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Unveiling the potential of deep eutectic solvents to improve the conformational and colloidal stability of immunoglobulin G antibodies†

Diksha Dhiman,[a] Ana S. C. Marques,[b] Meena Bisht,[a,b] Ana P. M. Tavares,[b] Mara G. Freire *[b] and Pannuru Venkatesu*[a]

Antibodies, such as immunoglobulin G (IgG), have a pivotal role as biopharmaceuticals capable of treating a wide variety of diseases. However, IgG is a protein, hence, it can easily lose stability and therapeutic efficiency, which may happen during its handling, transportation and preservation. The current work shows, for the first time, the positive effect of deep eutectic solvents (DESs) on the conformational and colloidal stability of IgG antibodies, thus opening the door for their use as novel solvents in IgG formulations. Here, aqueous solutions of cholinium-based DESs were applied to enhance the conformational and colloidal stability of IgG, with no need to add excipients. A series of DESs were prepared through the combination of cholinium chloride ([Ch]Cl), as a hydrogen-bond acceptor (HBA), and various hydrogen-bond donors (HBD), such as urea, glycerol (Gly) and ethylene glycol (EG), and investigated in detail. The effect of [Ch]Cl–urea at different molar ratios (1 : 1, 1 : 2, 1 : 3 and 2 : 1) was also analysed. Conformational stability was checked by thermal fluorescence spectrometry, and it was found that selected DESs allowed increasing the transition temperature (T_m) of IgG by *ca.* 4 °C. The observed increase in the conformational stability of IgG in the presence of DESs was in agreement with the results of other spectroscopic studies, including FTIR and Raman spectroscopies. In the presence of DESs, there was a minimum exposed surface of IgG with water molecules, thereby improving its stability. Dynamic light scattering (DLS), size-exclusion high-pressure liquid chromatography (SE-HPLC) and sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) experiments were additionally performed to analyse the aggregation rate of IgG, which was found to decrease in the presence of appropriate DESs. Finally, the long-term stability of IgG in the presence of DESs was investigated at room temperature. All the results obtained from the conformational and colloidal studies of IgG demonstrated the outstanding potential of cholinium-based DESs as novel solvents for IgG formulations, with the DESs comprising [Ch]Cl–urea or [Ch]Cl–Gly noted as the most promising candidates. All the described studies were also performed with the DESs' individual components, demonstrating that the full DESs (HBD + HDA) are needed to improve the stability of IgG.

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Introduction

Antibodies, *i.e.* immunoglobulins, are globular proteins whose function is to specifically bind to foreign substances in the host organism and thus to ward off and eliminate invaders. They are a crucial part of the humoral immune system and are mainly produced by B lymphocytes upon stimulation.[1,2] Most antibodies perform their best biological functions under physiological conditions, *i.e.* pH 6–8 and from room to body temperatures (25–37 °C).[3] There are two major forces responsible for the native structure of proteins: one corresponds to chemical forces within the protein functional groups and the other to the protein's interaction with the surrounding environment (mostly water).[4] Hence, the liquid-surrounding environment plays a crucial role in the stability of proteins. The hydration layer stabilizes protein structures through hydrogen bonding, which is mainly responsible for controlling the molecular dynamics.[5] However, at the same time, water is highly labile to hydrolysis and oxidation, thus destabilizing protein's structure and function at elevated temperatures.[6] Therefore, there is a crucial need to find novel excipients and/or biocompatible solvents that could stabilize proteins and enhance their storage capabilities.

[a]Department of Chemistry, University of Delhi, Delhi-110 007, India. E-mail: venkatesup@hotmail.com
[b]CICECO-Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal. E-mail: maragfreire@ua.pt

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Immunoglobulin G (IgG) antibodies are pharmaceutically active biomolecules and their conformational and colloidal stability have high relevance. However, due to various stresses during manufacturing, shipping and storage, these proteins tend to form aggregates, which is not desirable from a clinical point of view since they can lead to serious and fatal health effects.[7]

The most common currently used excipients in vaccine formulations (for stabilization during storage) are L-serine, glycine, sucrose, trimethylamine *N*-oxide and surfactants, such as Tween 80.[8,9] Specifically for IgG, glycine is used in the Cuvitru vaccine for its stabilization effect. Cuvitru is composed of concentrated IgG, and is used as a replacement therapy for primary humoral immunodeficiency in adult and paediatric patients.[8] Despite the clinical relevance of the excipients, recent studies have shown that some of these excipients are not environmentally friendly.[10,11]

Several research groups have shown the impact of the toxic solvent systems used in pharmaceutical industries.[10,11] In particular, in protein stability studies, it has been reported that solvents not only interact with the structure of proteins but also play a dominant role in their toxicity profile.[12,13] Thus, there is a critical need to find biocompatible excipients or solvents that could help in maintaining the structure of proteins, and in particular the IgG protein. Hallet and co-workers[14] studied different formulations for IgG4, some of them containing ionic liquids (ILs). The authors found that the formulation containing the IL cholinium dihydrogen phosphate ([Ch][Dhp]), with excipients like sugars, amino acids, and surfactants, is promising for stabilizing proteins against conformational destabilisation and aggregation.[14] Recently, we also studied the interactions between cholinium-based ILs and IgG antibodies.[15] We observed that cholinium chloride ([Ch]Cl) unfavourably interacts with the IgG antibody, increasing IgG's stability.[15] Based on these results, it seems promising to address the influence of deep eutectic solvents (DESs) comprising [Ch]Cl to improve the stability of IgG. Cholinium-based DESs are defined as type-III DESs, and are composed of a quaternary ammonium salt ([Ch]Cl) as a hydrogen-bond acceptor (HBA) combined with a hydrogen-bond donor (HBD), usually an amide or alcohol (EG, urea, Gly).[16] Compared to cholinium-based ILs, these liquids are simple to prepare, frequently inexpensive and biodegradable.[17–19] DESs were first reported in 2003 by Abbot *et al.*,[18] comprising a mixture of [Ch]Cl and carboxylic acids. DESs have been described as compounds with negligible toxicity, good biodegradability and non-volatile.[20–23] Nevertheless, it should be borne in mind that these properties are not general and depend on the specific DES and its constituents and composition. A large proportion of research has been focused on the application of DESs, with several works showing the use of DESs for the extraction and storage of proteins.[24–26]

There are also available reports on therapeutic deep eutectic solvents (THEDESs) comprising active pharmaceutical ingredients (APIs), and different categories of DESs have demonstrated their applicability for drug delivery.[13,26] Mitragotri *et al.*[27] recently used cholinium-based ILs and DESs for the gastrointestinal delivery of therapeutics, using rats as an *in vivo* model. A one-week repeat dose study followed by histology and serum biochemistry analysis indicated that these could be well tolerated by the rats.[27] Cholinium-based DESs were also evaluated for targeting cancer cell lines;[16] and it was found that the selectivity index for the studied DESs varied in the range >2 or ≤2, fulfilling an essential criterion to avoid any serious side effects of the therapeutics.[16] Although a long path (*in vitro* and in *in vivo* assays, followed by human clinical trials) still lies ahead to have the studied DESs approved by the regulatory agencies, all these scientific works, together with the current work, support the belief that DESs are promising excipients and solvents for therapeutic formulations.

Yadav and Venkatesu[28] recently investigated the effect of DESs on the activity and stability of several proteins. In addition to proteins, DESs have also been proven to have utility as potential media for other biomolecules, such as DNA. Mondal *et al.*[29] reported the enhanced solubility of DNA, with long-term structural and chemical stability, in recyclable and reusable bio-based DESs. From all these observations it can be concluded that DESs are indeed emerging compounds for therapeutic applications.

Results and discussion

The DESs investigated in this study, namely [Ch]Cl–urea, [Ch]Cl–Gly, and [Ch]Cl–EG, differ in the HBD, allowing us to evaluate to what extent the replacement of the HBD affects the strength of the DES's behaviour on the conformational, colloidal, and time-dependent stability of IgG. The discussion of the obtained results is structured as follows: (i) the effect of the DESs' nature on the stability of IgG as a function of the HBD; (ii) the effect of the DESs on the stability of IgG as a function of the DES concentration; and (iii) the long-term stability of IgG in the presence of the most promising DESs and the effect of the DES molar ratio. All these studies were also performed for the DESs' individual components.

Effect of the DESs' nature and concentration on the conformational stability of IgG

To analyse the influence of the DESs on the conformation stability of IgG, UV absorption, fluorescence emission, far-UV circular dichroism (CD) spectroscopy, FTIR and Raman spectroscopy analyses of IgG in the presence of all the DESs and their individual components were performed. The wavelength (λ_{max}) for the absorbance maxima (A_{max}) of IgG in the presence of the buffer (control) was observed at 280 nm. This peak was due to the absorption of aromatic residues, such as tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe).[30–34] From Fig. 1 (a–c), it can be seen that there was no wavelength shift, indicating that the aqueous solutions of all the DESs did not affect the polypeptide environment of IgG. Furthermore, there was no particular sequence of the absorbance change of IgG as a function of the concentration of the DESs. However, a hypochromic shift (A_{max}) was observed with respect to the control

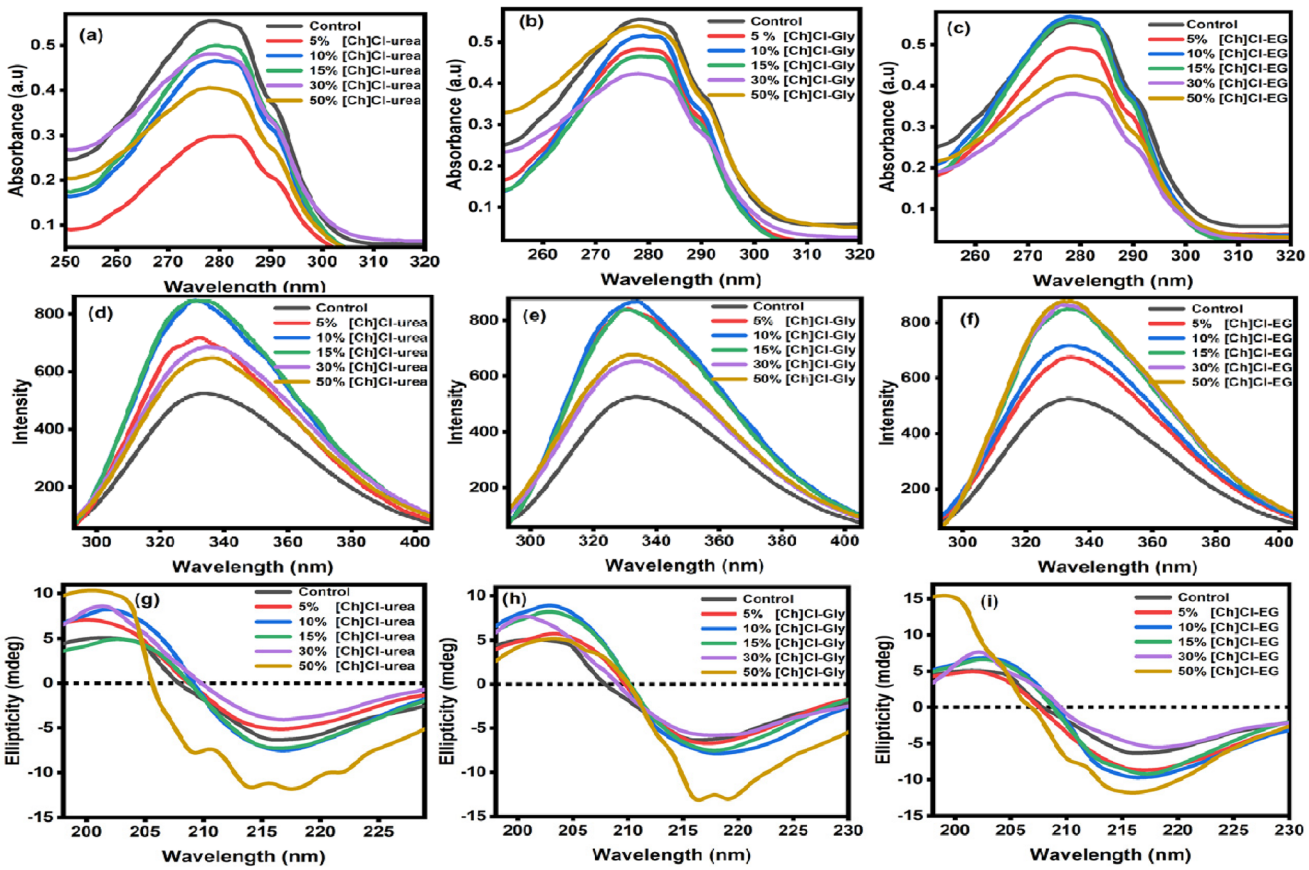


Fig. 1 Spectroscopic analysis of IgG: (a)–(c) UV-visible absorption spectra; (d)–(f) fluorescence emission spectra; (g)–(i) far-UV CD spectra in the presence of sodium phosphate buffer pH 7.0, 10 mM (control) and at various concentration of DESs.

in the presence of the DESs. The decrease in A_{max} of IgG in the presence of the DESs could be attributed to the shielding of the aromatic residues, which further limited the resonance of the aromatic ring as well as its π–π interaction with the solvent system.[33]

Changes in the intrinsic fluorescence were monitored to probe the global structural alterations of IgG. Trp residues in native IgG are often quenched internally by local contact with either disulphide bridges or each other.[35–37] However, Fig. 1 (d–f) showed there was an increase in the intensity of emission in the presence of DESs as compared to the control, which could be attributed to the increase in viscosity in the presence of the DESs.[38] The maximum wavelength of emission (λ_{max}) of IgG was found to be ~330 nm[35] in the presence of phosphate buffer as well as in the presence of the DESs. No major shift in λ_{max} was observed, showing that the Trp and Tyr residues remained intact in the hydrophobic core–shell in presence of DESs and help in stabilizing the IgG structure.

Far-UV CD was performed to study the secondary structure stability of IgG. IgG, being rich in a β-pleated structure, showed a positive ellipticity at 200 nm and a negative ellipticity at 218 nm.[39,40] IgG retained both peaks after incubation in all the DESs; nevertheless, at lower DES concentrations, the intensity was substantially stronger. Fig. 1(g–i) demonstrated that the ellipticity became higher than the control at 30% DES and began to deform again at 50% DES. These observations suggest that the secondary structure of IgG was retained up to 30% DES but started to be disturbed at higher DES concentrations.

From all the spectroscopic results, it could be concluded that the IgG conformational stability depends on the concentration of the DES and the nature of the DES (type of HBD). At higher concentrations of DESs (50%), IgG conformation starts deforming, whereas at lower concentrations (up to 30%) DESs behave as excellent stabilizers. The impact of the HBD on the stability of IgG follows the order: [Ch]Cl–Gly > [Ch]Cl–Urea > [Ch]Cl–EG.

The UV absorbance, fluorescence emission, and far-UV CD data of the individual components of the DESs are provided in Fig. S1 in the ESI.† The results in Fig. S1† show that urea and EG behave as destabilizers and Gly and [Ch]Cl behave as stabilizers, whereas the mixture of the components (HBD and HBA) behaves always as a stabilizer.

To further ascertain the effect of the HBD of each DES, FTIR and Raman spectroscopy of IgG in the presence of 30% DES were performed. Fig. 2 depicts the measured transmittance and scattering intensity as a function of the wavenumber (frequency of transition (ν)) in different solvent environments. Both Raman and FTIR spectroscopies were used to interpret

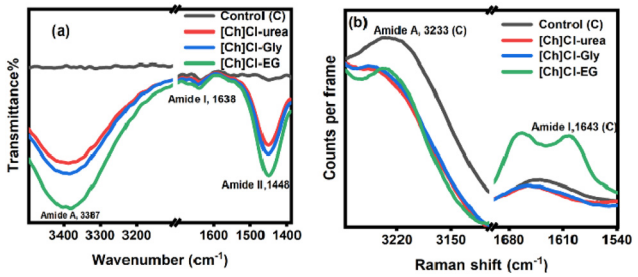


Fig. 2 (a) FTIR spectra of IgG at 25 °C in the presence of the control and 30% (w/w) [Ch]Cl–urea, [Ch]Cl–Gly and [Ch]Cl–EG. (b) Raman spectra of IgG at 25 °C in the presence of the control and 30% (w/w) [Ch]Cl–urea, [Ch]Cl–Gly and [Ch]Cl–EG.

the vibrational transition modes of the molecules, and both techniques provided complementary results.[38] The proteins majorly showed amide A and amide B bands between 3500 to 3200 cm^{-1}. The NH stretching gave rise to the amide A band and was exclusively localized on the NH group, being therefore insensitive to the conformation of the protein's backbone.[41] The vibration (ν) for amide A depends on the strength of hydrogen bonding. Amide I and II bands appeared in the 1700–1500 cm^{-1} region, and were due to the groups responsible for the peptide linkages in proteins.[42] Due to the C═O stretching vibrations of the peptide bonds, amide I appeared in the region of 1625–1695 cm^{-1}.[43,44]

Fig. 2(a) presents the changes in the transmittance intensities of IgG in the control and in the presence of DESs. For the IgG aqueous solution, three transmittance bands could be observed at 3387, 1640 and 1448 cm^{-1} (spectra in black). All three bands were consistent with those reported in the literature.[45] In the presence of all the DESs, the frequency of the amide A, amide I and amide II bands did not shift, but there was an increase in the intensity of transmittance in the presence of [Ch]Cl–Gly, [Ch]Cl–EG and [Ch]Cl–urea. This could be explained by the increasing polarity of the vibrating bonds of IgG (all the wavenumber values of IgG in the presence of DESs and the respective components are provided in Table S1 in the ESI†). The strength of transmittance increased with the increasing polarity of the vibrating bond.[46] Hence, in the presence of DESs, the side chain N–H bonds became more polar, which could be due to the extended electrostatic interaction, which also caused the increase in the intensity. From the FTIR studies, it could be interpreted that in the presence of the DESs there was an increase in the NH polarity. This increase in polarity could be due to the favourable influence of the DESs on the surface electrostatic interactions of the molecule.

Fig. 2(b) provides the laser Raman spectra of IgG in the presence of the DESs (*versus* the control). To avoid degradation of the sample, a 532 nm laser beam was used. For the IgG aqueous solution, two scattering bands were observed between 3233 and 1640 cm^{-1} (spectra in black), consistent with those reported in the literature.[47] [Ch]Cl–urea and [Ch]Cl–Gly showed the amide A frequency at 3252 cm^{-1}. Also, an increment of 20 cm^{-1} was observed concerning the control (all the values are provided in Table S2, in the ESI†). This increment in frequency for the amide A band in the presence of DES solvent may have been due to exclusion of the N–H bond in hydrogen bonding and the transformation of N–H···H–O–H to free N–H bonds.[48] On the other hand, N–H···H–O–H bonding was retained in the presence of [Ch]Cl–EG. There was also an increase by 12 cm^{-1} of the amide I peak in the presence of [Ch]Cl–urea and [Ch]Cl–Gly, whereas in the presence of [Ch]Cl–EG, the ν was approximately similar to the control. The increase in ν could be due to the shielding of IgG's C═O backbone from water molecules. The free stretching of C═O appeared in the region of 1660–1665 cm^{-1} in the spectrum; due to C═O···O–H–O stretching, while the ν for C═O was shifted to a lower wavenumber (1640 cm^{-1}).[49] Overall, from the laser Raman spectroscopy results, it could be inferred that the DESs helped in retaining the conformation of all the four polypeptide chains of IgG, which further stabilized the Fab segments and Fc segment through exclusive interactions with the hydrophobic core of the protein.

From the vibrational spectroscopic results, it could be concluded that cholinium-based DESs are suitable solvent media for retaining the 3D structure of IgG. Further, the DES HBD plays a crucial role in the conformational stability of proteins. The impact of the DES HBD on the stability of IgG followed the order: [Ch]Cl–Gly > [Ch]Cl–urea > [Ch]Cl–EG. Similar results were obtained by the UV, fluorescence, and far-UV CD studies. The FTIR and Raman spectra of the individual components of the DESs are provided in Fig. S2,† with the results listed in Tables S1 and S2 in the ESI.† The results in Fig. S2† show that both the FTIR and Raman bands got disturbed in the presence of urea (there was a decrement in the amide I band from 1640 to 1593 cm^{-1}), which was consistent with the literature.[46] All the bands of IgG were retained in the presence of [Ch]Cl, Gly and EG with a decrease in ν compared to the DESs, which could be attributed to the interactions (electrostatic or H-bonding) between the components and the IgG structure and the overall effect on the compact structure of the protein.

Effect of the DESs' nature on the conformation of IgG (temperature perturbation)

The thermal unfolding of IgG in the presence of all three DESs was further analysed by thermal fluorescence spectroscopy. The most important parameters to study the thermodynamic stability of proteins are the unfolding transition temperature (T_m) and the change in Gibbs free energy ($\Delta_{fu}G$). T_m is the point at which 50% of the protein is unfolded and the remaining 50% is folded.[50,51] Proteins with a higher T_m are more stable because a greater input of energy is required to reach the unfolding transition.[52] For a given event, proteins with a more positive $\Delta_{fu}G$ require higher energy to unfold. All the thermodynamic equations required to determine these thermodynamic parameters are provided in the ESI.†

To ascertain the temperature perturbation effect on the IgG antibody in the presence of the DESs, we performed thermal fluorescence spectroscopy and determined the T_m, the $\Delta_{fu}G$,

change in enthalpy ($\Delta_{fu}H$) and the change in heat capacity (ΔC_p). The thermodynamic parameters of IgG in the presence of the DESs are presented in Fig. 3, and the values are provided in Table S3 in the ESI.† The T_m of IgG in the control/buffer (pH 7) was 76 °C (Fig. 3a), which was in agreement with the literature.[15,53] It could be seen, however, that when adding all three DESs there was an increase in T_m values, changing from 76 °C in the control (the case without adding any DESs) to 78.5 °C, 80.0 °C and 76.5 °C in the presence of 30% [Ch]Cl–urea, [Ch]Cl–Gly and [Ch]Cl–EG, respectively. When compared with the DESs individual components, the T_m value of IgG in the presence of urea and EG decreased from 76 °C in the control to 58 °C in the presence of 30% urea and to 70 °C in the presence of 30% EG, respectively. On the other hand, Gly and [Ch]Cl behaved as excellent stabilizers, with the T_m values changing from 76 °C in the control to 86 °C in the presence of 30% Gly and 83.5 °C in the presence of [Ch]Cl. The graphical representation of these results is provided in Fig. S3 in the ESI.† The obtained results explicitly indicate that the DESs increased the T_m values of IgG and helped keep the folding form beyond 76.5–80 °C, which was higher than that obtained in the control. Interestingly, the T_m order showed that [Ch]Cl–Gly was the strongest stabilizer, while [Ch]Cl–urea and [Ch]Cl–EG were moderate thermal stabilizers. Bhojane *et al.*[54] evaluated some naturally occurring osmolytes, such as betaine, sarcosine, ectoine and hydrixyectoine, as potential stabilizers of IgG1 biotherapeutics. Sarcosine, a glycine derivative, showed the best IgG1 stabilization effect, and similarly to the results discussed above, an increase in T_m was obtained.[54] On the contrary, urea and EG depressed the T_m by 18 °C and 6 °C, with respect to the control, which could distinctly be related to its denaturation power. T_m studies of IgG were also performed for 50% DESs; nevertheless, at this concentration, a general destabilizing behaviour of the DESs over IgG was observed. These values are reported in Table S3 (ESI†).

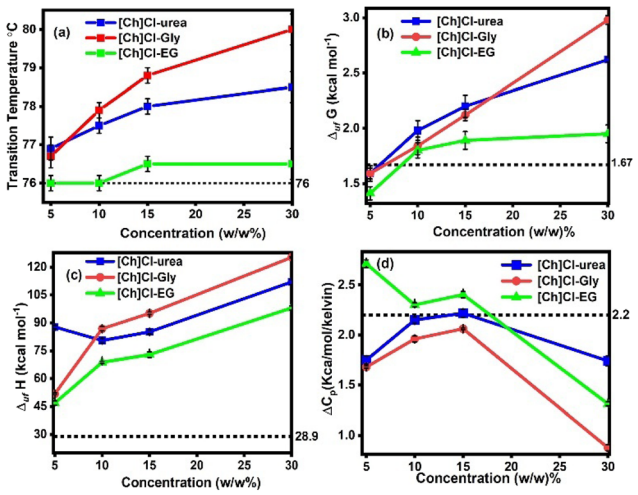


Fig. 3 Thermodynamic studies of IgG at 25 °C in the presence of sodium phosphate buffer pH 7.0, 10 mM (control) and as a function of the concentrations of the DESs. (a) Transition temperature (T_m); (b) Gibbs free energy changes ($\Delta_{fu}G$); (c) enthalpy change ($\Delta_{fu}H$) of unfolding; and (d) heat capacity change of unfolding (ΔC_p).

To gain further insights into the conformational transition, we additionally determined the $\Delta_{fu}G$, $\Delta_{fu}H$ and ΔC_P. The $\Delta_{fu}G$ and $\Delta_{fu}H$ values of IgG in the control were 1.57 kcal mol^{-1} and 40 kcal mol^{-1} (Fig. 3(b) and (c)). The highest values of $\Delta_{fu}G$ and $\Delta_{fu}H$ were found at 30% with the DESs [Ch]Cl–urea and [Ch]Cl–Gly (2.34, 2.98; 112, 120 kcal mol^{-1}). Overall, all three DESs unfavourably interacted with the surface of IgG and stabilized the folded native structure of IgG.

From Fig. 3d and a clear decrease in the C_P values of IgG as a function of the concentration of DESs could be observed, with the minimum value found at 30% of DES. The contrasting behaviour of ΔC_P values for IgG in the DESs reveals that the DESs did not alter the hydrophobic forces of the protein, which was further responsible for the compactness of the IgG's native structure. Overall, these studies further strengthen the observation that 30% DESs would make extraordinary solvent systems to improve the stability of IgG.

Effect of the DESs' nature and concentration on the colloidal stability of IgG

Colloidal stability corresponds to the dispersion state of the antibody. A higher colloidal stability means that the antibody molecules mainly exist as a stable monomer in a native or partially denatured state, not suffering aggregation. The aggregation of IgG is particularly relevant since it affects its therapeutic efficiency.[50] Moreover, IgG aggregation may be fatal for patients with a variety of diseases involving protein aggregation.[55] The effect of the DESs on the colloidal stability of IgG was evaluated using SE-HPLC, DLS and SDS-PAGE.

To elucidate the attenuation effects of cholinium-based DESs on IgG, DLS measurements were performed as a function of the concentration of the DESs and their individual components. All the samples were filtered using a 0.22 μm syringe. The size distribution *versus* the intensity of IgG in the buffer and DESs was plotted and is displayed in Fig. 4(a–c). The d_H value of IgG in phosphate buffer (control) at pH 7.0 under physiological conditions was found to be 15.07 nm, which was in agreement with the literature.[14,56] The polydispersity index of IgG was found to be 0.51, *i.e.* higher than 0.5, showing that the control solution was polydisperse. The results in Fig. 4(a) explicitly show that the d_H value of IgG in [Ch]Cl–urea increased as a function of the concentration (13.31, 15.98, 16.03, 18.83 and 24.53 nm), with the following PDI values: 0.44, 0.37, 0.47, 0.59 and 0.69 for 5%, 10%, 15%, 30% and 50% (w/w) of DES, respectively. For [Ch]Cl–Gly, the following d_H 10.9, 13.23, 14.37, 17.86 and 31.34 nm and PDI values 0.33, 0.35, 0.33, 0.66 and 0.94 were found (Fig. 4b). For [Ch]Cl–EG, these corresponded to 14.96, 14.99, 18.04, 20.08 and 30.73 nm and 0.24, 0.32, 0.59, 0.87 and 0.94 nm (Fig. 4e). The particle-size distribution of IgG in the presence of the DESs showed a decrement in d_H values up to 15% (w/w) of DES, whereas an increase in the d_H values was seen at 30% and 50% (w/w) DES. The PDI values showed the same behaviour. The increase in d_H and PDI values at higher concentrations of DESs could be

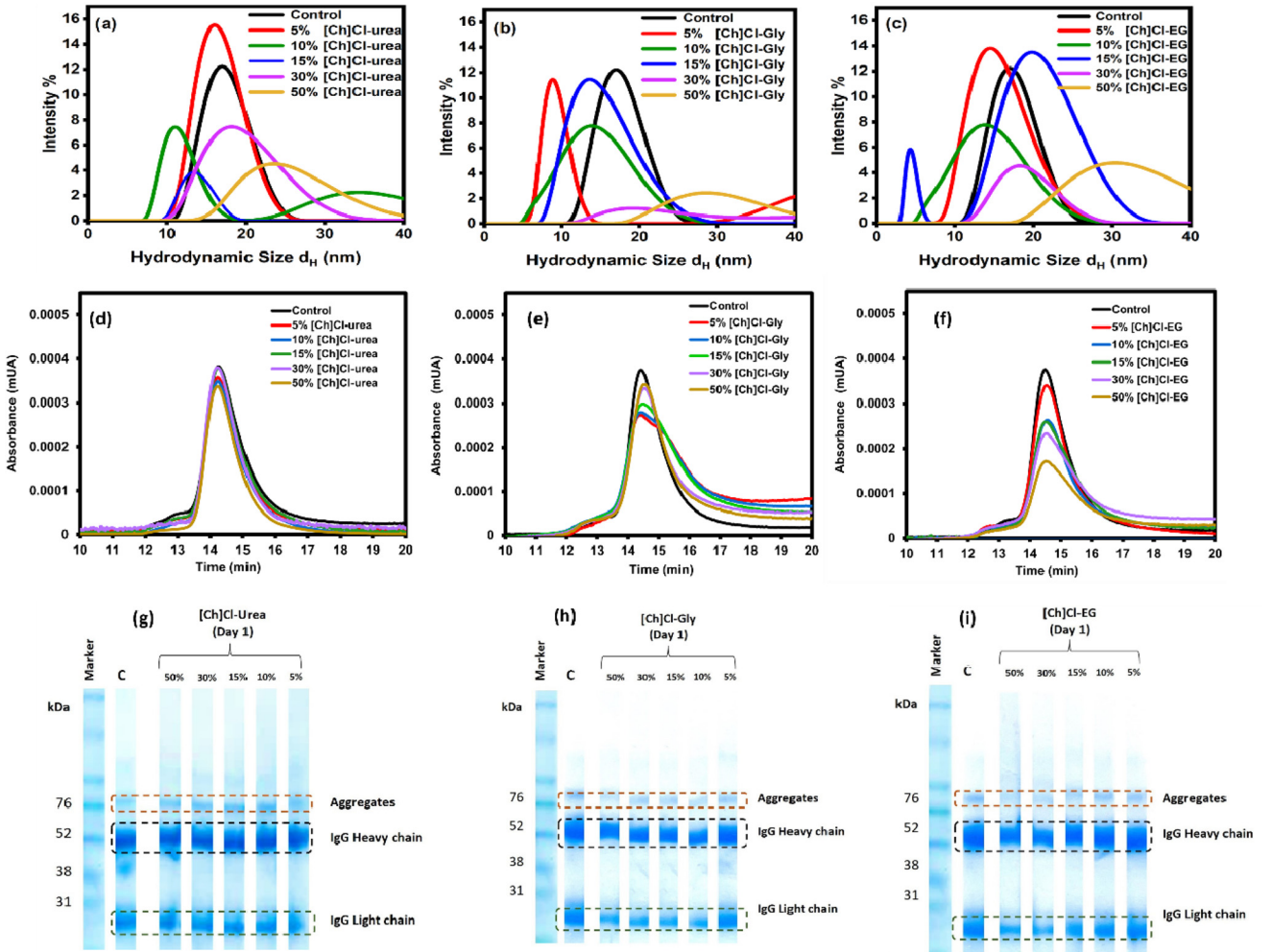


Fig. 4 Analysis of the aggregation of IgG: (a)–(c) size-distribution plots; (d)–(f) SE-HPLC spectra; (g)–(i) SDS-PAGE results in the presence of sodium phosphate buffer pH 7.0, 10 mM (control) and at various concentrations of [Ch]Cl–urea, [Ch]Cl–Gly and [Ch]Cl–EG at 25 °C.

attributed to the formation of polydisperse media in the presence of higher concentrations of the DESs, whereas at lower concentrations of monodisperse media, less IgG aggregation was observed. Similar results were provided by the individual components of the DESs (data provided in Table S4 in the ESI†).

Although SE-HPLC is usually used to quantify antibodies and identify the presence of other proteins, this technique can be also used to evaluate the structure of IgG by the changes in the elution profile and retention time. SE-HPLC chromatograms of IgG in the presence of sodium phosphate buffer pH 7.0, 10 mM (control) and all DESs at different concentrations are provided in Fig. 4(d–f). The chromatograms of IgG in the presence of individual DES components are presented in the ESI (Fig. S4†). According to the obtained chromatograms given in Fig. 4(d), the control IgG sample displayed 2 peaks: one peak at a retention time of ~14.3 min, corresponding to IgG in its monomeric form, and a second peak at ~13.4 min, corresponding to IgG aggregates. Compared to the control sample, there were no major changes in the IgG aggregates peak for [Ch]Cl–Gly and [Ch]Cl–EG, whereas there was a decrease in the aggregates peak with the increase in the [Ch]Cl–urea concentration. These results imply that the DESs can potentially avoid and even reduce IgG aggregation. Regarding the main chromatographic peak of IgG, it could be seen from the chromatogram in Fig. 4(e) that the absorbance of the main peak increased with the concentration of [Ch]Cl–Gly, increasing from 5% to 50%. However, comparing the peaks of the [Ch]Cl–Gly samples at 5%, 10% and 15% with the peaks of the control and the [Ch]Cl–Gly samples at 30% and 50%, we could observe that these peaks were wider and asymmetric, and consequently the peak area was not as high compared to the symmetrical peaks (control and [Ch]Cl–Gly 30%, 50%). Thus, to precisely determine the effect of the [Ch]Cl–Gly concentration, the peak areas for all the concentrations were calculated using PeakFit® software. According to these results, the values of the peak areas decreased with the [Ch]Cl–Gly concentration increasing. In addition, the SDS-PAGE gel results (Fig. 4h) also confirmed that there was a slight decrease in the amount of IgG with the increasing concentration of [Ch]Cl–Gly, since the intensity of

the IgG light chain slightly decreased. The values of the peak areas are provided in Table S5 in the ESI.† Additionally, no peaks were observed at higher retention times, indicating that no IgG degradation occurred.

The structural stability of IgG in the presence of sodium phosphate buffer pH 7.0, 10 mM (control) and all the DESs at different concentrations was also evaluated by SDS-PAGE, and the results are displayed in Fig. 4(g–i). The SDS-PAGE of the DESs' individual components is provided in Fig. S4(i, j, l and m) in the ESI.† According to the SDS-PAGE results in reduced conditions, the control IgG was composed of 2 heavy chains with a molecular weight of ~50 kDa and 2 light chains with a molecular weight of ~25 kDa (Fig. 4(g–i)). These two bands were identified in the presence of all the cholinium-based DESs investigated and at all concentrations, indicating that there were no significant changes and no complete degradation of the IgG structure. Moreover, as verified by the SE-HPLC chromatograms, a band with a higher molecular weight was observed, indicating the presence of IgG agglomerates in the control and in all the DES solutions. Comparing the three DESs, a slight decrease in the intensity of the IgG heavy and light chains was observed for the higher DES concentrations, namely 30% and 50%, corroborated with the decrease in the peaks of the respective SE-HPLC chromatograms.

From the colloidal stability studies, it can be concluded that all the DESs behaved as efficient media for increasing the monodispersity of IgG, particularly for concentrations up to 15%. At higher concentrations of DES (30% and 50%) the rate of aggregation was higher. Similar results were shown by the individual components of the DESs (Fig. S5, ESI†). Therefore, DESs are preferred over their components for maintaining the colloidal stability of IgG.

Long-term stability of IgG in the presence of the most promising DESs and effect of the DES molar ratio

For examining the long-term storage efficiency of IgG antibodies in the presence of cholinium-based DESs, DLS, far-UV CD, SE-HPLC and SDS-PAGE analyses of IgG in the presence of the DESs (at 30%) and their components were performed as a function of time for 20 days. All the assays were performed at temperatures varying from 30–35 °C, except for SE-HPLC and SDS-PAGE, which were performed at 25 °C.

Far-UV CD spectroscopy was performed on day 1 and day 20 for IgG in the presence of 30% DESs and their isolated components. The respective results are displayed in Fig. 5(a–d). IgG showed a significant broad peak at 218 nm on day 1 in the presence of phosphate buffer and the DESs. After 20 days, it could be seen that the peak becomes completely deformed in the presence of phosphate buffer, together with a shift in the wavelength in the presence of [Ch]Cl–EG. On the other hand, the peak at 218 nm was retained in the presence of [Ch]Cl–urea and [Ch]Cl–Gly, although with a slight decrement in the intensity. From these results, it was shown that [Ch]Cl–urea and [Ch]Cl–Gly are efficient solvent systems for storing IgG for longer periods. Consecutively, the variation in the β-pleated

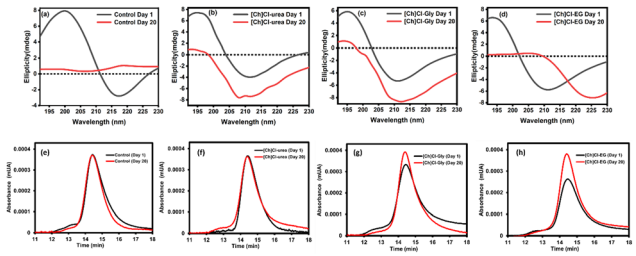


Fig. 5 Ellipticity calculated from time variable far-UV CD for IgG in (a) phosphate buffer, (b) 30% [Ch]Cl–urea, (c) 30% [Ch]Cl–Gly, (d) 30% [Ch]Cl–EG. SE-HPLC chromatograms of IgG as a function of the time, (e) control (f) [Ch]Cl–urea, (g) [Ch]Cl–Gly, (h) [Ch]Cl–EG.

structure percentage was calculated with the software dichroweb,[57] allowing us to confirm the retainment of the percentage of the β-pleated structure in the presence of [Ch]Cl–urea at day 20. The respective results are presented in Fig. 5(a–d).

The chromatograms for the individual DES constituents are provided in the ESI, Fig. S5.† All the IgG samples displayed similar retention times for both the monomeric and aggregated forms of IgG. Furthermore, there was an increase in the monomer corresponding peak in the presence of [Ch]Cl–Gly and [Ch]Cl–EG and a decrease in the aggregate's corresponding peak in the presence of [Ch]Cl–Gly after 20 days of incubation, Fig. 5(e–h). From Fig. S5 in the ESI,† it can be seen that in the presence of the DES's individual components, mainly [Ch]Cl, urea and EG, IgG's structure was not retained after 20 days of incubation. The stored IgG was further evaluated by DLS studies and SDS-PAGE, Fig. 6. The samples were not filtered when performing the time-dependent studies because of the formation of large aggregates after day 20. The results obtained are illustrated in Fig. 6(a). On day 1, the order of d_H was: control (238 nm) > [Ch]Cl–EG (213 nm) > [Ch]Cl–Gly (177 nm) > [Ch]Cl–urea (166 nm), and the order of the PDI values was approximately the same. On day 20, there was no change in the orders of d_H and PDI, varying as follows: control (1696 nm) > [Ch]Cl–EG (1610 nm) > [Ch]Cl–Gly (1584 nm) > [Ch]Cl–urea (989 nm). The increase in d_H and PDI values (Fig. 6(b)) was due to the long-term aggregation of the protein. However, it was here shown that the DESs are efficient solvent systems for decreasing the rate of aggregation of IgG antibodies, as the increase in d_H values after 20 days was found to be less in the presence of the DESs as compared to the control. When comparing with the components Gly and [Ch]Cl, Fig. S6 in the ESI,† a larger decrement in d_H could be observed compared to the control; however, this decrement was less than the one obtained with [Ch]Cl–Gly. Also, in the presence of urea (30%), the d_H value went to around 3323 nm (all the data are provided in Table S6 in the ESI†). Overall, it can be concluded that [Ch]Cl–urea and [Ch]Cl–Gly are better solvents to reduce the aggregation rate of IgG for long-term storage.

Finally, the effect of the molar ratio of the DES [Ch]Cl–urea (1 : 1, 1 : 3, 2 : 1) on the stability of IgG's structure was investigated. This DES was chosen since it is one of the most promis-

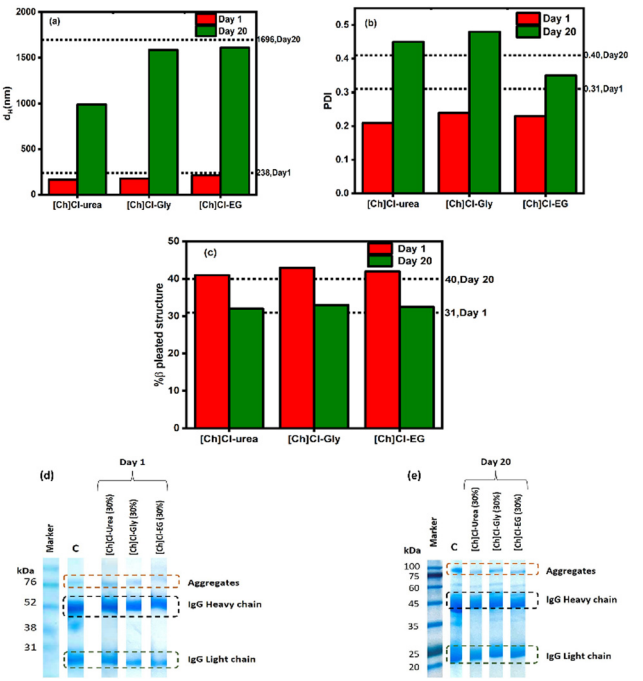


Fig. 6 (a) and (b) Hydrodynamic size and polydispersity index calculated from time variable DLS for IgG in the presence of the DESs. (c) Percentage secondary structure analysis of IgG in the presence of phosphate buffer and at 30% DESs as function of time. (d) and (e) SDS-PAGE spectra of IgG as a function of time: a1. day, 1, a2. day 20. Lane 1 – molecular weight marker; lane 2 – IgG in sodium phosphate buffer pH 7.0, 10 mM (control (C)) and DESs at 30%.

ing to improve the IgG stability. The results are provided in Fig. S7 and Table S7 in the ESI.† The obtained results demonstrated that the [Ch]Cl–urea system performed better at the 1 : 2 composition, which was the general composition used in the previously described assays and results.

All our results systematically revealed that all three DESs interacted unfavourably with the IgG antibodies, hence stabilizing the IgG structure. DESs tend to be excluded from the IgG surface, forcing the polypeptide to adopt a compact folded structure with a minimum exposed surface area to water molecules. [Ch]Cl and Gly also unfavourably interact with the IgG structure, causing an enhancement in the stability of the antibody. On the other hand, urea and EG interact more favourably with the protein than water, leading to a destabilization of the IgG structure. These results are in agreement with the literature, where it has been shown that urea behaves as a denaturant for proteins and destabilizes their conformation.[58] Remarkably, [Ch]Cl–urea was composed of [Ch]Cl and urea and behaved as an excellent stabilizer due to the extended hydrogen bonding between both components. A similar situation was the case of [Ch]Cl–EG, where EG behaved as a destabilizer individually, whereas in [Ch]Cl–EG the structure of IgG was retained in its native conformation.

Baker and co-workers[59] revealed that the stability of ribonuclease A is a function of the DES concentration, with the structure of the protein being retained with up to 35 (w%) [Ch]Cl–Gly. Our results corroborated these findings but with a different protein. All three studied DESs behaved as IgG stabilizers at up to 30 (w%), whereas at higher concentrations the structure of IgG was disrupted. When addressing the nature of the DES HBD, Gly-containing mixtures are the most promising for enhancing the stability of IgG. Kim *et al.*[60] acquired similar results for lipase. The activity, as well as stability of lipase, was enhanced in Gly-containing DES mixtures compared to other DESs. Similar results were provided by Venkatesu and co-workers,[61] with [Ch]Cl–Gly reported to behave as a better solvent system than [Ch]Cl–urea when addressing the thermal stability of α-chymotrypsin. Furthermore, Wu *et al.*[62] revealed that ChCl-based DESs were superior to cholinium-acetate-based ones for enhancing the activity and stability of horseradish peroxidase. When comparing the effect of cholinium-based ILs[16] and [Ch]Cl-based over IgG, a slightly higher T_m was obtained in the presence of [Ch]Cl than in presence of [Ch]Cl-based DESs; however, IgG showed a higher colloidal stability in the presence of the [Ch]Cl–Gly DES than in [Ch]Cl alone. DESs can efficiently enhance the conformational and colloidal stability of IgG and deserve to be further investigated as a potential solvent in the pharmaceutical area.

Experimental

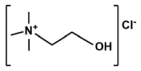
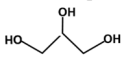
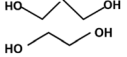
Materials

IgG from human serum (reagent grade ≥95%), lyophilized powder, glycerol and ethylene glycol were purchased from Sigma Aldrich, USA. Cholinium chloride (≥98%) was purchased from Alfa Aesar. Urea, sodium dihydrogen phosphate and disodium hydrogen phosphate were purchased from SRL (Sisco Research Laboratories). The water used for the preparation of phosphate buffer (pH 7.0) solution was double distilled by treatment with a Milli-Q plus 185 water purification apparatus. NovexTM WedgeWellTM 16% Tris-Glycine gels and NovexTM Tris-Glycine SDS running buffer were purchased from Thermo Fisher Scientific. The SDS-PAGE molecular weight standard used was the NZYColour Protein Marker II, from Nzytech.

Synthesis and characterization of cholinium-based DESs

Three DESs, namely [Ch]Cl–urea, [Ch]Cl–EG and [Ch]Cl–Gly, were prepared in the present work, as described in Table 1, following the protocol outlined by Zhu *et al.*[31] The process involved mixing the hydrogen-bond acceptor (HBA) with one hydrogen-bond donor (HBD) (urea/glycerol/ethylene glycol). All three DESs were mixed in 1 : 2 molar ratios, and the DES [Ch]Cl–urea in the additional molar ratios of 1 : 1, 1 : 3 and 2 : 1. To obtain a clear homogeneous liquid, the mixture was magnetically stirred at 80 °C for 1–2 h in an oil bath. Thereafter, the formed solution was cooled at room temperature and kept in a desiccator. The FTIR spectra of [Ch]Cl–urea, [Ch]Cl–EG and [Ch]Cl–Gly are presented in Fig. S8 (ESI).† The spectra of all the DESs showed that they were not degraded and were in

Table 1 HBA and HBDs used in the studied DESs

Hydrogen-bond acceptor (HBA)	Hydrogen-bond donor (HBD)		pH
[Ch]Cl[a]		Urea 	7.7
		Gly[b] 	6.3
		EG[c] 	6.9

[a] [Ch]Cl: cholinium chloride. [b] Gly: glycerol. [c] EG: ethylene glycol.

agreement with the available literature.[32,33] The investigated DESs are summarized in Table 1.

The effect of the DESs (solvation shell) on the conformation of IgG, *i.e.* the secondary and tertiary structures of IgG, was studied by UV visible spectroscopy, steady-state fluorescence emission spectroscopy, far-UV CD and FTIR.

The UV absorption spectra of 3.0 μM IgG (pH 7.0) and various concentrations of the DESs (5, 10, 15, 30, 50 (w/w%)) were measured with a Shimadzu UV-1800 (Japan) spectrophotometer with the highest resolution (1 nm) in the wavelength range of 200–350 nm, using quartz cells with a path length of 1 cm at 25 °C. All the samples were equilibrated at 25 °C for 30 min.

A Cary Eclipse spectrofluorometer from Varian optical spectroscopy instruments (Mulgrave, Victoria, Australia) equipped with a thermostat cell holder was used to study the fluorescence emission, using the same concentrations of IgG and DESs. To investigate the contribution of the tyrosine (Tyr) residue to the overall fluorescence emission, the sample was excited to 275 nm wavelength and the emission spectra were recorded between 280 to 400 nm.[15] The slit widths for excitation and emission were set at 5 nm.

The thermal stability of IgG in sodium phosphate buffer as a function of the concentration of the DESs (5, 10, 15, 30 and 50 (w/w%)) was analysed by fluorescence spectroscopy, from 20 °C to 90 °C, at an exciting wavelength of 275 nm. A 1 cm path length cuvette was used for the thermal unfolding analysis. The temperature was increased at a heating rate of 2 °C min^{-1} using a Peltier thermocouple and a time constant of 16 s. All the unfolding transitions of the IgG were determined by employing the two-state unfolding mechanism.

The far-UV CD measurements of IgG as a function of the DES concentration were carried out at 25 °C using a Jasco J-815 spectrophotometer, with a quartz cuvette of a path length of 0.1 cm. The wavelength range was set from 190 to 240 nm. Other parameters were fixed as follows: response time, 1 s; bandwidth, 1 nm and scan rate, 50 nm min^{-1}. The spectrum of the buffer alone was taken and deducted from the final scan for baseline correction.

The FTIR spectra of IgG in the presence of the DESs at 5, 10, 15, 30 and 50 (w/w%) were recorded using an IRAffinity-1S SHIMADZU FTIR spectrometer. The concentration of IgG was 3 μM. The FTIR spectra were obtained in the wavenumber range of 3000–1000 cm^{-1} by accumulating 256 scans, with a resolution of 4 cm^{-1} in transmittance mode. All the samples were prepared in D_2O buffer.

A laser Raman spectrometer (Enspectr R532 Raman spectrometer), working in a confocal mode, with an inVia Raman spectrometer coupled to a microscope was used for the measurement of the Raman spectra. The beam from a 523 nm HP NIR (high power near IR) diode laser was focused to avoid sample degradation. Raman light was dispersed using a diffraction grating with 1200 grooves mm^{-1}. The laser power was kept low, *ca.* 1–3 mW. The desired power was set using the spectrometer software by introducing appropriate filters in the laser beam.

Colloidal stability of IgG

Colloidal stability was analysed using the technique DLS, with a Zetasizer Nano instrument (ZS90) from Malvern Instruments Ltd, (UK), equipped with a He–Ne laser (4 mW, 632.8 nm). The scattering angle was set to 90° with a fixed operating wavelength of 633 nm. The hydrodynamic diameter (d_H) of IgG at a concentration of 1.5 μM in (10 mM, pH 7.0) sodium phosphate buffer was determined as a function of the concentration of the DESs (5, 10, 15, 30 and 50 (w/w%)) in sodium phosphate buffer pH 7.0, 10 mM. All the samples were equilibrated at 25 °C for 30 min.

Aggregation/degradation of IgG

Size-exclusion high-pressure liquid chromatography (SE-HPLC) was used to evaluate the aggregation/degradation of IgG by its changes in retention time and peak intensity. The equipment used was a Chromaster HPLC system (VWR Hitachi) equipped with a DAD detector, binary pump column oven (operating at 40 °C), temperature-controlled auto-sampler (operating at 10 °C) and a column Shodex Protein KW-802.5. The samples consisted of IgG (3.0 μM), sodium phosphate buffer (pH 7.0, 10 mM) and various concentrations of DESs (5, 10, 15, 30 and 50 (w/w%)). The mobile phase consisted of an aqueous sodium phosphate buffer solution (10 mM, pH 7.0 with NaCl 0.3 M) and was run isocratically with a flow rate of 0.5 mL min^{-1}. The sample injection volume was 25 μL and the wavelength was set at 280 nm.

Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to infer the protein profile and evaluate the structural integrity/stability of IgG. All the samples contained IgG and DESs at the previously describe concentrations. Samples were diluted in Laemmli sample buffer with dithiothreitol (DTT), under reducing conditions. These solutions were heated at 95 °C for 5 min for denaturation. A volume of 20 μL of each prepared sample was loaded into a polyacrylamide gel (stacking 4%; resolving 20%) and ran for 2 h at 100 V using a running buffer containing 250 mM Tris-HCl, 1.92 M glycine and 1% SDS. The molecular weight standard used Rainbow™ molecular weight markers from Sigma Aldrich. The gels were stained with InstantBlue® Coomassie Protein Stain from Abcam for 3–4 h at room temperature in an orbital shaker. The gels were then destained in

distilled water for 3–4 h at room temperature in an orbital shaker.

Conclusions

Biopharmaceuticals play a crucial role in the treatment of several diseases. However, by being bio-based, such as proteins, they suffer from low stability and require low temperatures and the use of excipients for storage and transportation. Therefore, there is a critical need to find new solvents and excipients to stabilize biopharmaceuticals, such as IgG. Accordingly, herein we report the remarkable potential of DESs to act as alternative solvent systems in IgG formulations. The DESs investigated were composed of [Ch]Cl as a common HBA and the following HBDs: urea, Gly and EG.

We addressed the conformation stability of IgG using UV absorbance, steady-state fluorescence spectroscopy, far-UV CD, FTIR, Raman spectroscopy and thermal fluorescence spectroscopy. Colloidal stability was investigated using SE-HPLC, SDS-PAGE and DLS. Furthermore, the long-term stability of IgG was determined using far-UV CD, DLS, SDS-PAGE and SE-HPLC. It was found that the DESs behaved as significant stabilizers of IgG, with [Ch]Cl–Gly as the most promising, followed by [Ch]Cl–urea and [Ch]Cl–EG. All the performed thermodynamic studies (increasing T_m and ΔG values) justified the enhancement of the conformational stability of IgG in the presence of the DESs. Time-dependent studies revealed the high potential of the DESs for the long-term storage of antibodies, majorly in [Ch]Cl–urea and [Ch]Cl–Gly. Furthermore, it was found that IgG showed different behaviour in the presence of the DES individual components (*e.g.* the structure of IgG was destabilized in the presence of urea and EG). Given the promising results here obtained, the impact of the DES HBA (*e.g.* betaine and amino acids) should also be studied and considered. Overall, the results here reported provide new insights into the design of new formulations of antibodies utilizing DESs.

Conflicts of interest

There are no conflicts to declare.

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