Ionic liquids as biocompatible stabilizers of proteins

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Abstract

Ionic liquids (ILs) have recently emerged as versatile solvents and additives in the field of biotechnology, particularly as stabilizers of proteins and enzymes. Of interest to the biotechnology industry is the formulation of stable biopharmaceuticals, therapeutic proteins, and vaccines which have revolutionized the treatment of many diseases including debilitating conditions such as cancers and auto-immune diseases. The stabilization of therapeutic proteins is typically achieved using additives that prevent unfolding and aggregation of these proteins during manufacture, transport, and long-term storage. To determine if ILs could be used in the formulation of stable therapeutic proteins, a thorough understanding of the effects of ILs on protein stability is needed, as well as understanding the toxicity of ILs on humans, and other considerations for formulation development such as viscosity and osmolality. In this review, we summarize recent developments on the stabilization of proteins and enzymes using ILs, with emphasis on identifying biocompatible ILs that may be suitable for the formulation of stable biopharmaceuticals in the future.

Keywords Protein aggregation · Stability · Toxicity · Formulations · Protein unfolding · Biotechnology

Abbreviations

ILs	Ionic liquids	HexSO ₄	Hexylsulfate
PIL	Protic ionic liquid	Tos	Tosylate
AIL	Aprotic ionic liquid	OH-EMIM	1-(2-Hydroxyethyl)-3-methylimidazolium
EAN	Ethylammonium nitrate	OH-PMIM	1-(3-Hydroxypropyl)-3-methylimidazolium
DHP	Dihydrogen phosphate	OH-HMIM	1-(6-Hydroxyhexyl)-3-methylimidazolium
Chol	Choline	BF ₄	Tetrafluoroborate
BMIM	1-Butyl-3-methylimidazolium	CT	α-Chymotrypsinogen
EMIM	1-Ethyl-3-methylimidazolium	BzMIM	1-Benzyl-3-methylimidazolium
PMIM	1-Methyl-3-propylimidazolium	TEAA	Triethylammonium acetate
HMIM	1-Hexyl-3-methylimidazolium	TEAP	Triethylammonium phosphate
MMIM	1,3-Dimethylimidazolium	TBPBr	Tetrabutylphosphonium bromide
SCN	Thiocyanate	Ac	Acetate
Cyt C	Cytochrome complex	Cit	Citrate
BSA	Bovine serum albumin	rHIL-2	Recombinant human interleukin-2
rPa	Recombinant plasminogen activator	TMA	Trimethyl acetate
MDEG	2(2-Methoxyethoxy)-ethyl	Lac	Lactate
		Prop	Propionate
		Hex	Hexanoate
		DMP	Dimethyl phosphate
		TFMS	Trifluoromethane sulfonate
		TF ₂ N	Bis(trifluoromethylsulfonyl)amide
		Glu	Glutarate
		GuHCl	Guanidine hydrochloride

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mAb	Monoclonal antibody
EGFR	Epidermal growth factor receptor
MD	Molecular dynamics
DBP	Dibutyl phosphate
BEH	Bis(2-ethylhexyl) phosphate
TMP	Bis(2,4,4-trimethylpentyl) phosphinate
DEP	<i>O,O'</i> -diethyl dithiophosphate
EOOEMIM	3-Methyl-1-(ethoxycarbonylmethyl) imidazolium
[EOOEMPy][C12SO4]	3-Methyl-1-(ethoxycarbonylmethyl) pyrrolidinium

Introduction

Ionic liquids (ILs) are organic salts with low melting points, typically below 100 °C. The interest in IL research began shortly after the synthesis of the first air- and moisture-stable ILs in 1992 (Wilkes and Zaworotko 1992). Initially developed as non-flammable alternatives to common organic solvents, ILs quickly found hundreds of applications spanning electrochemistry (Alvarez-Guerra et al. 2015; Angell et al. 2017; Fedorov and Kornyshev 2014), energy (MacFarlane et al. 2016; MacFarlane et al. 2014; Smiglak et al. 2014), organic synthesis and catalysis (He et al. 2014; Qureshi et al. 2014) to pharmaceuticals (Jouannin et al. 2014; Marrucho et al. 2014; Taha et al. 2015), biotechnology (Byrne et al. 2012; da Costa Lopes et al. 2013; Haykir et al. 2013; Quental et al. 2015; Sivapragasam et al. 2016; Wang et al. 2012), and more (Egorova et al. 2017; Wei and Danielson 2011). It is estimated that there are over a million combinations of cations and anions that can form an IL (Weingartner et al. 2012). Consequently, these versatile solvents which are often termed “designer solvents” can access a wide range of physicochemical properties which can be tailored for almost any application; such properties include low vapor pressure, high thermal stability, high conductivity, non-flammability, and biocompatibility (Rogers and Seddon 2003).

The use of ILs in biotechnology has gained significant attention recently, particularly for the enhanced stabilization of proteins, enzymes (Egorova et al. 2017; Greaves and Drummond 2015; Kumar et al. 2017a, 2017b), and viruses (Byrne et al. 2012). Proteins are highly sensitive to changes in their vicinity induced by temperature, pH, humidity, and salt concentration, in addition to any shear stress applied during manufacturing (Elgundi et al. 2017). As they are exposed to these “stresses” they undergo gradual changes in structure, or unfold, and form protein aggregates over time (Kayser et al. 2011). In addition, the poor stability of proteins and enzymes might result in loss of function and activity, significantly limiting their use in biological processes and as biopharmaceuticals.

ILs have been utilized as “neat” solvents or as solutes in an aqueous solution (“aqueous ILs”) to stabilize proteins (Persson and Bornscheuer 2003; Vrikkis et al. 2009). Although both approaches have led to improved protein stability, most neat ILs may not be suitable for biopharmaceutical applications due to the high viscosity of most ILs, and lack of solubility of proteins at high concentrations in neat ILs, which is particularly important for biologics. Consequently, dissolving ILs in an aqueous solution as an additive/co-solvent has been a more favorable approach in this field and will be the focus of this review.

The emergence of ILs as potential stabilizers of proteins is a significant finding that requires review, particularly for the development of stable biopharmaceuticals, an application of ILs which has not been addressed adequately in previous literature. Biologics have revolutionized management of cancers, auto-immune diseases, infections, and other difficult conditions to treat since their introduction as therapeutic products more than 30 years ago (Elgundi et al. 2017). The stabilization of biopharmaceuticals is more challenging because of additional considerations required to determine the suitability of a formulation additive, such as the toxicity of the additive, and particularly in the case of ILs, the influence on the viscosity of the final protein solution, especially if the product is to be injected intravenously, which is the case for many biopharmaceuticals.

Our aim is to review the current literature on the application of ILs for the stabilization of proteins and assess the suitability of the promising IL candidates for the stabilization of biopharmaceuticals.

Current literature on the stabilization of proteins using ILs

ILs are often classified as protic (PILs) or aprotic ILs (AILs). PILs act as hydrogen bond donors and often include ammonium or phosphonium derivatives as a cation, combined with a phosphate, sulfate or nitrate anion (Angell et al. 2007; Greaves and Drummond 2015; Smiatek 2017). Examples of protic ILs include ethylammonium nitrate (EAN) and choline dihydrogen phosphate ([Chol][DHP]) (Fig. 1). AILs act as hydrogen bond acceptors and typically include a bulky cation such as pyrrolidinium, pyridinium, or imidazolium or their derivatives, in combination with a halide anion, or hexafluorophosphate, tetrafluoroborate, or other such anions (Angell et al. 2007; Smiatek 2017). Examples of AILs include 1-butyl-3-methylimidazolium chloride ([BMIM][Cl]) and 1-benzyl-3-methylimidazolium tetrafluoroborate ([BzMIM][BF₄]) (Fig. 1). Of both classes of ILs, we will review some of the findings on imidazolium- and choline-based ILs which have received the most attention in recent years (Egorova et al. 2017; Kumar et al. 2017a, 2017b).

Fig. 1 Examples of protic and aprotic ILs. **a** 1-Benzyl-3-methylimidazolium tetrafluoroborate ([BzMIM][BF₄]). **b** 1-Butyl-3-methylimidazolium chloride ([BMIM][Cl]). **c** Triethylammonium

acetate (TEAA). **d** Choline dihydrogen phosphate ([Chol][DHP]). **e** Choline serinate ([Chol][Ser]). **f** Ethylammonium nitrate (EAN). **g** Tetrabutylphosphonium bromide (TBPBr)

Research on the imidazolium class of ILs has helped to gain insight into the mechanism behind the observed effects on protein stability, as well as develop better ILs. Additionally, choline ILs have emerged as one of the most suitable candidates for stabilization of biopharmaceuticals due to their biocompatibility and pronounced effect on protein stability and are therefore of primary relevance to this review. For a quick summary of the literature findings, see Table 1.

Imidazolium-based ILs

Imidazolium-based ILs are the most extensively researched family of ILs till date and are widely used in many of the applications mentioned previously (Fang et al. 2015; Gao et al. 2015; Ngo et al. 2000). Many studies which investigated the use of imidazolium ILs on protein stability have found that they act as protein denaturants or chaotropes; however, they have been extremely useful in gaining mechanistic insight into the complex interactions of ILs with proteins and are worth investigation (Patel et al. 2014; Sivapragasam et al. 2016).

Kumar et al. (2017b) studied the effect of three imidazolium ILs—[BMIM][Cl], [BMIM][Br], and [BMIM][I]—on the conformational structure and thermal stability of stem bromelain. All three ILs were found to be denaturing above concentrations of 0.01 M, while having no stabilizing effect below that concentration. The order of thermal stability was [BMIM][Cl] > [BMIM][Br] > [BMIM][I], with [BMIM][I] being the most denaturing. Tertiary structure was also found to be disrupted at concentrations above 0.01 M and followed the same order; however, [BMIM][Cl] caused significantly less disruption of tertiary structure above 0.01 M than the other two ILs. The order of destabilization was found to follow the Hofmeister series; that is, the chaotropic (structure-breaking) anion [I] was the most destabilizing, while the kosmotropic (structure-making) [Cl] was the least destabilizing (Kumar et al. 2017b; Schneider et al. 2011).

Takekiyo et al. (2017) studied whether 1-butyl-3-methylimidazolium thiocyanate ([BMIM][SCN]) and ethylammonium nitrate (EAN) could be used as cryoprotectants of cytochrome complex (cyt C) and lysozyme. Both ILs significantly decreased the activity of cyt C and lysozyme and induced unfolding. However, following the removal of the ILs by dialysis after cryo-storage and subsequent heating, the activities of both cyt C and lysozyme returned to >90% and structural changes were recovered (i.e. refolded) at IL concentrations >10% (Takekiyo et al. 2017). This study demonstrated that the denaturant effect of imidazolium ILs (and EAN) is predominantly reversible.

Some imidazolium ILs have been shown to act as protein refolding agents. For example, Yamaguchi et al. (2008) investigated numerous hydrophobic imidazolium cations coupled with a Cl anion and found that refolding was best achieved with short N-alkyl chain cations, with [BMIM][Cl] achieving the highest refolding yield of 84%. Increasing the length of the alkyl side chain and hydrophobicity of the cation moiety of the ILs reduced the refolding yield. The authors suggested that the hydrophobic moieties of these ILs with long alkyl side chains strongly interact with hydrophobic patches of the protein structure driving destabilization and preventing intramolecular interactions required for refolding. This was shown to be the case for bovine serum albumin (BSA) (Shu et al. 2011).

Buchfink et al. (2010) looked at the influence of the anion on the ability of EMIM-ILs to induce refolding of recombinant plasminogen activator (rPa) and stability of lysozyme. A series of anions were tested including 2(2-methoxyethoxy)-ethyl sulfate (MDEGSO₄), ethyl sulfate (EtSO₄), and hexyl sulfate (HexSO₄), Cl, Ac, Tosylate (Tos), and diethyl phosphate (Et₂PO₄). The ability of the anion to promote refolding of rPa while coupled to EMIM followed the order of [Cl] > [MDEGSO₄] > [EtSO₄] > [Ac] > [Tos] > [Et₂PO₄] > ≈ [HexSO₄]; [EMIM][Cl] was the only IL superior to ArgHCl as a refolding agent. Meanwhile the melting temperature (*T_m*)

Table 1 Summary of the literature on ILs and protein stability discussed in the review

Ionic liquid	Proteins tested	Effect on protein stability	References
Imidazolium-based ILs			
[MMIM][I]	Lysozyme crystals	+	Wang et al. (2010)
[BMIM][Cl]	Stem bromelain, lysozyme, lysozyme crystals	–, –, +	Kumar et al. (2017b), Lange et al. (2005), Wang et al. (2010)
[BMIM][Br]	Lysozyme crystals	+	Wang et al. (2010)
[BMIM][I]	Stem bromelain	–	Kumar et al. (2017a, 2017b)
[BMIM][SCN]	Lysozyme, cyt C	–, –	Takekiyo et al. (2017)
[BMIM][BF ₄]	Lysozyme crystals	+	Wang et al. (2010)
[EMIM][Ac]	rPa ^a	~	Buchfink et al. (2010)
[EMIM][Cl]	rPa ^a , lysozyme ^a	+, –	Buchfink et al. (2010), Lange et al. (2005)
[EMIM][EtSO ₄]	rPa ^a	+	Buchfink et al. (2010)
[EMIM][MDEGSO ₄]	rPa ^a	+	
[EMIM][Et ₂ PO ₄]	rPa ^a	+	
[EMIM][Tos]	rPa ^a	–	
[EMIM][HexSO ₄]	rPa ^a	–	
[OH-EMIM][Cl]	Lysozyme	–	Lange et al. (2005)
[OH-PMIM][Cl]	Lysozyme	–	
[HMIM][Cl]	Lysozyme	–	
[BzMIM][Cl]	CT	~	Attri et al. (2011)
[BzMIM][BF ₄]	CT	~	
Ammonium-based ILs			
EAN	Lysozyme, cyt C	–	Takekiyo et al. (2017)
TEAA	CT	++	Attri et al. (2011)
TEAP	CT	++	
[Chol][Ac]	CT	++	Bisht and Venkatesu (2017)
[Chol][DHP]	CT, lysozyme, rHIL-2, cyt c	++, +, ++, +++	Bisht et al. (2017), Bisht and Venkatesu (2017), Fujita et al. (2006), Fujita et al. (2007), Rodrigues et al. (2011), Weaver et al. (2012)
[Chol][Cit]	CT	–	Bisht and Venkatesu (2017)
[Chol][OH]	CT	–	Bisht and Venkatesu (2017)
[Chol][Cl]	CT, lysozyme	++	Bisht and Venkatesu (2017)
[Chol][TMA]	Lysozyme	+	Rodrigues et al. (2011)
[Chol][Lac]	Lysozyme	–	
[Chol][Prop]	Lysozyme	–	
[Chol][Hex]	Lysozyme	–	
[Chol][DMP]	Lysozyme	+	
[Chol][TFMS]	Lysozyme	–	
[Chol][TF ₂ N]	Lysozyme	–	
[Chol][Glu]	Cyt c	++	Bisht et al. (2017)
[Chol][Thr]	Collagen	–	Tarannum et al. (2018)
[Chol][Ser]	Collagen	–	
[Chol][Lys]	Collagen	–	
[Chol][Phe]	Collagen	–	
Others			
TBPBr	CT	~	Attri et al. (2011)
[EOEMIM][C ₁₂ SO ₄]	BSA	–	Wang et al. (2012)
[EOEMPY][C ₁₂ SO ₄]	BSA	–	

^a Only refolding was measured

of lysozyme in the same EMIM-ILs followed the order $[Cl] > [EtSO_4] \approx [MDEGSO_4] > [Et_2PO_4] > [Ac] \approx [Tos] > [HexSO_4]$. Interestingly, this finding did not obey the Hofmeister series, which predicts that $[Ac]$ is more stabilizing than $[Cl]$ (Schneider et al. 2011). To some degree, the ability of the anion to promote refolding was related to the hydrophobicity of the anion. More specifically, the authors found that ILs which had intermediate ability to solubilize tryptophan were most effective at promoting refolding of rPa.

Interestingly, imidazolium-based ILs have been shown to act as denaturants, refolding enhancers and suppressors of insoluble aggregates at the same time. Lange et al. (2005) studied the effect of $[BMIM][Cl]$, $[OH-EMIM][Cl]$, and $[OH-PMIM][Cl]$ on the stability of lysozyme and found that the T_m of lysozyme decreased significantly with increasing alkyl chain length of the cation. The alkyl-ILs were found to be stronger denaturants than their OH-IL counterparts. They also tested the effects of $[EMIM][Cl]$, $[BMIM][Cl]$, and $[HMIM][Cl]$, again confirming that increasing the alkyl chain length and hydrophobicity of the cation moiety significantly decreases the conformational stability of lysozyme. However, insoluble aggregates were suppressed in the presence of 2 M of each IL after denaturation of lysozyme and dilution into each IL, and at 0.5 M concentrations, $[HMIM][Cl]$ was the most effective suppressor of insoluble aggregates, while less hydrophobic cations were not as effective. Additionally, $[EMIM][Cl]$, $[OH-EMIM][Cl]$, and $[OH-PMIM][Cl]$ were found to be effective refolding enhancers, while $[BMIM][Cl]$, $[HMIM][Cl]$, and $[OH-HMIM][Cl]$ were ineffective.

While imidazolium ILs appear to act as protein denaturants, refolding enhancers, and suppressors of insoluble aggregation, other studies have found that some imidazolium ILs improved the thermal stability of proteins tested under certain conditions.

Wang et al. (2010) found that the T_m of lysozyme crystals produced in various imidazolium ILs as additives (1% w/v) increased from 65.6 to 71.4 °C ($[BMIM][Cl]$), 78.2 °C ($[MMIM][I]$), 81.2 °C ($[BMIM][BF_4]$), and 81.9 °C ($[BMIM][Br]$). Thus, the improved thermal stability obtained with the ILs followed the order of $[BMIM][Br] > [BMIM][BF_4] > [MMIM][I] > [BMIM][Cl]$ (Wang et al. 2010). The enhanced thermal stability of lysozyme crystals was attributed to enhanced crystal contacts, changes in conformational stability, and intermolecular interactions between lysozyme molecules, and lysozyme and each IL, which influenced lysozyme nucleation and crystal growth and morphology (Wang et al. 2010).

Attri et al. (2011) studied the thermal stability of α -chymotrypsinogen (CT) in five ILs based on ammonium, phosphonium, and imidazolium salts. Both imidazolium ILs tested (1-benzyl-3-methylimidazolium chloride ($[BzMIM][Cl]$), 1-benzyl-3-methylimidazolium

tetrafluoroborate ($[BzMIM][BF_4]$)) mildly improved the thermal stability of CT. On the other hand, triethyl ammonium acetate (TEAA) had the most stabilizing effect on CT increasing the T_m from 42 to 65 °C, followed by triethyl ammonium phosphate (TEAP) which increased its T_m to 62 °C. Tetra-butyl phosphonium bromide (TBPBr) only mildly improved thermal stability in comparison. However, all ILs reduced the activity of CT compared to buffer; TEAA retained the highest enzyme activity of all ILs. The stabilization of the native structure of CT was found to be due preferential expulsion of the ILs from the protein surface (the interaction between the ILs and disulfide bonds of CT is unfavorable) leading to the formation of a more compact CT structure which is more stable (Attri et al. 2011).

IL concentration is another critical factor as it determines the nature of interactions and subsequent effects of ILs on proteins and protein stability. For instance, Takekiyo et al. (2012) found that at 5 M concentrations of $[BMIM][NO_3]$ the tertiary structure of lysozyme was significantly disrupted, while at 6–10 M concentrations, partial refolding of the secondary structure was observed. At concentrations > 10 M, this partial refolding was maintained but the tertiary structure was significantly disrupted again. These observations were attributed to the changes in the spatial arrangement of water molecules around the protein structure, mediated by the concentration of IL in solution (Takekiyo et al. 2012).

Thus, we can conclude that the effects of ILs on protein stability are multi-faceted; whether imidazolium ILs act as protein denaturants, stabilizers, or refolding agents depends on the type of protein being investigated, the anion coupled to the imidazolium ion, the hydrophobicity and length of the alkyl side chain of the cation, and the concentration of the IL used. These factors are most likely relevant to all families of ILs.

Choline-based ILs

The ammonium family of ILs, especially choline ILs and their derivatives, has gained significant attention in the last decade for their pronounced effect on protein stability and apparent biocompatibility (Greaves and Drummond 2015; Jha et al. 2014; Pereira et al. 2015).

One of the earliest reports of the effect of choline-based ILs on protein stability by Fujita et al. (2006); Fujita et al. (2007) showed that the long-term stability of cyt C was significantly improved when dissolved in a solution of 80% w/v $[Chol][DHP]$. Even at this saturated concentration of $[Chol][DHP]$, up to 37 mg/mL of protein was soluble. However, it is not necessary to use the saturated concentration of $[Chol][DHP]$ or other choline-based ILs to observe significant improvements in protein stability, thus allowing for significantly higher protein solubility.

Bisht and Venkatesu (2017) studied the effect of five choline-based ILs on the conformational stability, thermal stability, and activity of CT. They tested 0.05–1.5 M concentrations of the following ILs: [Chol][Ac], [Chol][Cl], [Chol][Cit], [Chol][DHP], and [Chol][OH]. The conformational and thermal stability of CT in the different choline ILs followed the order [Ac] > [Cl] > [DHP] || >> [Cit] >> [OH] with [Chol][Ac] having the most overall stabilizing effect on CT, increasing transition temperature from ~49 to 63 °C; those to the right of “||” reduced the stability of CT. The activity of CT in the choline ILs also followed a similar order, except [Chol][Cit] reduced the proteolytic activity of CT more than [Chol][OH], and a mild increase in activity was observed with [Chol][Ac]; such that the order was as follows: [Ac] > [Cl] > [DHP] || >> [OH] >> [Cit]. The destabilizing effect of the [Cit] and [OH] anions was attributed to direct strong H-bonding with the CT structure, stripping off essential water molecules which normally stabilize the conformation of CT, while the stabilizing effect of anions such as [Ac], [Cl], and [DHP] was attributed to modification of water structuring around the protein, leading to its stabilization (Bisht and Venkatesu 2017).

Weaver et al. (2012) studied the effect of [Chol][DHP] on the thermal and structural stability of lysozyme and recombinant human interleukin-2 (rHIL-2) at various [Chol][DHP] concentrations and protein concentrations ≤1 mg/mL. Increased concentrations of [Chol][DHP] (20 to 40% w/v) resulted in increased alpha-to-beta transition in the secondary structure of lysozyme at pH 4 and 7.2. On the other hand, increasing concentrations of [Chol][DHP] (0.5 to 12% w/v) improved the secondary order of rHIL-2 by decreasing the percent of unordered content and increasing the percent of alpha-helical content. The T_m of rHIL-2 was also found to be higher with increasing [Chol][DHP] concentrations, especially at the highest concentration tested (12% w/v or 680 mM), increasing from 61.7 to 74.2 °C. Lysozyme T_m was also increased with increasing concentrations of [Chol][DHP]; 40% w/v [Chol][DHP] at pH 7.2 shifted the T_m from 73.7 to 88.8 °C. At lower pH (e.g., 3.85), the increase in T_m was lower (76.5 to 84 °C), alluding to the importance of surface charge for stabilization with [Chol][DHP]. The results illustrate that [Chol][DHP] inhibits denaturation of lysozyme and rHIL-2 via interactions with charged residues on the protein surface but modifies the secondary structural elements in a favorable or unfavorable way depending on the protein. Additionally, thermal denaturation of rHIL-2 and lysozyme formulated with [Chol][DHP] resulted in the formation of irreversible aggregates.

Rodrigues et al. (2011) studied the stability of lysozyme in a series of choline-based ILs—choline trimethylacetate [Chol][TMA], choline lactate [Chol][Lac], choline propionate [Chol][Prop], choline hexanoate [Chol][Hex], choline dihydrogen phosphate [Chol][DHP], choline dimethyl

phosphate [Chol][DMP], choline trifluoromethane sulfonate [Chol][TFMS], choline bis(trifluoromethylsulfonyl)amide [Chol][TF₂N], and choline chloride [Chol][Cl]. Lysozyme was best stabilized in [Chol][DHP] and destabilized in [Chol][TF₂N], following the order: [DHP] > [DMP] > [Lac] > [Cl] >> || [Prop] > [TFMS] ~ [TMA] > [Hex] > [TF₂N]. The effect of choline ILs was also found to be concentration-dependant; for example, [Chol][DHP] had a stabilizing effect above 0.5 M and a destabilizing effect below this concentration (Rodrigues et al. 2011).

Bisht et al. (2017) studied several choline ILs coupled to di/tri-carboxylate-based anions and dihydrogen phosphate as potential media for cyt C. Among the several ILs studied, >50-fold catalytic activity of cyt C was observed with choline glutarate ([Chol][Glu]) at 50% w/v of the IL, compared to PBS, and >25-fold compared to [Chol][DHP] ~ 33% w/v. Additionally, the catalytic activity of cyt C was maintained against chemical denaturants (H₂O₂ and GuHCl) and temperatures of up to 120 °C, as well as long-term storage of up to 21 weeks. It is important to note however that the activity of cyt C is increased with partial unfolding of the tertiary structure, unlike most other proteins. Thus, it is not clear if the increased activity in this case is due to increased structural stability, although some secondary structural elements were stabilized in the presence of [Chol][Glu]. The authors concluded from molecular docking studies that the enhanced activity is due to H-bonding and electrostatic interactions between the ILs and specific residues in the active site of cyt C, as ILs which did not significantly enhance activity, such as [Chol][DHP], did not interact with the active site.

Mazid et al. (2015) published a short study characterizing the fragmentation of an epidermal growth factor receptor (EGFR) monoclonal antibody (mAb) in the presence of buffered [Chol][DHP]. Twenty and 50% w/v concentrations of buffered [Chol][DHP] prolonged the biological activity of the EGFR mAb and stabilized it against fragmentation in the presence of proteinase K. Higher stability was observed with 50% w/v buffered [Chol][DHP]. These effects were attributed to stabilization of the EGFR mAb alpha-helices in the presence of buffered [Chol][DHP]. This structural ordering is likely due to the [DHP] anion inducing water restructuring around the protein. Interestingly, the aggregation index of the formulations containing buffered [Chol][DHP] was found to be higher than control PBS by 20–30%, suggesting a modest increase in aggregation propensity.

Amino acid-based ILs are another promising class of biocompatible ILs that have received some interest in recent years (Chevrot et al. 2015; Guncheva et al. 2015; Sankaranarayanan et al. 2012; Tarannum et al. 2018). Amino acids have been mostly incorporated as anions, especially with imidazolium or choline cations to produce a biocompatible or “green” IL. Guncheva et al. (2015) tested a series of choline-amino acid ILs on a hemocyanin and found that the thermal stability was

lower in all ILs tested than in the control. Tarannum et al. (2018) recently studied the effect of four choline-based amino acid ILs (choline serinate, threoninate, lysinate, and phenylalaninate) on the structural stability of collagen and had similar findings; they determined that competing hydrogen bonds between the ILs and OH groups on the collagen may be responsible for the destabilization observed.

Unfortunately, research into the stabilization of proteins in amino acid ILs is still in its early stages. Additionally, research is lacking on the formulation of ILs with therapeutically relevant proteins such as mAbs, and other novel formats of antibodies such as antibody-drug conjugates and bi-specifics (antibody products that bind to two different epitopes), which are receiving considerable attention and undergoing intense research. Investigating the effect of ILs on such antibody formulations is important, as they are more complex multi-domain proteins with high therapeutic value and will certainly increase our understanding of the mechanisms behind protein stabilization with ILs.

Simulation studies of ILs: mechanisms of protein stabilization or destabilization

To determine the mechanism of stabilization or destabilization of ILs on proteins, an understanding of the interactions of ILs with water (Hayes et al. 2012; Khan et al. 2014) and ILs with proteins is necessary. Molecular dynamic (MD) simulations have been important in reaching some understanding of these interactions (Benedetto and Ballone 2016; Hayes et al. 2012; Khan et al. 2014). A comprehensive review of the findings of MD simulations is beyond the scope of this review; Schröder (2017) and Smiatek (2017) recently summarized important aspects. In brief, several important mechanisms of interaction and stabilization or destabilization of proteins have been identified with the help of MD simulations which will be discussed below.

As a solute, ILs share properties with inorganic salts and other stabilizing solutes, especially in how they interact with water and the protein surface (Khan et al. 2014; Smiatek 2017). However, unlike simple inorganic salts which cannot typically form hydrogen bonds, IL interactions with water or proteins are in fact dominated by extensive hydrogen bonding. Thus, the influence of ILs on protein stability is twofold: the effect of the ions on water structure and direct hydrogen bonding to the protein surface residues.

IL interactions with water are based to some degree on the nature of the individual ions comprising the IL—i.e., if they are kosmotropic (structure-making/stabilizing) or chaotropic (structure-breaking/destabilizing). In turn, the effects of IL ions on water structure affect protein solvation and stability. Although the Hofmeister series—which ranks anions based on their ability to precipitate proteins—is not always followed

(Kumar and Venkatesu 2014), understanding the effects of IL ions on water structure gives some insight into the complex mechanisms involved in protein stabilization or destabilization.

In most cases, ILs are composed of a chaotropic cation such as imidazolium and a kosmotropic anion such as chloride or acetate. Interactions between ions and water are typically limited to their direct environment, i.e., they only influence the first hydration shell (Batchelor et al. 2004); however, these interactions may still impact protein stability. If there is strong involvement of the anions in the hydrogen bond network of water, the cations are often expelled from the water network and then either accumulate at the surface of the protein (Fig. 2) (Haberler et al. 2011; Klahn et al. 2011) or cluster together and form small aggregates or micelles (Hayes et al. 2015); it is not clear if or how these micelles affect protein stability, but they do not disrupt the water network as significantly as individual ions dispersed throughout (Hayes et al. 2015; Schröder 2017).

Secondly, the observed effects on protein stability exhibited by ILs are significantly influenced by the type of interactions with the protein surface. IL cations which are expelled from the anion-water network may directly interact with protein residues resulting in water removal from the surface and increased or decreased stability (Fig. 2) (Micaêlo and Soares 2008; Schröder 2017). These cations may interact with polar, non-polar (through alkyl side chains), or negatively charged residues, while anions interact with positively charged and polar residues only (Fig. 2) (Schröder 2017). MD simulations have shown that hydrophobic IL cations are inserted into

Fig. 2 Ionic liquid (IL) interactions with water and the protein surface. Purple = protein; red = IL cation; green = IL anion; blue = water. **a** Anions interact with water network and with positively charged regions on the protein surface electrostatically and via hydrogen bonding. **b** Cations interact electrostatically with negatively charged regions on the protein surface. **c** Cations and anions interact with polar regions on the surface of the protein electrostatically. **d** IL cations expelled from water network cluster around non-polar or hydrophobic regions on the protein surface IL

phospholipid membranes through their alkyl side chains (Lim et al. 2014). Micaêlo and Soares (2008) performed molecular simulations on the interaction of two [BMIM] ILs formulated with low water content, with a serine protease cutinase enzyme. They found that the two ILs directly interacted with the enzyme surface, stripping off most of the water from the surface of the enzyme, and that the remaining water molecules formed many small clusters. Furthermore, molecular docking studies of BSA in various BMIM ILs revealed that the imidazolium cations of these ILs interact with hydrophobic residues in the subdomains of BSA via hydrophobic interactions causing protein unfolding (Shu et al. 2011). Long alkyl chains would likely promote these hydrophobic interactions, contributing further to water removal from the surface, promoting further denaturation, as evident by the experimental results discussed in the previous section.

In cases where poorly soluble hydrophobic ILs have exhibited stabilizing effects, this stabilization has been attributed to the formation of two-phase systems, which results in less water depletion from the surface of the protein, enhancing its solubility and its overall stability (Weingartner et al. 2012).

MD simulations also show that the influence of the IL anion on protein stability is stronger at higher concentrations of ILs. At higher concentrations, anions interact with positively charged residues of the protein, promoting refolding (Summers and Flowers 2000). Additionally, because of the strong interaction of an anion with many positively charged residues, these residues are forced into a more compact conformation, facilitating the formation of H-bonds and restructuring of secondary structural elements, resulting in increased thermal stability of the protein (Summers and Flowers 2000); this is likely the case with choline ILs such as [Chol][DHP] which are better stabilizers at higher concentrations.

Recent computer simulations have suggested that the nucleophilicity and hydrogen bond basicity of the ions of an IL are the primary contributors to protein stability in ILs rather than their hydrophobicity, as postulated by numerous experimental studies, especially for enzymes. (Schröder 2017; Smiatek 2017). The body of work on modeling IL interactions with proteins is in its early stages—as more computational studies are performed, a deeper understanding of the specific interactions of ILs on protein stability will be possible.

Considerations for IL use in formulations of biologics and vaccines

Toxicity of ILs

The widespread interest in the use of ILs, particularly as industrial solvents, necessitated studies into the toxicity of these compounds on environmental systems and various organisms,

including humans. While the ecotoxicity of “green” ILs has been studied extensively and summarized elsewhere (Thuy Pham et al. 2010), of significant interest in biological applications of ILs is understanding their toxicity to humans, particularly their cytotoxicity (Frade and Afonso 2010). Numerous cytotoxicity assays have been performed on animal cell lines, particularly mouse leukemia cells (IPC-81) (Ranke et al. 2007; Torrecilla et al. 2009) and various human cell lines (Garcia-Lorenzo et al. 2008; Wang et al. 2007). The high toxicity of some ILs is even being exploited for the development of IL-based cytotoxic drugs and antibacterial agents (Pandey et al. 2017). Given that ILs can exhibit a range of toxicities, it is important to identify which class of ILs may be considered biocompatible or “safe” to use in formulations of biopharmaceuticals (Fig. 3).

The toxicity of ILs is a product of both the cation and anion (Frade and Afonso 2010; Pandey et al. 2017; Thuy Pham et al. 2010), despite earlier claims that the cation moiety was a more significant determinant of toxicity. Both cation and anion toxicities are influenced by several factors; most significantly is the length of the alkyl side chain and branching and the hydrophobicity of the ion. Several studies have shown that increasing the alkyl chain length of the cation significantly increases the toxicity of the IL (Kumar et al. 2009; Peric et al. 2013; Thuy Pham et al. 2010; Wang et al. 2007). For example, Wang et al. (2007) found that the half maximal effective

Fig. 3 Important considerations for the use of ILs in protein formulations for therapeutic use. Asterisk: considerations for injectable formulations

concentration (EC_{50}) of 1-ethyl-3-methylimidazolium bromide in a human cervical cancer cell line (HeLa) decreased from 8.4 to 2.8 mM when the ethyl was substituted for a butyl group and further to 0.3 mM when substituted for an octyl group.

It has also been shown that introducing hydrophilic functional groups such as hydroxyls, nitriles, and polar ethers to the side chains of ILs reduces the toxicity of the IL cation when compared with a non-functionalised alkyl side chain (Kumar et al. 2009; Stasiewicz et al. 2008; Stolte et al. 2007). This is attributed to a reduction in hydrophobicity and ability to interact with and disrupt the lipid bilayer of cell membranes (Jing et al. 2016). Additionally, some perfluorinated anions may be cytotoxic if they decompose and release fluoride ions (Steudte et al. 2012); however, this is dependent on the organism tested and whether the anion is hydrolysable or not. Nonetheless, most AILs without modification would not be an ideal choice of biocompatible ILs especially at high concentrations due to toxicity issues.

In most cases, PILs have been shown to exhibit less cytotoxicity than commonly used AILs, as they typically lack the bulky hydrophobic cations and perfluorinated anions (Gouveia et al. 2014; Peric et al. 2013). Choline-based ILs are a promising class of biocompatible PILs for the stabilization of biopharmaceuticals. At least, the choline cation is significantly less toxic than an imidazolium or pyridinium-based cation as shown by Gouveia et al. (2014). The authors found that the EC_{50} of choline IL in HeLa cells were at least 10-fold lower than imidazolium and pyridinium ILs tested. Additionally, choline is an FDA approved-molecule.

As the choline cation is typically considered a “non-toxic” essential micronutrient, the overall cytotoxicity of choline ILs is mostly dependent on the anion it is coupled with.

Weaver et al. (2010) studied the cytotoxicity of choline cation paired with five phosphate-based anions: DHP, dibutyl phosphate (DBP), bis(2-ethylhexyl) phosphate (BEH), bis(2,4,4-trimethylpentyl) phosphinate (TMP), and *O,O'*-diethyl dithiophosphate (DEP). The cytotoxicity of the choline ILs was assessed on a J774 murine macrophage cell line. Of all the choline ILs tested, [Chol][DHP] exhibited the lowest cytotoxicity to J774; the EC_{50} values (mM) of the ILs were 0.25 [Chol][TMP], 0.3 [Chol][BEH], 8.2 [Chol][DEP], 9.1 [Chol][DBP], 20 [Chol][DHP] compared with values of 63 for NaCl and 34 for [Chol][Cl] (Weaver et al. 2010). Increased anion size and the presence of moderately long and/or branched alkyl chains increased the cytotoxicity of the IL, as previously shown with cations of AILs.

Following on from work by Weaver et al. (2012), Foureau et al. (2012) investigated the potential of [Chol][DHP] as a biocompatible additive for the stabilization and formulation of recombinant human interleukin-2 (rHIL-2). [Chol][DHP] in vitro concentrations < 80 mM were found to have no cytotoxic activity towards primary splenocytes nor mouse

melanoma cells (B16-F10), suggesting that [Chol][DHP] is biocompatible at these physiological concentrations in an in vitro mouse model. Thus, it appears that the choline family of ILs is one of the most promising candidates for biocompatible ILs that exhibit a strong protein-stabilizing effect.

Viscosity of ILs

ILs are typically quite viscous and range in viscosities from tens to thousands in centipoise (Table 2); thus, it is integral to assess the viscosity of typical IL solutions, to ensure they are below the acceptable viscosity for injectables (< ~20 cP), which is still one of the most common methods of formulating and administering biopharmaceuticals (Fig. 3). Since these ILs are often used in concentrations $\leq 50\%$ w/v, their viscosity would be expected to be significantly lower than in their “neat” form. Using [Chol][DHP] as an example, we observe that the viscosity of the 80% w/v as measured by Fujita et al. (2009) was 440 cP; however, at 65% w/v IL concentration, the viscosity decreases significantly to 25 cP (Table 2). Thus, it is expected that the viscosity of [Chol][DHP] solutions $\leq 50\%$ w/v to be below 20 cP—the generally acceptable viscosity of IV formulations. However, it is important to consider the contribution of the high protein concentrations often formulated in these injectables to the total viscosity of the solution; the final formulation viscosity would need to be measured in a case-by-case basis to determine the suitability of the IL used.

Osmolality of IL solutions for injection

When choosing ILs for use in IV formulations of biologics, the final solution osmolality must be measured (Fig. 3). If ILs are used in large concentrations (for, e.g., > 50% w/v), they would undoubtedly result in hypertonic formulations that may not be suitable for IV injection. For example, Foureau et al. (2012) measured the osmolality of rHIL-2 formulations containing 680 mM (12% w/v) [Chol][DHP] and found that the solutions were hypertonic with an osmolality of 1520 mOsm/

Table 2 Viscosities of some common ILs

Ionic liquid	Viscosity (cP) at 25 °C	Reference
[BMIM][I]	1110	Huddleston et al. (2001)
[BMIM][TF ₂ N]	69	
[BMIM][BF ₄]	219	McEwen et al. (1999)
[HMIM][Cl]	716	
[EMIM][TF ₂ N]	28	
[Chol][DHP] 80% w/w	440	Fujita et al. (2009)
[Chol][DHP] 65% w/w	25	

L, approximately 2.5–5-fold higher than near-isotonic levels suitable for IV injection (~300–600 mOsm/L) (Wang 2015). Since ILs have concentration-dependant effects on protein stability and are often stabilizing in higher concentrations, issues with osmolality are inevitable. One approach to address the hypertonicity of the solutions is to introduce an intermediate dilution step prior to injection. Alternatively, if lower concentrations of IL are used as with Foureau et al. (2012), the product may be formulated in other types of injectables or infused; for example, Nony et al. (2001) reported that intramuscular injections comfortably administered 0.5 mL of up to 1100 mOsm/L hypertonic vaccine-like solutions. Such considerations are becoming less significant as we head towards the development of non-invasive formulation routes for the administration of biopharmaceuticals.

Conclusion

ILs have demonstrated potential as novel versatile protein-stabilizing additives albeit with complex mechanisms of action. However, most studies up to date have focused on the thermal or chemical denaturation of proteins in IL solutions. While partial protein unfolding may lead to protein aggregation and influence overall protein stability, it is not sufficient to describe protein aggregation mechanism, as the aggregation process is distinct and is driven by protein-protein interactions. It is possible for an additive to inhibit unfolding while also promote aggregation, resulting in a limited overall benefit. Additionally, few of the studies on protein stability with ILs were performed on therapeutically important and multi-domain proteins such as mAbs, which are extremely valuable, and few at high protein concentrations. With the development of biocompatible ILs, toxicity issues are becoming less of a concern for their use in therapeutic formulations. Additionally, the viscosity and osmolality of protein-IL formulations is not an issue for long-term storage of biotherapeutics as long as the product is diluted prior to administration and only if the product is injected. ILs are exciting versatile liquids—with high potential yet to be exploited fully for rationally developed formulations of biologics and vaccines—certainly with more research in this field, as well as computational MD simulations for deeper mechanistic insights, we may see the incorporation of ILs in biotherapeutic formulations within the next decade.

Compliance with ethical standards

Conflict of interest Mouhamad Reslan declares that he has no conflict of interest. Veysel Kayser declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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