (AP **4**), while still showing high production yield of APG **3**. When lower amounts of water (up to 5% v/v) were used with ChCl:glycerol (1:2), no enzyme activity was seen; however, when 10% (v/v) water was used, high enzymatic activity was seen with the production of APG **3** in 90% yield. This high yield continued to be seen in solutions containing 25% (v/v) water, which differed somewhat from the aforementioned paper, showing that the amount of water affects systems differently. Conversions between 80-90% were seen with ChCl:urea (1:2) and ChCl:isosorbide (1:2) with 10% (v/v) water; however, ChCl:xylitol (1:1) with the same amount of water only yielded conversions around 40%.

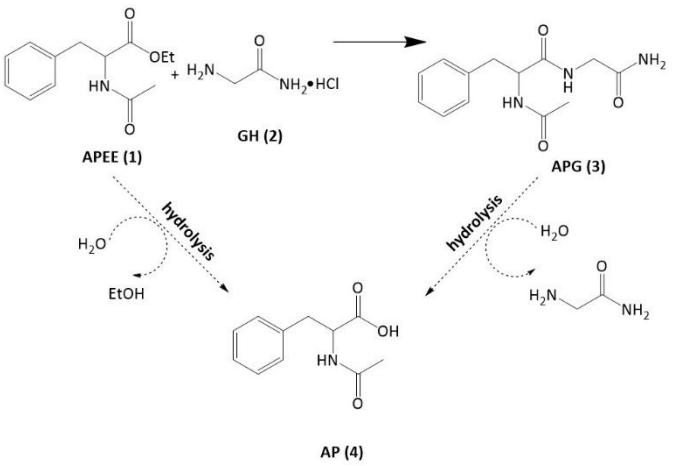

Fig. 9 Chymotrypsin-catalyzed reaction adapted with permission from reference[99]. Copyright © 2013 Wiley.

Interestingly, a higher water content (40%) was necessary with ChCl:glycerol when thiamine-diphosphate dependent lyase (ThDP-lyase), specifically benzaldehyde lyase (BAL) from *Pseudomonas fluorescens*, was used in a biocatalytic reaction to produce chiral α-hydroxy ketones.[100] The conversions and enantiomeric excesses were 96% (52% *R*), 98% (27% *R*), 95% (>99% *R*), and 75% (63% *R*), when butyraldehyde, valeraldehyde, benzaldehye, and 2-furaldehyde, were used as the starting substrates, respectively.

Haloalkane dehalogenases (HLDs) are also enzymes that have been investigated with DESs. Specifically, ChCl:ethylene

glycol (1:2) was used in the hydrolysis reaction of 1-iodo-hexane and its effect on the structure, stability, and enantioselectivity of three hydrophobic haloalkane dehalogenases (HLDs), including DbjA from *Bradyrhizobium japonicum* USDA1110, DhaA from *Rhodococcus rhodochrous* NCIMB13064, and LinB from *Sphingobium japonicum* UT26 was determined.[101] The most DES-tolerant enzyme was determined to be DhaA, followed by DbjA and then LinB as can be seen if Fig. 10. Even at a very high concentration of ChCl:ethylene glycol (90% v/v), activities in all three enzymes were still observed, which was much improved from the lack of activity seen when traditional solvents of methanol or acetone were used with concentrations of 50% or above. When the individual DES components were studied alongside the enzymes, the activity of DhaA was decreased, which is consistent with previous literature which stated that lipases are more stable in the DES as compared to its individual components.[69]

DESs have also been used as solvents in reactions involving both ketoreductases (KREDS) and oxidoreductases. Cicco *et al.* investigated multiple DESs in the bioreduction of propiophenone to 1-phenyl-1-propanol catalyzed by ten different KREDs.[102] All of the KREDS were inactive in 50% (w/w) ChCl:urea (1:2) and ChCl:lactic acid (1:2); however, higher conversions were seen (39-99%) when 50% (w/w) ChCl:sorbitol (1:1) and ChCl:glycerol (1:2) were used. Similar reports have shown steady activity of the alcohol dehydrogenase *Ralstonia* sp. ADH (RasADH) in 70% (v/v) ChCl:glycerol (1:2), an enantiomeric excess between 60-90% at higher DES concentrations, and the enzyme retained half of its original activity in very concentrated (95% v/v) ChCl:glycerol.[103] Additionally, ChCl:glycerol (1:2) has been successfully used alongside other ADH-catalyzed reactions.[104] The water-immiscible enzyme laccase (benzenediol oxygen oxidoreductase) from *Bacillus* sp. HR03 has also been studied with DESs.[105] Khodaverdian *et al.* found that 20% (v/v) betaine:glycerol (1:2) resulted in an enzyme activity of 300% and 50% (v/v) malic acid:betaine:water (1:1:1) showed an activity of 150%. It was also observed that laccase retained about 92% and 85% of its initial activity in sorbitol:betaine:water (1:1:1) and betaine:glycerol (1:2), respectively, after 120 min at room temperature.

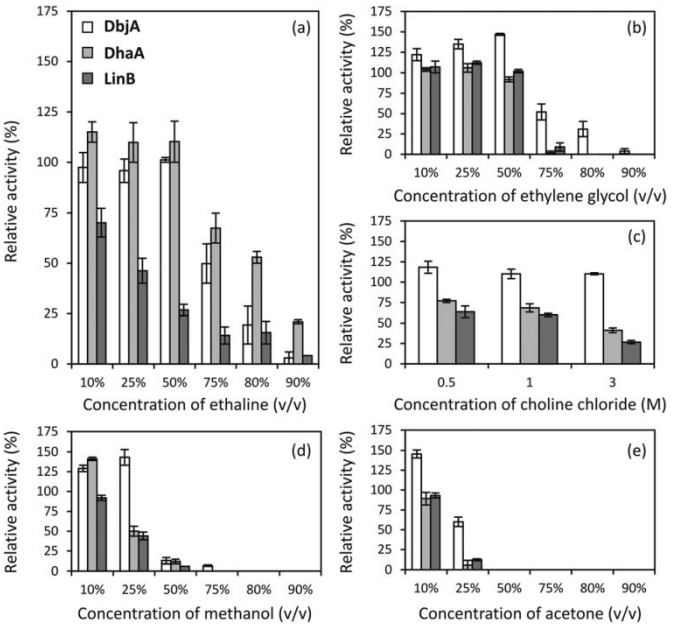

Fig. 10 The relative activities of DbjA, DhaA, and LinB measured in the presence of various concentrations of (a) ChCl:ethylene glycol (ethaline), (b) ethylene glycol, (c) choline chloride, (d) methanol, and (e) acetone at 37 °C and pH 8.6. Reprinted with permission from reference[101]. Copyright © 2014 Royal Society of Chemistry.

In a recent study, Toledo *et al.* swapped out the chloride ion of ChCl with either dihydrogen citrate (DHC) or dihydrogen phosphate (DHP) in various NADESs and showed that an increase in laccase activity could be achieved over ChCl.[106] When DHP was used, the highest enzyme activity was seen in ChDHP:xylitol (1:2), yielding 170% activity at both 10 and 25 wt% DES. Similar laccase activities were obtained with ChDHC:erythritol (between 120-150% at molar ratios of 2:1, 1:1, or 1:2) and ChDHC:xylitol (between 120-200% at molar ratios of 2:1, 1:1, or 1:2) at DES concentrations of 25 and 50 wt%. Comparing all ChDHC-based DESs at a molar ratio of 2:1 and at 50 wt%, the increase in the enzyme activity followed the order of increasing number of hydroxyl groups in the polyol: ChDHC: ethylene glycol < ChDHC: glycerol < ChDHC: erythritol <ChDHC: xylitol. Interestingly, the activity of laccase in betaine:glycerol regardless of molar ratio and DES concentration was less than 150%, which was lower than the aforementioned report of 300% laccase activity in 20% (v/v) betaine:glycerol (1:2).[105]

Decarboxylases have also been incorporated alongside DESs to improve their reactions. Phenolic acid decarboxylase from *Bacillus subtilis* (*Bs*PAD) was used in the enzymatic decarboxylation of *p*-hydroxycinnamic acid followed by Pd-catalyzed Heck-type C-C coupling to produce biaryl derivatives.[107] The use of ChCl:glycerol (1:2) allowed for much higher yields initially (95% after 20 h) as compared to water which resulted in 87% yield after 24 h. It was also reported that the Pd-catalyzed Heck-type coupling in DES gave an isolated yield of 65% of (*E*)-4-hydroxystilbene after 8 h. Similar results have been observed in a fully integrated two-step continuous flow process resulting in full conversions and yielding 20% product in 16 h.[108] Interestingly, 3D printing techniques have been used to construct custom-built reactors that were shown

to be applicable for the decarboxylation of *p*-coumaric acid (via *Bs*PAD) and subsequent Pd-catalyzed Heck cross-coupling reaction in a continuous flow method. The use of ChCl:glycerol (1:2) allowed for excellent selectivity (99%), conversion of 70%, and an overall yield of (*E*)-4-hydroxystilbene to be 15% after > 4 h.[132]

4.4 Whole-cell biocatalysis

As compared to the use of isolated enzymes, a major advantage of using whole cell catalysts is that they provide a natural environment for enzymes, preventing denaturation and inactivation that might occur more easily in nonaqueous reaction media. Additionally, they do not require any enzyme purification which allows for more cost-efficient applications. Although researchers have begun incorporating DESs into whole cell biotransformations, the amount of water necessary has still been a valid concern. As mentioned earlier, hydration up to a certain percent has been shown to destroy the supramolecular structure of DESs resulting in solutions of their individual components, therefore losing their unique characteristics.[26, 133] To this, Gutierrez *et al.* devised a unique approach to bypass this issue by incorporating freeze-dried bacteria solutions (*Escherichia coli*; strain TG1/pPBG11) in neat ChCl:glycerol (1:2).[16] Overall, only a few damaged bacteria were observed resulting in a 2% loss of fluorescence intensity indicating that this system retained the bacteria's viability and integrity. Similar results have been seen with NADESs acting as cryoprotective agents against lactic acid bacteria. The NADESs had a stronger ability to inhibit ice crystal formation during the freeze-drying process, resulting in minimal disruption of cellular metabolic enzyme activities and cell structures after long-term cryostorage.[134]

Hydrated ChCl:glycerol (1:2) has been used with baker's yeast in the enantioselective reduction of ethyl acetoacetate.[109] The presence of DESs with varying water contents (0-50% v/v) led to low conversions after 72 h (4-25% conversion); however, the cells were active for at least 200 h. Therefore, low conversion rates could be related to low concentrations of enzymes in the whole-cell and not due to any type of degradation by the DES. Interestingly, varying the water content in the system led to complete inversion of the enantioselectivity of the reduction reaction, as can be seen in Fig. 11. In pure water, (no DES) the baker's yeast whole cells displayed high stereoselectivity of the (*S*) enantiomer with an enantiomeric excess (*ee*) of 95%. As the amount of water decreased (therefore increasing DES content), it was noticed that a high (*R*) enantioselectivity emerged, while still maintaining a 95% *ee*. At 30% (v/v) water in the DES, a racemic mixture could be achieved.

Similar results have been seen with Vitale *et al.* who observed that at higher water contents (40-50%) with DESs (either ChCl:glycerol or ChCl:fructose), the (*S*) enantiomer was favoured in a baker's yeast bioreduction of aryl-containing ketones. Upon decreasing the water content to ~10%, the stereoselectivity was inverted, favouring the (*R*)enantiomer. [110] When baker's yeast was used alongside various DESs in the reduction of ethyl-3-oxobutanoate to ethyl-3-hydroxybutanoate, the (*S*) enantiomer was predominantly seen regardless of water content (10-90% w/w), although the (*R*) enantiomer was observed when 10% (w/w) water in ChCl:fructose (3:2) was used. [111]

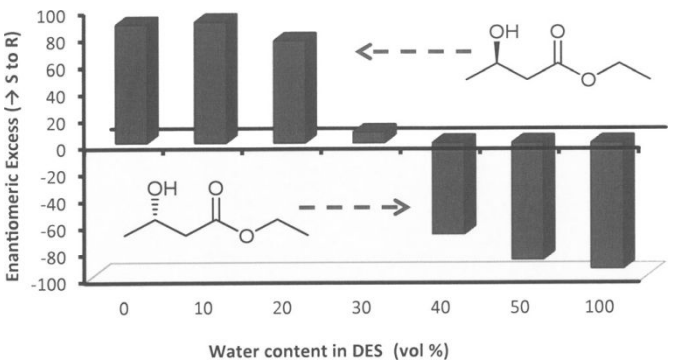

Fig. 11 Enantioselectivity of the reductive reaction depending on the different water/DES mixtures. Conditions include 50 mM ethyl acetoacetate and 200 mg mL^{-1} baker's yeast in 1:2 molar ratio of ChCl:glycerol at room temperature for 72 h. Reprinted with permission from reference[109]. Copyright © 2014 Wiley.

In another interesting report, Yang *et al.* studied the bioconversion of isoeugenol to vanillin catalyzed by Lysinibacillus fusiformis CGMCC1347 cells.[112] Twenty-four DESs were synthesized either with ChCl or ChAc and the HBA along with various HBDs of acetamide, urea, glycerol, ethylene glycol at various molar ratios. All DESs (except for 2:1 ChCl:urea) resulted in high conversions of isoeugenol to vanillin at 1% (v/v) DES concentration, and although the yields did not seem to have any obvious correlation with either the HBD or the salt:HBD molar ratio, the yields obtained in the presence of ChAc-based DESs were overall slightly higher than those obtained with ChCl-based DESs. In addition, the optimum yield (142%) was obtained when the volume of DES added reached 20% (v/v). When NADESs were investigated on the bioconversion, 20% (v/v) ChCl:lactose (4:1) and ChCl:raffinose (11:2) resulted in high conversions of 132% and 131%, respectively. It is interesting to note that cell viability studies were performed and agreed with similar literature that stated that increasing the amount of DES to over 50% disrupts the cell membranes and leads to apoptosis.[111] Interestingly, the cells treated with ChCl-based DESs survived better than the cells treated with ChAc-based DESs.

Lastly, as a model reaction, Li *et al.* studied the synthesis of (R)-[3,5-bis-(trifluoromethyl)phenyl]ethanol ((R)-BTPE) by asymmetric reduction of 3,5-bis(trifluoromethyl)acetophenone (3,5-BTAP) using whole cells of *Trichoderma asperellum* ZJPH0810 as a biocatalyst in a DES of ChCl:glutathione (1:1).[113] A solution containing 1% DES (w/v) resulted in a yield of 90.7% and enantiomeric excess >99%. Since glutathione is composed of the amino acids γ-glutamine, cysteine, and glycine, DESs consisting of ChCl and those amino acids were also synthesized in 1:1 molar ratios. As to be expected, when 1% (w/v) ChCl:γ-glutamine, ChCl:cysteine, and ChCl:glycine were investigated in the bioreduction reaction, they resulted in yields lower than the

ChCl:glutathione DES (66.0%, 80.7%, 63.1%, respectively), and all enantiomeric excesses were >99%, which shows that the amino acids had an enhanced effect in glutathione as compared to their individual results.

4.4.1. *Acetobacter* whole cells

Much research has been done regarding various DESs as cosolvents to help improve the biocatalytic performance of immobilized *Acetobacter* sp. CCTCC M209061 cells. For example, Xu *et al.* implemented DESs to improve the performance of these cells for the asymmetric oxidation of 1-(4-methoxyphenyl)ethanol (MOPE).[114] The DESs ChCl:urea, ChCl:ethylene glycol and ChCl:glycerol (molar ratios 1:2, 20% v/v) all resulted in similar conversions (42.9-49.4%) to the buffer (47.5%); however, stability of the immobilized *Acetobacter* sp. CCTCC M209061 cells were greatly enhanced with the incorporation of DESs and the enantiomeric excess increased from 91.4% to 98.7% in ChCl:glycerol. Additionally, ChCl:glycerol resulted in the highest cell membrane integrity and was shown to be very biocompatible, as well as allowed the reaction to proceed in only 9 h as compared to 11 h in buffer. Similar results were seen when the same DESs and cells were used in another reduction, specifically, the asymmetric reduction of 3-chloropropiophenone (CPE) to (*S*)-3-chloro-1-phenylpropanol (CPL).[115] In this reaction, the buffer produced an 85.2% yield and 5% (v/v) DESs yielded results between 79.9-86.0%. Interestingly, ChCl:urea resulted in the highest yield (86.0%) and shortened the reaction time from 10 h in buffer to only 6 h, as well as being the most biocompatible to the cells. The DESs and substrates for both reactions showed slight toxicity against the immobilized *Acetobacter* sp. CCTCC M209061 cells, although the DESs reduced the substrate toxicity when incorporated into the reaction system. Furthermore, the biocatalyst retained nearly 80.0% of its initial activity and the product enantiomeric excess was consistently >99% in systems containing ChCl:urea after five consecutive batches as compared to the system with no DES, which resulted in 50.4%.

Interestingly, when the aforementioned DESs were used with *Acetobacter pasteurianus* GIM1.158 cells for the biocatalytic reduction of 2-octanone to (*R*)-2-octanol, 10% ChCl:ethylene glycol (1:2) resulted in the highest conversion of 85.5% as compared to the buffer, which yielded results of 80.1%.[116]

4.5 Biomass processing

Industrial applications for biomolecules using DESs have become increasingly popular. Since there are multiple reviews that discuss biomass pretreatment and conversions of lignocellulosic material, some of which involve the use of DESs, [135,136] this review herein will discuss only the most recent literature and compare results from previous reports. Lignocellulosic biomass is a renewable, widely available raw material for fuels (e.g. bioethanol), chemicals and polymeric materials production. It can be obtained from various agricultural sources including wastes such as corn stalks, corn cobs, and sugar cane bagasse, and forest residues such as sawdust, bark, and waste left from paper and wood products. Lignocellulosic material consists of a complex matrix of cellulose, hemicellulose and lignin which makes it highly resistant to depolymerization. Once these components are separated, typically by pretreatment and fractionation processes, they can be converted to valuable fuels and chemicals via various reactions such as hydrolysis, hydrogenation and oxidation. The pretreatment and fractionation steps generally require excessive use of organic and inorganic acids to aid in loosening the components from the matrix. Some of the most common pretreatment methods include steam/steam explosion, grinding/milling/ hot water/autohydrolysis, acid treatment and alkali treatment.[137-140] These pretreatment methods can be energy intensive and may require additional steps for solvent recovery. Therefore, DESs have been implemented to enhance the biomass pretreatment process as they can effectively break down the complex matrix and enhance cellulose enzymatic hydrolysis.[118, 141, 142] A modified liquid hot water (MLHW) pretreatment technique was proposed that involved hemicellulose-derived acids (acetic, formic, and gluconic acid) being produced during the LHW process, which could then synthesize a DES *in situ* when ChCl was loaded into the system due to hydrogen bonding of the substituents to improve the delignification of garden waste. [143]

More than 50 million tons of lignin are produced annually by the global paper industry, but only a small proportion is used; the rest typically gets burned away, which unnecessarily depletes resources and causes environmental pollution. Therefore, developing ways to use lignin effectively is an important project for environmental and commercial reasons. Cellulose and hemicellulose can be converted into fermentable sugars by pretreatment and enzymatic hydrolysis processes. However, due to the recalcitrant structure of lignin in the lignocellulose, the direct conversion of lignocellulosic biomass into products is not energy-favorable because lignin serves as a protective barrier, causing the biomass to be strongly resistant against biological and chemical attacks. Hence, the removal of lignin from the lignocellulosic biomass by pretreatment is necessary to facilitate the conversion into other bio-based products.

Throughout the literature, it has been determined that acidic DESs exhibit a larger affinity toward lignin and hemicellulose dissolution during the pretreatment process. [144, 13] Additionally, Satlewal *et al.* found that ChCl:lactic acid (1:5) successfully removed 50.6% and 63.0% lignin and hemicellulose, respectively, from sugar cane bagasse, as compared to dilute alkali (NaOH) and acid (H_2SO_4) pretreatment which resulted in 59.7% and 10.4% lignin removal, respectively.[117] These results agree with previous literature that have reported high lignin removal with ChCl:lactic acid pretreatment of rice straw and corncob.[118, 119] In another study, when the molar ratio of ChCl:lactic acid was changed to 1:10, the DES yielded a maximum lignin removal of 64.0% in the pretreatment of *Eucalyptus camaldulensis*. [120] The DES has also been used in the pretreatment of oil palm empty fruit punch to extract 33.5% lignin when the molar ratio was 1:1; however, when it was changed to 1:15, the lignin yield increased to 61.0%.[121] In addition to high lignin extractions, ChCl:lactic acid

has been shown to enhance enzymatic hydrolysis/ saccharification of cellulose to produce glucose yields between 20-94%,[120,117,145] which is similar to or better than the typical dilute alkali and acid pretreatments. [146,147] Both conventional acid and alkaline pretreatments are not eco-friendly, as high cost is required on chemical recovery, equipment and neutralization processes. Therefore, implementing DESs can be used as an alternative environmentally friendly solvent for lignocellulosic biomass pretreatment.

Other acidic DESs have shown some lignin and hemicellulose dissolution, namely ChCl:oxalic acid, ChCl:malonic acid and ChCl:succinic acid. [121,126,128] More specifically, Lee *et al.* found that when the above DESs (neat) were used in a 1:1 molar ratio, enhanced dissolution of hemicellulose was observed to eventually result in furfural from oil palm fronds, yielding the product of 9.74 mol%, 0.36 mol%, and 0.19 mol%, respectively, over 120-300 minutes. [122] When varying water contents were added to each DES, the furfural yields remained fairly consistent with ChCl:malonic acid and ChCl:succinic acid; however, the yields were greatly enhanced to 26.34 mol% in ChCl:oxalic acid with 16.4 wt% water, most likely due to decreased mass transfer limitations. Similar results have been reported when ChCl:urea (1:2) was used in the pretreatment process on the delignification of oil palm fronds.[123] The neat DES only allowed 11.10% lignin removal, but the addition of 30% water with the DES led to an increase of 16.31% lignin removal. Liang *et al.* implemented ChCl:ethylene glycol (1:2) in a hydrothermal pretreatment to fractionate hemicellulose and lignin from Eucalyptus globulus wood.[124] In this study, biomass pretreatment involved the hemicellulose removal by hydrothermal treatment followed by lignin removal by DES treatment from the hydrothermal residue. After the two-step pretreatment, the removal ratios of lignin and hemicellulose were 90.2% and 97.4% while cellulose could be effectively retained with 94.5% of original cellulose in the DES-treated residue.

Chen *et al.* used ChCl:*p*-coumaric acid (PCA) as a DES in a 1:1 molar ratio to effectively pretreat herb residues of *Cortex albiziae* (HRCA).[125] It was observed that neat ChCl:PCA successfully removed 85.25% of hemicellulose at 180 °C in only 1 h. When the reaction proceeded for 7 h, 64.03% lignin could be removed. However, at 180 °C, >50% cellulose was removed which hindered enzymatic hydrolysis reactions. When the optimized parameters were determined (ChCl:PCA 1:1, 160 °C, 5 h), the enzymatic hydrolysis increased from 48.08 to 84.62%. Interestingly, hemicellulose removal increased from 69.68% to 79.78% with the addition of 5% water in the newly optimized system and the removal of lignin increased to 39.89% with the addition of 50% water.

Alkaline DESs have been shown to enhance the solubility of cellulose due to their ability to break down the intermolecular hydrogen bonding of cellulose. For instance, successful fractionation of cellulose from lignin and hemicellulose was observed when ChCl:KOH (1:4) was used in the pretreatment of beechwood.[126] Lim *et al.* investigated the DES K_2CO_3:glycerol (1:7) as an alternative solvent to the typical kraft pulping pretreatment process that usually involves high temperatures and pressure.[148] Although kraft pulping is an efficient process, it releases volatile sulphur compounds such as hydrogen sulfide (H_2S), sulfur dioxide (SO_2), dimethyl sulfide (C_2H_6S) and methanethiol (CH_4S) into the atmosphere, in addition to contaminating water sources. Cellulose isolation was successfully achieved from rice straw using the alkaline-DES pulping technique with 73.8% at somewhat lower temperatures (140 °C) and 100 minutes. Another example involving the use of 20% (v/v) ChCl:ethylene glycol and ChCl:glycerol (both 1:2 molar ratios) led to increased halophilic cellulase activity, which led to a high *in situ* saccharification glucose yields in rice husks. [127] Another interesting pretreatment approach has been to synthesize a ternary DES with two HBD's. Jablonsky *et al.* found that malonic acid:ChCl:propanediol (1:1:3) solubilized 2.09% of cellulose, whereas betaine:urea:glycerol, (1:2:3), betaine:propanediol:lactic acid (1:3:1), and ChCl:acetamide:lactic acid (1:2:3) all solubilized 1.24%, 0.87%, and 0.66% of cellulose, respectively.[128] Additionally, the ternary DESs were investigated in the delignification process of unbleached beech pulp and the authors found that ChCl:acetamide:lactic acid effectively removed 33.80% lignin.

In another example, the addition of P-toluenesulfonic acid (PTSA) as a second HBD to the DES of guanidine hydrochloride (GH) and ethylene glycol (EG) was investigated on the pretreatment of switchgrass. The resulting DES GH-EG-PTSA was shown to remove 79% lignin and 82% xylose from switchgrass and <2% cellulose was lost during this pretreatment, which produced a glucose yield of 90% after enzymatic cellulose hydrolysis after 36 h. [129] Similar sugar yields and lignin removal percentages have been seen with binary DESs in the pretreatment of switchgrass,[118, 149-151] although longer reaction times were necessary.

It has been determined that DESs with lower density have an easier time penetrating through the complex lignocellulosic matrix, allowing for better dissolution and fractionation of lignin, hemicellulose and cellulose. Additionally, it has been observed that the delignification efficiency of a HBD containing a dicarboxylic acid is generally lower than when monocarboxylic acid HBDs are used in various pretreatment processes.[152] D'Agostino *et al.* explained that in DESs containing dicarboxylic acid HBDs, the two COOH groups can form extensive chains of dimers, which can restrict the mobility of solvent molecules.[153] Additionally, it has been observed that lower carbon chain-length acids (such as formic and lactic acid) result in higher lignin removal yields as compared to increasing aliphatic chain length (such as butyric, malic and citric acid) that typically result in lower lignin removal yields.[153,130] Although the fractionation of lignocellulose biomass using aqueous DES solutions has shown to be successful, it is still somewhat poorly understood. It is thus important to achieve a deeper understanding of the role of DESs and their aqueous solutions on the selective solubilization of lignin.

5. Biomedical and pharmaceutical applications

5.1 Drug solubilization and delivery

Drug delivery is a crucial process in the treatment of illnesses and diseases. There have been many strategies and methods employed to ensure drugs arrive at the location of interest, although the water-solubility of drugs continues to be an issue. Additionally, some drugs such as aspirin can undergo hydrolysis upon prolonged storage in water. Organic solvents have been used to increase drug solubility; however, due to toxicity, flammability, volatility, and environmental concerns, the use of organic solvents is not a practical or a desirable alternative in most pharmaceutical settings. Therefore, DESs have been investigated as a versatile alternative solvent for the solubilization and delivery of pharmaceutical drugs.

To this, Morrison *et al.* were among the first to investigate the solubilization potential of two DESs: one composed of ChCl:urea (1:2 molar ratio) and one of ChCl:malonic acid (1:1 molar ratio).[154] These eutectic systems were studied alongside several poorly water-soluble compounds including benzoic acid, griseofulvin, danazol, itraconazole, and an experimental drug AMG517. Solubility was measured in the neat DES, mixtures of DES and water (75:25 and 50:50 by weight) and pure water. The results indicated that the solubility of the compounds increased by 5 to 22,000-fold in neat DESs when compared with their solubility in water. The solubility of the compounds in aqueous solutions of the individual DES components (ChCl, urea, and malonic acid) were also investigated and, although solubilization of drugs did occur, it was significantly lower than the drugs in the DESs, which confirm the symbiotic nature of the DES components. Li *et al.* also studied the solubilization of itraconazole, along with posaconazole, lidocaine, and piroxicam in several ChCl-based DESs.[155] The solubilities of all four drugs significantly increased in ChCl:glycolic acid (1:2) compared to that of water. For example, lidocaine had a solubility in water of 3.63 mg mL^{-1}, but increased to 100.6 mg mL^{-1} in ChCl:glycolic acid. When a third component (oxalic acid) was introduced into the DES to make a ternary DES composed of ChCl:glycolic acid:oxalic acid, (1:1.7:0.3), the solubility of lidocaine was further enhanced to 295.4 mg mL^{-1}. Additionally, the solubilities of itraconazole, piroxicam, and posaconazole all increased in both binary and ternary DESs as compared to their solubilities in water.

The solubility of non-steroidal anti-inflammatory drugs (NSAIDS) in DESs has also been studied. Lu *et al.* studied nineteen different DESs as possible vehicles for drug solubilization and stability of aspirin, acetaminophen, ketoprofen, naproxen and ibuprofen.[156] Overall, the solubility of these drugs increased by 17- to 5,477-fold in various neat DESs as compared to their solubility in water, with tetrapropylammonium bromide:1,2-propanediol (TPAB:P; 1:2 molar ratio) resulting in the highest solubility of 383.4 ± 4.03 mg mL^{-1} for ibuprofen. Although high drug solubilization was seen when TPAB was used as the HBA, the DES was not considered to be a viable solvent due to its safety issues in both food and pharmaceuticals. Although the DESs ChCl:levulinic acid (1:2) and ChCl:1,2-propanediol (1:2) resulted in somewhat lower solubilities for acetaminophen and ibuprofen (183.3–324.0 mg mL^{-1}), they were ~10-fold higher than the reported solubilities in ILs 1-butyl-3-methylimidazolium hexafluorophosphate [BMIM][PF_6] and 1-hexyl-3-methylimidazolium hexafluorophosphate [HMIM][PF_6] of 10.1-37.2 mg mL^{-1} at room temperature.[157] The solubility of acetaminophen has also been investigated in DESs of ChCl:urea (1:2), ChCl:malonic acid (1:1), and ChCl:oxalic acid (1:1).[158] The mole fraction of solubility of acetaminophen increased with increasing weight fractions of DESs up to 0.15, yielding solubilities of 3.9887-9.8765, 3.7999-5.8970, and 3.6050-5.2959 in ChCl:malonic acid, ChCl:oxalic acid, and ChCl:urea, respectively, from T = 298.15–313.15 K. In a subsequent paper, the authors were able to increase the weight fraction of DES to 0.90 which showed a >25-fold enhancement in the acetaminophen solubility in all DESs, with solubility mole fractions of 74.444–140.519, 62.988–103.052, and 52.584–97.302 for ChCl:malonic acid, ChCl:oxalic acid, and ChCl:urea, respectively, in the temperature range of T = 298.15–318.15 K.[159] Interestingly, when the DESs ChCl:glycerol and ChCl:ethylene glycol (molar ratios both 1:2) were implemented, the maximum solubility of acetaminophen was observed when weight fractions of the DESs were 0.80, yielding results of 22.1743–51.6381 and 62.5752–92.2144, respectively from T = 298.15–313.15 K.[160] Structurally speaking, the dicarboxylic acids on malonic and oxalic acid could provide increased hydrogen bonding of the drug to the DES, increasing its solubility, whereas the extra hydroxyl group on glycerol might potentially sterically hinder the acetaminophen-DES interactions, ultimately resulting in lower solubility.

These DESs have been observed to enhance the solubility of other pharmaceutical compounds. For instance, ChCl:malonic acid, ChCl:ethylene glycol, and ChCl:urea all strongly enhanced naproxen solubility up to 3,300-fold with DES weight fractions up to 0.90 and temperatures up to 313.15 K.[161] In another study, neat ChCl:urea, ChCl:glycerol, and ChCl:ethylene glycol have successfully enhanced the solubility of the anticonvulsant drug, lamotrigine.[162] Lamotrigine belongs to the bio-pharmaceutics classification system Class II of drugs due to its low water solubility, the generic scheme of which can be seen in Fig. 12. However, upon the incorporation of the DESs alongside the drug, the results showed a 341-fold solubility enhancement and lamotrigine could be classified as Class I due to the improved solubility.

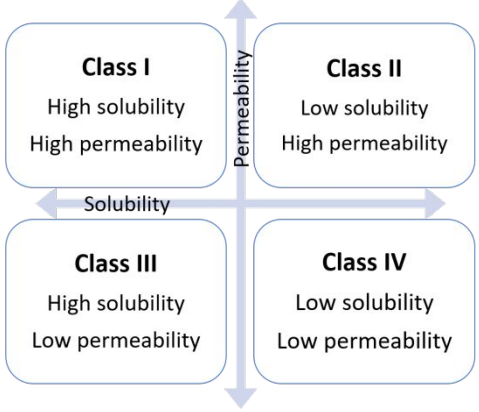

Fig. 12 Biopharmaceutical classification system of APIs reprinted with permission from reference[163]. Copyright © 2017 Elsevier.

Natural DESs (NADESs) have also been shown to improve the solubility of drugs. β-lactam antibiotics have a very unstable chemical structure that are prone to β-lactam ring opening resulting from nucleophilic or electrophilic attacks, which can lead to antibiotic degradation or loss of antimicrobial activity. Therefore, a NADES consisting of betaine:urea (1:1.5) has been used to successfully stabilize two β-lactam antibiotics, namely clavulanic acid (CLV) and imipenem (IMP).[164] Both antibiotics displayed no reduction in antimicrobial activity when dissolved in the NADES over at least seven days, as compared to the antibiotics in water which showed significantly decreased activity, further proving that these viable solvents can improve solubility and stability of very important pharmaceuticals.

Lastly, resveratrol, a dietary water-insoluble anti-oxidant, has been studied with a DES to inhibit matrix metalloprotease-9 in the tumour necrosis factor (TNF- α) activated human leukemia cell line.[165] Resveratrol was successfully dissolved in the DES composed of 1,2-propanediol:ChCl:water (1:1:1), and was able to inhibit matrix metalloprotease-9 activity by 52% at a remarkable DES concentration of 0.5%. DMSO, the most commonly used solvent for *in vitro* studies, did not reduce the matrix metalloprotease 9 activity at all when the concentration was 0.5%. Overall, this successful resveratrol/DES formulation could allow for lower dosage requirements in future treatments.

5.2. Antimicrobial photodynamic therapy (aPDT)

Antibiotic resistance is becoming an increasingly bigger issue due to the misuse and overuse of antibiotics in this modern world.[166] New treatments and techniques are being developed in order to combat this potentially dangerous problem. To this, antimicrobial photodynamic therapy (aPDT) is a relatively new treatment modality that has been shown to overcome several antibiotic resistant strains of bacteria with much success. aPDT utilizes a combination of visible light, a photosensitizer (PS) and oxygen to produce reactive oxygen species which can damage cellular structures of microorganisms and led to apoptosis. Porphyrins are aromatic heterocyclic compounds that are present in nature and have been widely investigated in photodynamic therapy of tumours and microbial infections as photosensitizers. Interestingly, neutral and anionic porphyrins have been shown to be phototoxic predominantly against Gram-positive bacteria, whereas cationic porphyrins effectively photoinactivate both Gram-positive and Gram-negative microorganisms. The solvents used in these treatments are typically organic in nature and, due to some of their previously discussed disadvantages, have been used less in recent reports. Researchers are now implementing DESs as alternative solvent systems as well as potential porphyrin solubilizers to evaluate these porphyrins as photosensitizers in aPDT treatments.

Some of the work that has come out of the Tønnesen group has investigated the solubilization ability of NADESs on two different porphyrins, specifically the hydrophobic, neutral porphyrin 5,10,15,20-tetrakis(4-hydroxyphenyl) porphyrin (THPP) and the anionic porphyrin *meso*-tetra-(4-carboxyphenyl)-porphine (TCPP). It was determined that the hydrophilic NADESs composed of citric acid:sucrose (1:1) and glucose:malic acid (1:1) were the best at solubilizing THPP due to increased hydrogen bonding between the constituents.[167] The photostability of THPP was enhanced in aqueous NADES solutions of containing 22% (w/w) water content as compared to the porphyrin in typical organic solvents. The high viscosity of the NADES resulted in slower reaction rates and helped form a more stable network between the NADES and THPP. Additionally, the NADESs were able to change the neutral porphyrin to a cationic porphyrin due to proton donor-acceptor interactions between the NADES and THPP. The protons in the acids of the NADES (citric acid and malic acid) donate a hydrogen atom to a pyrrole in THPP, thus changing the neutral porphyrin to a cationic porphyrin. This aPDT system resulted in complete photoinactivation of the Gram-negative bacteria *Escherichia coli* and the Gram-positive bacteria *Enterococcus faecalis* using only nanomolar amounts of THPP (0.5-5 nM) in NADES. Interestingly, the fungus *Candida albicans* was least susceptible to any photoinactivation treatment regardless of the NADES or nanomolar THPP amounts used.[168] Further studies implementing higher concentrations of THPP, a different porphyrin, or different NADESs need to be investigated in order to successfully photoinactivate this fungal strain.

Typically, anionic porphyrins have been shown to be ineffective against Gram-negative bacteria due to a thick permeability barrier of the outer membrane surrounding these bacteria. However, 40 nM TCPP and blue light combined with the NADES citric acid:sucrose (1:1) or malic acid:fructose:glucose both induced *E. coli* reductions of >90% due to the NADES effectively solubilizing TCPP and changing the anionic porphyrin to cationic.[169] Additionally, >90% bacterial reduction was seen in Gram-positive microorganisms (including *E. faecalis* and *Staphylococcus aureus*) when nanomolar concentrations of TCCPP (1–10 nM) were solubilized in the NADES with 22% (w/w) water content. The resulting phototoxicities from THPP and TCPP were enhanced when incorporated with NADES and these solvents proved to be viable porphyrin solubilizers that could allow them to be excellent photosensitizers in future aPDT treatments.

5.3. Drug Delivery and Extraction

5.3.1. Targeted drug delivery

Successful delivery to the appropriate target of interest in the human body is always a challenge when designing drugs. A hurdle of oral administration of pharmaceuticals is the release of the drug too early and not reaching the area of interest or the degradation of the drug itself in the digestive system. Polymers can behave as vehicles for drug delivery since they can polymerize and self-assemble into micelles which can then be used to encapsulate drugs. DESs have been implemented as functionalizing agents and act as the hydrophobic moiety in the inner core of the micelle allowing for incorporation of hydrophobic drugs while the polymeric carrier forms the hydrophilic outer shell of the polymer allowing it to navigate through the biological environment.[170, 171] Pradeepkumar *et al.*

have synthesized micelles using polycaprolactone functionalized with DESs to investigate the controlled release of hydrophobic drugs. Specifically, micelles functionalized with ChCl:citric acid (either 1:1 or 1:2 molar ratio) were used to entrap the anticancer drug camptothecin for successful delivery.[172] It was determined that 93%, 85%, 84%, and 67% of camptothecin was released from the micelle carrier at pH values of 2.6, 5.5, 6.8, and 7.4, respectively, over 8.5 h at 37 °C. The drug release was predominantly influenced by the hydrolysis of the ester bonds that were formed between polycaprolactone and the citric acid component of the DES. The cytotoxicity of the camptothecin-loading micelles was studied in human lung cancer cell line (A549) as well as normal human cell line (L929). The drug-loaded micelles were more cytotoxic to the A549 cells most likely due to the strong negative surface charge on the micelles which disrupted the cancer cell membrane and induced early and late apoptosis as can be seen in Fig 13.

Polycaprolactone has also been conjugated with folic acid and β-alanine to encapsulate the drug doxorubicin using the DES (3-(4-(4-(bis(2-chloroethyl)amino)phenyl)butanoyloxy)-N,N,N-trimethylpropane-1-aminium chloride) (CABAL):1,4-butanediol in a 1:6 molar ratio.[173] Again, the DES was used to help form the hydrophobic moiety of the micelle to help encapsulate the hydrophobic drug. It was determined that 98% of doxorubicin was released at pH 2.5 within 225 minutes whereas <50% of the drug was released at pH 5.5 and 6.8, agreeing with the previous results that show that acidic environments can improve drug release. When tested against the breast cancer cell line MDA-MB-231 and normal human cell line L929, the doxorubicin-loaded micelle was more cytotoxic to MDA-MB-231 cells. Mechanistically, once the carrier reached the cancer cells, the ester bonds in the micelle were degraded by lysozyme, which has been shown to be highly present in cancer cells. This degradation allowed for the release of doxorubicin into the cancer cell and lead to apoptosis.

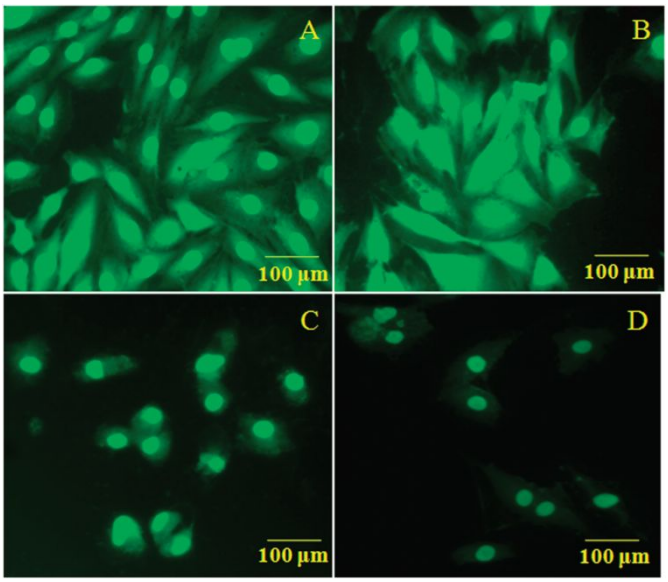

Fig. 13 Apoptosis of A549 cells cultured with CPT-loaded poly-cp-*co*-CA micelles for (A) 0 min, (B) 24 h, (C) 48 h and (D) 72 h. Reprinted with permission from reference[172]. Copyright © 2018 Royal Society of Chemistry.

Another interesting example comes from Mukesh *et al.* who have reported the self-polymerization of 2-hydroxy ethyl methacrylate (HEMA) in the NADES of ChCl:fructose (2:1) in the presence of the NSAID drug indomethacin, where indomethacin was immobilized in the resulting DES/HEMA ion gel.[174] The nontoxic and biocompatible gel was then tested for slow release of the drug at pH 1.2, 4.0, 6.8, and 7.4 in PBS. Pure indomethacin was insoluble in PBS buffer regardless of pH; however, when indomethacin was in incorporated into the DES/HEMA ion gel, it was released up to 96% and 88% at pH 7.4 and 6.8 in 15 h and 20 h, respectively, and the NSAID was found to be structurally stable in the gels for at least six months. Interestingly, indomethacin in the DES/HEMA ion gel was found to be insoluble in acidic pH (pH 1.2 and 4). Due to the specificity of these pH-sensitive micellar systems, they show promise as effective delivery systems to target and treat various ailments and diseases.

5.3.2. Microextraction techniques

Many of the extraction techniques used to extract pharmaceuticals including dispersive liquid-liquid extraction, solid-phase extraction, and hollow-fiber-based liquid-liquid extraction are labour intensive and involve the use organic solvents which violate the principles found in green chemistry. Additionally, these solvents are limited to extracting compounds that have low polarity, which prove to be ineffective at extracting polar media. Microextraction techniques implementing DESs as the extraction solvent have become increasingly popular. For instance, ChCl:phenol (1:4) has been used to successfully extract various anti-depressant drugs from biofluid and pharmaceutical wastewater samples using an efficient air-agitated emulsification microextraction technique.[175] In this extraction process, the water-miscible DES molecules aggregate and form a cloudy state after the completion of 14 air-agitation cycles in a home-made extraction cell, after which the target analytes become trapped in the aggregated DES and extracted. The percent extraction recovery (%ER) and enrichment factor (EF) are then calculated. At a spiked concentration level of 100 ng mL^{-1}, the %ER and EF values were determined to be 68% and 40 for escitalopram, 42% and 25 for desipramine and 64% and 38 for imipramine, respectively with a standard deviation lower than 5.7%. When this DES microextraction technique was implemented to test biofluid and pharmaceutical wastewater, the relative recovery percentages were determined to be 88.75%-99.45%. Interestingly, this air-agitation microextraction process was also successful in extracting amphetamine and methamphetamine from human plasma and pharmaceutical wastewater, although the DES composed of ChCl:phenethyl alcohol (1:4) was used.[176] The resulting extraction percentages of 63% and 66% and enrichment factors of 47 and 50 were seen in spiked samples for amphetamine and methamphetamine, respectively, with relative recoveries between 80.5-103.3% for real biofluid and pharmaceutical wastewater samples.

The DES of ChCl:phenethyl alcohol (1:4) has been incorporated into another microextraction technique, namely a

hollow-fiber based liquid phase microextraction (HF-LPME) to assist in extracting antiarrhythmic agents.[177] For this technique, the target compounds were penetrated into a supported liquid matrix containing the DES that was fixed inside the pores of a hollow fiber which effectively trapped the drugs of interest, followed by immediate back-extraction into an aqueous phase that was located in the lumen of the hollow fiber. The extraction recoveries of spiked concentration levels of 150 ng mL^{-1} propranolol, carvedilol, verapamil, and amlodipine were determined to be 54%, 48%, 44%, and 44% respectively, with enrichment factors of 135, 120, 110, 110, which were all higher as compared to the traditional solvent of 1-octanol. Additionally, the relative recoveries of these drugs in real samples of urine, plasma, and pharmaceutical wastewater were found to be in the range of 96.8-103.7%.

Liquid-liquid microextraction and solid-phase microextraction are other techniques that have shown enhanced extraction of antibiotics using DESs. The DES of ChCl:glycerol (1:2) mixed with chloroform successfully extracted 87.23% and 83.17% of chloromycetin and thiamphenicol, respectively, using liquid-liquid microextraction. Interestingly, however, these values increased when the DES alone was used in a solid-phase extraction to purify and extract these antibiotics from tainted milk, resulting in extraction percentages of 91.23% and 87.02% for chloromycetin and thiamphenicol, respectively.[178]

Additionally, Tang *et al.* successfully extracted the antibiotics levofloxacin and ciprofloxacin using the DES of tricaprylmethylammonium chloride:1-octanol (1:1) in a vortex-assisted liquid-liquid microextraction to yield extraction efficiencies >80% and relative recoveries up to 94.5% in real water samples.[179] Lastly, a DES composed of choline chloride and itaconic acid (1:5) was a functional monomer to synthesize a polymer monolithic column inside a poly (ether ether ketone) (PEEK) tube in order to perform an in-tube solid phase microextraction of NSAIDs.[180] The recoveries of three NSAIDs, specifically ketoprofen, flurbiprofen and diclofenac sodium were determined by HPLC to be between 87.3–113.7% in real lake water samples, while also completing extraction in as little as 17 minutes, as compared to other systems which have reported taking anywhere from 30–120 minutes.[181, 182]

5.4. Therapeutic deep eutectic solvents (THEDES)

5.4.1. Drug solubilization in THEDESs

The ability to form a DES with an active pharmaceutical ingredient (API) as one of the DES components is known as a therapeutic deep eutectic solvent (THEDES). These systems allow for the possibility of developing drug delivery vehicles. The eutectic can prevent recrystallization of the active pharmaceutical ingredient when dispersed in water, leading to more stable and active APIs. Additionally, the preparation of THEDES (and DESs in general) yields 100% pure product, without the need for any purification steps. Stott and coworkers demonstrated for the first time, transdermal drug delivery of ibuprofen through DESs composed of ibuprofen and a range of terpene enhancers including menthol, thymol, menthone, 1,8-cineole, d-Limonene, p-Cymene.[183] These DESs showed increased solubility, permeability, and absorption of the API of interest.

Aroso *et al.* developed THEDES based on ChCl or menthol as the HBAs conjugated with three different APIs, namely acetylsalicylic acid, benzoic acid, and phenylacetic acid.[184] Specifically, the DESs investigated were ChCl:phenylacetic acid (1:1), ChCl:acetylsalicylic acid (1:1), menthol:acetylsalicylic acid (3:1), menthol:benzoic acid (3:1), menthol:phenylacetic acid (2:1) and menthol:phenylacetic acid (3:1). Interestingly, the dissolution rate was enhanced in the majority of menthol-based THEDES (15-45 min) as compared to ChCl-based THEDES (>24 h), as well as a few of the individual APIs in buffer (>24 h). The resulting dissolution efficiencies for all samples tested were in the range of 10-87%, with menthol:benzoic acid providing the highest dissolution efficiency of 87%, although it required 24 h. Interestingly, menthol:phenylacetic acid (3:1) displayed a high efficiency of 81% in only 15 minutes. Additionally, all menthol-based THEDES showed wide inhibitory zones of growth when tested against both Gram-positive and Gram-negative bacteria, as compared to the pure APIs, showing enhanced antibacterial properties when APIs are incorporated into a DES. In a later work, menthol was combined with ibuprofen in a 3:1 ratio to make a THEDES that enhanced ibuprofen solubility from 2.1 mg mL^{-1} in PBS to 26.8 mg mL^{-1}.[163] Ibuprofen in its native form falls into the Class II type of drugs when fitted with the biopharmaceutical classification system, as can be seen in Fig. 12; however, when it is in combination with menthol, it can now be classified as a Class I type due to its increased solubility and permeability in a THEDES.

The anti-tuberculosis drug ethambutol and the amino acid L-arginine have been incorporated as an API into THEDES using citric acid as the HBA and water to further increase solubility.[185] Impressively, ethambutol solubility increased from 4.64 ± 0.07 mg mL^{-1} in PBS to 127.60 ± 0.69 mg mL^{-1} when incorporated into the THEDES (citric acid:ethambutol:water; 2:1:10). Additionally, the permeability was shown to have a 1.5-fold increase, which altered its classification from a Class III compound (high solubility, low permeability) to Class I (high solubility, high permeability) when in THEDES. The incorporation of ethambutol in a THEDES could be a potentially better anti-tuberculosis treatment due to the lower dosage that could be implemented for patients. For L-arginine, a slight solubility increase was observed from 99.35 ± 0.54 mg mL^{-1} in PBS to 102.17–107.86 mg mL^{-1} in the THEDES (citric acid:L-arginine) regardless of the molar ratio used. Arginine-based DESs have also been investigated to increase the API lidocaine solubility in various THEDES. Density functional theory and molecular dynamic methods have shown that high solvation of lidocaine with arginine in a THEDES is based on strong solute-solvent intermolecular interactions, with hydrogen bonding playing the key role in the solubilization process.[186, 187]

Another interesting study came from Abbott *et al.* who employed ChCl as the HBA along with the following HBDs: phenol, catechol, resorcinol, hydroquinone, 2-methyl-phenol, 3,4-dimethyl-phenol, 2,4,6-trimethyl-phenol, thymol, 2,6-di-tert-butyl-4-methylphenol, salicylic acid, 4-chloro phenol, 4-methoxy phenol, paracetamol, benzoic acid, and aspirin.[188]

Among these, ChCl:resorcinol (1:1) displayed the highest solubility of 1110 g dm^{-3}, while 2,6-di-tert-butyl-4-methylphenol was insoluble and did not even form a DES. Additionally, the THEDES incorporating benzoic acid and aspirin showed the lowest solubilities of 3.4 g dm^{-3} and 4.6 g dm^{-3}, respectively. Two commonly prescribed drugs, adiphenine and ranitidine, were also investigated in this study and were able to form eutectics with HBDs of glycerol, urea, and aspirin, the latter showing that two active pharmaceutical ingredients can be incorporated together in a DES. Oddly enough, when adiphenine was mixed with glycerol, urea, or aspirin, the solubility remained the same throughout, at 70 g dm^{-3}, showing that, in this case at least, the HBD did not make a difference.

As this section has discussed, drug solubilization and permeability can be greatly enhanced when the APIs of interest are incorporated into the deep eutectic solvent system. Dissolution and permeation of the APIs depends on the formulation of the THEDES, which, due to their strong intermolecular hydrogen bonding in solution, allows for higher transport rates through membranes than in their solid forms. Additionally, the stoichiometries of THEDES can be fine-tuned to allow for greater specificity and show great potential as new delivery systems.[189] Overall, it's been shown that a wide array of pharmaceuticals can form THEDES that increase the APIs solubility and improve drug delivery while also decreasing the dosage requirements for future treatments.

5.4.2. THEDES in polymers

Biodegradable elastomers have demonstrated a tremendous versatility as polymeric networks for regenerative medicine as well as efficient systems for controlled drug release in biomedical applications.[190] The coupling of THEDES with polymeric material brings new possibilities for alternative pharmaceutical systems. Supercritical fluid technology has opened the door to a variety of applications in recent years. This sintering process takes place at subcritical conditions using carbon dioxide ($scCO_2$) where polymeric particles are fused together by plasticization to create a lightweight and porous 3D structure. The main requirement for the CO_2–foaming process is that a sufficient amount of CO_2 needs to be dissolved in the polymer. By doping polymers with THEDES, this foaming/sintering process can still take place due to the plasticizing properties of the THEDES. These potential plasticizing agents can improve the foaming ability by decreasing the melting point of the polymers or by enhancing CO_2 solubility in the matrix, while also improving the overall flexibility of the polymer. Additionally, the resulting polymeric matrices can display enhanced porosity that can be exploited as potential alternatives as drug delivery systems.

Martins *et al.* have evaluated various NADESs to enhance $scCO_2$ foaming process to promote the synthesis of 3D porous structures.[191] Although the final polymer porosity increased with all NADESs investigated, it was highly dependent on which NADES was used. In the case of the polymer blended with ChCl:sucrose (1:1) and ChCl:citric acid (1:1), the foamed structures presented large open pores, high homogeneity, and high pore interconnectivity, with ChCl:sucrose resulting in a 52% increase in porosity. As expected, the polymer blend foamed without the presence of NADES showed very low levels of porosity.

Aroso *et al.* have created a polymer based on 70% corn starch and poly-ε-caprolactone impregnated with the THEDES menthol:ibuprofen (3:1) as a potential drug delivery system.[192] The presence of 20% THEDES impregnated in the polymers enhanced the porosity to 44% and increased the interconnectivity to 28%, which was shown to be 3-fold and 9-fold higher, respectively, than the polymer alone. Additionally, ibuprofen displayed a faster release from the polymeric matrix when it was impregnated with THEDES (~85%) as compared to the polymer containing just ibuprofen (~65%) in 125 h. Similar supercritical fluid sintering results have been shown with ChCl:ascorbic acid (2:1) containing solubilized dexamethasone.[193] An incredible increase in dexamethasone solubility and diffusion rate was seen when the polymer was doped with 10 wt% THEDES (~4000 mg L^{-1}) as compared with the drug in powder form (60 mg L^{-1}).

Poly(diol-*co*-citrates) (PDC) polymers were first described by Yang *et al*. in 2004 as new types of biodegradable and biocompatible polyesters.[194] Over the years, there have been many challenges in regards to incorporating APIs into these polymers mostly due to the prepolymer synthetic step because the high temperatures that are required (several minutes at 160-165 °C) can be detrimental to APIs, leading to inactivation or denaturation of these pharmaceuticals. In addition, during the post-synthesis steps, it is difficult for the APIs to be homogeneously distributed. Nonetheless, Serrano *et al.* have prepared DESs based on lidocaine and 1,8-octanediol (molar ratio 1:3) to create poly(octanediol-*co*-citrate) elastomers loaded with lidocaine.[195] The DES provided not only one of the polymer precursors and the API, but was also the synthetic media where the second polymer precursor (citric acid) could be solubilized in order for polymerization to take place. The prepolymer synthesis was able to be carried out at 90 °C (instead of the typical 160 °C), ensuring no lidocaine inactivation or degradation. A burst release of lidocaine occurred within the first hour of the elastomer being in water or PBS after which it continued to slowly increase up to 300 h.

6. Emerging biomedical applications

Alginates, specifically calcium alginates, have been used in the pharmaceutical industry for controlled drug release; however, DESs are more recently being used as reinforcement fillers in calcium alginate beads to further enhance the slow release of drugs. More specifically, ChCl:thiourea (1:2) has been mixed with chitin powder to produce chitin nanofibers in calcium alginate beads to enhance the release of the anticancer drug 5-fluoroaurascil.[196] These DES-chitin nanofibers were able to release 70% of the drug after 24 h at pH 7.4, as compared to the typical DES-free calcium-alginate beads that only released 39% of the drug after 3 h.

Shrimp shells contain calcium carbonate (25-50%), protein (35-50%), and chitin (15-25%), among which, chitin has many

beneficial properties such as biocompatibility, biodegradability, bioactivity, and non-toxicity, and can be applied in numerous fields such as biomedicine, agriculture, water treatment and cosmetics. Moreover, chitin derivatives including deacylated, acylated, thiolated, and grafted chitin have attracted more interest because of their high solubility and possess unique properties compared with chitin. The conventional methods to retrieve chitin and obtain its acylated derivative involve removing the calcium carbonate and protein which typically involve time-consuming processes and the use of harsh chemicals that are hazardous to the environment. Hence, DESs have been implemented as a triple threat solvent where decalcification, deproteinization, and acylation of chitin all occur in a one-step process. For instance, ChCl:DL-malic acid (1:2) has been shown to successfully remove calcium and proteins from shrimp shells while simultaneously causing an acylation reaction in chitin at 130 °C in 3 h.[197] The DES had the ability to release H^+ which reacted with calcium carbonate to produce carbon dioxide and water-soluble calcium salts and the acidity of the DES allowed for protein dissolution and degradation. Additionally, the H^+ released from the DES was able to catalyze the acylation of chitin in a nucleophilic type of reaction to produce the acylated product O-malate chitin. The obtained acylated chitin was shown to have good antibacterial and antitumor activity against both *E. coli* and C6 glioma, respectively. Interestingly, Zhao *et al.* found that pre-treating shrimp shells first with 10% citric acid to help with demineralization allowed the DESs of betaine hydrochloric acid:urea, ChCl:urea, ChCl:ethylene glycol, and ChCl:glycerol (all in 1:2 molar ratios) to all successfully retrieve chitin in similar yields of around 20-25%, which was higher than the typical acid/alkali extraction method (17.7%), while also drastically reducing the extraction time to as little as seven minutes using microwave irradiation.[198]

A transdermal drug delivery system (TDDS) has many advantages, such as non-invasiveness, sustained release, and enhanced therapeutic efficiency. However, a low permeation rate of drugs through the skin still continues to be an issue. To this, a DES composed of choline bicarbonate and geranic acid (molar ratio 1:2) exhibited broad-spectrum antimicrobial activity against a number of drug-resistant bacteria, fungi, and viruses including *Mycobacterium tuberculosis*, *Staphylococcus aureus* and *Candida albicans* as well as strains of Herpes Simplex Virus.[199] Interestingly, *Pseudomonas aeruginosa* was the most resilient to treatment, requiring ~25% (v/v) CAGE to neutralize it. *Mycobacterium tuberculosis* (TB), on the other hand, was the most susceptible out of all 47 strains tested against CAGE, needing <0.195% (v/v) CAGE for complete neutralization. Overall, the majority of strains tested including Gram-positive and Gram-negative bacteria, aerobes and anaerobes, and fungi and viruses only required <1% (v/v) CAGE treatment. Additionally, CAGE resulted in an ~180-14,000-fold improved efficacy/toxicity ratio over currently used antiseptic agents and was shown to penetrate deep into the dermis layer of skin to treat a *Propionibacterium acnes* infection *in vivo*, while also displaying good biocompatibility and no toxicity with healthy tissues. Similar studies have been reported for CAGE to act as an antibacterial agent against the Gram-negative bacteria *Escherichia coli* (*E. coli*) while also being successful in topical delivery for skin diseases and transdermal drug delivery.[200-202] CAGE has also been used to deliver BSA, ovalbumin, and insulin into the dermis of porcine skin to enhance topical delivery of therapeutic proteins.[203] A significant enhancement of BSA in CAGE was delivered to the epidermis and dermis of rats (2.84 ± 0.59 μg cm^{-2} and 1.61 ± 0.09 μg cm^{-2}, respectively) after only 12 hours as compared to BSA in PBS (0.08 ± 0.01 μg cm^{-2} and 0.04 ± 0.04 μg cm^{-2} for epidermis and dermis, respectively), as can be seen in Fig. 14.

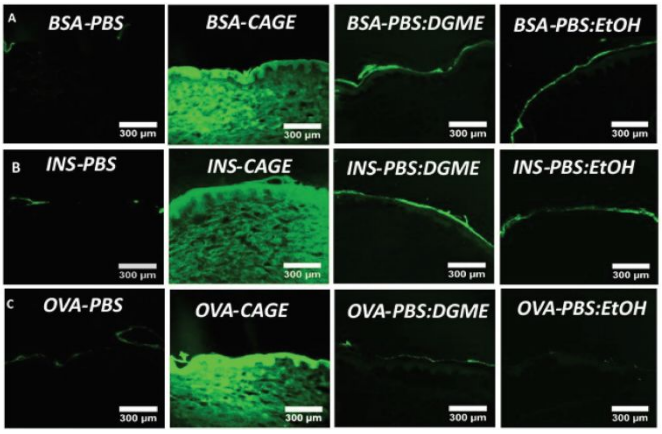

Fig. 14 Confocal images of skin penetration of fluorescently labeled proteins in PBS, CAGE, 50:50 (v/v) PBS:DGME, or 50:50 (v/v) PBS:ethanol. Reprinted with permission from reference[203]. Copyright © 2017 Wiley.

When rats were given 10 U mL^{-1} insulin-CAGE (25 U kg^{-1} body weight), an immediate 25% decrease in the blood glucose level was observed within two hours of administration, with the highest decrease in blood glucose seen around 40% (59.5 ± 5.6%) after four hours. In comparison with typical insulin injections, when 1 U kg^{-1} of insulin was administered, an immediate 45% decrease in blood glucose was seen; however, it returned to its original value within four hours. Other reports have observed increased absorption and delivery of insulin and small molecule drugs in CAGE,[204-209] although there have been some conflicting reports as to whether CAGE is a DES or IL. It is important to note that CAGE could be used as a standalone drug in the case of mild infections or in synergistic combinations with antibiotics in the case of severe or life-threatening infections.

Deep eutectic gels have become increasingly popular over the years in ion gel reports. One of the first involves a resorcinol-formaldehyde gel that was prepared using a polycondensation reaction inside DESs to synthesize carbon monoliths.[210] Interestingly, the ion gel was not the product of the reaction but served only as a template that was discarded after synthesis. Subsequently, publications started to increase where the ion gel was synthesized by incorporating a DES into the synthesis and these "eutectogels" showed good flexibility, conductivity, and shape recovery. [174, 211-213] In a recent report, a eutectogel was formed by incorporating the DES ChCl:glyceline (1:2) within bacterial cellulose.[214] The very high crystallinity of bacterial

cellulose remained unaffected by the DES contained in the gels even at high loadings (98.8% by weight). Overall, the structural behaviors and dynamic properties of this gelatin-like self-supported material was investigated through a multitude of techniques, including X-ray diffraction, nuclear magnetic resonance (NMR), small-angle neutron scattering (SANS), and molecular dynamics (MD) simulations.

7. Conclusions and outlook

We have reviewed the use of DESs in a wide array of biological or biotechnology-oriented applications. These emergent and potentially-green solvents have been shown to greatly improve the thermostability of proteins, to aid in high value biological extractions, and to open the door to steering the self-assembly of various DNA topologies, including the emergence of unique, non-native architectures. In many cases, DESs strongly increase product yields and conversion rates when incorporated as the solvent, co-solvent, substrate, or catalyst in various biocatalytic scenarios. DESs also allow for the enhanced pretreatment of recalcitrant biomass (*e.g.*, removal of chitin from shrimp shells), effectively separating and breaking down complex lignocellulosic matrices to better utilize the resulting products as fuels or synthetic feedstocks. Furthermore, the improved stability and solubility of various pharmaceuticals and the introduction of therapeutic DESs (THEDES) indicate future promise in oral and transdermal drug delivery, potentially at lower dosing of APIs.

In addition to some established avenues for the application of DESs in biologics, there are some less-explored but highly exciting avenues that will see increased activity in the coming years. Because DESs frequently comprise natural primary metabolites known to aid in survival at negative temperatures (*e.g.*, sugars, sugar alcohols, organic acids, amino acids, amines), they hold intriguing potential as cryoprotective agents[134, 215], conversely, as room-temperature preservation media to eliminate the cold-chain storage of therapeutic proteins and vaccines.[33] DESs are likely to be a great asset to the biotechnologist in other ways as well, including as artificial, small-molecule chaperonins aiding in protein refolding[216], as promoters of rapidly controlled protein fibrillation[27], and as media for the design of artificial biosystems[217] and synthetic protocells.

Despite the obvious importance of water content (*e.g.*, key structural waters) in the behavior of DESs, the key molecular details are still awaited. And while, in principle, these solvents can be optimized to the application of interest, the lion's share of studies remain focused on a handful of DESs, particularly the "notorious three" (*i.e.*, reline, ethaline, glyceline). Given the enormous interest in these solvents, the ongoing challenges and opportunities discussed herein will very likely be met and exceeded in real time.

Conflicts of interest

There are no conflicts to declare.

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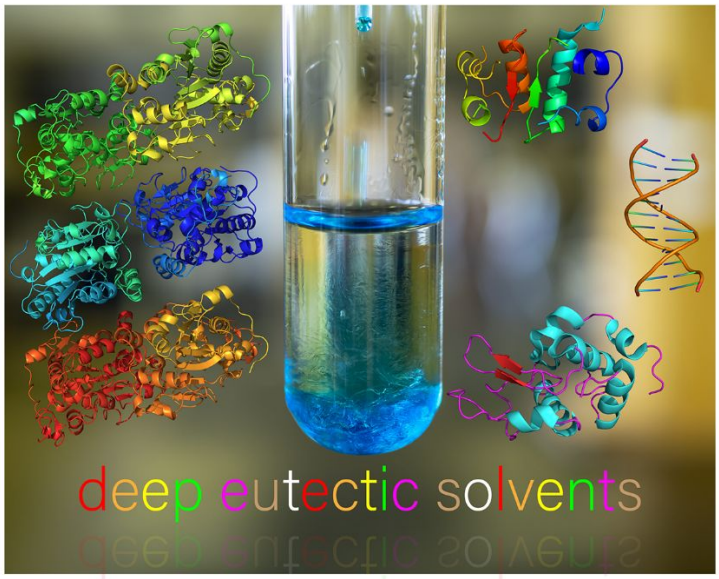

Deep eutectic solvents offer stimulating possibilities for biomolecular stabilization and manipulation, biocatalysis, bioextraction, biomass processing, and drug delivery and therapy.