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Characterization of DNA-protein Complex Ionogels using Small Angle Neutron Scattering, Differential Scanning Calorimetry and Rheology

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Abstract — We have studied the structural and thermo-mechanical properties of DNA-protein (gelatin A, GA) complex gels formed in imidazolium based ionic liquid solutions called ionogels generated has a result of first order phase transition from a complex coacervate. We probed the microscopic structure of these ionogels using Small Angle Neutron Scattering (SANS), differential scanning calorimetry (DSC) and rheological measurements. Data show that around 0.1% (w/v) GA concentration stiffening of DNA-GA complex takes place (optimum binding concentration). At higher temperature, the GA-DNA binding weakens and GA-GA interaction facilitates the reorganization of the material which on heating turns into ionosols. Ionosols when cooled to room temperature formed ionogels. The typical size of the complexes is a 150 nm (radius of gyration, R_g). SANS experiments indicate a mesh size, $\xi \approx 3.8 \pm 0.2$ nm to these gels independent of protein concentration C_{GA}. Viscoelastic studies reveal that the storage and loss moduli (G′ and G″) values that are comparable, and the viscoelastic length ξ_{el} is typically double the mesh size of the network. It is found that these ionogels were associated with higher gel strength, and specific heat at optimum binding conditions. It is concluded that the DNA-gelatin complex ionogels comprise a unique class of designer soft material stable with respect to protein concentration change, higher gel strength and melting temperature compared to conventional gelatin gels.

Keywords : *DNA, gelatin, ionogels, SANS, rheology, specific heat.*

INTRODUCTION

There has been a lot of interest in soft matter systems in the recent years. Materials

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such as colloidal gels, glasses, ionogels, organogels, coacervates etc have been successfuly made and their properties are comprehensively studied [1–12]. These materials exhibit unique thermo-viscoelastic properties, and interestingly can be tuned to suit the requirement of the user. Biomacromolecules such as proteins and DNA may be associated in aqueous solution to form supramolecular structures via intermolecular non-covalent interactions. Proteins, with a wide range of molecular weight, large number of derivatizable groups, wide availability, stablility, non-toxicity, hydrophilicity and bio-degradablility, warrant their use as important carriers in various drug delivery systems [13]. A number of studies have been conducted on protein gels in the past. The physiological property, functionality and application of a gel are dependent on degree of crosslinking. The most pressing need however, is the design of new biodegradable polymeric carriers, that can be used at relatively high molecular concentration to promote enhanced permeability, Retention and EPR-mediated targeting and then get safely eliminated. Efforts are focused to develop novel gel-based materials to improve biological effectiveness, reduce general toxicity and broaden the spectrum of activity.

The last decades has seen a considerable amount of interest in the gelatin/water based systems that include : physical and crosslinked gels, micro and nano spheres, simple [14] and complex coacervates [15] etc. Gelatin, a biodegradable polymer is derived from the protein collagen, isolated from animal skin, bones and fish skin, either by alkali process or acidic process, and thus, the gelatin obtained differs in its isoelectric pH value 9.0 to 9.4 for alkali and 4.8 to 5.1 for acidic processed samples. The net result of this diverse process is a final product-containing component with a wide range of molecular weights [16]. Gelatin is a heterogeneous mixture of single or multi-stranded polypeptides, each with extended left-handed proline helix conformations, containing between 300–4000 amino acids. Investigation on skin collagen suggest that the chains in the unmodified materials are either very long or are cyclic. The gelatin based systems have been exploited for use in drug delivery applications, in personal care products, as food additives and for its therapeutic potential. This polyampholyte molecule has pH-dependent net charge and light scattering measurements have assigned the following dimensions [17] (radius of gyration, R_g, and hydrodynamic radius, R_h) to the chains of gelatin-B, R_g = 55 nm and R_h = 23 nm. Thus, one can estimate the chain stiffness from the ratio $R_h/R_g \approx 0.67$. This clearly attributes a fully flexible conformation to gelatin chain. Gelation of this biopolymer has been exhaustively investigated in the past that attribute a typical gelation concentration, $C_g \approx 2\%$ (w/v) and gelation temperature, $T_g \approx 30^oC$. Considering the fact that the biopolymer, in its gel form, has found applications in pharmacy, food, and cosmetic industry etc., Thus modifiction of its thermal and

rheological properties is important.

It has been realized that proteins can interact with DNA either specifically or non-specifically. Interestingly, in the case of non-specific interactions, the sequence of nucleotides does not matter, as far as the binding interactions are concerned. Histone (protein) - DNA interactions are examples of such interactions, and they occur between functional groups on the protein and the sugar-phosphate backbone of DNA. Specific DNA - protein interactions, however, depend upon the sequence of bases in the DNA and on the orientation of the bases that can vary with twisting and super-coiling. These DNA - protein interactions are strong, and are mediated by : (i) Hydrogen bonding mediated by water molecules, (ii) Ionic interactions like formation of salt bridges, protein side chains - DNA backbone interaction and (iii) van der Waals and hydrophobic interactions. The formation of supramolecular protein-DNA complexes with characteristic hydration provides an unique phase to this material. Under specific conditions, intermolecular interactions lead to coacervation where the strongly interacting biopolymers exhibit liquid-liquid phase separation which have been extensively probed and reported earlier [18]. However, studies related to DNA-protein gels in ionic liquid solutions are not reported hitherto. Gels prepared in ionic liquid environment (ionogels) display unusual properties [9–29]. In ionic liquid (IL) solutions, one introduces mobile ions (in the form of light anions and heavy cations), hydrophobocity (alkyl chain length) and hydrogen bonding (N---H) interaction simultaneously into the continuous phase.

Room temperature ionic liquids (RTIL) have received considerable attention in the recent past due to their unique physico-chemical attributes in the field of chemical and process engineering [20–24]. These liquids, comprising inorganic anions and organic cations, are associated with negligible vapour pressure, high thermal, chemical and electrochemical stability that enable these to be considered as green solvents. A variety of possible combinations of anions and cations can, in principle, generate a wide spectrum of designer solvents. Imidazolium based ILs are quite hygroscopic and miscible with water. So it is important to understand the modified properties of biopolymer gels due to their interaction with ILs in water environment before using them for any specified purpose. It is important to understand the thermodynamic, viscoelastic, mechanical and structural properties of ionogels and their interactions within the dispersion constitutes the principal objective of this report. Further, the phase stability of these gels with respect to their composition requires attention. This report attempts to answer these questions in a comprehensive manner.

MATERIALS AND METHODS

In this study, IL refers to [C8mim][Cl]. Gelatin A in powder form and 1-octyl-3-methyl imidazolium chloride ([C8mim][Cl]) in viscous form were bought from Sigma-Aldrich and used as received. DNA (200 bp) was procured from Acros Organics and used as received. Gelatin A (300 bloom) from porcine skin had maximum nominal impurities as follows : sulphate ash = 1.5%; $SO_2 = 2 \times 10^{-4}$ % and heavy metals (Zn, Cu, Pb) in the concentration lower than the SO_2 concentration. This preparation was devoid of any E-coli and liquefier presence. The protein was not subjected to further purification by dialysis. Both the biopolymers had comparable molecular weights.

Five concentration of DNA-IL-GA complex coacervates and their respective ionogels (fixed C_{DNA} = 0.005% (w/v and C_{IL} = 0.05% (w/v) with variable C_{GA} = 0 to 0.25 % (w/v) were prepared following the exact protocol described in ref. [29] using doubled distilled deionised water. For Small Angle Neutron Scattering (SANS) measurements, samples having fixed DNA 0.005% (w/v), fixed IL concentration (0.05% w/v) and variable GA concentrations of 0.075, 0.10, 0.15 and 0.20% (w/v) were prepared in heavy water. All the experiments were performed at room temperature, 20°C and relative humidity less than 50%. These samples were stored in air tight borosilicate glass bottles for further analysis10.

Small angle neutron scattering measurements were carried out at the SANS-II facility, Swiss Spallation Neutron Source SINQ, Paul Scherrer Institut, Switzerland. The wavelength of the monochromatic neutron beam used was 6 Å. The experiments were performed at sample-to-detector distance of 1.2 and 5 m to cover the wave vector transfer q ($= 4\pi \sin\theta/\lambda$, where 2θ is the scattering angle and λ is the wavelength of incident neutron) in the range of 0.0043–0.25 $Å^{-1}$. The scattered neutrons were detected by using a two dimensional 64 × 64 cm detector. All the samples were made in D_2O and measurements were performed in 2 mm thick quartz cells. The data were corrected for background, empty cell, transmission etc as per standard protocols.

Rheological measurements, using small amplitude oscillatory shear, were performed on the ionogels samples using controlled stress AR 500 rheometer (TA Instruments, Surrey, England). For all the tests, the storage modulus (G′) data was computed from raw dynamic oscillatory data using TA Instrument Rheology Advantage Data Analysis software (version 3.0.1). All measurements were performed in triplicate to ensure data reproducibility (relative standard deviation less 5%) for each sample. Rheological experiments were performed with the objective to interrelate the stiffness

and thermal stability of the networks in frequency and temperature sweep modes. In the frequency sweep experiments, ionogel samples of different GA concentrations were loaded onto the rheometer plate and cooled to 20°C. Measurements were carried out with a cone plate geometry using a constant oscillation stress of 6.3 Pa. The effect of oscillatory frequency on the dynamic rheological properties of the ionogel network was evaluated at these temperatures after the samples were allowed to equilibrate for 10 min. The mechanical spectra were characterized by observing the in-phase (G′) and out of-phase (G″) storage modulus as function of angular frequency (ω) in the range of 0.1 to 100 rad/s.

DSC experiments were performed by using a DSC 4000 (Perkin-Elmer, USA) instrument. Here, the objective was to determine and correlate the melting temperature of the ionogels with the results obtained from rheology. The endotherms obtained were used to probe ionogel melting (phase transition). In a typical experiment, 10 mg of the sample was taken on an aluminium pan and hermetically sealed. Temperature sweep was performed with the heating rate maintained at 1°C/min. The measurement protocol was as specified by the manufacturer of the instrument. Pyris software supplied by the manufacturer was used to analyze the data.

RESULTS AND DISCUSSION

Physical properties of DNA and gelatin

The biopolymers used in this work possessed the following physical properties. The DNA polyanion had 200 bps, persistence length ≈ 50 nm, effective hydrodynamic radius, R_{DNA} ≈ 140 nm, cross sectional diameter of the DNA helix R_c ≈ 2–3 nm and the associated zeta potential = (–140 mV). We used physiological protein, gelatin which is a polyampholyte with pI ≈9, persistence length ≈ 10 nm and effective hydrodynamic radius, R_{GA} ≈ 55 nm. Thus, the zeta-potential ratio was (DNA : GA) 16 : 1, persistence length ratio was 5 : 1 and binding ratio was 1 : 20. DNA and gelatin A had comparable molecular weights. As has been already mentioned the thermo-viscoelastic and structural properties of DNA-GA ionogels were investigated through an array of experimental techniques like SANS, rheology and DSC.

Small Angle Neutron Scattering

SANS is a powerful technique to probe internal structure of soft matter systems; however the system needs to have a large neutron scattering length density in order to express a good signal to noise ratio. The typical structure factor data I(q) versus q obtained from a ionogel sample is shown in Fig. 1(a).

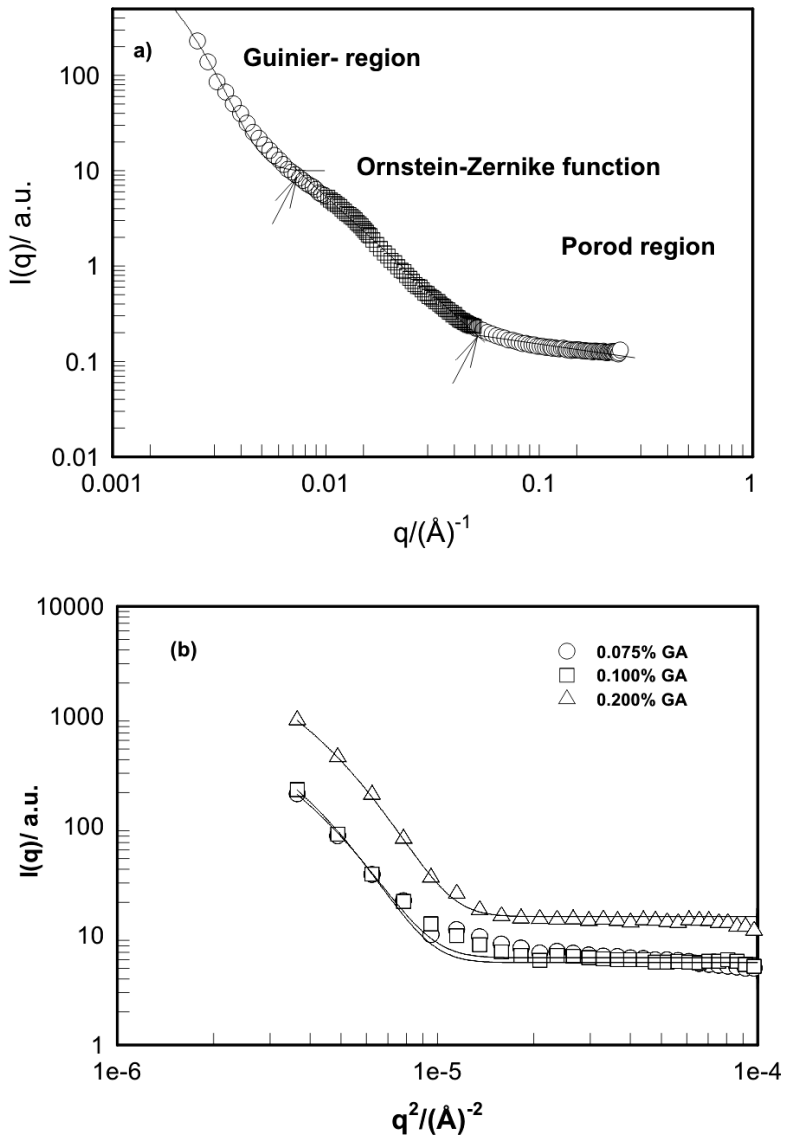

Fig. 1. a) Structure factor of DNA-GA ionogels determined from SANS data. The various scattering regions clearly marked here. b) Structure factor data in the low-q region was fitted to the function defined by eq. (1). This yielded the radius of gyration value of the scattering centers. The DNA concentration was fixed at 0.005% (w/v). The GA concentration is mentioned in the graph as legend. Experimental data pertain to 20°C.

The structure factor data could be spliced into three distinct domains that characterize three distinguishable length scales. This was carried out in the following manner. It must be realized that in the solution state, the scattering entity having radius of gyration R_g contributes a component I_G (q) to the structure factor in the low q-region ($1.91 \times 10^{-3} < q < 9.87 \times 10^{-3}$) given by the Guinier function

$$I_G (q) = I_G (0)\exp (-q^2R_g^2/3) \quad (1)$$

If the scattering material has gel-like attributes, the density fluctuations is characterized by the correlation length ξ (also called the mesh size of the network) and a contribution I_{OZ} (q) arises to the structure factor in the intermediate q-region ($9.87 \times 10^{-3} < q < 0.0496$) given by the Ornstein-Zernike function

$$I_{OZ} (q) = I_{OZ} (0)/(1 + q^2\xi^2) \quad (2)$$

In the high q-region ($0.0496 < q < 0.24$), the Guiner-Porod regime prevailed and an additional contribution to the structure factor I_p (q) was observed that defined the cross-sectional radius of the scattering object R_c given by

$$I_p (q) = I_p (0)\exp (-q^2R_c^2/2) \quad (3)$$

Thus, a plot of 1/I(q) versus q yielded a distinct linear plot, one each for low, intermediate and high q-regime with positive slope and a finite intercept from which the characteristic length scales R_g, ξ and R_c were determined (Fig. 3). The fitting of the structure factor data to the Guinier ($1.91 \times 10^{-3} < q < 9.87 \times 10^{-3}$) and Ornstein-Zernike ($9.87 \times 10^{-3} < q < 0.0496$) region are depicted in Figs. 1(b) and 2(a).

The Guiner-Porod equation was fitted in the high q-region ($0.0496 < q < 0.24$), this is shown in Fig. 2(b). The variation of R_g, R_c, ξ and ξ_{el} with concentration of protein C_{GA} is clearly shown in Fig. 3. The analysis concluded three distinct facts : (i) the typical size of the scattering unit had a size $R_g \approx 150$ nm, (ii) the correlation length was $\xi \approx 3.8 \pm 0.2$ nm and (ii) the cross-sectional radius of the scattering unit was $R_c = 1.8$ nm. Interestingly, none of these length scales changed with protein concentration. It is inferred that there exists a stoichiometric binding ratio between DNA and protein beyond which the protein chains do not participate in the complexation and, hence, in gelation mechanism.

Earlier studies have established that when these coacervates were subjected to slow heating (to 50°C) followed by cooling to room temperature (20°C) cycle, weakening of the DNA-GA binding occurred favoring preferential GA-GA interaction,

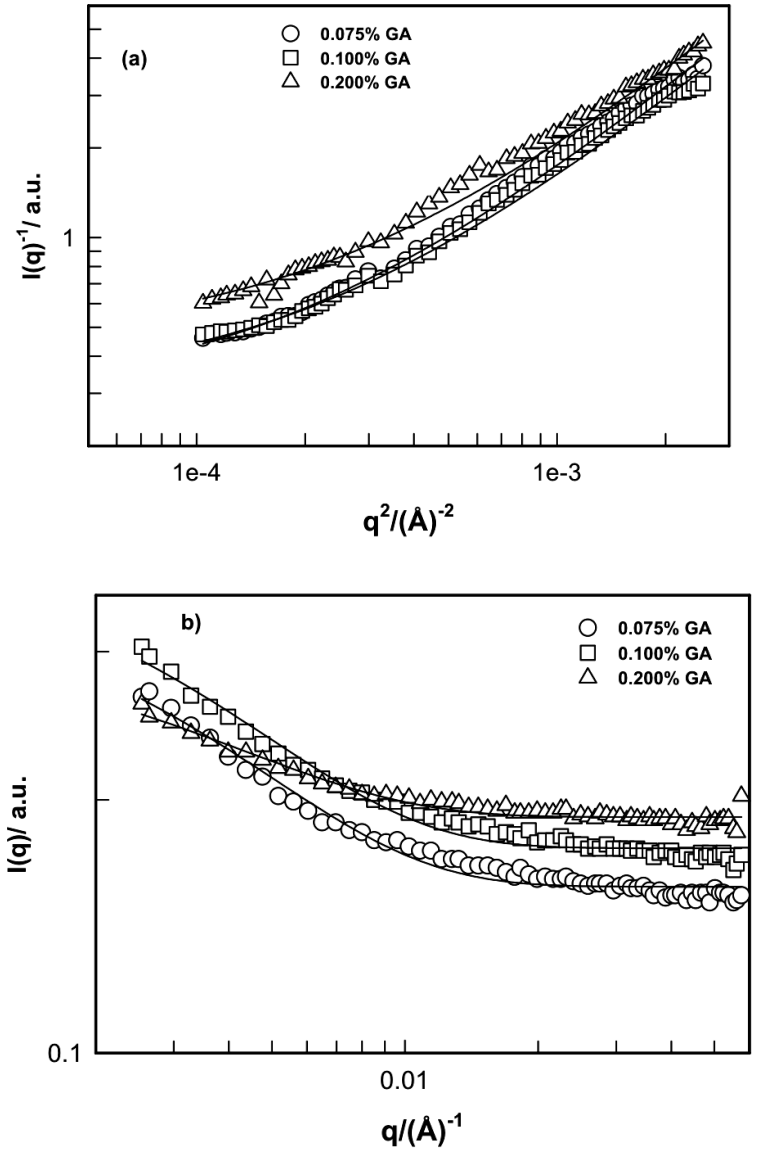

Fig. 2. Structure factor data in the a) intermediate-q region was fitted to the Ornstein-Zernike function defined by eq. (2). This yielded the correlation length (mesh size) values of the ionogels. b) high-q region was fitted to the Guinier-Porod region defined by eq. (3). This yielded the cross-sectional radius of the biopolymer. The DNA concentration was fixed at 0.005% (w/v). The GA concentration is mentioned in the graph as legend. Experimental data pertain to 20°C.

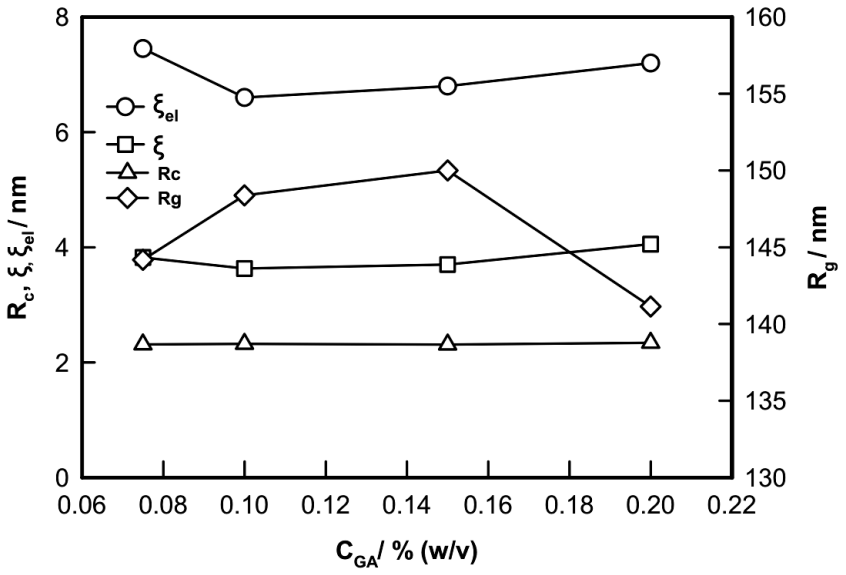

Fig. 3. Variation of the characteristic length scales radius of gyration (R_g), radius of gyration (R_c), viscoelastic length (ξ_{el}) and mesh size (ξ) as function of protein concentration. Notice the invariance of these parameters with GA concentration implying saturation binding between DNA and GA. The DNA concentration was fixed at 0.005% (w/v). Experimental data pertain to 20°C.

followed by reorganization of labile coacervate material into rigid, self-sorted and anisotropic ion-GA gel with DNA molecules acting as scaffolds. Such a material will be spatially heterogeneous and anisotropic due to preferential alignment of DNA molecules. Beyond this protein concentration addition of more GA did not alter the mesh size of the networks.

Rheology

The viscoelastic response of the material is best captured from the frequency dependent dispersion behavior of the in phase storage modulus $G'(\omega)$ and out-of-phase loss modulus $G''(\omega)$. The explicit frequency dependence is given by the power-law function

$$G'(\omega) \sim \omega^{n'} \text{ and } G'(\omega) \sim \omega^{n''} \tag{4}$$

The exponents n' and n'' describe the network rigidity to a good extent. For instance, for solid-like samples these assume very low values whereas for viscoelastic substances they exhibit values in the range 1-2. In the Maxwell model, pertaining to ideal

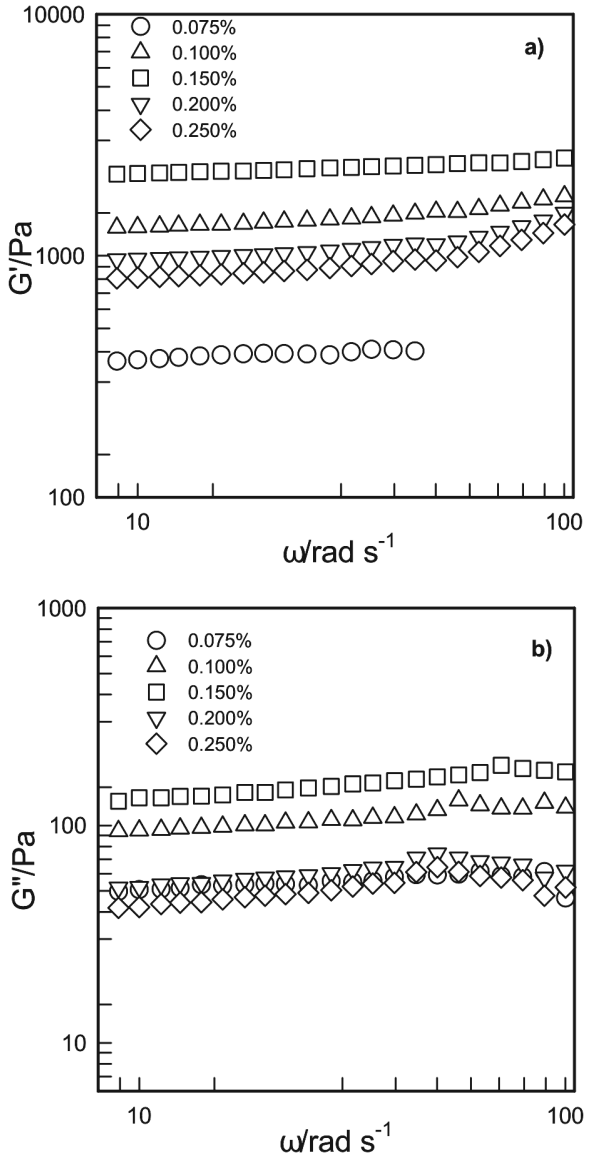

Fig. 4. Variation of a) storage modulus G′(ω) and b) loss modulus G″(ω) as function frequency ω. Notice the weak dependence of these parameters on frequency with implying solid-like behavior of ionogels. Note that above 0.150% (w/v) of GA concentration the gel rigidity decreased. The DNA concentration was fixed at 0.005% (w/v). The GA concentration is mentioned in the graph as legend. Experimental data pertain to 20°C. See text for details.

viscoelastic materials, it has been shown that $n' = 2$ and $n'' = 1$. At the gelation transition point both the exponents assume identical value, $n' = n''$. The $G'(\omega)$ and $G''(\omega)$ data are presented in Figs. 4(a) and (b) respectively.

It is possible to make the following observations from the rheology data : (i) the value of the exponents n' and n'' was very small (≈ 0.1) and (ii) $G'(\omega) >> G''(\omega)$, implying $\tan\delta = G''(\omega)/G'(\omega) << 1$. Viscoelastic theory will classify this material as gel residing deep inside the gelation phase. Considering the origin of these DNA-GA ionogels such an attribute is plausible. It is to be noted here that gelatin gel is associated with a typical gel strength ≈450 Pa in contrast these ionogels are at least ten fold stronger.

In a network of transiently connected chains, the shear modulus is proportional to the concentration of inter-molecular bonds. The value of the length of elastically active strands, calculated from Eqn. 5 is similar to the characteristic viscoelastic network size, $\xi_{el,}$ estimated from the low-frequency shear modulus, G_0. This is a measure of elastic free energy stored per unit volume of a characteristic viscoelastic network of size, ξ_{el}. Hence[30]

$$G_0 \sim k_B T/\xi_{el}^3 \quad (5)$$

Experiments revealed that shear storage modulus of ionogels was weakly dependent on frequency (Fig. 3) which reaffirms the validity of Eqn. 5. Thus, typical viscoelastic length scale prevalent in these materials becomes easily accessible from dynamic rheology measurements. The values obtained from the data shown in Fig. 3 were $\xi_{el} \approx 2\xi \approx 6.5$ nm independent of protein concentration.

DSC

The specific heat, ΔC_p data obtained from the measurements is depicted in Fig. 5.

The specific heat maxima was observed at $T_g \approx 42 \pm 2°C$, and there was no dependence on protein concentration which could be ascribed to the already cited saturation binding between the two biopolymers. This temperature can be assumed to be the gelation temperature of these gels. Thus, in comparison with gelatin gels, ionogels exhibited melting temperature 12°C higher. Ionogels once formed maintained rigid interpenetrating network structures that did not permit excess protein molecules to get embedded into it. Further, the gelation enthalpy (area under the specific heat peak) of all the samples was not too different. This requires more probing.

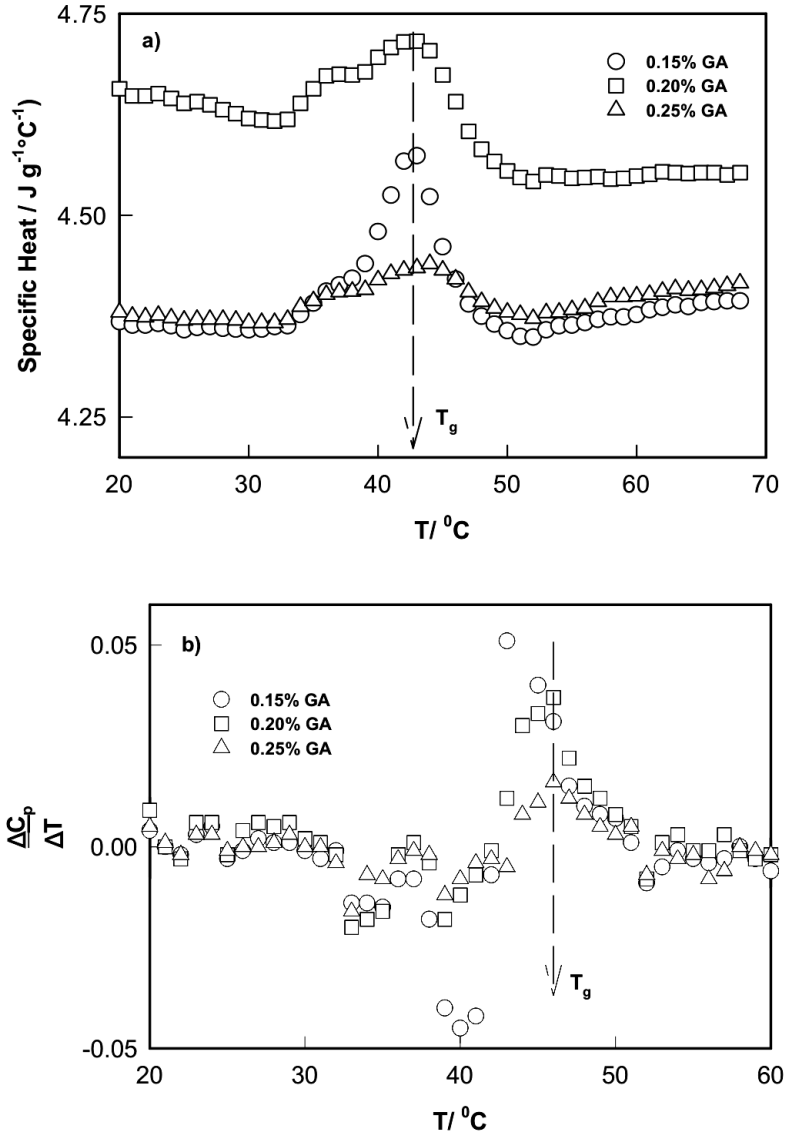

Fig. 5. (a) Variation of specific heat DC_p as function temperature, T. Notice the weak dependence of the gelation temperature T_g on protein concentration implying saturation binding between DNA and GA. (b) Derivative of ΔC_p with respect to temperature where the invariance is seen clearly. The DNA concentration was fixed at 0.005% (w/v). The GA concentration is mentioned in the graph as legend. Experimental data pertain to 20°C. See text for details.

CONCLUSIONS

The ionogels formed as a result of first order phase transition from a coacervate phase have been investigated in the present work. We reported comprehensive thermo-mechanical properties of DNA-gelatin A complex ionogels using results obtained from rheology and DSC experiments. It is found that an interpenetrating network of nucleic acid strands firmly bound to protein chains at a well defined stoichiometric ratio informed. In the random phase approximation, the density fluctuations are defined through a characteristic correlation length (also called mesh size). The data presented in Fig. 3 reveals no change in the value of this length with protein concentration. Such an observation indicates that the interpenetrating network of DNA-GA strands comprise a saturation binding between the two polyelectrolytes. Further addition of protein chains to such an optimized gel does not change its correlation length. These ionogels exhibit saturation behavior with respect to addition of more protein chains as far as their gelation temperature and gel strength are concerned. These complex ionogels are found to be associated with higher gel strength and gelation temperature. Thus, these constitute a better soft matter system in contrast to gelatin gels that are known for their low gel strength and melting temperature. These ionogels are found to be very stable. Interestingly, the mesh size of the networks also indicate saturation binding between the two biopolymer components. Clearly more experiments over a wide range of compositions is required to provide further insight into the physical properties of these gels. DNA-IL-gelatin is indeed a complex mixture of three intricately interacting constituents. However, in the present work, we explore the effect of protein content on gel behavior keeping the concentration of other two constituents fixed. Perhaps a future study will address this issue. Considering the fact that physical attributes of these gels can be customized to the specific requirements of a given application, further development of such materials highly important.

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REFERENCES

1. S. S. Jena and H. B. Bohidar, *J. Chem. Phys.*, 100, 6888–6895 (1994).
2. S. S. Jena and H. B. Bohidar, *J. Chem. Phys.*, 98, 8970–8977 (1993).
3. I. Pezron, M. Djabourov and J. Leblond, *Polymer*, 32, 3201–3209 (1991).
4. M. Djabourov, J. Leblond and P. Papon, *J. Phys.*, 49, 319–332 (1988).
5. M. Djabourov, J. Leblond and P. Papon, *J. Phys.*, 49, 333–343 (1988).
6. L. Guo, R. H. Colby, C. P. Lusignan and T. H. Whitesides, *Macromolecules*, 36, 9999–10008 (2003).
7. S. Boral and H. B. Bohidar, *J. Phys. Chem. B.*, 116, 7113–7121 (2012).
8. S. Sanwlani, P. Kumar and H. B. Bohidar, *J. Phys. Chem. B.*, 115, 7332–7340 (2011).
9. K. Rawat, J. Pathak and H. B. Bohidar, *Soft Matter*, 10, 862–872 (2014).
10. A. B. Kayitmazer, H. B. Bohidar, K. W. Mattison, A. Bose, J. Sarkar, A. Hashidzume, P. S. Russo, W. Jaeger and P. L. Dubin, *Soft Matter*, 3, 1064–1076 (2007).
11. Y. Yan, E. Kizilay, D. Seeman, S. Flanagan, P. L. Dubin, L. Bovetto, L. Donato, and C. Schmitt, *Langmuir*, 29 (50), 15614–15623 (2013).
12. E. Kizilay, S. Maccarrone, E. Foun, A. D. Dinsmore, and P. L. Dubin, *J. Phys. Chem. B.*, 115 (22), 7256–7263 (2011).
13. A. Kamra, K. Sachin, A. Saxena and H. B. Bohidar, *Curr. Pharm. Biotech.*, 6 121–130 (2005).
14. B. Mohanty and H. B. Bohidar, *Biomacromolecules*, 4, 1080–1086 (2003).
15. J. Pathak, K. Rawat and H. B. Bohidar, *Int. J. Biol. Macromols.*, 63, 29–37 (2014).
16. A. Veis, The macromolecular chemistry of gelatin, Academic Press, New York, 1964.
17. A. Tiwari, Sonal Bindal and H. B. Bohidar, *Biomacromolecules*, 10, 184–189 (2009).
18. N. Arfin and H. B. Bohidar, *J. Phys. Chem. B.*, 116, 13192-131-99 (2012).
19. M. A. B. H. Susan, T. Kaneko, A. Noda and M. Watanabe, *J. Am. Chem. Soc.*, 127, 4976–4983 (2005).
20. N. Winterton, *J. Mater. Chem.*, 16, 4281–4293 (2006).
21. J. L. Bideau, L. Viaub and A. Vioux, *Chem. Soc. Rev.*, 40, 907–925 (2011).
22. Y. Hea and T. P. A. Lodge, *Chem. Commun.*, 2732–2734 (2007).
23. Y. Lei and T. P. Lodge, *Soft Matter*, 8, 2110–2120 (2012).
24. M. Kofu, T. Someya, S. Tatsumi, K. Ueno, T. Ueki, M. Watanabe, T. Matsunaga, M. Shibayama, V. G. Sakai, M. Tyagi and O. Yamamuro, *Soft Matter*, 8, 7888–7897 (2012).

25. K. Matsumoto and T. Endo, *Macromolecules*, 41, 6981–6986 (2008).
26. J. F. Stanzione, R. E. Jensen, P. J. Costanzo and G. R. Palmese, *ACS Appl. Mater. Interfaces*, 4, 6142–6150 (2012).
27. J. Fuller, A. C. Breda and R. T. Carlin, *J. Electrochem. Soc.*, 144, L67–L701997.
28. J. C. Ribot, C. Guerrero-Sanchez, R. Hoogenbooma and U. S. Schubert, *Chem. Commun.*, 46, 6971–6973 (2010).
29. K. Rawat, V. K. Aswal and H. B. Bohidar, *J. Phys. Chem. B.*, 116, 14805–14816 (2012).
30. A. Ajji and L. Choplin, Macromolecules, 24, 5221–5223 (1991).