Cholinium-based Ionic Liquids as an Efficient Media for Improving the Structural and Thermal Stability of Immunoglobulin G Antibodies

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ABSTRACT: Among antibody-based biopharmaceuticals, immunoglobulin G (IgG) plays a central role. However, IgG is a protein and as such easily loses stability, thus compromising its use as an effective therapeutic. To overcome this drawback, in this work, we evaluated cholinium-based ionic liquids (ILs) as effective stabilizers of IgG. We have investigated four ILs in aqueous solution, namely cholinium acetate ([Ch][Ac]), cholinium chloride ([Ch]Cl), cholinium dihydrogen citrate ([Ch][Dhc]) and cholinium dihydrogen phosphate ([Ch][Dhp]). We used a quantitative approach using different spectroscopic and chromatographic techniques to evaluate the thermal and structural stability of IgG. Thermal fluorescence spectroscopy studies were performed to obtain the transition temperature (T_m) and the change in Gibbs free energy (ΔG) of IgG in all cholinium-based ionic liquid (IL) aqueous solutions. The results indicate an appreciable increase in T_m in presence of [Ch][Ac] and [Ch]Cl. The thermodynamic parameters obtained from thermal fluorescence are compared with the structural stability of IgG by UV, fluorescence, CD and FT-IR spectroscopies, as well as with SE-HPLC and SDS-PAGE, showing that all results are in good agreement. Furthermore, the hydrodynamic diameter (d_H) of the IgG as a function of the concentration of IL was analyzed using dynamic light scattering (DLS), showing favorable interactions between protein residues. Finally, molecular docking studies for IgG in ILs using Molegro Virtual Docker (MVD) have been performed, reinforcing the main interactions ruling the IgG stability. This work highlights the potential validity of using cholinium-based ILs in IgG formulations for enhancing its thermal and structural stability, and thus preservation of IgG antibodies.

Key words: Antibodies, Cholinium-based Ionic liquids, Immunoglobulin G, Structural stability, Thermal stability

INTRODUCTION

Antibodies are proteins naturally present in mammal's serum, being of high relevance for therapeutic purposes.[1] In particular, immunoglobulin G (IgG) is an antibody and a glycoprotein present in plasma and extracellular fluids, which constitute the humoral branch of the animal immune system.[2] IgG is a large molecule with a molecular mass of approximately 150 kDa. It consists of four peptide chains composed of two heavy (H, 50 kDa) and two light (L, 25 kDa) providing Y-shape confirmation.[3] IgG contains two fragments; the site that can bind to antigens is called the F_{ab} fragment and the site which acts as a base of the protein is known as the Fc fragment of IgG.[4] Polyclonal IgG obtained from human serum is currently used as IgIV (immunoglobulin intravenous) solutions to treat several disorders and immunodeficiency.[5,6] On the other hand, monoclonal IgG antibodies can be obtained through mammalian cell cultures and recombinant technology, requiring the transfection of cells and culture media.[7] The main reasons for the increasing popularity of IgG as therapeutics correspond to their specific binding and few side effects. Furthermore, antibodies may be conjugated to another therapeutic entity for efficient delivery to a target site, thus reducing potential side effects.[8] However; the widespread use of IgG is still challenging due to the difficulty in maintaining antibody stability over time to guarantee therapeutic efficacy when called upon.

IgG is a protein of very high molecular mass and therefore, is a large and complex molecule, thus, sustaining inherited physicochemical complexity as compared to small pharmaceutical molecules.[3] Proteins are highly labile molecules, which may suffer degradation under conditions that do not correspond to their native environment, such as by changing the pH, ionic strength, temperature, etc. However, to have therapeutic efficiency, IgG must be highly stable. Therefore, there is a crucial need for developing novel cost-effective methods to maintain its stability and biological activity in the available formulations. It is well-known that the solvent formulation has a key role in maintaining the hugely variable complex of antibodies[9] since the solvent

surrounding the protein causes differences in electrostatic and hydrophobic interactions. Furthermore, protein-solvent interactions are equally important as protein-protein interactions, leading to a large variance in the thermal stability and higher-order structure of proteins.[10] The critical destabilizing factors that limit the therapeutic use of antibodies are the change in the structure of the protein in the presence of water as a solvent.[11] As we know, water is a natural media for vaccine transportation but unfortunately water is not independent of the effect of the temperature.[11] Therefore, we want to explore the effect of solvents on antibodies which can behave as excipients and helps with the stabilization of the antibodies in the presence of water.

Numerous solvents have been investigated as excipients in formulations to improve the stability of IgG, however, most of its effective therapeutic utilization; however, most of these solvents may not be suitable for biomedical applications or do not confer enough stability on these biopharmaceuticals.[12] In this field, IgG stability might be improved by employing ionic liquids (ILs) as protective media, while envisioning their use as excipients in IgG formulations. ILs are molten salts, consisting of large size cations such as quaternary ammonium, imidazolium, pyridinium, piperidinium, pyrrolidinium, combined with an anion of weak coordination properties, for instance, halogen, fluorinated or alkyl chain substituents.[13] Compared to conventional organic solvents, ILs have found widespread applications due to their superior physical and chemical properties and remarkable solvating power.[12] However, the studies showed that ILs with larger anions possess higher hydrophobicity and hence high toxicity levels whereas, the smaller anionic moieties, such as acetate and chloride decrease the toxicity.[14] Although all the cholinium-based ILs are treated as harmless liquids, Ventura et al. have demonstrated the increasing trend of eco-toxicity where [Ch][Dhc] is found to be the toxic, whereas [Ch][Ac] is found better with respect to toxicity index as compared to other cholinium-based ILs.[15] Clearly, the anion Dhc has longer chain as compared to acetate which can be responsible for more toxic behavior of larger anion. Aiming at designing excipients that may increase the stability of antibody-based pharmaceuticals and extend their self-life, cholinium-based ILs were chosen in this work. Cholinium-based ILs, if properly selected, have high biocompatibility and biodegradability[16,17], and they have been proved to be efficient co-solvents for different proteins, like stem bromelain and cytochrome C. Kumar et al.[18] have demonstrated that the concentration range of cholinium-based ILs has a high impact on the overall stability of

proteins. It has also been shown that chaotropic cation (cholinium) and various kosmotropic anions of ILs show excellent properties to stabilize the native structure of α-chymotrypsin.[19] Furthermore, bio-based cholinium-based ILs have been used for the extraction and purification of IgG, with the stability of IgG confirmed after the extraction step.[20]

Hallett and co-workers[21] recently studied the effect of different excipients in the presence and absence of cholinium dihydrogen phosphate ([Ch][Dhp]) on the physical and thermal stability of IgG. The authors showed that the propensity of IgG for conformational change is driven by the preferential binding of dihydrogen phosphate anion to the antibody fragment. They further concluded that formulations containing [Ch][Dhp] with sugars, amino acids and surfactants are promising candidates for stabilizing IgG against conformational destabilization and aggregation. Ramalho et al.[22] have also separated IgG using an aqueous biphasic system (ABS) composed of cholinium-based ILs, finding that in all ABS, IgG preferentially partitions to the IL-rich phase, unveiling preferential interactions between IgG and cholinium-based ILs. Although all these shreds of evidence show that formulations composed of cholinium-based ILs may be promising candidates to improve the stability, more biophysical studies are still needed to confirm their true potential as excipients in IgG formulations.

In this work, we have studied the impact of cholinium-based ILs at different concentrations on IgG. The thermal and structural stabilities of IgG were explored using various biophysical techniques, such as UV-visible, steady-state fluorescence, thermal fluorescence, circular dichroism (CD) spectroscopy, Fourier-transform infrared spectroscopy (FTIR), size exclusion high performance liquid chromatography (SE-HPLC), sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and dynamic light scattering (DLS). We are trying to analyze the effect of ILs on the hydration layer of IgG. We have also studied the time-dependent stability of IgG in the presence of ILs which further explores the effect of ILs for storage of IgG in the presence of ILs. Ultimately these results provide the useful information regarding the stabilization of antibodies and increasing packaging and storage capabilities of IgG in presence of cholinium-based ILs.[23]The applied methodology allowed us to deliver key insights into the impact of cholinium-based ILs on the structural and thermal stability of IgG. The data obtained will help researchers to design better excipients to improve the structural and thermal stability of IgG and other biopharmaceuticals. Figure 1 shows the chemical structure of the studied cholinium-based ILs.

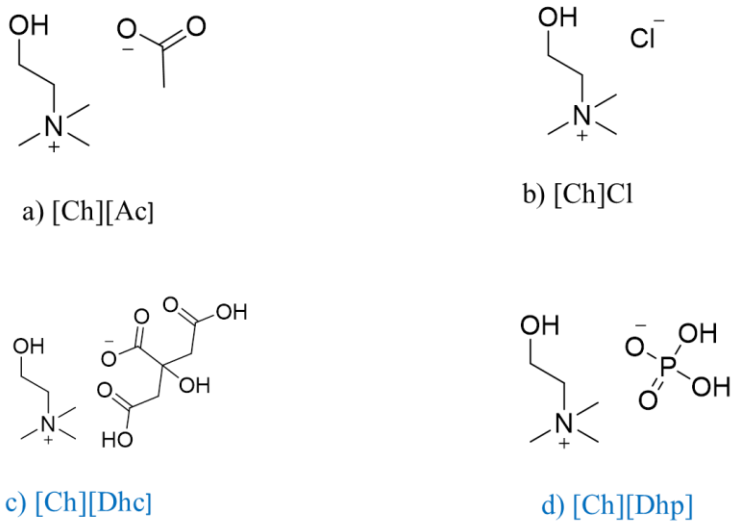


Figure 1. Chemical structure of the investigated cholinium-based ILs a) [Ch][Ac] b) [Ch]Cl c) [Ch][Dhc] d) [Ch][Dhp].

MATERIALS AND METHODS

IgG from human serum (reagent grade ≥ 95%) was purchased, as a lyophilized powder, from Sigma Aldrich, USA. Cholinium acetate ≥ 95% and cholinium dihydrogen citrate ≥ 98% were also purchased from Sigma Aldrich, USA. cholinium chloride, ≥ 98%, was purchased from Alfa Aesar and cholinium dihydrogen phosphate,>98% was purchased from Lolitech company. The water content of all ILs were measured using a Metrohm 831 Karl Fisher coulometer (Table S1) to guarantee the correct concentration of ILs. Moreover the pH values of all ILs are also included in Table S1.

Sodium phosphate buffer pH 7.0 (10 mM) was used as a reference solvent for preparing IgG solution and ILs solutions in double-distilled water. The IgG concentration was fixed to 3.0 μM for all measurements, except for the DLS measurements in which the IgG concentration was kept at 1.5 mM. The concentration of IgG was confirmed by UV visible spectroscopy using an extinction coefficient of 1.41 cm^2/mg. The stock concentration of all the ILs taken was 5 mM,

which was further diluted from 0.5 to 2.5 mM. All samples were incubated for 15–20 min at 25 ºC in order to attain equilibrium.

INSTRUMENTATION AND MEASUREMENTS

The UV absorption spectra of IgG 3.0 μM consisting of sodium phosphate buffer pH 7.0, 10 mM and various concentrations of ILs (0.5-2.5 mM) were measured by 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 ºC.

SE-HPLC (Chromaster, VWR Hitachi) equipped with a size exclusion column (Shodex Protein KW-802.5; 8 mm x 300 mm) and DAD detector was used to evaluate the aggregation/degradation of IgG structure by changes in the retention time. The samples consisted of IgG 3.0 μM pH 7.0 buffer (control) with different concentrations of ILs (0.5-2.5 mM). The mobile phase consisted of 10 mM sodium phosphate buffer pH 7.0 and NaCl 0.3 M. The sample injection volume was 25 μL and the wavelength was set at 280 nm. The samples ran isocratically with a flow rate of 0.5 $mL.min^{-1}$. The autosampler and column oven were kept at 10 and 40 ºC, respectively.

The structural stability of IgG was also evaluated by SDS-PAGE. All samples containing IgG 3.0 μM in sodium phosphate buffer pH 7.0, 10 mM in the presence of various concentrations of ILs (0.5-2.5 mM) were diluted in Laemmli sample buffer with dithiothreitol (DTT) (reduced conditions). The effect was to load ca. 200 μg of IgG per mL per lane prior to loading in the gel wells. These solutions were heated at 95 °C for 5 min for denaturation followed by loading into the wells of the SDS-PAGE gel containing 20% polyacrylamide. SDS-PAGE molecular weight markers from VWR were used. Gels were electrophoresed for 1.5 h at 135 V on polyacrylamide gels (stacking 4%; resolving 20%) with a running buffer constituted by 250 mM Tris-HCl, 1.92 M glycine and 1% SDS. After the run, the gels were stained with Coomassie Brilliant Blue G-250 0.1% (w/v), methanol 50% (v/v), acetic acid 7% (v/v) and water 42.9% (v/v) for 3–4 h at room temperature in an orbital shaker. The gels were then de-stained in a solution of acetic acid at 7% (v/v), methanol at 20% (v/v) and water at 73% (v/v) in an orbital shaker at 50 rpm for 3–4 h at 40 °C.

A Cary Eclipse spectrofluorometer from Varian optical spectroscopy instruments (Mulgrave, Victoria, Australia) equipped with a thermostat cell holder was used to monitor the fluorescence emission spectra of 3.0 μM IgG in 10 mM sodium phosphate buffer at pH 7.0 and in the presence of various concentrations of ILs (0.5-2.5 mM). The excitation wavelength was set at 275 nm and the emission spectra recorded were between 280 to 400 nm in order to calculate the contribution of the tyrosine (Tyr) residue to the overall fluorescence emission.[24] The slit widths for excitation and emission were being set at 5 nm. The thermal stability of IgG in sodium phosphate buffer as a function of the concentration of ILs (0.5-2.5 mM) was analyzed by the 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 3.0 μM in 10 mM sodium phosphate butter at pH 7 and as a function of the concentration of ILs (0.5-2.5 mM) carried out at 25 ºC using the Jasco J-815 spectrophotometer with a quartz cuvette of the path length of 0.1 cm at 25 ºC. The wavelength range was set from 190 to 240 nm at 25 ºC. The other parameters which were fixed are response time, 1 s; bandwidth, 1 nm and scan rate, 50 nm/min. The spectrum of the buffer alone was taken and was deducted from the final scan, functioning as a baseline correction.

Fourier-transform infrared (FTIR) spectra of IgG in the absence and presence of various concentrations of ILs (0.5, 1, 1.5, 2.5mM) were recorded by using an IRAffinity-1S SHIMADZU FTIR spectrometer. The FTIR spectra were obtained in the wavenumber range of 4000-400 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. The concentration of IgG was 3μM.

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 in sodium phosphate buffer pH 7.0, 10 mM was measured as a function of the concentration of ILs (0.5-2.5 mM). The DLS was of the company Zetasizer Nano instrument (ZS90), (Malvern Instruments

Ltd., UK), equipped with He-Ne laser (4 mW, 632.8 nm). The scattering angle was set to 90° with a fixed operating wavelength of 633 nm.

Molecular docking was performed to find out the potential binding site of ILs F_{ab} and F_c fragments of IgG. The trial version of Molegro virtual docker (MVD) was downloaded from Molegro to carry out the docking studies. All the default parameters were set. Three-dimensional structures of Fab (light and heavy chain) and Fc fragments of IgG were acquired from the RCSB in protein databank format (PDB entry 1N0X and 4BYH, respectively). Yasara online available software was used for optimizing the energy of both the fragments. For structure preparation and energy minimization, Chem Draw and Chem Draw 3D were used. The binding sites were restricted to the sphere of the radius of 15 Å, and all the other parameters including the maximum (1500 A) of the size iterations, a number of runs 60, maximum population size (60), scaling factor 0.5, grid resolution 0.30, and crossover rate 0.9, were fixed.

RESULTS AND DISCUSSION

Effect of Cholinium-Based ILs on the Structure of IgG as a function of concentration and the nature of anion

Up to date, the mechanisms of IgG behavior and a complete study of its structural and thermal stabilities in the presence of a wide range of cholinium-based ILs ([Ch][Ac], [Ch]Cl, [Ch][Dhc], and [Ch][Dhp]) has not previously been investigated. In this work, we have tried to explore the interactions between cholinium-based ILs and IgG, by applying a series of biophysical techniques, thus we can investigate the potential of these ILs to act as potential excipients to preserve IgG biopharmaceuticals. In addition, our examination of the temperature derived folding/unfolding has been carried out as a function of a concentration gradient. Overall, different ILs may have different significance, which is established by the physical and chemical properties of each IL in relation to the protein under study.

UV Spectral Analysis. UV absorption measurement is a successful method to explore conformational changes of proteins. Figure 2 shows the absorption spectra of IgG in the presence of various concentrations of cholinium-based ILs (0.5-2.5 mM) at 25 °C. The maximum

wavelength (λ_{max}) for the absorbance maxima of IgG is observed at 280 nm. The peak around 280 nm is due to the absorption of aromatic residues such as tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe).[24,26]

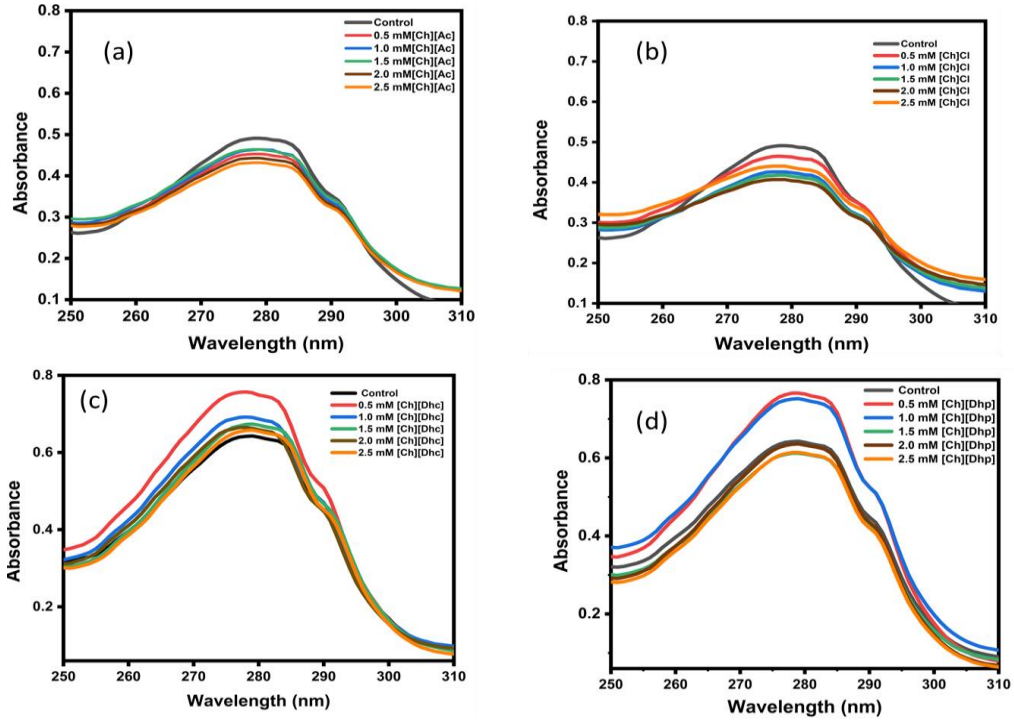


Figure 2. UV absorption spectra analysis of IgG at 25 °C in the presence of sodium phosphate buffer pH 7.0, 10 mM (control) and at various concentrations of ILs: (a) [Ch][Ac], (b) [Ch]Cl, (c) [Ch][Dhc], (d) [Ch][Dhp].

From Figure 2, UV absorbance results suggest that IgG shows a lead hypochromic (Figure 2 a and b) and hyperchromic (Figure 2c and d) shift in presence of ILs. As can be seen in Figure 2a, the A_{max} of IgG slightly decreases (Table S2) with the increasing concentration of [Ch][Ac], which indicates that the aromatic residues of IgG may not be significantly exposed to the solvent, resulting in a lower absorbance. In the case of [Ch]Cl (Figure 2b) the absorbance of IgG first decreases up to 2.0 mM, and then starts to increase at 2.5 mM (Figure 2b, Table S2). However, this increase is lower than in the control (IgG in phosphate buffer). On the other hand, in the presence of [Ch][Dhc] and [Ch][Dhp] (Figures 2c and 2d) IgG shows a sharp increase in the absorbance values at lower IL concentrations (0.5 and 1 mM), and then the absorbance decreases

as the concentration of [Ch][Dhp] and [Ch][Dhc] increases (Table S2). This trend indicates protection of aromatic residues of IgG at higher concentrations of [Ch][Dhp] and [Ch][Dhc]. Interestingly, there is no alteration in the λ_{max} of IgG in all the studied concentration of ILs (Table S2). Hence, from UV studies it can be assumed that the IgG can sustain a more compact conformation in these ILs as compared to that in the buffer solution.

SE-HPLC Analysis. The structural changes of IgG were evaluated by SE-HPLC. Even though SE-HPLC may be used to quantify IgG, and identify and quantify the presence of other proteins, this method can be used to evaluate the structure of antibodies since peaks corresponding to proteins aggregation or fragmentation will appear in the respective chromatograms.[20,22,25] The importance of evaluating the presence of IgG aggregates is due to its biological activity, which is related to the therapeutic efficiency that is usually reduced by the formation of aggregates.[25] Moreover, IgG aggregation may be fatal for patients with a variety of diseases involving protein aggregation.[22]

SE-HPLC chromatograms of IgG in the presence of sodium phosphate buffer pH 7.0, 10 mM (control) and at various concentrations of the ILs [Ch][Ac], [Ch]Cl, [Ch][Dhc] and [Ch][Dhp] are shown in Figure 3. According to the obtained results, under the chromatographic conditions applied, IgG samples show 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, indicating the presence of some IgG aggregates present in the commercial IgG from human serum. Compared to the control sample in buffer, after the IgG incubation in all cholinium-based ILs, showed no increase in protein aggregates, implying that these cholinium-based ILs have a good potential to avoid IgG aggregation. Additionally, no shift in the retention time is observed for all samples and no peaks at higher retention times are observed, meaning that no IgG degradation occurs. In a previous study reporting the use of 1-alkyl-3-methyl imidazolium chloride (alkyl: ethyl, butyl, hexyl and octyl), it was found that ILs were able to dissociate IgG aggregates, being 1-ethyl-3-methylimidazolium chloride the best solvent towards this end.[25] In a similar work, the aggregation of IgG was studied in the presence of different concentrations of 1-octyl-3-methylimidazolium chloride, and it was found upon increasing IL concentrations from 0.05 to 0.20% w/v that the size of the non-aggregated fraction was invariant at different pH values.[25] These findings are in agreement with the stability of other model proteins (hen egg white

lysozyme and the single-chain antibody fragment ScFvOx) in the presence of imidazolium-based ILs, where the selected ILs suppress protein aggregation.[26]

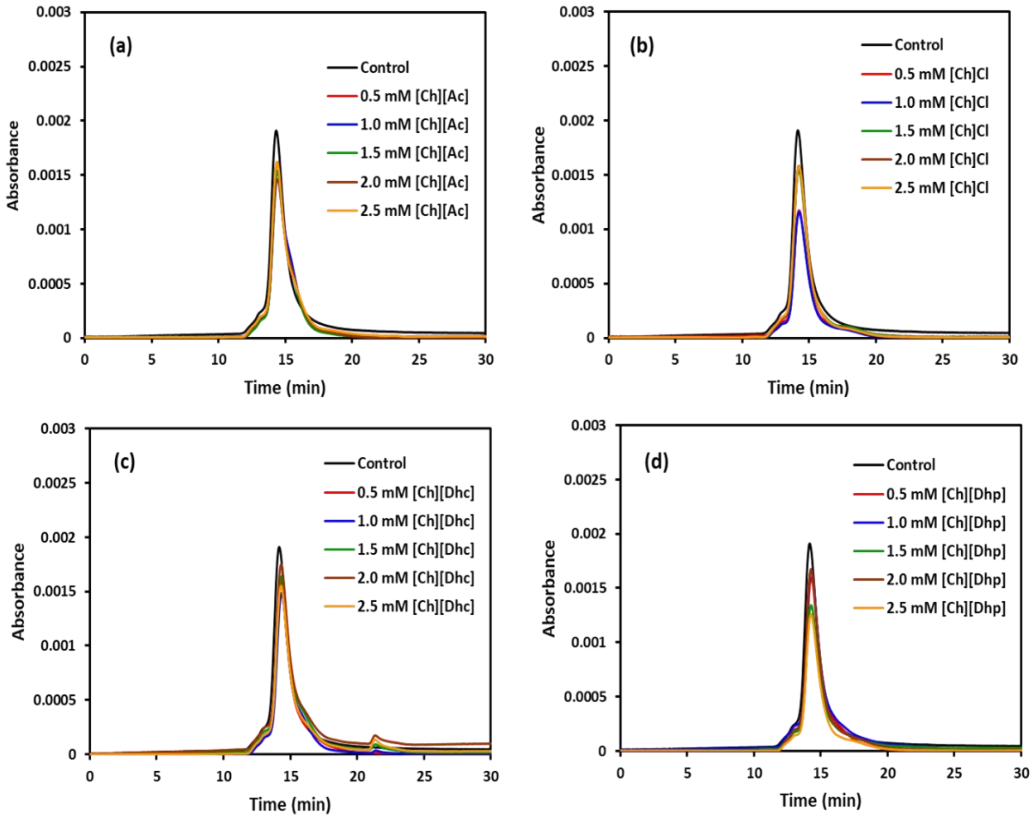


Figure 3. SE-HPLC chromatograms of IgG in the presence of phosphate buffer pH 7.0, 10 mM (control) and in the presence of cholinium based-ILs at various concentrations: (a) [Ch][Ac], (b) [Ch]Cl, (c) [Ch][Dhc], (d) [Ch][Dhp].

SDS-PAGE Analysis. The structural stability of IgG in the presence of [Ch][Ac], [Ch][Dhc], [Ch][Dhp] and [Ch]Cl at different concentrations (0.5- 2.5 mM) was evaluated by SDS-PAGE, whose results are displayed in Figure 4. SDS-PAGE for IgG in sodium phosphate buffer (10 mM, pH 7.0; control) was performed for comparative analysis. According to the SDS-PAGE in reduced conditions, 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 (Figure 4). These two bands are

identified in the presence of all cholinium-based ILs investigated and at all concentrations, indicating that there are no changes and no degradation of the IgG structure. Moreover, as verified by the SE-HPLC chromatograms, a band with high molecular weight is observed, indicating the presence of IgG agglomerates in the control and IL solutions. However, the intensity of this band is not observed to increase, thus corroborating the SE-HPLC results, *i.e.*, no new IgG aggregates other than originally present are formed.[28,29] In the previous studies the structural stability of IgG was evaluated in presence of imidazolium-, ammonium- and phosphonium-based ILs by SDS-PAGE, after its extraction from rabbit serum, no degradation of IgG was detected, except in presence of tetrabutylphosphonium bromide. In this previous work, Ferreira et al.[30] concluded that the high hydrophobicity of phosphonium-based ILs was the main reason behind its stronger negative effect on the integrity of IgG. These and the current results combine to reinforce the conclusion that cholinium- based ILs, by being more hydrophilic, behave as more suitable and biocompatible media for keeping the IgG structure stable[31,32].

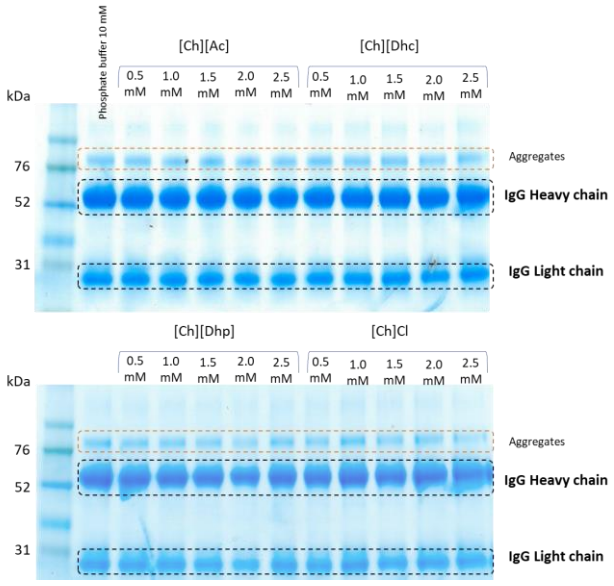


Figure 4. SDS-PAGE of IgG at different concentrations of [Ch][Ac], [Ch][Dhc], [Ch][Dhp] and [Ch]Cl. Lane 1 – molecular weight marker; lane 2 – IgG in sodium phosphate buffer pH 7.0, 10 mM (control).

Steady-State Fluorescence Spectral Analysis. The aromatic amino acids Trp, Tyr, and Phe serve as intrinsic fluorescence probes to study protein conformation, dynamics, and intermolecular interactions. The fluorescence properties such as maximum intensity (I_{max}) and maximum emission wavelength (λ_{max}) are highly sensitive to the polarity change in the microenvironment around the aromatic residues that can be used to investigate IgG−IL interactions.[17]

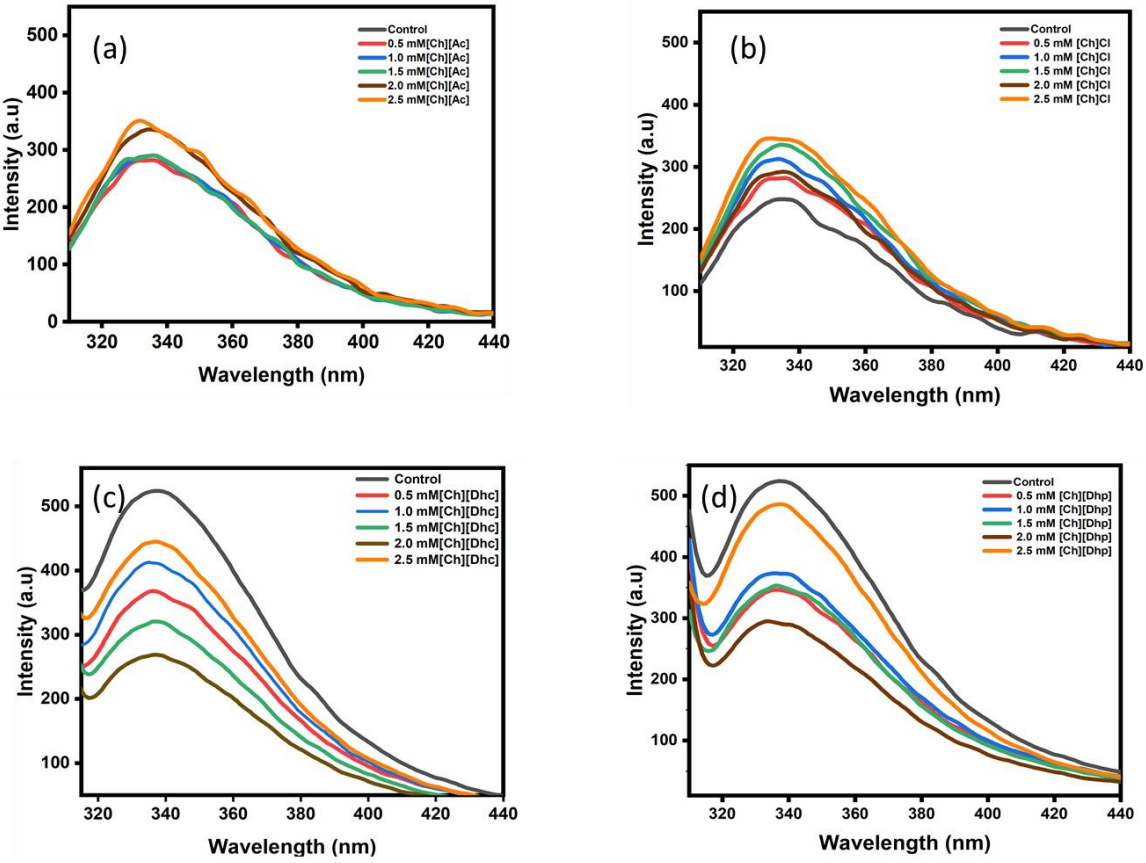


Figure 5. Fluorescence spectra analysis of IgG at 25 °C in the presence of phosphate buffer (pH=7) and various concentrations of ILs: (a) [Ch][Ac], (b) [Ch]Cl, (c) [Ch][Dhc], and (d) [Ch][Dhp]**.**

Figure 5 reveals the variation of emission spectra of IgG in the presence of various concentrations of different ILs when excited at 275 nm.[24] The change in the Tyr fluorescence is indicative of disturbance in the conformational dynamics of IgG. As can be clearly observed in Figure 5, in the case of IgG in [Ch][Ac], the I_{max} is gradually increasing with the increase in the

concentrations whereas in the case of [Ch]Cl the I_{max} increases with increase in the concentration of IL except at concentration 2.0 mM (Table S3) ILs do not cause any shift in λ_{max}. The increase in the intensity can attribute to higher quantum yield (ratio of the photon emitted to photon absorbed) of IgG in the presence of [Ch][Ac] and [Ch]Cl as fluorophore's environment can impact quantum yield, usually resulting from changes in the rates of non-radiative decay.[33] On the other hand, a sharp decrease in the I_{max} is observed in presence of [Ch][Dhp] (Figure 5d) at 0.5, 1 and 2 mM. A slight increase in I_{max} was observed on further addition of [Ch][Dhp] (Table S3). However, this increase is lower when compared to the control value. In the case of [Ch][Dhc] (Figure 5c), a sharp decrease in the I_{max} is observed even at the lowest concentration (0.5 mM) (Table S3), which is an indication of decreased hydrophobicity around the Tyr residue.[34] Nevertheless, it should be remarked that the trend of fluorescence is not uniform in the case of [Ch][Dhc] as well as there is no appreciable shift in the λ_{max} value of IgG in the presence of all ILs. All the values of λ_{max} and I_{max} are provided in Table S3. From the fluorescence results, it can be anticipated that Tyr of IgG may be getting exposed to the polar environment in the case of [Ch][Dhc] and [Ch][Dhp]. Thereby, there is more quenching of Tyr by [Ch][Dhc] and [Ch][Dhp] in comparison to [Ch][Ac] and [Ch]Cl. This behavior of the protein can be attributed to either the burial of Tyr inside the core of protein or the decreased quenching by neighboring charged residues.

Fluorescence Quenching Effect Shown by [Ch][Dhc]. In order to ascertain fluorescence quenching effect, fluorescence quenching of IgG in the presence of [Ch][Dhc] is studied. The Stern−Volmer plot of fluorescence Quenching is displayed in Figure 6.

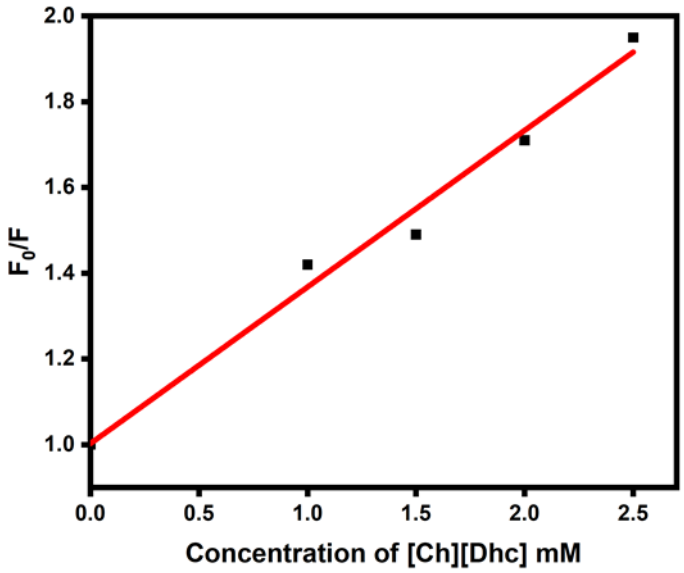


Figure 6. Stern−Volmer plot for quenching of IgG fluorophore by [Ch][Dhc]. The black square points represent the F_o/F values of IgG at various concentrations of [Ch][Dhc] and red line represents the linear curve fit.

Generally, in fluorescence spectroscopy, two types of quenching are possible, static quenching or dynamic quenching. In static quenching ground state complex form between fluorophore and quencher whereas, in dynamic quenching excited state collision of fluorophore and quencher takes place. [35] Hence, the quenching ability of [Ch][Dhc] is predicted with the help of Stern−Volmer plot which reflects the binding of [Dhc] with protein in the vicinity of fluorophore and protein backbone. The linear relationship between the fluorescence intensity of IgG and the concentration of [Ch][Dhc] is obtained from following equation.

$$F_0 / F = 1 + k_{sv} [Q] = 1 + \tau_o k_q [Q] \quad (1)$$

Where, F_0 is the intensity of fluorophore in the absence of quencher, F is the intensity of the fluorophore in the presence of quencher, [Q] is the concentration of the quencher, k_q is Quenching constant, τ_o is average life time of fluorophore that is 10^{-8} and k_{sv} is calculated from the slope of F_0/F versus concentration of [Ch][Dhc]. The k_{sv} values for IgG in the presence of [Ch][Dhc] was obtained from the slope of the Stern−Volmer plot and was found to be 0.36 M^{-1} and k_q values were obtained from average life-time of fluorophore and the value was found 3.6 ×

10^7 s^{-1}. The k_q value calculated from Stern−Volmer equation was found to be in the order of 10^7 s^{-1} which is less than the maximum dynamic quenching constant value (i.e., 2×10^{10} M^{-1} s^{-1}) revealing that fluorescence quenching of IgG in the presence [Ch][Dhc] arises due to dynamic diffusion. Hence, it can be concluded that [Dhc] and [Dhp] anions form complex with the excited tyrosine residue of the protein in the presence of UV absorbance which is responsible for the quenching in the fluorescence intensity.

CD Spectral Analysis. To gain more insight into the possible structural changes of IgG, CD spectroscopy studies were carried to study the changes in the secondary structure of the protein in the presence of cholinium-based ILs at different concentrations (Figures 7 and 8). Far UV CD spectra (180-250) can provide qualitative information about the presence of secondary structural element analysis in the protein.[36]

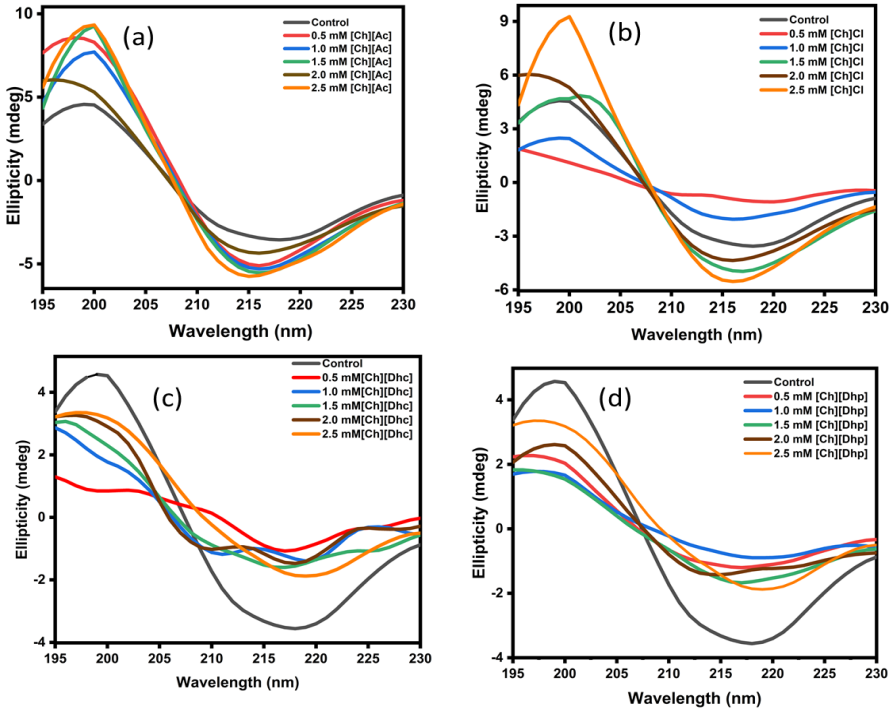


Figure 7. Far-UV CD analysis of IgG at 25 °C in the presence of phosphate buffer (pH=7.0, 10 mM) and at various concentrations of ILs: (a) [Ch][Ac], (b) [Ch]Cl, (c) [Ch][Dhc] and (d) [Ch][Dhp]**.**

In the Far-UV CD studies, a broad minimum at 218 nm and maximum at 199 nm suggests that the β-sheet structure dominates in the IgG (Figure 7).[36] As can be seen from Figure 7 (a), both positive and negative band ellipticity of IgG increases with the addition of [Ch][Ac]. From Figure 7 (b), it is clear that in presence of [Ch]Cl, the secondary structure of IgG is first disturbed at lower concentrations (0.5 and 1 mM), while with the increase in concentration (after 1.5 mM) the negative, as well as positive ellipticity, starts to increase which corresponds to increase in the β-bleated conformation.[37] However, similar to UV and fluorescence results, contrasting CD trends occur in the case of [Ch][Dhp] and [Ch][Dhc]. Results in Figure 7 (c) reveal that in presence of [Ch][Dhc] the secondary structure of IgG is disturbed even at low concentrations. Nevertheless, at a higher concentration of 2.5 mM, IgG starts to restore its secondary structure. In Figure 7 (d), it can be observed that with the increase in concentration (up to 1.5 mM) of [Ch][Dhp], there is a decrease in the ellipticity value with a slight blue shift. A further increase in concentrations (at 1.0 and 1.5 M) leads to the ellipticity starting to increase, however, but still lower than in the control. A reduction in the ellipticity indicates partial unfolding through the conversion of β-sheet into unordered structures. The percentage of secondary structure was calculated using Dichroweb software and it was concluded that overall, in presence of [Ch][Ac] and [Ch]Cl (Figures 8a and 8b) the percentage of β-sheet increases as compared to the control. On the other hand, in presence of [Ch][Dhc] and [Ch][Dhp] (Figures 8c and 8d), there is an alteration in secondary structure at a concentration lower than 2.5 mM with a reduction of β-sheet conformations accompanying an increase in unordered structure. In summary, the data suggest that [Ch][Ac] and [Ch]Cl help in retaining the secondary structure of IgG and to form a more compact structure at all studied concentrations as compared to [Ch][Dhc] and [Ch][Dhp].

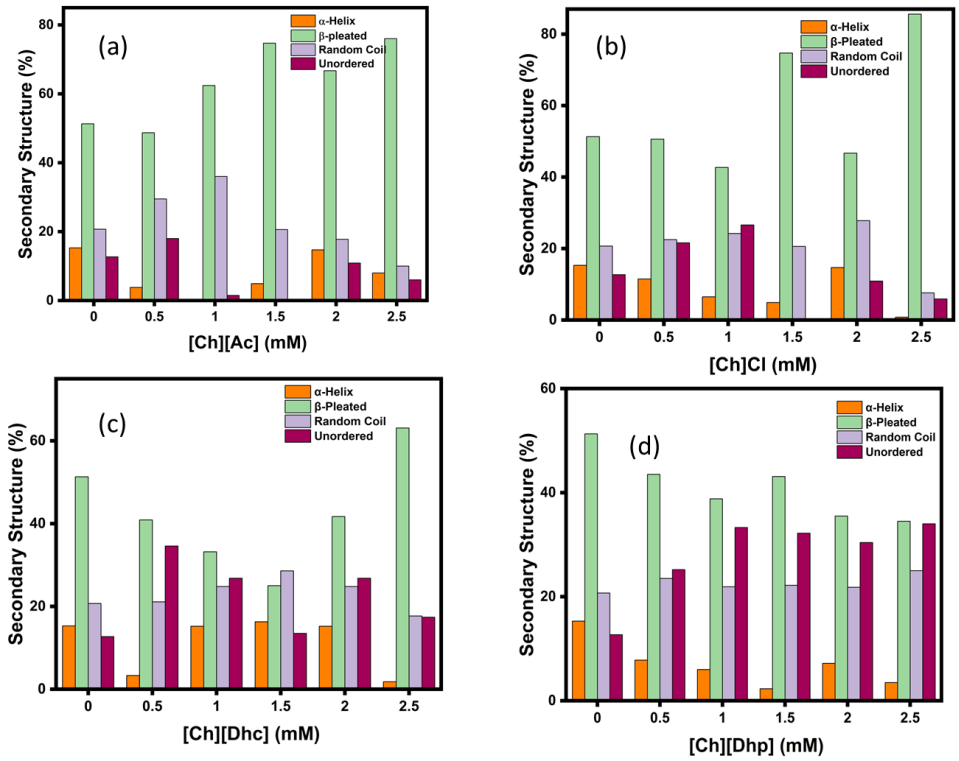


Figure 8. Secondary structure (%) of IgG in presence of varying concentrations of ILs: (a) [Ch][Ac], (b) [Ch]Cl, (c) [Ch][Dhp], and (d) [Ch][Dhc]**.**

FT-IR Analysis. FTIR is concerned with the vibration of molecules absorbing in the infrared region of the electromagnetic spectrum. For proteins, the vibrational bands studied are mostly the amide I at 1640 and amide II band occurs at 1550 cm^{-1}. Amide I come in the region of 1625-1695 cm^{-1}, and is primarily due to the amide C=O stretching vibrations of the peptide bonds, whereas amide II covers the region 1550 cm^{-1} due to CN stretching and NH bend.[38] Since the vibrational frequency depends upon the bond strength while the intensity depends upon the bond polarity, the absorption depends upon the bond vibrations which are excited. The increase in bond strength increases the frequency of vibration, whereas the increase in bond polarity increases the intensity of absorption.[39]

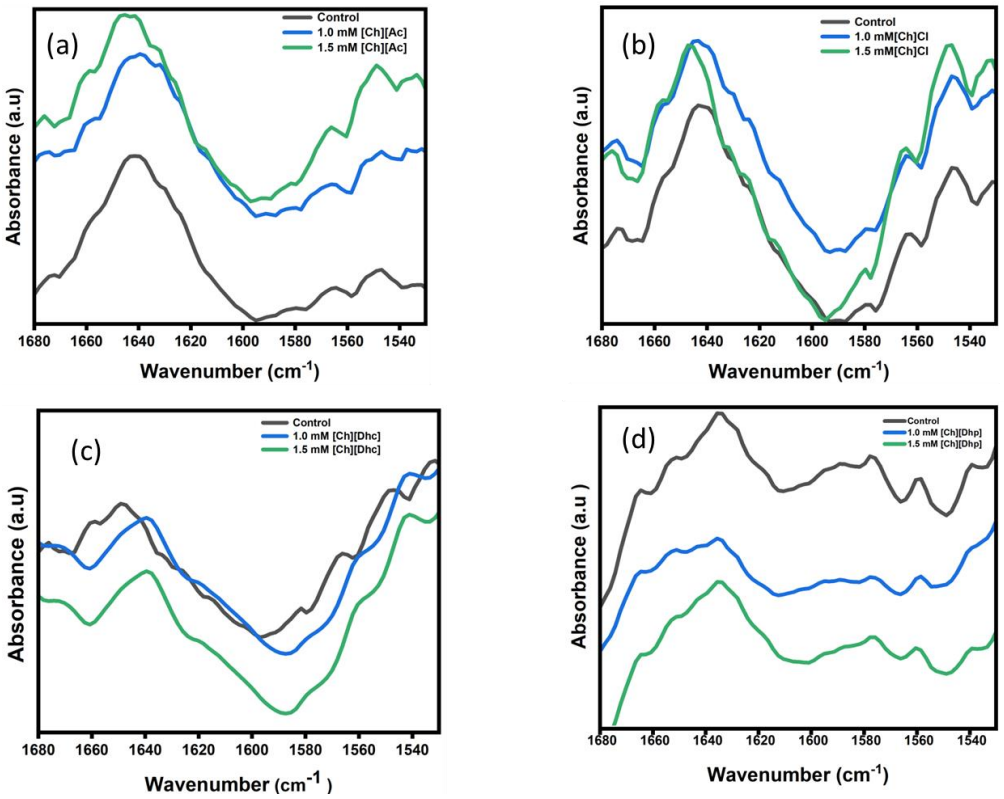


Figure 9. FTIR spectra of IgG in the amide I and amide II regions at 25 ºC in the presence of the ILs: (a) [Ch][Ac],(b)[Ch]Cl, (c) [Ch][Dhc] and (d) [Ch][Dhp] at various concentrations. Control: IgG in sodium phosphate buffer, pH 7.0, 10 mM.

From Figure 9, it can be seen that IgG shows both amide I and amide II bands. Indeed, in the presence of [Ch][Ac], with increasing concentration in the absorbance of both bands of IgG with the increase in the concentration of [Ch][Ac], which highlights the increase in the polarity of the carbonyl and N-H vibrational band due to hydrogen bonding. Similarly, in the presence of increasing concentration of [Ch]Cl, the strength of the absorbance of IgG increases (Figure 9b), whereas in the presence of [Ch][Dhc] and [Ch][Dhp] the maximum absorbance is shown by the control point and then the sequence is in the increasing order of the concentration of the IL (Figure 9c and d). Coming to the wavenumber shift, in the presence of [Ch]Cl there is no major shift, where as in the presence of [Ch][Dhp] and [Ch][Dhc] the amide I peak of IgG at 1640 cm^{-1} is shifted towards shorter wavelengths, thus implying an increase in the hydrogen bond formation between IgG and [Ch][Dhp] and [Ch][Dhc]. Therefore, it can be concluded that the secondary structure of IgG is not altered by the addition of any IL (as both the peaks were

retained in all the ILs). Nevertheless, extended hydrogen bonding may be seen in the case of [Ch][Dhc] and [Ch][Dhp].

Hydrodynamic size studies. Dynamic light scattering (DLS) is a technique used to calculate the hydrodynamic diameter (d_H) of a molecule. d_H can be defined as the diameter of a hypothetical sphere that diffuses at the same rate as the molecule under investigation.[40] Hence, in order to elucidate the attenuation effects of cholinium-based ILs on IgG, the DLS measurements were performed as a function of the concentration of the ILs [Ch][Ac], [Ch][Cl], [Ch][Dhc] and [Ch][Dhp], being the results are illustrated in Figures 10 and 11. Additional data can be found in Table S4 in the Supporting Information.

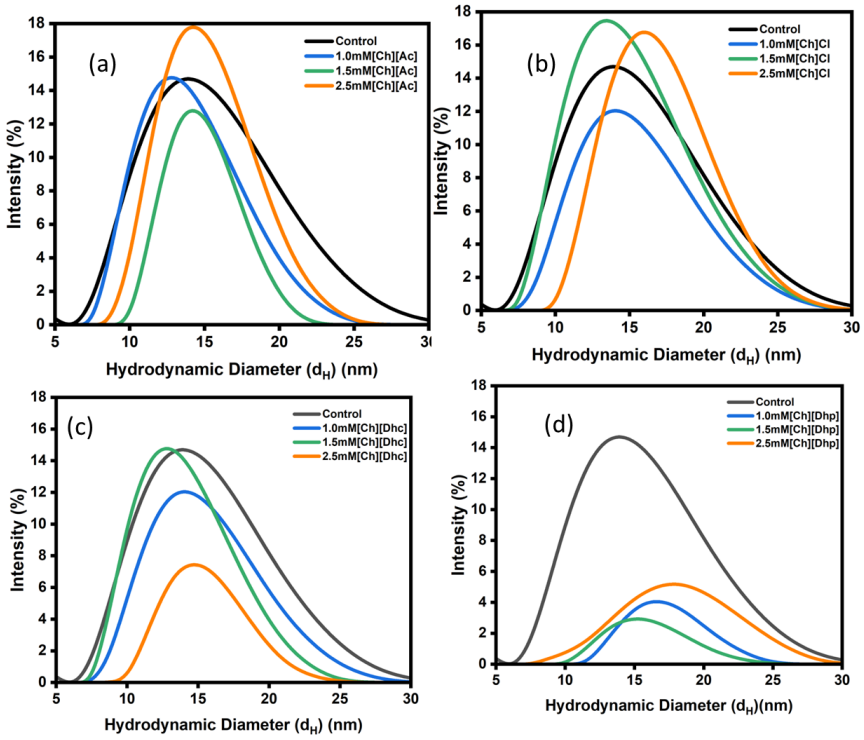


Figure 10. The hydrodynamic diameter (d_H) analysis of IgG at 25 °C in the presence of phosphate buffer (pH=7.0, 10 mM) and at various concentrations of ILs: (a) [Ch][Ac], (b) [Ch]Cl, (c) [Ch][Dhc] and (d) [Ch][Dhp]**.**

The d_H value of IgG in phosphate buffer (Control) at pH 7.0 under physiological conditions was 13.86 nm, which is in good agreement with reported data.[41] Figure 11 explicitly shows that the

d_H values of IgG in all the ILs are not appreciably changed up to the concentration 1.5 mM. For example, d_H values are 13.48, 13.52, 15.90, 15.86 for concentrations 0 (control), 1.0, 1.5, and 2.5 mM of [Ch][Ac] given in Figure 10 and 11. The ILs [Ch]Cl, [Ch][Dhp] and [Ch][Dhc], behave in a similar fashion. Any slight increment in the d_H can be explained on the basis of an increase in the number of ion-ion repulsions, which can be expected to increase the value of d_H in protein. Therefore, it can be concluded that in the presence of all four ILs, IgG retains its compact structure and shows no significant change in the d_H values as compared with the control whereas there is increase in aggregation formation in case of [Ch][Dhp] explained by appearance of wider peaks.

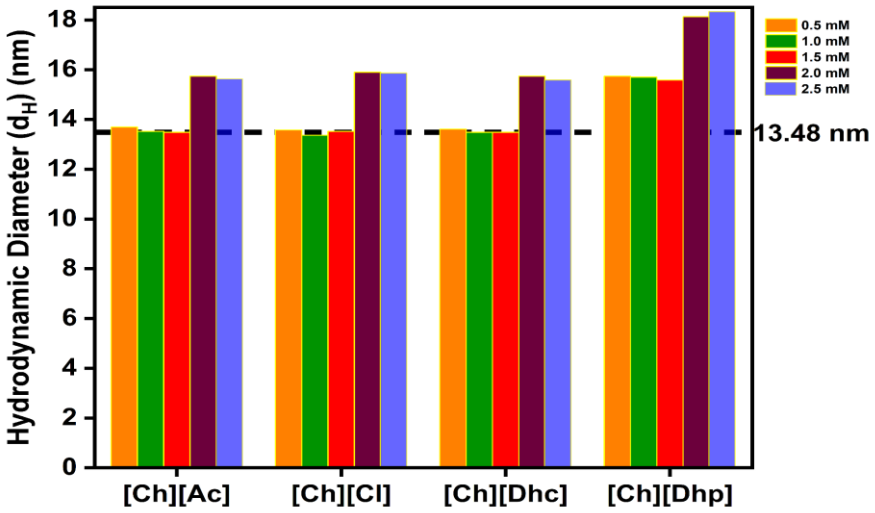


Figure 11. The hydrodynamic diameter (d_H) at 25 °C for IgG in 0.5 mM (orange), 1.0 mM (green), 1.5 mM (red), 2.0 mM (violet) and 2.5 mM (blue) [Ch][Ac], [Ch]Cl, [Ch][Dhc] and [Ch][Dhp]. The dashed line at 14.4 nm means the control (IgG in sodium phosphate buffer pH 7.0, 10 mM).

Time-dependent Stability Study of IgG in the Presence of Cholinium-based ILs

For studying the storage capability of IgG in different ILs, the SDS-PAGE, CD, and DLS analysis of IgG in all the ILs was performed for four weeks at 2 and 2.5 mM concentration of ILs.

SDS-PAGE Study: The structural stability of IgG for four weeks in the presence of phosphate buffer (control) and in the presence of [Ch][Ac], [Ch][Dhc], [Ch][Dhp] and [Ch]Cl at 2.0 and 2.5 mM was evaluated by SDS-PAGE in reduced conditions. The results depicted in Figure 12 shows that, except for [Ch]Cl, the light and heavy chains of IgG can be detected for both concentrations and for the control after four weeks. For [Ch]Cl at both concentrations it is possible to observe the presence of a new band at lower molecular weight after the second week of incubation and a significant decrease in the intensity of IgG heavy chain when compared to the control. These results indicate the reduction of aggregation of heavy chain of IgG in the presence of [Ch]Cl after week 1. Thus, all the ILs are favorable to the structural stability of IgG for a long period of time with [Ch]Cl being the favorable one.

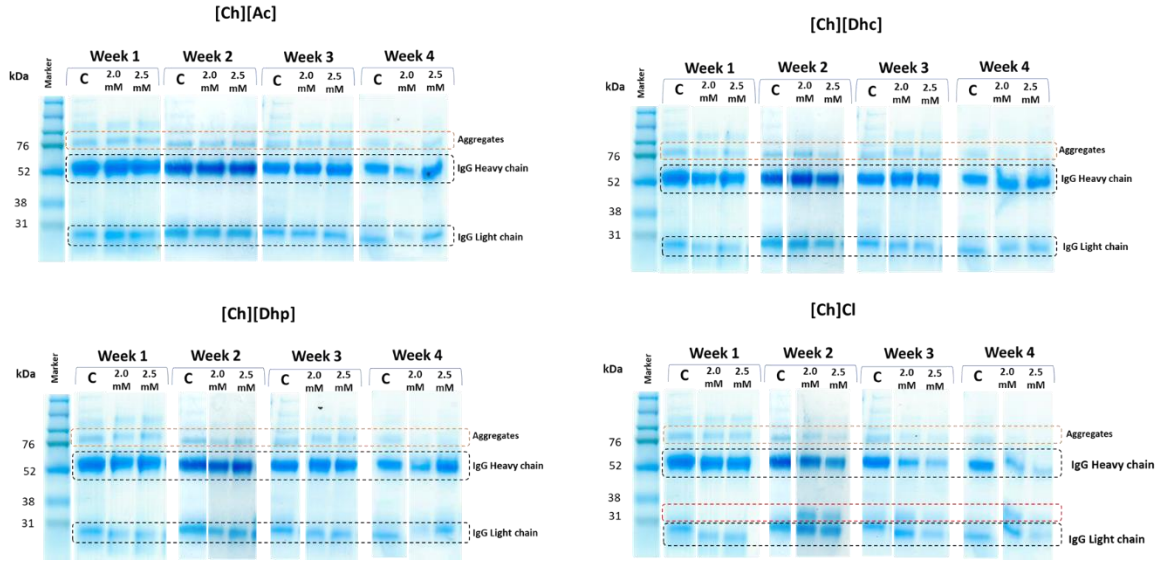


Figure 12 - SDS-PAGE of IgG for four weeks of incubation at 25 °C in the presence [Ch][Ac], [Ch][Dhc], [Ch][Dhp] and [Ch]Cl at 2.0 mM and 2.5 mM. Lane 1 – molecular weight marker; lane 2 – IgG in sodium phosphate buffer pH 7.0, 10 mM (control (C)).

CD Spectra Analysis: To explore the long-term storage and possible structural changes of IgG, far-UV CD studies were carried out in the presence of different cholinium based ILs, at 2 and 2.5 mM concentration for four weeks (Figure 13). As can be seen from Figure13 (a) IgG in phosphate buffer (control) shows a signature peak of β-pleated at 218 nm up to 2weeks whereas, on weeks 3 and 4, there is a blue shift of negative CD band which can be caused due to change in the secondary structure of the IgG due to longer storage. Figure 13 (b) and (f) show the CD

spectra of IgG in [Ch][Ac] solution at concentration 2.0 mM and 2.5 mM and the result shows that on week 4, there is a slight blue shift to the ellipticity values at concentration 2.0 mM whereas at concentration 2.5 mM the structure is retained with a slight decrease in negative ellipticity values. As can be seen from Figure 13 (c) and (g), the secondary structure of IgG in presence of [Ch]Cl is more stable at 2 mM than 2.5 mM as peaks at 218 becomes wider at 2.5 mM on week 3 and 4 at higher concentration. Figure 13 (d) and (h) shows CD spectra of IgG in [Ch][Dhc] at 2 mM and 2.5 mM respectively, as can be seen from spectra that on longer storage the structure become destabilized, however, in case of [Ch][Dhp] IgG retained its structure at 2.5 mM on longer storage as compared to 2.5 mM.

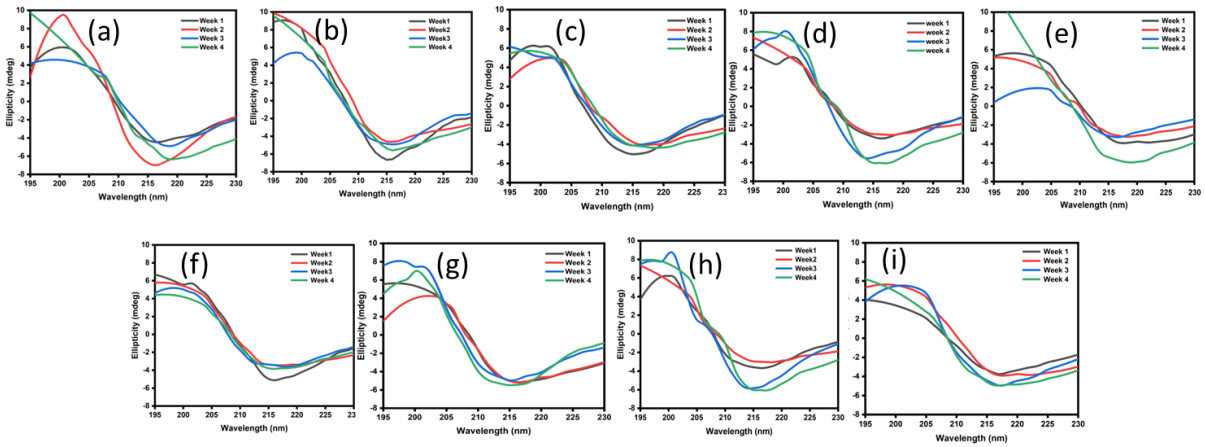


Figure 13. Far-UV CD analysis of IgG for four weeks at 25 °C in the presence of (a) phosphate buffer (pH=7.0, 10 mM) alone, and at 2.0 mM (b) [Ch][Ac], (c) [Ch]Cl, (d) [Ch][Dhc] and (e) [Ch][Dhp] and at 2.5 mM (f) [Ch][Ac], (g) [Ch]Cl, (h) [Ch][Dhc] and (i) [Ch][Dhp]

Hydrodynamic size studies. DLS was utilized to further observe the variation of d_H of IgG as a function of time and the results are illustrated in Figure 14. The plot of size distribution by the intensity of IgG in the buffer is displayed in Figure 14(a). One sharp peak at 13.95 nm has been observed for IgG monomer while another peak at 313.2 nm has occurred due to the oligomerization of the IgG monomer. . On week 2, the monomer's hydrodynamic size shifted to higher-value that is 16.82 with low intensity and the second peak appeared at 155.6, showing the increase in aggregation of the protein. After two more weeks, the protein samples were again

analyzed and two wider peaks can be seen that is the due formation of aggregates of the IgG. Hence, it can be concluded that IgG is deactivated after 2 weeks of storage in buffer, which causes the formation of higher aggregation. In [Ch][Ac] at 2.0 mM the aggregation pattern is almost similar like in buffer alone whereas at 2.5 mM the d_H value is retained and the aggregation decreases (as curves become sharper) for weeks 3 and 4, hence showing better stability for long-term storage (Figure 14b). Whereas, [Ch]Cl displayed a decrease in aggregation at both concentrations (Figure 14c). For [Ch][Dhc] the aggregation increases for week 3 and week 4(Figure 14d). In presence of [Ch][Dhp] the aggregation is less pronounced as comparison to the IgG in buffer alone solution. Hence, it can concluded that due to long-term storage the aggregation IgG decreases in the presence of ILs with the sequence [Ch][Cl]>[Ch][Ac]>[Ch][Dhp]>[Ch][Dhc]>buffer alone. All the DLS results are well consisted with CD spectroscopic results.

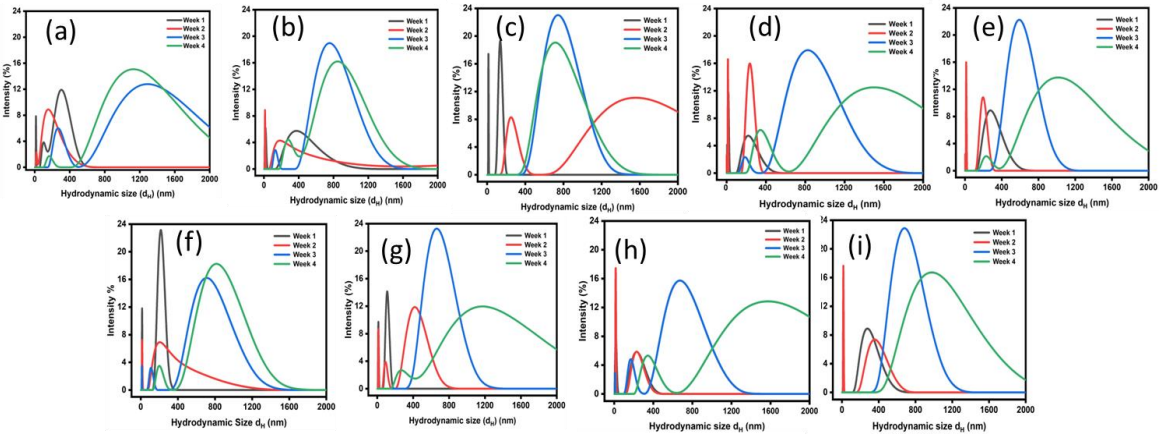


Figure14. The hydrodynamic diameter (d_H) analysis of IgG for four weeks at 25 °C in the presence (a) phosphate buffer (pH=7.0, 10 mM) alone, and at 2.0 mM(b) [Ch][Ac], (c) [Ch]Cl, (d) [Ch][Dhc] and (e) [Ch][Dhp] and at 2.5 mM (g) [Ch][Ac], (h) [Ch]Cl, (i) [Ch][Dhc] and (j) [Ch][Dhp].

Thermal Stability Analysis of IgG in Cholinum based ILs. A protein-solvent system is a thermodynamic system composed of the solute (i.e., the protein and the solvent (liquid water, and buffer ions)). The native states and assemblies of the protein are stabilized by the intermolecular and as well as intramolecular interactions of the protein in the solvent system.[42] Hence, when we unfold a protein, the interior regions become exposed to the surroundings that

tend to change the thermodynamic forces that underlie the structure and function of the biomolecules.[43] Thermodynamic parameters, free energy (ΔG) are useful in describing the global protein folding phenomenon, and hence, is helpful in understanding protein stability whereas the enthalpy change (ΔH), heat capacity change (ΔC_p), and transition temperature (T_m) are helpful to understand protein stability in terms of non-covalent forces of different structural states.[44] The thermodynamic studies of denaturation of IgG were performed using a two-state mechanism as shown in equation 1, where the native (N) is the folded state and the denatured (D) state is unfolded state of the protein, and determined by thermal fluorescence.[45]

Sigmoidal fluorescence intensity curves were obtained for IgG in the presence of the various concentrations of ILs. As indicated by equations (1) - (3) below, the fractions of folded protein (f_f) and unfolded protein are (u_f) are determined by combining the intensity, Y, of protein found at a temperature, T, with the experimentally observed intensities of the pre-and post-transition values, and, respectively, of the native and denatured Y_f and Y_u protein. The latter values are obtained by extrapolating the pre-and post-transition baseline values using a linear fitting method. Upon each change in temperature, the system is assumed to be at thermal equilibrium. It is then possible to use equation 4 to define an effective equilibrium constant, K. This equilibrium constant is related to the standard free energy of formation, according to eq 5. The melting temperature can be defined by the condition, Y=1/2 ($Y_f + Y_u$), for which K=1 and $\Delta_{fu} G = 0$.

$$N \leftrightharpoons D \quad (1)$$

$$f_u = (Y_f - Y) / (Y_f - Y_u) \quad (2)$$

$$f_u + f_f = 1 \quad (3)$$

$$K = f_u / f_f = (Y_f - Y) / (Y - Y_u) \quad (4)$$

$$\Delta_{fu} G = -RT \ln K \quad (5)$$

$$\Delta_{fu} H = T_m \Delta_{fu} S \quad (6)$$

If we let ΔC_p represent the difference in the isobaric heat capacities of the two forms of the protein, the integral of the Gibbs – Helmholtz equation leads to the result,

$$\Delta_{fu} G(T) = \Delta_{fu} H(T_m)[1 - (T/T_m)] + \Delta C_p[(T-T_m) - T \ln (T/T_m)] \quad (7)$$

The thermodynamic data obtained from the 25ºC unfolding data are collected in Tables S5 and S6. The intensity versus temperature data of sigmoidal curve of IgG in ILs are represented in Figures S1-S4. All the obtained thermodynamic results are graphically depicted in Figure 15.

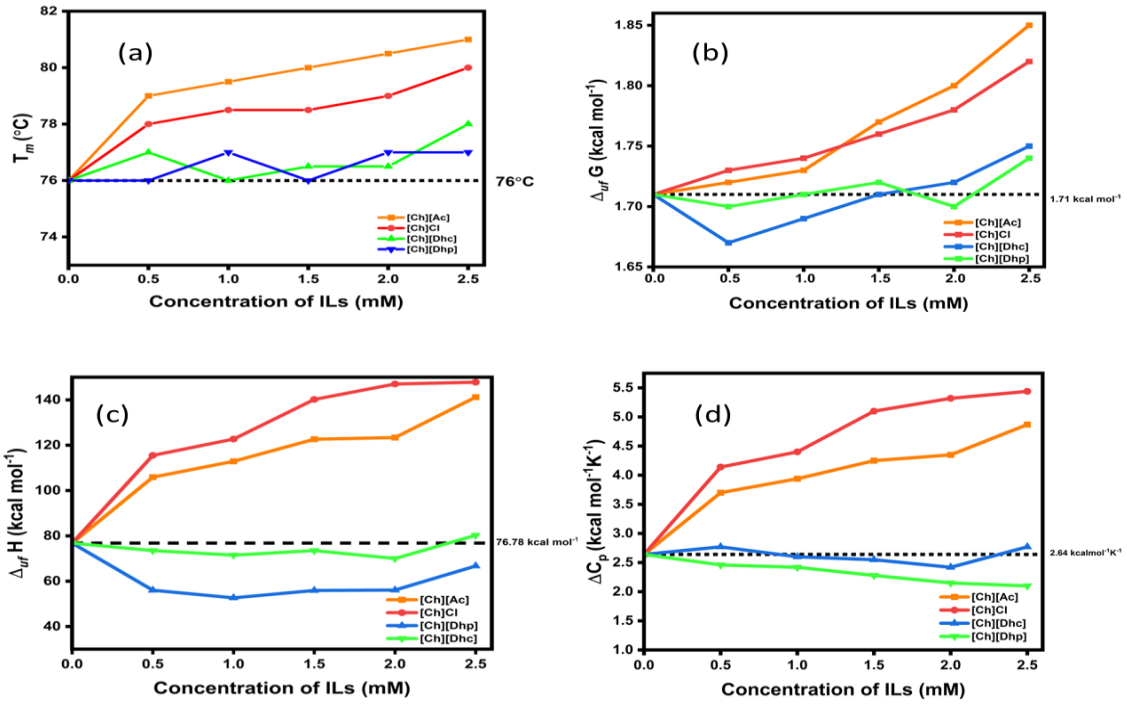


Figure 15. Effect of ILs on the thermal stability of IgG using fluorescence spectroscopy: (a) transition temperature (T_m); (b) Gibbs free energy changes ($\Delta_{uf}G$); (c) enthalpy change ($\Delta_{uf}H$) of unfolding; and (d) heat capacity change of unfolding (ΔCp) for IgG in presence of [Ch][Ac] (orange), [Ch]Cl (red), [Ch][Dhc] (blue) and [Ch][Dhp] (green). The dashed line shows the value of control (IgG in sodium phosphate buffer, pH 7.0, 10 mM).

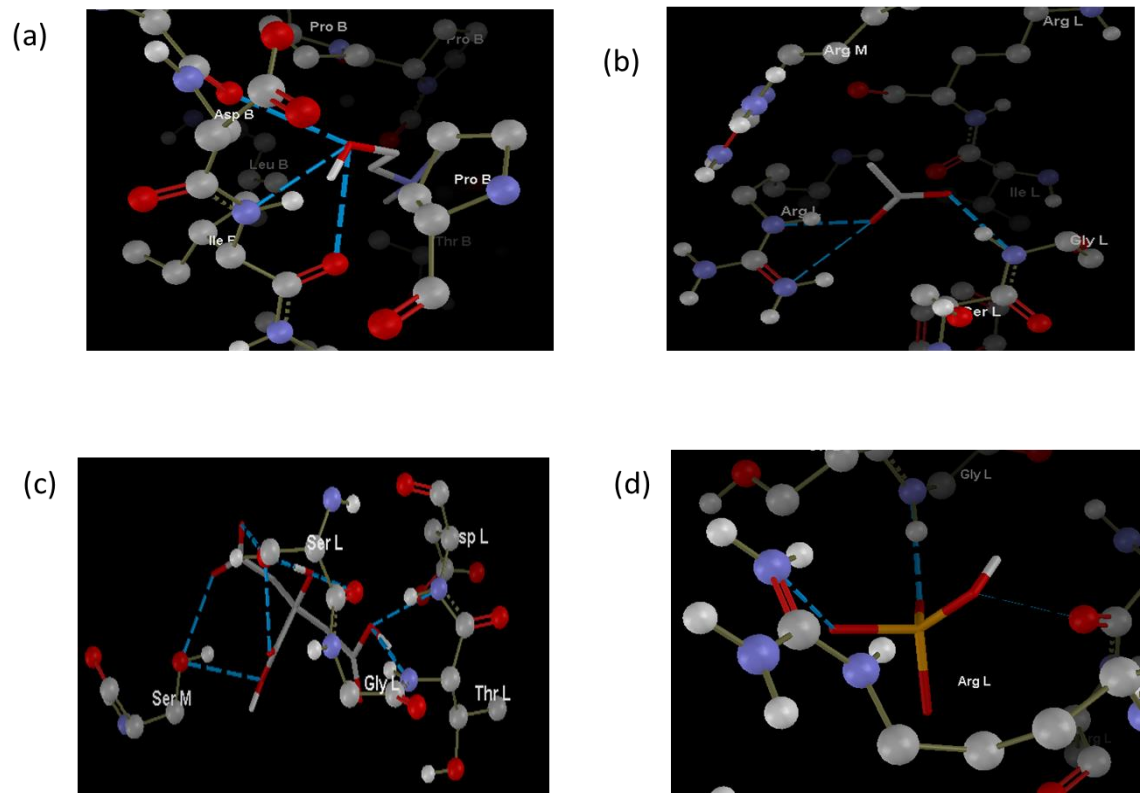
The results in Figure (15a) summarize the change of T_m values, which correspond to the transition of IgG to the unfolded state, as a function of the ILs concentration. It can be seen that in the presence of ILs, the T_m value of IgG was equal or exceeded the control point. In the

presence of [Ch][Ac], the T_m value of IgG increases linearly from 76 to 81°C with an increase in the IL concentration (0 to 2.5 mM). Similar is the case of [Ch] Cl, where the T_m value increases from 76 to 80 °C. For [Ch][Dhc] and [Ch][Dhp] there is no linear dependence of T_m of IgG with the concentration of IL; however, as mentioned earlier, in the presence of all ILs the T_m of IgG is always higher than in the control. Therefore, these observations evidently reveal that the IgG structure of the folded state is not altered by the addition of any studied IL and the stability abilities of the native state of IgG varies with the IL anion, following the trend: [Ch][Ac] > [Ch]Cl > [Ch][Dhc] > [Ch][Dhp. Temperature is one of the main parameters; an increase in temperature causes the unfolding of the protein (antibody) and further aggregation. Hence, increase of any degree of transition temperature is beneficial for longer storage of the protein.

It emerges from Figure 15 (b, c and d) that the $\Delta_{fu}H$, $\Delta_{fu}G$ and ΔC_p values of IgG in presence of [Ch][Ac] and [Ch]Cl increase linearly as the concentration increases. Perhaps there is an increment in the values of these parameters in the presence [Ch][Dhc] and [Ch][Dhp]; however, the increment is below the control point. Therefore, the results from thermodynamic studies indicate that [Ch][Ac] and [Ch]Cl ILs interact unfavorably with the surface of IgG and that these ILs stabilize the folded native structure of IgG and do not interfere with the functional groups of IgG. On the other hand, [Ch][Dhc] and [Ch][Dhp] moderately interact with the surface of the protein, being slightly less favorable than [Ch][Ac] and [Ch]Cl.

Molecular Docking Studies. Molecular docking was performed to study the potential binding sites of [Ch]Cl, [Ch][Ac], [Ch][Dhc] and [Ch][Dhp] at the F_{ab} and F_c fragments of IgG. There can be different types of interactions between F_{ab} and F_c fragments and ILs; however, for the sake of simplicity, only hydrogen bonding (H-bond) and hydrophobic interactions are studied under this section.

1



2 **Figure 16.** Molecular docking of F_{ab} fragment with (a) cholinium cation; (b) acetate anion; (c)
3 dihydrogen citrate anion; and (d) dihydrogen phosphate anion obtained from Molegro software.

4

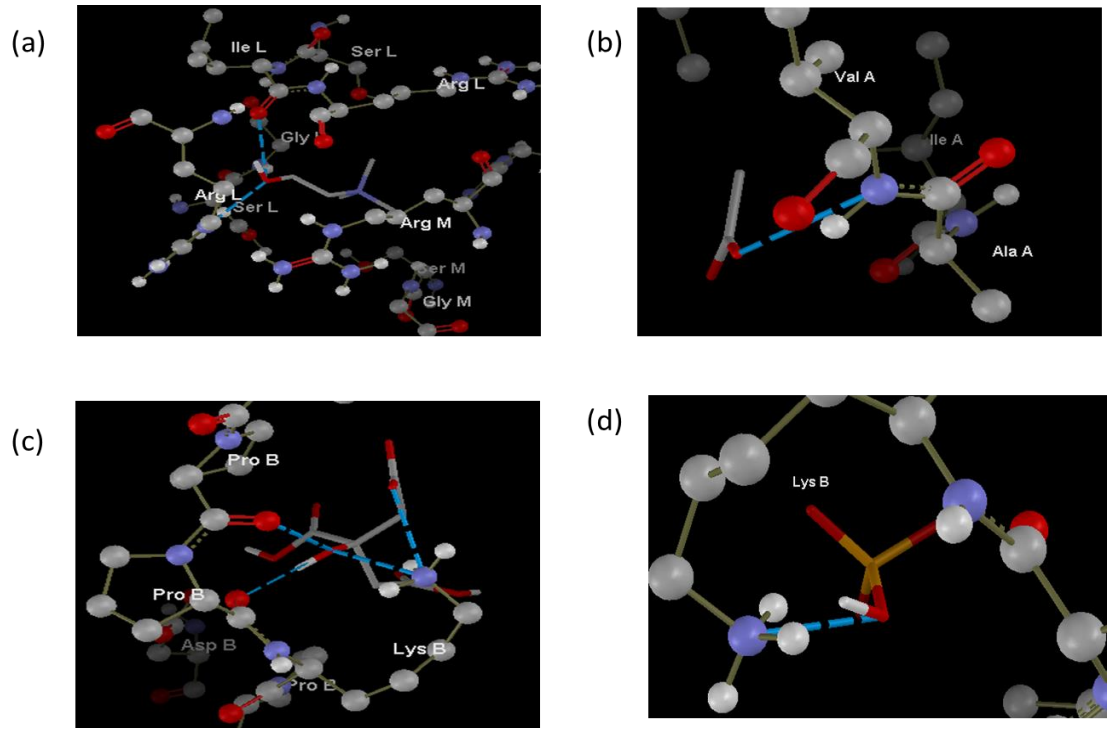


Figure 17 Molecular docking of F_c fragment with (a) cholinium cation; (b) acetate anion; (c) dihydrogen citrate anion; and (d) dihydrogen phosphate anion obtained from Molegro software.

Figures 16 and 17 show the specific binding of cholinium, acetate, dihydrogen phosphate and dihydrogen citrate ions with F_{ab} and F_c fragments of IgG through molecular docking. However, the docking results for chloride could not be displayed as the Cl anion does not form a H-bond with the protein molecules.[46] Figure 16 (c, d) and Figure 17 (c,d) show that hydrophobic interactions in the case of dihydrogen citrate and dihydrogen phosphate are higher with three amino acid residues of F_{ab}, whereas three amino acid residues of F_c fragments show hydrophobic interactions with dihydrogen phosphate, which has also been seen in the FT-IR studies. Whereas dihydrogen citrate is interacting with five hydrophobic residues of F_{ab} and one hydrophobic residue of F_c, these interactions further explain the perturbation of the secondary structure of IgG at higher concentrations of [Ch][Dhp] and [Ch][Dhc]. However, the strong binding of cholinium-based ILs with IgG is evident by the formation of hydrogen bonds with F_{ab} (Ile 28, Arg 31) and F_c (Ile 377) fragments with the cholinium cation. All further results are summarized in Table 1.

Table 1: Summary of molecular docking studies of F_{ab} and F_c fragments of IgG with cholinium-based ILs.

Ions	Hydrogen bonding interactions		Hydrophobic interactions	
	F_{ab}	F_c	F_{ab}	F_c
Cholinium	Ile 28, Arg 31	Ile 377	Ile 28, Arg 31	Ser 375
Acetate	Arg 29, Arg 31	Arg 29, Arg 31	Lys 248	Lys 248
Dihydrogen Phosphate	Thr 69, Asp7, Ser 67		Thr 69, Asp70, Ser 67	Lys246, Pro 244, Pro 245
Dihydrogen Citrate	Ser 67, Ser 67, Asp 70, Thr 69	Lys 69	Ser 67, Ser 67, Asp 70, Thr 69, Gly 68	Lys 69

Spectroscopy has shown that the hyperchromic/hypochromic shift seen in UV and fluorescence spectroscopy has exhibited that IgG is more stable in the presence of [Ch][Ac] and [Ch]Cl, which is further verified by analyzing the secondary structure of IgG using Far-UV CD and FT-IR spectroscopy. The secondary structure of IgG has been retained in the presence of all the ILs; however, when comparing all the four ILs the trend of IgG secondary structure stability is as follows: [Ch][Ac] > [Ch]Cl > [Ch][Dhc] > [Ch][Dhp]. Furthermore, thermal denaturation studies showed that the T_m increases with increasing the concentration of ILs, denoting that the overall thermal stability of the IgG

Our parallel (UV absorbance, fluorescence, SE-HPLC, SDS-PAGE, far-UV CD, thermodynamic Fluorescence, DLS, FTIR and Molecular Docking) results show that cholinium-based ILs are a highly important class of excipients to be considered in IgG formulations. In general, the anion variation is thought to have a superseding effect on the structure and the stability of protein than that of the cation. The interactions between proteins and IL solutions are controlled by the complex interactions depending on some microscopic properties such as ion hydration, ion effect on protein hydration and direct interactions between ions and proteins. Our results have revealed that the effect of anions on the stability of protein seems to be much more complicated and does not always follow the Hofmeister series. Hence, the most promising ILs for IgG stability are [Ch][Ac] and [Ch]Cl.

Antibodies-mediated therapeutic drug stability depends on the distinct formulation environment and various excipients like arginine and arginine hydrochloride (ArgHCl), which are effective additives to improve protein stability.[47] Cholinium-based ILs are well known to improve proteins stability and their ability to extensively reduce aggregation, which makes them potential solvent additives for the formulation development of therapeutic proteins. Recently, Hallet and co-workers have studied the formulation consisting of [Ch][Dhp] and different excipients.[18] They have found that in the presence of [Ch][Dhp], the structure of IgG is less stabilized, although the destabilizing effect of [Ch][Dhp] is overcome by adding the excipients. Similarly, in our studies, [Ch][Dhp] is also not completely helping the IgG to retain its confirmation, whereas ILs like [Ch][Ac] and [Ch]Cl are potential solvent additives for the formulation development of IgG. These results reinforce the potential of cholinium-based ILs paired with appropriate anions to be considered as potential excipients of biopharmaceutical formulations.

CONCLUSIONS

Most of the studies on protein−IL mixtures have specifically targeted to enzymes. However, there is a range of protein-based biopharmaceuticals, such as IgG, in which ILs may play a relevant role in their stabilization. The present study delivered a comprehensive overview of the outcome of cholinium-based ILs on the stability and thermodynamic profile of IgG antibodies. The results indicate an appreciable increase (around 5 ^{o}C) in T_m values of IgG in presence of [Ch][Ac] (81 °C), [Ch]Cl (80 °C), [Ch][Dhc] (78°C) and [Ch][Dhp] (77 °C) as compared to buffer (76 °C). SE-HPLC and SDS-PAGE results have shown that cholinium-based ILs do not lead to the formation of IgG aggregates or IgG fragmentation. The spectroscopic studies concluded that [Ch][Ac] and [Ch]Cl are preferred ILs for providing the conformational and thermal stability of IgG. Time dependent studies of IgG have also been performed to study the effect of ILs on long-term stability and the results shows the high potential of ILs as storage media for IgG for longer period of time. Hence, from our study we can interpret that cholinium-based ILs can be explored more in future as these ILs have shown more structural and thermal stability of IgG. Taking a step forward, these ILs should be investigated to stabilize other relevant antibodies, like IgA and IgM, or other biopharmaceuticals. Finally, the required studies to validate their use in biopharmaceutical formulations must be conducted.

ASSOCIATED CONTENT

*S Supporting Information

The Supporting Information is available free of charge at

water content and pH of ILs, the tables of UV-Vis spectroscopy, Fluorescence spectroscopy (values), Hydrodynamic size (DLS studies), Thermal fluorescence (Sigmoidal graphs of T_m), all the thermodynamic parameters in buffer and in various concentration of ILs.

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CONFLICTS OF INTEREST

There are no conflicts to declare.

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