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ARTICLE

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

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Antibodies, among which Immunoglobulin G (IgG), have a pivotal place as biopharmaceuticals capable of treating a wide variety of diseases. However, IgG is a protein; hence, it easily loses stability and therapeutic efficiency, which may happen during 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. It is here shown that aqueous solutions of cholinium-based DESs enhance the conformational and colloidal stability of IgG, with no need of adding excipients. A series of DESs were prepared and investigated, viz. through the combination of cholinium chloride ([Ch]Cl) as hydrogen bond acceptor (HBA), with various hydrogen bond donors (HBD) such as urea, glycerol (Gly) and ethylene glycol (EG). The effect of [Ch]Cl-urea at different molar ratio (1:1, 1:2, 1:3 and 2:1) was also analysed. Conformational stability was checked by thermal fluorescence spectrometry, with selected DESs allowing increasing the transition temperature (Tm) of IgG by ca. 4 °C. The observed increase in the conformational stability of IgG in presence of DESs is in agreement with other spectroscopic studies, i.e., FT-IR and Raman spectroscopies. In presence of DESs, there is a minimum exposed surface of IgG with water molecules, 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 decreases in the presence of appropriate DESs. Finally, the long-term stability of IgG in presence of DESs was investigated at room temperature. All the results obtained from conformational and colloidal studies of IgG demonstrate an outstanding potential of cholinium-based DESs as novel solvents for IgG formulations, with the DES comprising [Ch]Cl and urea or [Ch]Cl and glycerol as the most promising candidates. All the described studies were also performed with the DESs individual components, demonstrating that DESs (HBD + HDA) are needed to improve IgG stability.

Introduction

Antibodies, i.e. immunoglobulins, are globular proteins whose function is to specifically bind to foreign substances in the host organism and thus eliminate invaders. They are a crucial part of the humoral immune system and are majorly produced by B lymphocytes upon stimulation.[1,2] Most antibodies perform their best biological functions at physiological conditions (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 molecular dynamics.[5] However, at the same time, water is very labile to hydrolysis and oxidation, thus destabilising protein's structure and function at elevated temperatures.[6] Therefore, there is a crucial need of finding novel excipients and/or biocompatible solvents that could stabilise proteins and enhance their storage capabilities.

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

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Current used excipients in vaccines 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 has been used in the Cuvitru vaccine for its stabilization. Cuvitru is composed of concentrated IgG, being a replacement therapy for primary humoral immunodeficiency in adult and paediatric patients.[8] Although the relevance of excipients, recent studies have shown that some of these excipients are not environmentally-friendly.[10,11]

Several research groups have shown the impact of toxic solvent systems in pharmaceutical industries.[10,11] Particularly, in protein stability studies, solvents do not only interact with the structure of proteins but also play a dominant role in toxicity profile.[12,13] Thus, there is a critical need to find biocompatible excipients or solvents that could help in maintaining the structure of 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 stabilising proteins against conformational destabilisation and aggregation.[14] Recently, we have 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 the IgG 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, composed of a quaternary ammonium salt ([Ch]Cl) as 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], with the mixture of [Ch]Cl and carboxylic acids. DESs have been described as compounds of negligible toxicity, biodegradable and non-volatile.[21-23] Nevertheless, it should be kept in mind that these properties are not general and depend on the 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 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 the *in vivo* model. One-week repeat dose study followed by histology and serum biochemistry analysis indicated that these are well tolerated by rats.[27] Cholinium-based DESs were also evaluated for targeting cancer cell lines[16]; it was found that the selectivity index for the studied DESs varied in the range > 2 or ≤ 2m, fulfilling an essential criterion to avoid any serious side effects of therapeutics.[16] Although a long path (*in vitro* and in *in vivo* assays, followed by human clinical trials) is still needed to have the studied DESs approved by the regulatory agencies, all these scientific works, together with the current work, support that DESs are promising excipients and solvents for therapeutic formulations.

Niketa and Vekatesu[28] have recently investigated the effect of DESs on the activity and stability of several proteins. In addition to proteins, DESs have also been proven 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, 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 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 DESs nature on the stability of IgG as a function of the HBD; (ii) the effect of DESs on the stability of IgG as a function of the DES concentration; and (iii) long-term stability of IgG in presence of the most promising DESs and 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 DESs on the conformation stability of IgG, UV absorption, fluorescence emission, far-UV CD spectra FTIR and Raman spectra of IgG in presence of all DESs and their individual components were performed. The wavelength (λ_{max}) for the absorbance maxima (A_{max}) of IgG in presence of buffer (control) is observed at 280 nm. This peak is due to the absorption of aromatic residues, such as tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe).[30-34] From Fig. 1 (a, b and c) it can be seen that there is no wavelength shift, indicating that the aqueous solutions of all DESs do not affect the polypeptide environment of IgG. Furthermore, there is no particular sequence of the absorbance change of IgG as a function of the concentration of DESs. However, there is a hypochromic shift (A_{max}) with respect to control in the presence of DESs. The decrease of A_{max} of IgG in the presence of DESs can be attributed to the shielding of aromatic residues, which further limits the resonance of the aromatic ring as well as its cation-π π interaction with the solvent system.[33]

Changes in intrinsic fluorescence were monitored to probe global structural alterations of IgG. Trp residues in native IgG are often quenched internally by local contacts with either disulphide bridges or each other.[35-37] However, Fig. 1 (d, e and f) shows the increase in the intensity of emission in presence of DESs as compared to control, which can be attributed to the increase in viscosity in presence of DESs.[38] The maximum wavelength of emission (λ_{max}) of IgG was find out to be ~330 nm[35] in the presence of phosphate buffer as well as in presence of DESs. No major shift in λ_{max} was observed, showing that the Trp and Tyr residues remain 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 β-pleated structure shows a positive ellipticity at 200 nm and a negative ellipticity at 218 nm.[39,40] IgG retained both peaks after incubation in all DESs; nevertheless, at

lower DES concentrations, the intensity is substantially stronger. Fig. 1 (g, h and i) demonstrates that the ellipticity becomes higher than the control at 30% of DES and begins to deform again at 50% of DES. These observations suggest that the secondary structure of IgG is retained up to 30% of DES and starts to be disturbed at higher DES concentrations.

From all the spectroscopic results discussed it can conclude that the IgG conformational stability depends on the concentration of DESs and 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 individual components of DES are provided in Fig. S1 in the Supporting Information. 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.

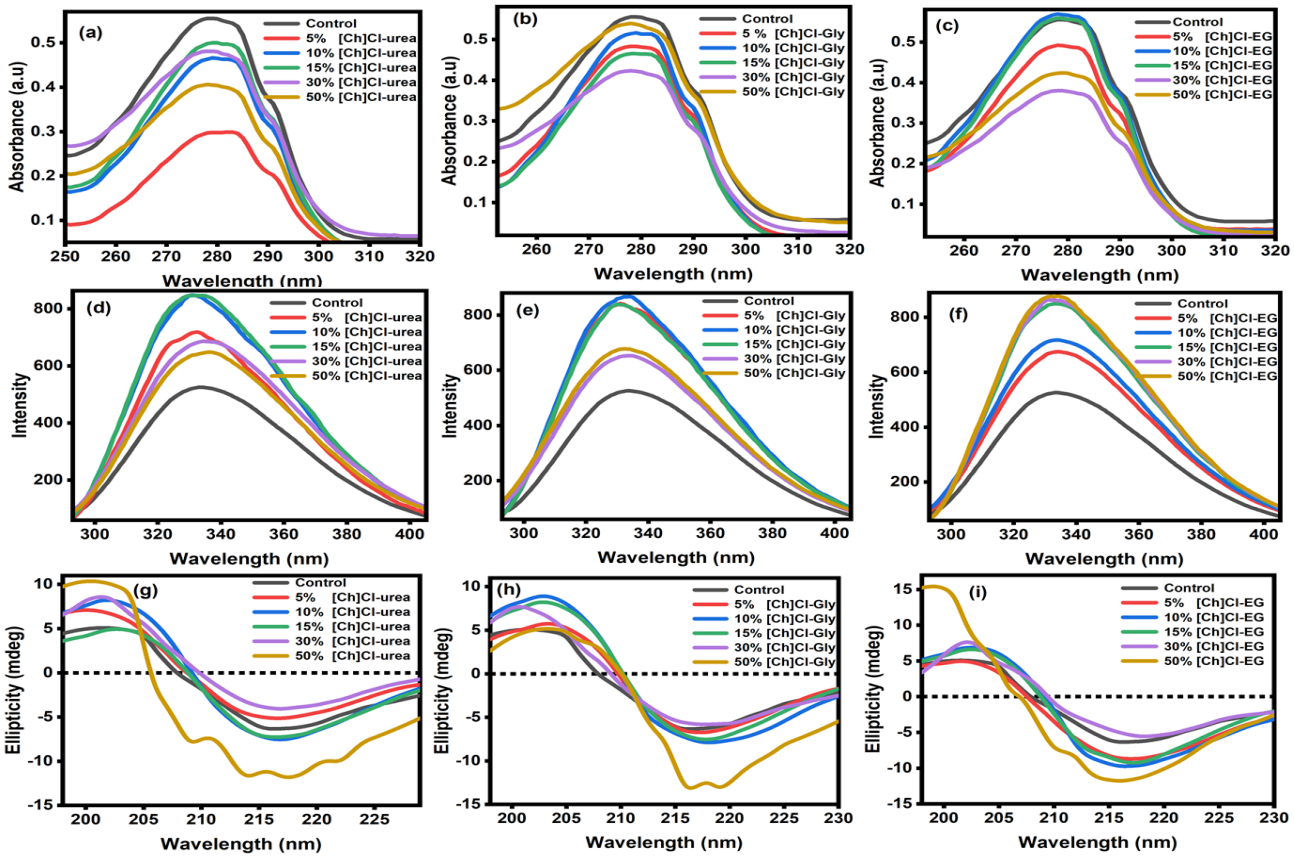


Fig. 1 Spectroscopic analysis of IgG: (a) (b) and (c) are UV visible absorption spectra; (d), (e) and (f) are fluorescence emission spectra; (g), (h) and (i) are far-UV CD spectra in the presence of sodium phosphate buffer pH 7.0, 10 mM (control) and at various concentration of DES's.

To further ascertain the effect of the HBD of each DES, FTIR and Raman spectra of IgG in the presence of 30% of DES were acquired. Fig. 2 depicts the measured transmittance and scattering intensity as a function of wavenumber (frequency of transition (ν)) in different solvent environments. Both Raman and FTIR spectroscopies are used to interpret the vibrational transition mode of molecules, and both techniques provide complementary results.[38] Proteins majorly show amide A and amide B bands between 3500 to 3200 cm^{-1}. The NH stretching gives rise to the amide A band and is exclusively localised on the NH group, being therefore insensitive to the conformation of the protein's backbone.[41] Amide A ν depends on the strength of hydrogen bonding. Amide I and II bands that appear in the 1700 – 1500 cm^{-1} region are defined by groups responsible for the peptide linkages in proteins.[42] Due to the C=O stretching vibrations of the peptide bonds, amide I appear 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 presence of DESs. For the IgG aqueous solution, three transmittance bands at 3387, 1640 and 1448 cm^{-1} (spectra in black) are observed. All three bands are consistent with those reported in the literature.[45] In the presence of all DESs, the frequency of amide A, amide I and amide II band do not shift, but there is an increase in the intensity of transmittance in the presence of [Ch]Cl-Gly, [Ch]Cl-EG and [Ch]Cl-urea. This can be explained by the increasing polarity of vibrating bonds of IgG (all the wavenumber values of IgG in presence of DESs and respective components are provided in Table S1 in the Supporting Information). The strength of transmittance increases with the

increasing polarity of the vibrating bond.[46] Hence, in the presence of DESs, the side chain N-H bonds become more polar, which can be due to extended electrostatic interaction that causes the increase in the intensity. From FTIR studies it can be interpreted that in the presence of DESs there is an increase in the NH polarity. The increase in polarity can be due to the favourable influence of DESs on the surface electrostatic interactions of the molecule.

Fig. 2 (b) provides the laser Raman spectra of IgG in the presence of DESs (versus the control). To avoid degradation of the sample, a laser beam of 532 nm was used. For IgG aqueous solution, two scattering bands are 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 show amide A frequency at 3252 cm^{-1}. There is an increment of 20 cm^{-1} concerning the control (all the values are provided in Table S2, in the Supporting Information). The increment of frequency of the amide A band in the presence of DES solvent can be due to exclusion of the N-H bond in hydrogen bonding and transformation of N-H···H–O–H to free N-H bonds.[48] On the other hand, N-H···H–O–H bonding is retained in the presence of [Ch]Cl-EG. There is an increase of the 12 cm^{-1} of amide I peak in the presence of [Ch]Cl-urea and [Ch]Cl-Gly, whereas in the presence of [Ch]Cl-EG the ν is approximately similar to the control. The increase in ν can be due to the shielding of IgG's C=O backbone from water molecules. The free stretching of C=O appears in the region of 1660-1665 cm^{-1} in the spectrum; due to C=O----O-H-O stretching, ν C=O shifted to the lower wavenumber (1640 cm^{-1}).[49] Overall, from laser Raman spectroscopy it can be interpreted that DESs help in retaining the conformation of all the four polypeptide chains of IgG, which further stabilizes the Fab segments and Fc segment by exclusive interactions with the hydrophobic core of the protein.

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

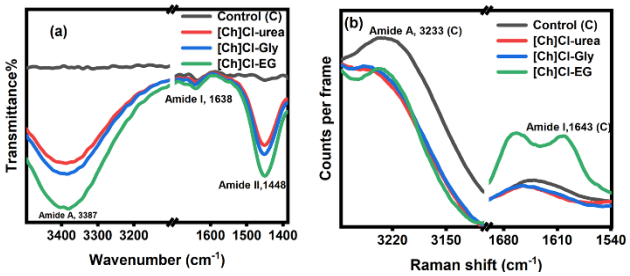


Fig. 2. (a) FT-IR spectra of IgG at 25 °C in the presence of control and 30% (w/w) [Ch]Cl-urea, [Ch]Cl-Gly and [Ch]Cl-EG. (b) Raman spectra of IgG at 25 °C in the presence of control and 30% (w/w) [Ch]Cl-urea, [Ch]Cl-Gly and [Ch]Cl-EG.

Effect of the DES nature on the conformation of IgG (temperature perturbation)

The thermal unfolding of IgG in the presence of all three DESs was further analysed using 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 (Δ_{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 more positive $\Delta_{fu}G$ require higher energy to unfold. All the thermodynamic equations required to determine these thermodynamic parameters are provided in the Supporting Information.

To ascertain the temperature perturbation effect on the IgG antibody in the presence of DESs, we have performed thermal fluorescence spectroscopy and determined the T_m, $\Delta_{fu}G$, change in enthalpy ($\Delta_{fu}H$) and change in heat capacity (ΔC_p). The thermodynamic parameters of IgG in presence of DESs are presented in Fig. 3, whose values are provided in Table S3 in the Supporting Information. The T_m of IgG in the control/buffer (pH=7) is 76 °C (Fig. 3a), which is in agreement with the literature.[15,53] It can be seen, however, when that adding all the three DESs there is an increase in T_m values, changing from 76 °C in the control (the case without adding any DESs) to 78.5, 80.0, and 76.5 °C in the presence of 30% of [Ch]Cl-urea, [Ch]Cl-Gly and [Ch]Cl-EG, respectively. When comparing with the DESs individual components, the T_m value of IgG in the presence of urea and EG decreases from 76 °C in the control to 58 °C in the presence of 30% of urea and to 70 °C in the presence of 30% of EG, respectively. On the other hand, Gly and [Ch]Cl behave as excellent stabilizers, with T_m values changing from 76 °C in the control to 86 °C in the presence of 30% of Gly and 83.5 °C in the presence of [Ch]Cl. The graphical representation of these results is provided in Fig. S3 in the Supporting Information. The obtained results explicitly indicate that DESs increase the T_m values of IgG and keep the folding form beyond 76.5-80 °C, which is higher than that obtained in the control. Interestingly, the T_m order shows that [Ch]Cl-Gly is the strongest stabilizer, while [Ch]Cl-urea and [Ch]Cl-EG are 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 Tm was obtained.[54] On the contrary, urea and EG depress the T_m by 18 and 6 °C, with respect to the control, which can distinctly be related to its denaturation power. T_m studies of IgG were also performed for 50% of DESs; nevertheless, at this concentration, there is a general destabilizing behaviour of DESs over IgG. These values are reported in Table S3 (Supporting Information).

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 are 1.57 kcal/mol and 40 kcal/mol (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 kcal/mol, 2.98 kcal/mol; 112 kcal/mol, 120 kcal/mol). Overall, all three DESs unfavourably interact with the surface of IgG and stabilise the folded native structure of IgG.

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

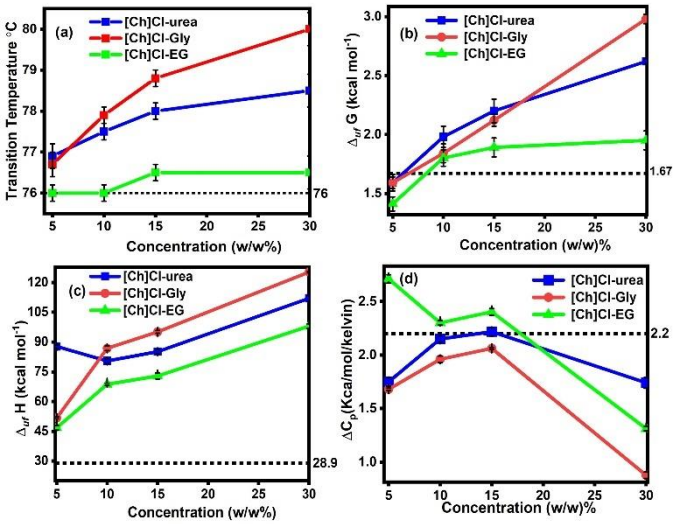


Fig. 3. Thermodynamic studies of IgG at 25 °C in the presence of sodium phosphate buffer pH 7.0, 10 mM (control) and as a function of concentrations of DESs. (a) Transition temperature (T_m); (b) Gibbs free energy changes ($\Delta_{fu}G$); (c) enthalpy change ($\Delta_{fu}H$) of unfolding; and (d) heat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

Effect of the DES 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 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 samples were filtered using a 0.22 µm syringe. The plot of size distribution by the intensity of IgG in the buffer and DESs is displayed in Fig. 4 (a, b and 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, in agreement with the literature.[14, 56] The polydispersity index of IgG was found to be 0.51, which is higher than 0.5 and shows that the control solution is polydisperse. The results in Fig. 4 (a) explicitly show that the d_H value of IgG in [Ch]Cl-urea increases as a function of 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 correspond 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). Particle size distribution of IgG in the presence of DESs shows a decrement in d_H values up to 15 % (w/w) of DES, whereas an increase in the d_H values is seen at 30 and 50 % (w/w) of DES. The PDI values show the same behaviour. The increase in d_H and PDI values at higher concentrations of DESs can be attributed to the formation of polydisperse media in the presence of higher concentrations of DESs, whereas at lower concentrations monodisperse media, i.e., less IgG aggregation, is observed. Similar results have been provided by the individual components of DESs (data provided in Table S4 in the Supporting Information).

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 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, e and f). The chromatograms of IgG in the presence of individual DESs components are presented in the Supporting Information (Fig. S4). According to the obtained chromatograms given in Fig. 4 (d), the control IgG sample has 2 peaks: one peak at a retention time of ~14.3 min, corresponding to the IgG in its monomeric form, and a second peak at ~13.4 min, corresponding to IgG aggregates. Compared to the control sample, there are no major changes in the IgG aggregates peak for [Ch]Cl-Gly and [Ch]Cl-EG, whereas there is a decrease in the aggregates peak with the increase in the [Ch]Cl-urea concentration. These results imply that DESs have a good potential to avoid and even reduce IgG aggregation. Regarding the main chromatographic peak of IgG, it can be seen from the chromatogram in Fig. 4 (e) that the absorbance of the main peak increases with the concentration of [Ch]Cl-Gly, increasing from 5% to 50%. However, if we compare 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 can observe that these peaks are wider and asymmetric, and consequently the peak area is not as high compared to the symmetrical peaks (control and [Ch]Cl-Gly 30%, 50%). Thus, to precisely determine the effect of [Ch]Cl-Gly

concentration, the peak areas for all concentrations were calculated using the PeakFit® software. According to these results, the values of the peak areas decrease with the [Ch]Cl-Gly concentration increase. In addition, the SDS-page gel results (Fig. 4 h) also confirm that there is 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 decreases. The values of the peak areas are provided in Table S5, in the Supporting Information. Additionally, no peaks at higher retention times were observed, meaning 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 DESs at different concentrations was also evaluated by SDS-PAGE, whose results are displayed in Fig. 4 (g, h and i). The SDS-PAGE of DESs individual components is provided in Fig. S4 (l, j, l & m) in the Supporting Information. According to the SDS-PAGE in reduced conditions, the control IgG is 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, h and i). These two bands are identified in the presence of all cholinium-based DESs investigated and at all concentrations, indicating that there are 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 is observed, indicating the presence of IgG agglomerates in the control and all DESs solutions. Comparing the three DESs, a slight decrease in the intensity of the IgG heavy and light chains was observed for the higher DESs concentrations, namely 30 and 50%, corroborating with the decrease in the peaks of the respective SE-HPLC chromatograms.

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

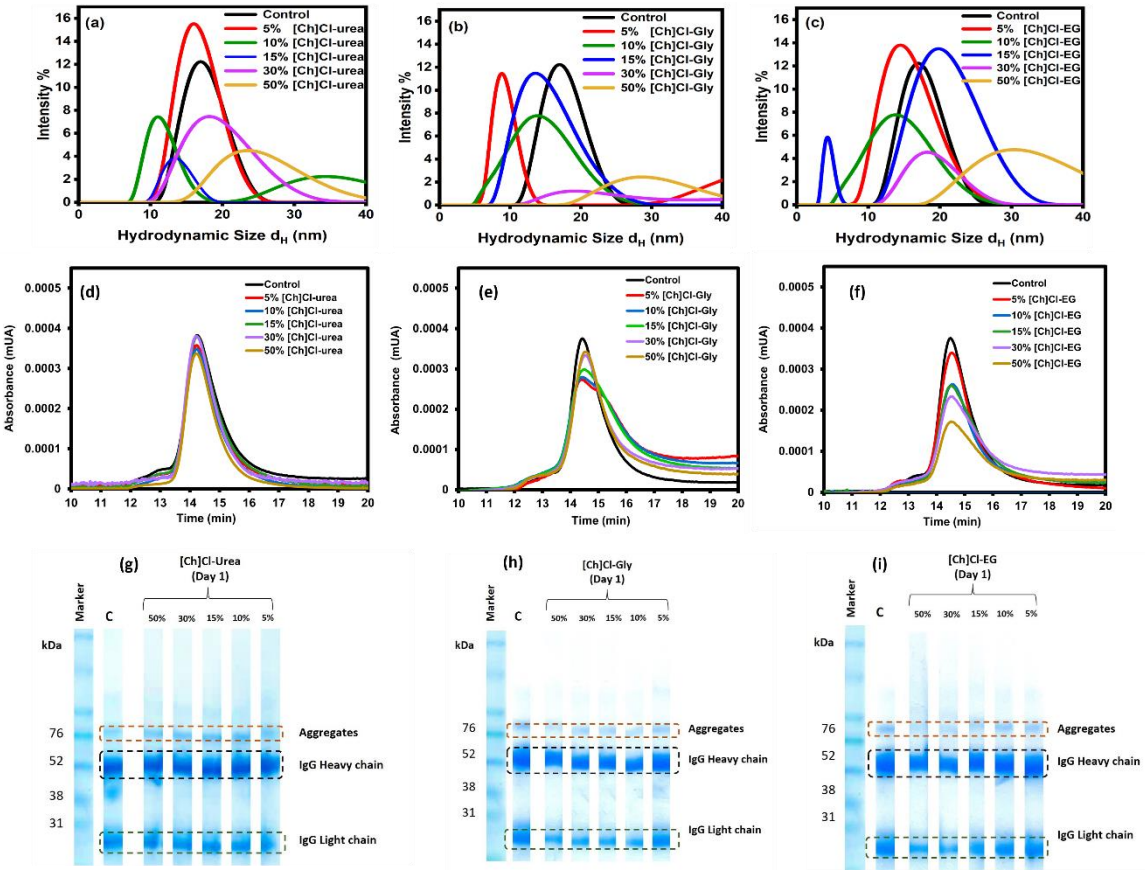


Fig. 4. Analysis of aggregation of IgG (a) (b) and (c) are size-distribution plot; (d), (e) and (f) are SE-HPLC spectra; (g), (h) and (i) are SDS-PAGE in the presence of sodium phosphate buffer pH 7.0, 10 mM (control) and at various concentrations of [Ch]Cl-urea, [Ch]Cl-Gly and [Ch]Cl-EG at 25 °C.

Long-term stability of IgG in 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 analysis of IgG in the presence of DESs (at 30%) and its components were performed as a function of time for 20 days. All assays were performed at temperatures varying from 30-35 °C, except for SE-HPLC and SDS-PAGE that were performed at 25 °C.

Far-UV CD spectroscopy have been performed on day 1 and day 20 for IgG in the presence of 30% of DESs and their isolated components. The respective results are displayed in Fig. 5 (a, b, c and d). IgG shows its significant broad peak at 218 nm on day one in

presence of phosphate buffer and DESs. After 20 days, it can be seen that the peak gets completely deformed in presence of phosphate buffer, and there is a shift in the wavelength in 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 is shown that [Ch]Cl-urea and [Ch]Cl-Gly are efficient solvent systems for storing IgG for longer periods. Consecutively, the variation in β-pleated structure percentage was calculated with the software dichroweb[57], allowing us to confirm the retainment of the percentage of the β-pleated structure in presence of [Ch]Cl-urea and day 20. The respective results are presented in Fig. 5 (a, b, c and d).

The chromatograms for the individual DES constituents are provided in the Supporting Information, Fig. S5. All IgG samples have similar retention times for both monomeric and aggregated forms of IgG. Furthermore, there was an increase in the monomer corresponding peak in presence of [Ch]Cl-Gly and [Ch]Cl-EG and a decrease in the aggregate's corresponding peak in presence of [Ch]Cl-Gly after 20 days of incubation, Fig. 5 (e, f, g and h). From Fig. S5 in the Supporting Information, it can be seen that in the presence of DES's individual components, mainly [Ch]Cl, urea and EG, IgG's structure is not retained after 20 days of incubation. The stored IgG was further evaluated by DLS studies and SDS-PAGE, Fig. 6. Samples were not filtered while 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 is Control (238 nm) > [Ch]Cl-EG (213 nm) > [Ch]Cl-Gly (177 nm) > [Ch]Cl-urea (166 nm) and the order of PDI values are approximately the same. On day 20, there was no change in the order 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)) is due to the long-term aggregation of the protein. However, it is here shown that 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 presence of DESs as compared to the control. When comparing with the components Gly and [Ch]Cl, Fig. S6 in the Supporting Information, there is a larger decrement in d_H as compared to the control; however, this decrement is less than the one obtained with [Ch]Cl-Gly. Also, in the presence of urea (30%), the d_H value goes around 3323 nm (all data are provided in Table S6 in the Supporting Information). 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 structure was investigated. This DES was chosen since it is one of the most promising to improve the IgG stability. Results are provided in Fig. S7 and Table S7 in the Supporting Information. The obtained results demonstrate that the [Ch]Cl-urea performs better at the 1:2 composition, the general composition used in the previously described assays and results.

All our results systematically reveal that all three DESs interact unfavourably with the IgG antibodies, hence stabilizing the IgG structure. DESs tend to exclude from the IgG surface, forcing the polypeptide to adopt a compactly 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 the 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 is composed of [Ch]Cl and urea and behaves as an excellent stabilizer due to extended hydrogen bonding between both components. Similar is the case of [Ch]Cl-EG, where EG behaves as a destabiliser individually whereas in [Ch]Cl-EG the structure of IgG is retained to its native conformation.

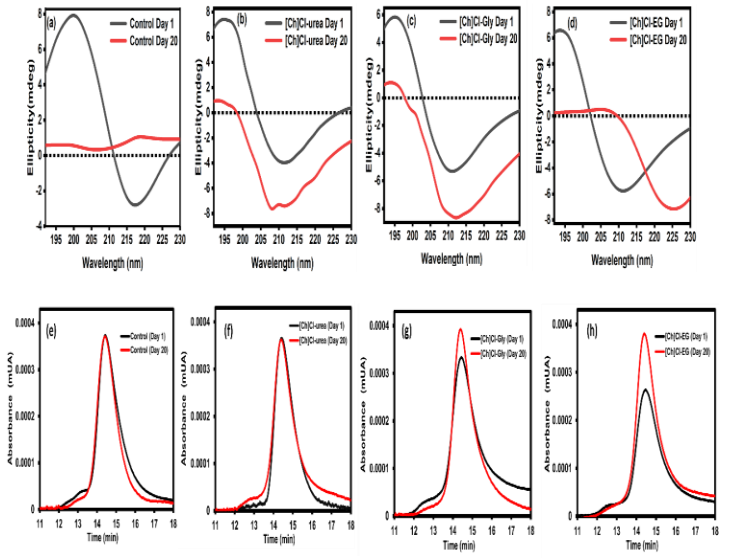


Fig. 5 Ellipticity calculated from time variable far-UV CD for IgG in (a) in phosphate buffer (b) 30% [Ch]Cl-urea (c) 30% [Ch]Cl-Gly (d) 30% [Ch]Cl-EG. SE-HPLC Chromatogram of IgG as a function of time, (e) Control (f) [Ch]Cl-urea) (g) [Ch]Cl-Gly (h) [Ch]Cl-EG.

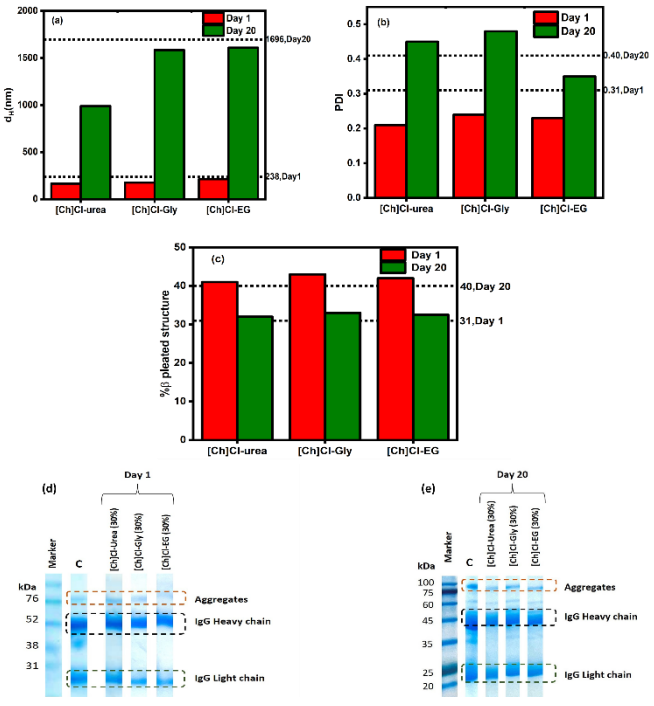


Fig. 6 (a), (b) Hydrodynamic size and polydispersity index calculated from time variable DLS for IgG in the presence of DES's. (c) Percentage secondary structure analysis of IgG in the presence of phosphate buffer and at 30% DES's as function of time. (d) and (e) SDS-PAGE spectra of IgG as a function 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%.

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 up to 35 (w%) of [Ch]Cl-Gly. Our results corroborate these findings with a different protein. All three studied DESs behave as IgG stabilizers 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 in 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 as compared to other DESs. Similar results were provided by Venkatesu and co-workers,[61] with [Ch]Cl-Gly behaving 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 are superior to the 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 is obtained in the presence of [Ch]Cl than in presence of [Ch]Cl-based DESs; however, IgG shows a higher colloidal stability in the presence of the [Ch]Cl-Gly DES than in the [Ch]Cl alone. DESs efficiently enhance the conformational and colloidal stability of IgG and deserve to be further investigated as a potential solvent in the pharmaceutical area.

Experimental

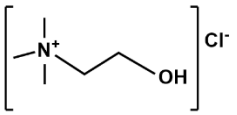
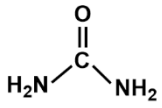
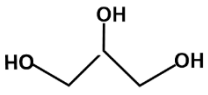
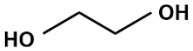
Materials

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 which was treated 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 delineated by Zhu *et al.*[31] The process involved mixing of 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 ratio 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 (Supporting Information). The spectra of all DESs show that they are not degraded and are in agreement with the available literature.[32,33] The investigated DESs are summarized in Table 1.

Table 1 – HBA and HBDs used in the studied DESs.

Hydrogen-bond Acceptor (HBA)		Hydrogen-bond donor (HBD)		pH
[Ch]Cl [(1)]		Urea		7.7
		Gly [(2)]		6.3
		EG [(3)]		6.9

[(1)] [Ch]Cl: Cholinium chloride; [(2)] Gly: Glycerol; [(3)] EG: ethylene glycol

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

The UV absorption spectra of 3.0 µM IgG (pH 7.0) and various concentrations of DESs (5, 10, 15, 30, 50 (w/w%)) were measured with a Shimadzu UV-1800 (Japan) spectrophotometer with the highest resolution (1 nm) in a wavelength range of 200–350 nm, using quartz cells of path length 1 cm at 25 °C. All 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 concentration of IgG and DESs. To investigate the contribution of the tyrosine (Tyr) residue to the overall fluorescence emission, the sample was excited to wavelength 275 nm and the emission spectra recorded were 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 DESs (5, 10, 15, 30, and 50 (w/w%)) was analysed by fluorescence spectra, from 20 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 using the 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 the 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. The spectrum of the buffer alone was taken and deducted from the final scan, as a baseline correction.

FTIR spectra of IgG in the presence of 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 samples were prepared in a D_2O buffer.

A laser Raman spectrometer (Enspectr R532 Raman spectrometer), working in a confocal mode, the inVia is a 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}. 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 the Zetasizer Nano instrument (ZS90), (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 DESs (5, 10, 15, 30 and 50 (w/w%)) in sodium phosphate buffer pH 7.0, 10 mM. All samples were equilibrated at 25 °C for 30 minutes.

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 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 2h 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 biobased 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 of finding 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 are composed of [Ch]Cl as the 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 has been 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 DESs behave 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 (increase in T_m and ΔG values) justified the enhancement of conformational stability of IgG in the presence of DESs. Time-dependent studies revealed the high potential of DESs for long-term storage of antibodies, majorly in [Ch]Cl-urea and [Ch]Cl-Gly. Furthermore, it was found that IgG shows different behaviour in presence of the DES individual components (e.g., the structure of IgG is destabilised in 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 resorting to DESs.

Conflicts of interest

There are no conflicts to declare.

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