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Heparin-like native protein aggregate dissociation by 1-alkyl-3-methyl imidazolium chloride ionic liquids

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ABSTRACT

At room temperature, ionic liquids (ILs) 1-alkyl-3-methyl imidazolium chloride (alkyl: ethyl, butyl, hexyl and octyl) are observed to exhibit aggregate dissociation behavior of native proteins. This is similar to the well known protein aggregation inhibitor and aggregate dissociation molecule heparin. Dynamic light scattering (DLS) experiments performed on three model proteins bovine serum albumin (BSA), β-lactoglobulin (β-Lg) and immunoglobulin (IgG) revealed that on addition of ILs the fractal aggregates of proteins (apparent maximum hydrodynamic radius R_{max} and fractal dimension $d_f = 1.5 \pm 0.2$) dissociated into oligomers (hydrodynamic radius R_h) following an exponential decay profile with time, $R_h = R_{max}\exp(-k_a t)$ The dissociation constant k_a has been correlated to hydrophobicity index (H-index) of the protein concerned. Thus, if the combined contributions of dissociation constant and hydration effect on secondary structure are taken into account together, [C8mim][Cl] with BSA, [C2mim][Cl] with β-Lg and IgG, rank as the best aggregation reversal agent (ARA) amongst all other ionic liquid samples examined. The additional advantage of the used ILs over heparin is the release of mobile Cl^- ions to the solution. This lead to the increased solution entropy, thereby, providing stability to the final dispersions.

1. Introduction

Proteins comprise the largest percentage of organic molecules in organisms. Most of the cellular activities are mediated by native proteins because these molecules have both structural and dynamic functions. Structural functions include the formation of collagen and elastin which constitute the bone and muscle matrices; keratin, for example, is present in the epidermis and the vascular system. Dynamic functions include kinetics of blood clotting, enzyme and hormone mediated biochemical reactions, antibody generation, membrane reception *etc.*, other than their role in genetic control and muscular functions. Thus, protein aggregation is an issue that affects the aforesaid biological activities. Protein aggregation is preferentially observed in their partially denatured conformation [1–4]. Protein aggregation is one of the major problems in biotechnology and pharmaceutical industries. In addition, this causes clinical conditions that belong to a well-known class of ailments like Alzheimer, Parkinson and Huntington diseases. They all involve protein aggregation *in vivo* and share common features such as the presence of insoluble fibrous protein aggregates in a specific structural motif characterized by a cross-β sheet structure [5]. Thus, it is not only necessary to understand the molecular level kinetics of protein aggregation, but also it is imperative to identify new protein growth inhibitors and aggregate dissociating agents (ARAs). Aggregation and unfolding are different physical processes that must be dealt exclusively.

Heparin, a mucopolysaccharide with chemical designation N-sulpho glucosamine 6-sulphate (Fig. 1), is found in the blood, lungs, liver, kidney and spleen, is a well known anticoagulant. It binds to many proteins to form proteoglycans or mucoproteins. Proteins–heparin interaction can lead to a variety of consequences. *In vitro*, heparin hinders the aggregation of recombinant human keratine growth factor at higher temperatures [6] and reverses insulin aggregation significantly [7]. Similarly, *in vivo*, it inhibits the association of fibrillin-1 [8], reduces aggregation induced by agrin [9] and obstructs the induction of amyloid [10]. On the other hand, heparin has been shown to cause rapid aggregation of lysozyme [1] and promote aggregation of recombinant prion protein [2]. Thus, there is enough evidence in the literature that concludes protein–heparin binding can cause aggregation in some proteins and hinder aggregation in others. This owes its origin to the fact that protein–heparin intermolecular complex formation is poorly

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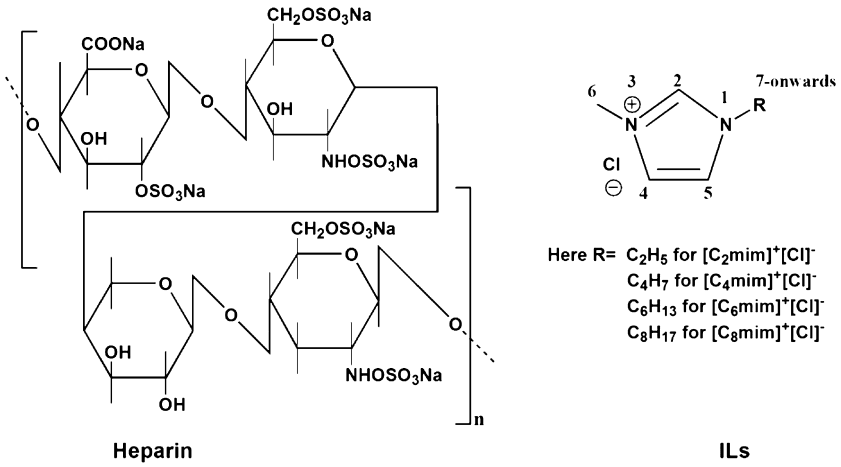

Fig. 1. Molecular structures of heparin and ionic liquids used.

understood *vis a vis* protein structure. In view of the undesirable effects this phenomenon creates in areas like biology [9], medical science [11–16] and molecular biophysics, the search for new anticoagulation agents of proteins is the need of the hour.

Ionic liquids (ILs) consist of poorly coordinating organic cations and inorganic anions which are generally liquid below the boiling point of water. They possess surprising features such as low melting points, negligible vapor pressure, non-volatility, no-flammability, wide electrochemical windows and so on [17–26]. Nucleic acids have been shown to remain in stable dispersion at room temperature in IL solutions [24]. In recent years, ionic liquids (ILs) have been used to stabilize the protein activity, to inhibit or reduce aggregation, and to improve the *in vitro* refolding of denatured proteins [26,27].

Anticoagulation as well as aggregation reversal attributes of heparin has been recently reported for BSA, β-Lg and Zn-Insulin proteins [28]. It was observed that the differential protein–heparin binding owed its origin to protein charge anisotropy. This raises a pertinent question how is the aggregated protein altered in IL solutions *vis a vis* heparin. This constitutes the main objective of the present work. The motivation was to explore the possibility of using IL as shelf life enhancing agent for protein dispersions. We have chosen three model proteins (having well defined protein database attributes) bovine serum albumin (BSA), β-lactoglobulin (β-Lg) and immunoglobulin (IgG) which form their aggregates at their respective p*I*s. More specifically, we have explored their dissociation of these aggregates using ionic liquids (ILs). ILs used in this study are 1-alkyl-3-methyl imidazolium chloride (alkyl: ethyl, butyl, hexyl and octyl). We make a systematic comparison between protein–heparin *versus* protein–IL binding and conclude that 1-octyl-3-methyl-imidazolium chloride ([C8mim][Cl]) has the same effect as heparin. Further, the aggregate dissociation constant could be correlated to protein hydrophobicity index. We shall hence forth refer to heparin and ionic liquids as aggregation reverting agents (ARAs).

2. Materials and methods

All of the proteins, bovine serum albumin (BSA), β-lactoglobulin (β-Lg), immunoglobulin (IgG), heparin and the ILs, 1-octyl-3-methyl imidazolium chloride ([C8mim][Cl]), 1-hexyl-3-methyl imidazolium chloride ([C6mim][Cl]), 1-butyl-3-methyl imidazolium chloride ([C4mim][Cl]), and 1-ethyl-3-methyl imidazolium chloride ([C2mim][Cl]), were bought from Sigma-Aldrich, USA and were used as received to prepare the samples. Triple distilled deionized water from Organo Biotech Laboratories, India, was used to prepare the solutions. The dispersions were prepared by dissolving protein in the aqueous solutions (5 mM salt) with required concentrations (0.25% BSA, 0.1% β-Lg and 0.025% (w/v) IgG) and these were allowed to homogenize by resorting to continuous stirring for 1 h at room temperature (25 °C). The pHs of these solutions were adjusted close to their p*I*s like 4.8 for BSA, 5.1 for IgG and 5.2 for β-Lg. After pH adjustments, these solutions were immediately subjected to dynamic light scattering studies at room temperature (25 °C). All concentrations are expressed in the unit of %.

Dynamic light scattering (DLS) experiments were performed at scattering angle of $\theta = 90^{\circ}$ and laser wavelength of $\lambda = 632.8$ nm on a 256-channel Photocor-FC (Photocor Inc., USA) that was operated in the multi-τ mode (logarithmically spaced channels). Details of the DLS are described elsewhere [29]. The DLS experiments were performed on the protein samples in two steps: (i) first the proteins were allowed to undergo self-aggregation in 5 mM NaCl solution close to their p*I* (pH 4.8 for BSA, 5.1 for IgG and 5.2 for β-Lg) for about 3.5 h until an aggregation plateau was noticed and (ii) at this point, ARA was added to the solution and the particle sizing was done continuously for next 3 h. Infrared spectra were recorded using Varian 7000 FT-IR instrument. Raman spectra from all samples were recorded on an FT-IR/Raman Spectrometer (1064 nm) attached with a Microscope (Varian 7000 FT-Raman and Varian 600 UMA). Circular dichroism (CD) experiments were performed using Applied Photophysics Chirascan Circular Dichroism Spectrometer (USA) to estimate the secondary structure of proteins using the standard operation procedure recommended by the manufacturer. We could not scan the protein stock dispersions as these yielded signals that crossed the saturation limit of the instrument. So, the stock solutions were diluted to 6.25 (β-Lg), 1.56 (IgG), and 15.6 mg/L (BSA). The path length of the cuvette used in the CD experiments was 0.1 cm.

3. Results and discussion

3.1. Protein properties

A set of three common proteins, bovine serum albumin (BSA), immunoglobulin (IgG) and β-lactoglobulin (β-Lg) were examined in four homologous IL solutions at room temperature in a systematic manner. This set was chosen using the following criteria: (i) the isoelectric pH (p*I*) should be $\approx 5.0 \pm 0.3$, (ii) apparent protein size and (iii) hydrophobicity indices are not too different. All the proteins used in this study are well characterized biomolecules with known protein data base (PDB) attributes. The zeta potential of these polyampholyte proteins is strongly dependent on solution