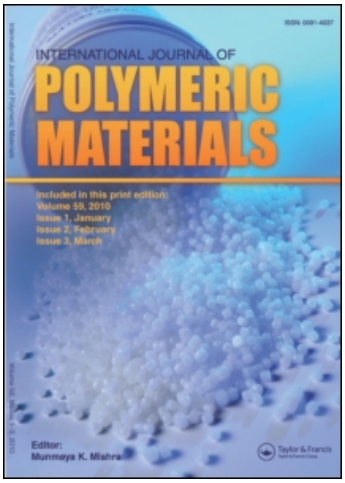

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AFM Study of Morphology of Ethanol Induced Gelatin Coacervation

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AFM studies have been carried out on coacervating 1% (w/v) aqueous alkali processed gelatin (Type-B) solutions titrated with ethanol close to its iso-electric $pH = 5.01$ *(*$pI = 4.9$*). Turbidity measurements performed at 450 nm clearly established the transition points in terms of the percentage of volume of alcohol added relative to that of the total volume corresponding to the first occurrence of turbidity* ($Vt = 47 \pm 2\%$ *v/v) and a point of turbidity maximum* ($Vp = 51 \pm 2\%$ *v/v). Addition of more alcohol drove the system towards precipitation. The values of Vt and Vp characterized the initiation of intramolecular collapse and intermolecular aggregate formation of the charge neutralized gelatin molecules, and the subsequent micro coacervate droplet formation. The gelatin molecules in the solution were observed to undergo a series of micro-structural transformations as seen by AFM until the coacervates phase was reached. At a critical ethanol concentration of* $V = 45 \pm 3\%$ *v/v fractal clusters of aggregated polypeptide molecules having fractal dimension* $d_f = 1.60 \pm 0.05$ *were observed.*

Keywords: atomic force microscopy (AFM), gelatin coacervates, fractal aggregates, surface morphology, self-assembly

INTRODUCTION

Coacervation is a process during which a homogeneous solution of charged macromolecules undergoes liquid-liquid phase separation, giving rise to a polymer-rich dense phase [1–3]. Coacervation has been

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classified into simple and complex processes depending on the number of participating macromolecules. In simple polyelectrolyte coacervation, addition of salt or alcohol normally promotes coacervation [1]. In complex coacervation, two oppositely charged macromolecules (or a polyelectrolyte and an oppositely charged colloid) can undergo coacervation through associative interactions. The charges on the polyelectrolytes must be sufficiently large to cause significant electrostatic interactions, but not so large to cause precipitation. The dilute liquid phase, usually the supernatant, remains in equilibrium with the coacervate phase. These two liquid phases are incompatible and immiscible. Protein-polyelectrolyte system is a special case of colloid-polyelectrolyte coacervates. In these systems, interactions primarily arising from electrostatic forces, lead to coacervation. Protein-polyelectrolyte coacervates are a novel state of matter where the concentration of bound protein can reach a level of $\approx$150 g/l normally unsustainable in aqueous solutions [1]. It must be noted here that coacervation is not precipitation. Coacervates are polymer-rich superconcentrated solutions that remain in equilibrium with the supernatant. Precipitation is a liquid to solid phase transition involving latent heat whereas coacervation is a liquid-liquid phase transition involving entropy gain. The investigation of the basic aspects of coacervation of polyelectrolyte complexes provides a foundation not only for the fundamental understanding of these supramolecular structures but also for their practical uses in protein-related applications and industrial processes [4–6].

In a recent work [7], turbidity and light scattering measurements, along with phase contrast microscopy were used to follow the processes leading to complex coacervation when aqueous solutions of bovine serum albumin (BSA) and poly (dimethyldiallylammonium chloride) (PDMDAAC) were brought from pH = 4 to 10. The state of macromolecular assembly of complexes formed between BSA and PDMDAAC prior to and during the pH-induced coacervation could be characterized by specific pH values at which recognizable transitions took place. Based on the pH-induced evolution of scattering intensity measurements, it was concluded that the formation of soluble primary protein-polymer complexes is initiated at pH_c and this proceeds until $pH\phi$. A maximum in scattering intensity at $pH\phi$ is observed coincident with the appearance of turbidity and also corresponding to the first microscopic observation of coacervate droplets. The temperature and protein to polymer concentration ratio dependence of pH_c and $pH\phi$ were studied explicitly.

A survey of the literature confirms the near total absence of systematic studies on simple coacervate systems. Gelatin, a polyampholyte

obtained from denatured collagen, is a polypeptide and is an ideal case for such studies. Aqueous solutions properties of gelatin have been well studied and characterized in the past [8–9]. Depending on the process of recovery the gelatin molecules bear different physical characteristics. Type-A gelatin is acid processed, has an isoelectric pH, pI $\approx$ 9 whereas the alkali processed type-B gelatin has pI $\approx$ 5 [10]. The monomeric representation of this polypeptide is $(Gly\text{-}X\text{-}Pro)_n$, where X is an amino acid. The detailed chemical composition of this biopolymer is as follows (as per Merck index): Glycine constitutes 26%, alanine and arginine are in 1:1 ratio together constitute $\approx$ 20%, proline is $\approx$ 14%, glutamic acid and hydroxyproline are in 1:1 ratio constituting $\approx$ 22%, aspartic acid $\approx$ 6%, lysine $\approx$ 5%, valine, leucine, and serine constitute $\approx$ 2.0% each, the rest (1%) is comprises isoleucine, threonine, and so on. In the past all the coacervation studies on gelatin involved complexation between type-A and type-B, or gelatin and acacia molecules [11–13].

In the present work, we have undertaken a qualitative and yet a systematic AFM study on simple coacervation of Type-B gelatin (a low charge density biomolecule) in aqueous environment close to the isoelectric pH of the protein with a long-term objective to understand the kinetics of simple coacervation. No such study has ever been undertaken despite the fact that such an understanding may serve as a precursor to the mapping of the complexities of coacervation phenomena as a whole.

MATERIALS AND METHODS

Ethanol was obtained from Merck, Germany. Gelatin (Type-B, microbiology grade devoid of E. Coli and liquifier presence) and sodium chloride were bought from E. Merck, India. The gelatin sample was bovine skin extract, bloom strength was 75 and had a molecular weight $\approx (90 \pm 10)$ kDa determined from SDS/PAGE. All other chemicals used were bought from Thomas Baker, India. All the chemicals were of analytical grade. The gelatin samples were used as supplied. The solvent used was deionized water. pH (using 0.1 M HCl or 0.1 M NaOH) and ionic strength of the solvent were first set as per the experimental requirement (0.1 M NaCl), and the gelatin solutions (1% w/v) were prepared by dispersing gelatin in this medium at 60°C and pH set to 5.01 after cooling to room temperature. The macromolecules were allowed to hydrate completely; this took 30 min to 1 hour. The final stock solution was a transparent liquid. The gelation concentration of gelatin in water is $\approx$2% (w/v), the gelatin concentration chosen in these experiments was deliberately kept lower than this to avoid

formation of gels. Samples were checked continuously by passing these through SDS/PAGE to ensure absence of gelatin degradation.

Tubidimitric Titration

Typically 100 ml of stock solution (1% w/v aqueous gelatin) were taken in a beaker kept on a magnetic stirrer and stirred at moderate speed with stir bars throughout the titration process. The change in transmittance of the solution was monitored continuously using a turbidity meter (Brinkmann-910, Brinkmann Instruments, USA) operating at 450 nm. Alcohol was taken in a calibrated burette and added in drops to the reaction beaker and the volume of alcohol added to produce the first appearance of turbidity was measured at $Vt = 47 \pm 2\%$ v/v and a point of turbidity maximum was seen at $Vp = 51 \pm 2\%$ v/v. Addition of more alcohol drove the system towards its precipitation point. The values of Vt and Vp characterized the initiation of intramolecular collapse and intermolecular aggregate formation of the charge neutralized gelatin molecules, and the subsequent micro coacervate droplet formation. This procedure was performed at temperature $T = 25^{\circ}C$ inside a thermostatic water bath providing a temperature stability of $\pm 1^{\circ}C$. The pH of the reaction mixture was monitored continuously during this process. A typical titration curve is shown in Figure 1. The coacervates were extracted from the solution at $V = 48 \pm 1\%$ v/v by repeated centrifugation of the solution at 10000 rpm and during this process the lower $\approx 30\%$ of the sample was collected and the supernatant was discarded. Normally, it took three such steps to collect the final product.

AFM Measurements

During the experiment, a drop of the solution was removed from the reaction beaker and allowed to spread out uniformly on a degreased glass cover-slip plate over a period of 30 minutes. Atomic Force Microscope (AFM) pictures were taken using a Autoprobe CP Research AFM system, model AP-2001 (Thermomicroscopes, USA) using a 90 μm scanner and tapping mode. During the course of these studies the complete mapping of coacervation phenomenon was followed by taking AFM micrographs at various volumes of added ethanol until coacervation was reached. The gelatin particles and aggregates could be visualized in the solvent environment because the hydration solvent has a refractive index different from that of the solvent background, which is responsible for providing the necessary optical contrast.

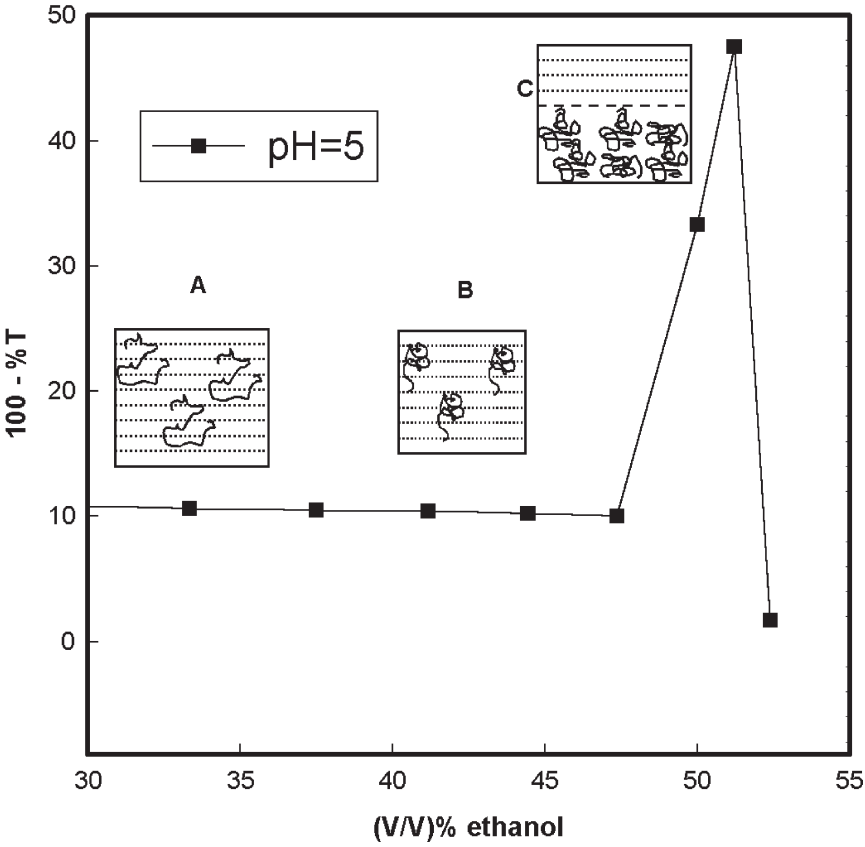

FIGURE 1 Titration profile is shown for ethanol/water system for a 1% (w/v) aqueous gelatin (Type-B) solution with $I = 0.1\,M$ and $pH = 5$ performed at 25°C. At low ethanol concentration the gelatin molecules have linear random coil conformation (inset A), as the solvent turns into a marginal one due to addition of alcohol the chain contracts creating intra- and intermolecular chain overlap, only intramolecular collapse is shown in inset B. Finally, at a threshold ethanol concentration V_t, the liquid-liquid phase transition ensues, giving rise coacervation (inset C).

DLS Measurements

The particle sizing measurements were done by dynamic laser light scattering (DLS) technique, using a Brookhaven-9000AT digital auto-correlator (Brookhaven Instruments, USA) and a homemade goniometer. The excitation source was a diode pumped solid state laser (Model-DPY 305-II, Adlas, Germany) emitting 50 mW of power at

532 nm in linearly polarized single frequency mode. The scattering angle was fixed at 90° and the data analysis was done using CONTIN software provided by Brookhaven Instruments. More on DLS and data analysis can be found elsewhere [14].

RESULTS AND DISCUSSIONS

As the gelatin stock solution was titrated with ethanol the gelatin molecules inside the solution went through a sequence of morphological changes as shown schematically in Figure 1. Here the authors focus on four distinct situations where AFM pictures were revealing shown as zones A, B, and C in Figure 1 and the narrow fractal zone that resides between B and C.

Ethanol Concentration < V_t

Here the authors probed the ethanol concentration range from 0–44% (v/v), shown as insets A and B in Figure 1. Gelatin is not soluble in alcohols whereas water is a good solvent. As ethanol is added to water, the water molecules will preferentially bind to the alcohol molecules through hydrogen bonding and the resultant binary mixture becomes a marginal solvent for gelatin molecules. Secondly, the dielectric constant decreases [15] significantly facilitating stronger electrostatic interactions between charged segments (both intra and inter) of gelatin molecules. The strength of electrostatic interactions between two oppositely charged particles increases as $\varepsilon^{-3/2}$ at a given temperature as per the Debye-Huckel theory. Because the solution pH was close to the iso-electric point of gelatin, there is no net charge on the polypeptide. Nonetheless, as mentioned already, the chemical structure of this biopolymer indicates almost a 1:1 positively and negatively charged patches along this linear random coil molecule. These overlap as the chain contracts due to the decrease in the Flory-Huggin's solute-solvent interaction, caused by the presence of ethanol, resulting in bringing charged segments to each other's vicinity through electrostatic interactions leading to chain collapse. This collapse can be intramolecular, yielding a typical hydrodynamic radius $\approx 30 \pm 5$ nm on intermolecular chain collapse due to electrostatic interactions between charged segments belonging to different molecules forming large aggregates of radius that are an order of magnitude larger. Such an effect will yield aggregates of gelatin molecules with large polydispersity, which continues up to ethanol concentration, $V \leq 44\%$ v/v.

This is clearly seen in AFM pictures shown as Figures 2 (ethanol concentration = 0) and 3 (ethanol concentration = 33.3% v/v). In

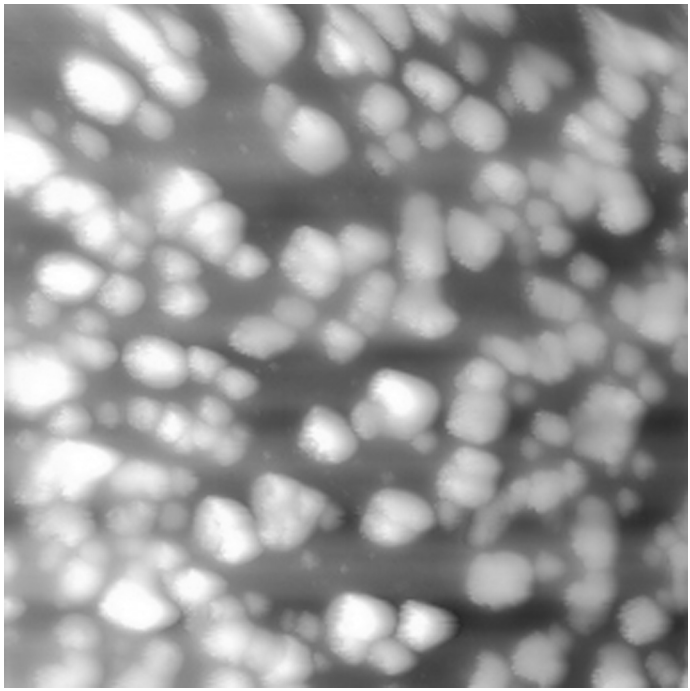

FIGURE 2 Scanning probe micrograph ($5\,\mu m \times 5\,\mu m$) of 1% w/v aqueous gelatin solution with 0.1 M NaCl taken at 25°C in the tapping mode. The picture shows gelatin aggregates of size 65 nm, 190 nm, 380 nm, and larger clusters. Tiny particles seen in the background are single molecules of typical size 30 ± 5 nm.

addition to $\approx 30 \pm 5$ nm gelatin molecules (intramolecular collapse), aggregates of sizes ≈ 65 nm, 180 nm, 380 nm (intermolecular aggregates) are clearly seen in Figure 2. As the alcohol concentration is increased to 33.3% v/v much larger aggregates are formed (Figure 3) at the cost of smaller ones. The general observation has been that as V_t is approached there is a propensity toward larger aggregates.

Ethanol Concentration Close to V_t

There is a very narrow zone between B and C of Figure 1 ($V = 45 \pm 2\%$ v/v) where fractal self-assembly of gelatin aggregates occurs (Figure 4). One observes beautiful fractal trees with branches emerging out of large aggregates. There is a swarm of small particles and a 650 nm aggregate near one end of these trees in Figure 4. Interestingly, in the close vicinity of these fractal trees no free particles are

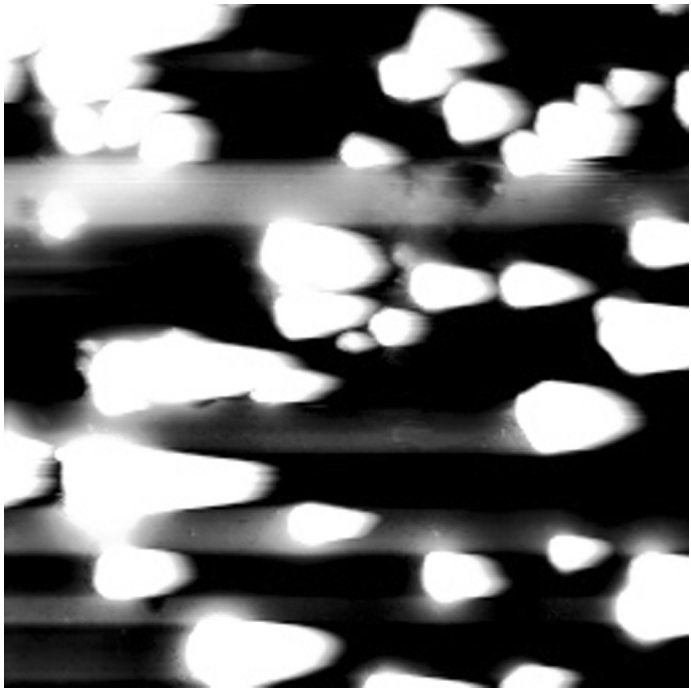

FIGURE 3 Scanning probe micrograph ($10\,\mu m \times 10\,\mu m$) of 1% w/v aqueous gelatin solution with 0.1 M NaCl taken at 25°C in the tapping mode in presence of 33.3% v/v ethanol. The picture shows gelatin aggregates giving rise to larger clusters of typical size 1000 nm. Tiny particles not clearly seen in the background are single molecules of typical size 30 ± 5 nm.

present implying the participation of all the smaller gelatin aggregates available in that space in the formation of the fractal structures. The swarm of approximately 200 nm size particles seen is plausibly in the process of joining the fractal tree or starting a new structure from the 1300 nm cluster available nearby. Figure 4 clearly exhibits direction dependent affinity.

The stochastic self-similarity and scaling of the random fractals observed in Figure 4 was ascertained from the fractal dimension, d_f [16]. The present analysis yielded $d_f = 1.60 \pm 0.05$.

Ethanol Concentration Equal to V_t and Coacervation

As the alcohol concentration reached $V_t = (47 \pm 2)\%$ v/v, the turbidity showed a sharp rise, as seen in Figure 1. Such a situation corresponds

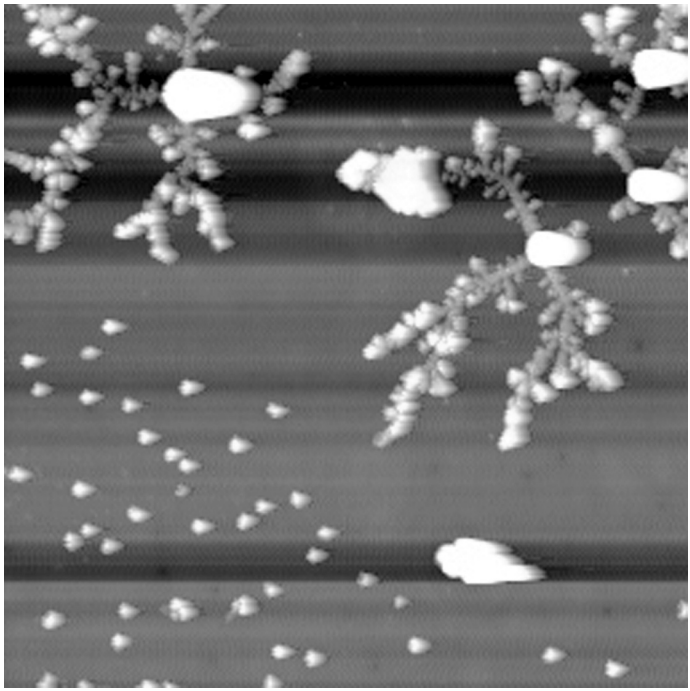

FIGURE 4 Scanning probe micrograph ($10\,\mu m \times 10\,\mu m$) of the solution adsorbed on glass substrate having ethanol concentration ≈45% (v/v). The fractal trees are seen to emerge from 1300 nm clusters. Swarm of free particles (size ≈ 200 nm) are seen along with a 1300 nm aggregate, which are yet to join to form another fractal tree. Notice the propensity of directional affinity and absence of isotropic self-similarity, the fractal arms have $d_f = 1.60 \pm 0.5$.

to a state where very large number of charge-neutralized inter- and intramolecular clusters of gelatin molecules are formed in a cooperative manner. The corresponding AFM picture is shown in Figure 5 where typically 1 μm clusters are clearly visible along with larger ones. Most of these are intermolecular clusters. At a threshold ethanol to water ratio the bulk of the matter is expelled out of the binary solvent mixture along with its hydration water into a separate equilibrium phase called coacervates (C in Figure 1). The polyions do not precipitate out of the solvent because of entropy gain achieved by random mixing of polyions in the coacervate phase, which is the final equilibrium state. Equilibrium images of the coacervates (Figure 6) show lumps of dense matter with immense heterogeneity spread in space having no definite geometric structures.

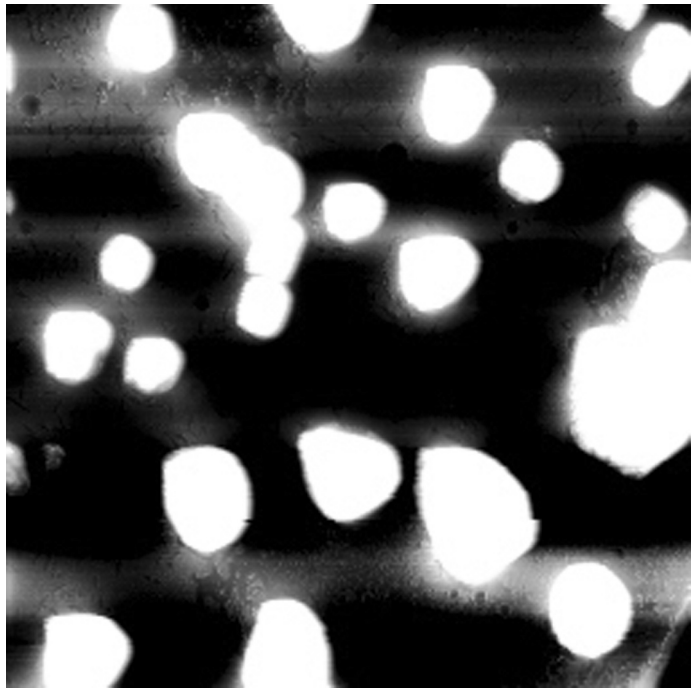

FIGURE 5 Scanning probe micrograph (10 μm × 10 μm) of 1% w/v aqueous gelatin solution with 0.1 M NaCl taken at 25°C in the tapping mode in presence of 47% v/v ethanol (at V_t). The picture shows gelatin aggregates giving rise to larger clusters of typical size 1000 nm and coalesced clusters.

Ethanol Concentration Equal to V_p

As more and more alcohol is added to the solution, one observes a liquid to solid phase separation giving rise to precipitation of the polypeptide from the bulk of the binary liquid mixture. Such a process is slow and gradual and involves latent heat. An AFM image taken very close to = 49% v/v shows 2–3 μm clusters that slowly phase separate as precipitates (Figure 7).

Size of Aggregates

During the titration process, a few milliliters of the sample were drawn from the reaction beaker and loaded into borosilicate cylindrical cell (volume = 5 ml) and DLS experiment performed. The data analysis revealed two particle sizes all throughout until Vp was

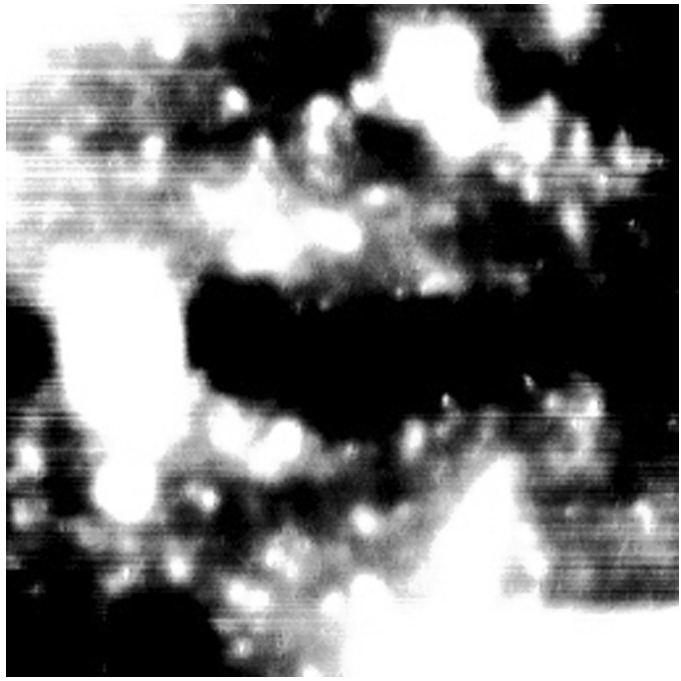

FIGURE 6 Scanning probe micrograph ($5\,\mu m \times 5\,\mu m$) of gelatin coacervates matter extracted from coacervating solution at 25°C in the presence of 51% v/v ethanol. Picture shows lumps of heterogeneous matter spread over the substrate.

reached. The smaller particles had a size of circa 20 ± 4 nm whereas the aggregates grew in size to reach a value of several hundred nanometers at Vp. This is shown in Figure 8. For comparison the value of dielectric constant [15] of the ethanol-water mixture is shown in the same plot. The results show spontaneous size segregation preceding coacervation, which is consistent with the results reported by Veis [17] . As alcohol is added to water, the water molecules will bind with alcohol molecules through hydrogen bonding and the resultant binary mixture becomes a poor solvent for gelatin molecules. As has already been mentioned, the strength of electrostatic interactions between two oppositely charged particles increases as $\varepsilon^{-3/2}$ at a given temperature as per the Debye-Huckel theory. The poor solvent quality compels the gelatin molecule to reduce its spatial expansion thereby bringing charged segments to each other's vicinity through electrostatic interactions. This results in the collapse of some of the single gelatin

FIGURE 7 Scanning probe micrograph ($5\,\mu m \times 5\,\mu m$) of 1% w/v aqueous gelatin solution with 0.1 M NaCl taken at 25°C in the tapping mode in presence of 49% v/v ethanol (close to V_p). Picture shows gelatin aggregates giving rise to coalesced clusters of typical size 1000–2000 nm.

molecules through intramolecular interactions yielding a typical hydrodynamic radius $\approx 20 \pm 4$ nm whereas most other molecules associate through intermolecular electrostatic interactions to form large aggregates of radius that are ten times larger consistent with earlier observations [17].

Simultaneously, intermolecular segments of complementary charge form aggregates of size ≈200 nm. Because these aggregates may not be fully charge neutralized, they can attract other gelatin molecules and, thus, grow in size. At a critical volume of added alcohol (Vt) the liquid-liquid phase separation ensues. These aggregates constitute the coacervate phase as seen from the AFM picture shown in Figure 7, which clearly shows clusters of sizes ≈200 nm and few microns. This observation is qualitatively identical to the model proposed by Veis [17] and Tainaka [18]. They argue that the aggregates escape the fate of precipitation because of the configurational entropy gain achieved

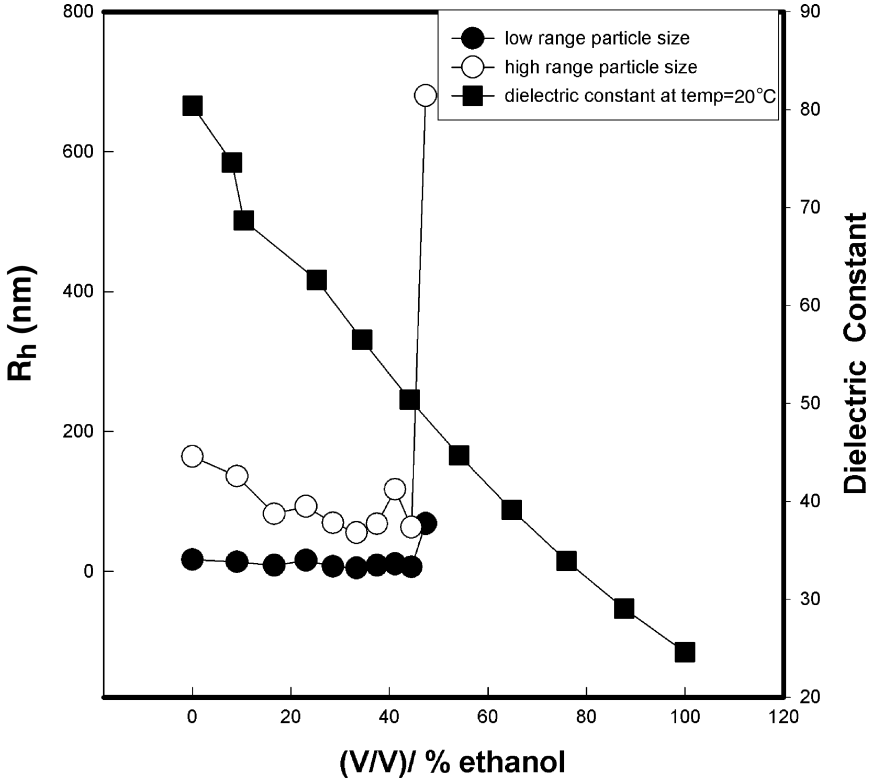

FIGURE 8 Size of various particles measured in terms of hydrodynamic radius (R_h) by DLS for a 1% (w/v) aqueous gelatin (Type-B) solution titrated with ethanol with I = 0.1 M and pH = 5 performed at 20°C (left hand scale). The dielectric constant (ε) data of ethanol/water binary mixture is shown for comparison (right hand scale).

by randomly mixed heavily concentrated gelatin molecules in the coacervate phase. The solvent in the concentrated phase largely constitutes the solvation liquid. The supernatant dilute polyelectrolyte solution largely containing the gelatin nano–particles (collapsed single chains). In the Veis model [17] the aggregates are referred to as symmetrical aggregate polymer (SAP) whereas Tainaka's revised model [18] refers to this as asymmetric aggregate polymer (AAP). Regardless, it is accepted that the coacervate phase owes its origin to these aggregates. The Tainaka model [18] assumes Gaussian distribution of segments in AAP aggregates independent of their size and that all the counter-ions are bound to the AAP aggregates.

CONCLUSION

The experimental data presented indicate that the electrostatic interactions between charged segments of this polyion is facilitated by the solute-solvent interactions, that turns the solvent from good to marginal, enabling closer approach between charged segments. These segments, in turn, overlap because of electrostatic interactions. Typical persistence length [8–9] of gelatin is 2 nm, imparting enough flexibility to the positively charged segments to overlap (both inter and intra) with negatively charged segments through electrostatic interactions thus neutralizing charges of the segments involved, and the subsequent micro coacervate droplet formation. It must be realized that the gelatin molecules do carry positive and negative segments at all pHs, although at some pHs there is excess of one type. This, however, does not change the overall picture of coacervation. It will only affect the degree of charge neutralization in a coacervates solution. It should also be realized that when two oppositely charged segments join together, some amount of counter ion is always released into the solvent, thereby increasing the entropy of the solution. This will assist the process to move toward coacervation. The size of single collapsed gelatin chains seen in DLS measurements was found to be 50% lower than that of AFM data. This discrepancy could be due to instrumental artifact. Realize that AFM measurements are 2D-pictures of the system on hydrophilic substrates, which can stretch out a soft spheroid to an oblate spheroid due to surface tension effects thus enlarging its equatorial diameter. These are hydrated particles, hence the size would be larger than for the corresponding dry size deduced from molecular weight.

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