Chapter 6

Structural Studies of Agar-Gelatin Complex Coacervates by Small Angle Neutron Scattering, Rheology and Differential Scanning Calorimetry

6.1 Introduction

Novel and smart biomaterials can be made through inter and intra-molecular complexation of bio-polyelectrolytes. Coacervates are a new class of polymeric materials formed via electrostatic interactions between complementary polyelectrolytes or a polyelectrolyte and colloid pair [1-6]. Such materials have found applications in protein purification [7], drug and enzyme immobilization [8] and in trapping organic plumes [9]. A variety of coacervates have been made in the past by suitably tuning and enhancing the electrostatic interactions between pairs of polyions. In these transitions, the screened electrostatic interactions existing between oppositely charged polyelectrolytes yield soluble intermolecular complexes that precede coacervation. During such a process, the homogeneous solution undergoes a liquid-liquid phase separation with the polymer-rich phase compartmentalizing itself into a dense state withdrawn from the bulk of the solvent, called the supernatant that co-exists thermodynamically with the coacervate. The solvent present in the concentrated phase largely constitutes the solvation liquid. The supernatant is a very dilute polyelectrolyte solution.

This phenomenon has been conceptualized in various models. In Veis model [10] the aggregates are referred to as symmetrical aggregate polymer (SAP) whereas Tainaka’s revised model [11] refers to these as asymmetrically aggregated polymers (AAP). In Nakajima-Sato model [12] the solute-solvent interactions was incorporated in to the earlier proposed Overbeek-Voorn model [13] through Flory-Huggins prescriptions. Regardless, it is accepted that the coacervate phase owes its origin to the formation of intermolecular soluble aggregates. Tainaka model [11] assumes Gaussian distribution
of segments in AAP aggregates independent of their size and that all the counter-ions are bound to the AAP aggregates. It should be realized that when two oppositely charged segments join together, some amount of counter ion is always released in to the solvent, there by increasing the entropy of the solution. This will assist the process to move towards coacervation. A more rigorous model describing coacervation [14] and its conceptualization in the free-energy landscape formalism was proposed recently [15].

In chapter 4, we have undertaken a qualitative, and yet a systematic study on intermolecular complexation and phase separation in aqueous solutions of gelatin (a low charge density polyanalyte molecule) interacting with agar, a polysaccharide, under various thermodynamic conditions of the solvent with an objective to understand the kinetics of complex coacervation [16]. Agar, a poly-anion was shown to undergo liquid-liquid phase separation (complex coacervation) in presence of a polyanalyte, gelatin, both for pH<pi and pH >pi of gelatin. Agar sols form thermo-reversible physical gels with large hysteresis between melting and gelling temperatures with the constituent unit being anti-symmetric double helices [17,18]. Gelatin, a polyanalyte obtained from denatured collagen, is a polypeptide. The gelation kinetics of gelatin has been well studied and characterized in the past [19,20]. The length scale hierarchy that exists in gelatin sol, gel and coacervate phases has been extensively discussed in an earlier work [21]. The mapping of microscopic structure of coacervates requires further probing in order to understand the spatial ordering of polyelectrolytes inside the coacervate phase. In the past very little attention has been paid to this aspect. In this chapter, we examine the physical characteristics of agar-gelatin coacervates by physically probing these samples through an array of experimental techniques like: Small Angle Neutron Scattering (SANS), rheology and Differential Scanning Calorimetry (DSC).

Thermal experiments were carried out using a Labsys TG-DSC16 differential scanning calorimeter instrument manufactured by Setaram, France. Samples (typically =50 mg) were heated in aluminum cups sealed with lids at a controlled rate of 1°C/min up to a maximum temperature of 100°C starting from room temperature (20°C).
6.2 Results and Discussions

6.2.1 Small Angle Neutron Scattering data (SANS)

The differential scattering cross section arising from a collection of scattering particles consists of two terms [22-25]. The first term depends on the intra-particle scattering which in turn depends on the shape, and size of the particle and in principle can be calculated for any geometry. The second term depends on the inter-particle scattering that is governed by the inter-particle interactions prevailing inside the system. Incoherent background correction in SANS data normally poses a serious challenge. Samples prepared in deuterated water helps in minimizing this effect.

The present objective was to study the microscopic structure of the agar-gelatin coacervates. The expression for scattering cross-section can be simplified for the experimental situation of the coacervate that permits the SANS data to be analyzed in random phase approximation, in two distinct ranges of scattering vectors (q): low q-range (Debye-Bueche behaviour [26]) and the high-q range (Ornstein-Zernike behaviour [27]). Analysis of SANS data within the model of mean field theory reveals that for the polymers in a good solvent, at equilibrium, the structure factor of concentration fluctuations in the Ornstein-Zernike (O-Z) region is given by

\[ S_L(q) = \frac{I_L(0)}{(1+q^2\xi^2)}; \quad q\xi \ll 1 \]  \hspace{1cm} (6.1)

Where, \( \xi \) defines is the correlation length of the concentration fluctuations or mesh size of the network and \( I_L(0) \) is related to the cross-link density and longitudinal osmotic modulus. Experiments carried out in the semi dilute regime of polymer solutions have shown deviations from the Ornstein-Zernike function.

Long wavelength concentration fluctuations in these systems often gives rise to excess scattering in the low-q region of the SANS data. The origin of these fluctuations remains debatable, and a clear cause that generates such excess scattering is yet to be established. However, Koberstein et al. [28] have suggested long-range random inhomogeneities with correlation length many times larger than the radius of gyration.
of the dissolved polymer to cause this excess scattering. If the spatial scale of density fluctuations due to the presence of inhomogeneities is large compared to the correlation length $\xi$, then the two contributions can be treated separately and added to give the total structure factor as

$$S(q) = S_L(q) + S_{ex}(q)$$  \hspace{1cm} (6.2)

Where $S_L(q)$ is the Ornstein-Zernike (O-Z) function, and the Debye-Bueche (D-B) structure factor has the form $S_{ex}(q)$ given by

$$S_{ex}(q) = \frac{I_{ex}(0)}{(1+q^2\xi^2)^2}$$  \hspace{1cm} (6.3)

where $I_{ex}(0)$ is the extrapolated structure factor at zero wave vector. It is possible to study low-q domain of the structure factor provided a high instrumental resolution SANS spectrometers is used to collect data. We have examined neutron scattering data obtained from agar-gelatin coacervate ($20^\circ$C) as function of salt concentration. The experimental data are presented in Figures 6.1 and 6.2 reveal structure factor profile for all the samples. Let us recall that coacervates are amorphous substances, and devoid of any spatial ordering (coacervation transition is a first-order phase transition). This would imply identical scattering profiles for all the samples, which was observed (Figure 6.2) if and only if the inter-molecular interactions are so strong that mobile ion induced Debye-Huckel screening is not too effective. Since, $I(q)$ depends on the square of the difference of neutron scattering-length densities of the scatterer and the solvent, it cannot distinguish between microscopic structures of two samples with identical concentrations, particularly, when both are amorphous materials.
Figure 6.1: Plot of static structure factor, \( S(q) \) versus scattering wave vector, \( q \) for agar-gelatin coacervates prepared in D\(_2\)O determined from SANS measurements performed at 20\(^\circ\)C. Notice the invariance of scattering profiles with ionic strength of the coacervate samples.

A least-squares fit of the structure factor data in the low-\( q \) range, 0.018 Å\(^{-1}\) nm\(^{-1}\) ≤ \( q \) ≤ 0.072 Å\(^{-1}\) (see Figure 6.2), to the D-B function yields size of the heterogeneities (\( \xi = 220 \pm 20 \) Å) present inside the coacervate phase. Secondly, the measured correlation length (Fig. 2) determined by fitting high-\( q \) region data to O-Z function gave, \( \xi = 12 \pm 2 \) Å much smaller than the persistence length of gelatin, which is \( \approx 25 \) Å\(^{-1}\). For gelatin coacervates, gels and sols the correlation length values reported [21] are 12 Å, 26 Å and 26 Å respectively (see Table-I). Size of heterogeneities reported for such systems were \( \approx 200 \) Å which is comparable to the size of the heterogeneities, \( \xi = 220 \pm 30 \) Å reported now. Thus, it can be inferred that the simple and complex coacervates of gelatin bear identical structural signatures. On the other hand, agar gels are associated with contrastingly different length scales; the correlation length and heterogeneity size are 59 Å and 700 Å respectively [29]. These values remained unchanged as the
concentration of CaCl$_2$ added to these gels was varied from 0.01 to 1M. The agar-gelatin samples did not exhibit any such dependence either.

**Figure 6.2:** Plot of reciprocal of static structure factor, $1/S(q)$ versus scattering wave vector, $q$ for agar-gelatin coacervates prepared in D$_2$O determined from SANS measurements; data is from Figure 1. Low-q data fitted to Debye-Bueche and high-q fitted to Ornstein-Zernike function. This defines a clear $q$-cut off.

### 6.2.2 Rheology data

Isochronal temperature sweep (at the rate 0.3°C/min.) measurements were undertaken on various samples to measure their melting behaviour. The first derivative (d$G'/dT$) of storage modulus ($G'$) with temperature (T) gave unambiguous values for two melting transition temperatures and the data are shown in Figure 6.3 for various salt concentrations. Let us compare these temperatures with the thermal properties of agar [30] and gelatin gels [31]. Agar has a gelation temperature $\approx 40^\circ$C and melting temperature $\approx 85^\circ$C. On the other hand, gelatin gels at $\approx 30^\circ$C. The data shown in Figure 6.3, thus, represents a linear combination of the thermal behaviour of agar and
gelatin gels. One can also argue that such behaviour owes it origin to the presence of gel-like (physical) network structures inside the coacervate phase. Agar and gelatin gels are known to comprise of hydrogen bond stabilized double [32] and triple helix [33] units that constitute the three dimensional interconnected network. The gelatin gels are rubbery and since, these molecules are rich in glycine, proline and hydroxyproline residues, inside the triple helix, the individual molecules feature in poly(L-proline II) (trans) helix conformation in contrast, agar gels are composed of micro-domains of polymer-rich spinodal phases that imparts it a non-rubbery structure [18]. The shear induced flow behaviour of coacervate samples were quite revealing. The shear rate dependent viscosity data of these samples is plotted in Figure 6.4 which reveals the non-Newtonian yield.

![Figure 6.3](image.png)

**Figure 6.3:** Plot of the first derivative of isochronal storage modulus in a temperature sweep experiment. Notice the near invariance of transition temperatures with ionic strength of the samples. It appears that the strong associative interactions present inside the dense medium are not effected by screening.
Figure 6.4: Plot of viscosity versus shear rate for samples prepared with different ionic strengths. The Carreau model provided excellent fitting to the data within experimental error. For clarity fitting is shown for one sample only having salt concentration =0.05M. The exponent, $k$ changed by less than 10% as the salt concentration increased from 0.01 to 0.1M.

In addition shear thinning behaviour was exhibited by these samples. The data presented in Figure 6.4 were fitted to Carreau model expression [34]

$$\eta(\dot{\gamma}) \sim (\dot{\gamma})^{-k}$$

with $k = 1.2 \pm 0.2$ independent of ionic strength. In fact, $k$ reveals the viscous response of the samples to applied shear: $k=0$ gives Newtonian, $k<0$ indicates shear-thickening and $k>0$ implies shear-thinning behaviour. Thus, shear-thinning features are clearly manifested in these samples. It is interesting to note that alike the SANS results, the rheology data did not exhibit any ionic strength dependence. Alcohol induced simple coacervates of gelatin formed due to self-charge neutralization has been shown to exhibit non-Newtonian behaviour in the past [21]. Thus, the agar-gelatin coacervate has rheological features not very different from that of gelatin coacervate indicating that the
Carreau model can be universally applied to describe such material. In contrast, the gels of agar and gelatin have been shown to exhibit viscoelastic response (see Table-I).

6.2.3 Differential Scanning Calorimetry data (DSC)

The DSC data pertaining to the coacervate samples prepared with different salt concentrations is displayed in Figure 6.5. These indotherms depict near invariance of data with sample ionic strength. The weak melting behavior observed at $T \approx 30^0C$ could be ascribed to melting of the network structures present inside the coacervate phase. The rheology data supported the existence of such network structures which did show a melting temperature around $32^0C$. Thus, the indotherms seen in Figure 6.5 can be identified as that corresponding to the gelation temperature of gelatin network. The area under the endotherm quantifies the enthalpy of the transition. A closer look at Figure 6.5 reveals that this enthalpy is invariant of ionic strength of the medium. A comparative data is presented in Table-I that distinguishes various phases of agar and gelatin. It is quite interesting to note that the endotherm observed at $T \approx 30^0C$ has direct correspondence to a similar melting temperature observed in gelatin gel and coacervate systems [21,31]. This can be safely attributed to the gelation temperature of gelatin.

In another study an exotherm was observed at the same temperature for agar gel samples that was attributed to local crystallization of helices in agar gels [35]. However, the agar gels, used by us, had two characteristic transition temperatures [30]: gelation temperature $\approx 40^0C$ and melting temperature $\approx 80^0C$. The agar-gelatin coacervate samples did not yield signature of such an exotherm. Thus, it appears that the thermal properties of this coacervate were completely governed by the same of gelatin regardless of the presence of agar that was expected to be strongly interacting with this polypeptide. Another anomaly pertains to the observation of invariance of the transition temperature with ionic strength of the coacervate. Gelation temperature of gelatin gels is strongly influenced by the nature and concentration of the salt present [36].

Table-I: Summary of physical characteristics describing various phases of agar and gelatin. Data reported pertain to $20^0C$ for samples prepared in aqueous medium (D$_2$O
for SANS experiments). The values listed are representative. The transition temperatures are associated with an uncertainty of ±3°C and the same with characteristic sizes is ±15% of the listed data, typically.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Parameter/Property</th>
<th>Agar Gel</th>
<th>Gelatin Gel</th>
<th>Gelatin Coacervate</th>
<th>Agar-gelatin Coacervate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Mesh size</td>
<td>59 Å c</td>
<td>26 Å a</td>
<td>12 Å</td>
<td>12 Å</td>
</tr>
<tr>
<td>2.</td>
<td>Size of heterogeneity</td>
<td>700 Å c</td>
<td>200 Å a</td>
<td>200 Å</td>
<td>220 Å</td>
</tr>
<tr>
<td>3.</td>
<td>First Transition Temp. (DSC)</td>
<td>41°C d, (30°C) g</td>
<td>28°C b</td>
<td>33°C</td>
<td>30°C</td>
</tr>
<tr>
<td>4.</td>
<td>Second Transition Temp. (DSC)</td>
<td>75°C d</td>
<td>—</td>
<td>—</td>
<td>75°C</td>
</tr>
<tr>
<td>5.</td>
<td>First Transition Temp. (Rheology)</td>
<td>33°C e</td>
<td>28°C d</td>
<td>34°C</td>
<td>33°C</td>
</tr>
<tr>
<td>6.</td>
<td>Second Transition Temp. (Rheology)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>80°C</td>
</tr>
<tr>
<td>7.</td>
<td>Viscoelastic feature</td>
<td>Viscoelastic</td>
<td>Viscoelastic</td>
<td>Viscous</td>
<td>Viscous</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td>( \eta(\gamma^<em>) \sim (\gamma^</em>)^k )</td>
<td>( \eta(\gamma^<em>) \sim (\gamma^</em>)^k )</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td>( k = 1.4 )</td>
<td>( k = 1.2 )</td>
</tr>
</tbody>
</table>

a: ref [21], b: ref [31], c: ref [29], d: ref [30] and e: ref [41], f: ref [36], g: ref [35]
Figure 6.5: DSC thermograms for agar-gelatin coacervates prepared in water. The rate of heating was $1^\circ C$/min. The initial concentration of each of these biopolymers was 0.1% w/v. Notice absence of any endotherm at the higher end of temperature. A single endotherm was located close to $30^\circ C$ invariant of ionic strength of the coacervate samples.

6.3 Conclusions

Agar-gelatin coacervate samples were probed by three techniques in order to determine the micro-structure of this phase. The experimental results reveal that the coacervate phase is a heterogeneous viscous material. The polymer-rich phase comprises physically crosslinked networks of agar and gelatin molecules. There is finite possibility of existence of asymmetric double helices of agar coexisting with triple helices of gelatin. The presence of inter-penetrating networks of these biopolymers can not be claimed with certainty at this stage. However, it will be appropriate to make a physical comparison between agar-gelatin complex coacervate with agar, and gelatin gels, and gelatin coacervates. Such a comparison has been provided in Table-I. The agar-gelatin coacervate has been found to retain the thermal properties of its
constituents to a remarkable extent. This indicates the presence of physically entangled networks of agar and gelatin in the coacervate phase. The viscoelastic response of this phase to external stress is dependent on the specific nature and density of these crosslinks. Thus, determination of the degree of helicity present in the coacervate phase will be of significant importance. Coacervates, like gels, are biphasic in nature comprising the solvated polymer and solvent in various structural forms. Thus, the exact determination of the amount of interstitial and free water present in this material is required to be evaluated which will provide a better understanding of the thermal and temporal stability of this material.

It is interesting to observe that gelatin coacervates (simple) and agar-gelatin coacervates (complex) share a generality as far as their microscopic structures are concerned, which is clearly evident from the data shown in Table-I. Though the characteristic temperatures differ by a few degrees, the rest of the physical signatures are identical. Thus it will be appropriate to argue that the microscopic structure of the coacervate material comprised of crosslinked polymer-rich zones separated by polymer-poor regions having characteristic viscoelastic length. Such systems are associated with two characteristic relaxation processes [37, 38]: one due to concentration fluctuation and another arising from viscoelastic relaxation. This has been adequately described by models and supported by experiments in the past. The model involves dynamic coupling between stress and diffusion in a complex and rigorous way [39,40]. Thus, the coacervate phase is in a dynamically evolving state that makes this system extremely interesting [41]. The results presented provide a significant insight into the distinctive microscopic features of this coacervate vis-à-vis gelatin and agar gels, and gelatin coacervates. It does not answer all the questions related to the structure of coacervates, yet it makes an attempt to give some foundation to its understanding.
6.4 References


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