Complex Coacervation and Overcharging during Interaction between Hydrophobic Zein and Hydrophilic Laponite in Aqueous Ethanol Solution

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ABSTRACT: In this paper, for the first time, we have reported the formation of complex coacervate during interaction between hydrophobic protein, zein, and hydrophilic nanoclay, Laponite, in a 60% v/v ethanol solution at pH 4. Dynamic light scattering and viscosity measurements revealed the formation of zein–Laponite complexes during the interaction between zein at fixed concentration, \( C_Z = 1 \text{ mg/mL} \), and varying concentrations of Laponite, \( C_L \) (\( 7.8 \times 10^{-4} \text{ to } 0.25\% \text{ w/v} \)). Further investigation of the zein–Laponite complexes using turbidity and zeta potential data showed that these complexes could be demarcated in three different regions: Region I, below the charge neutralization region \( (C_Z = 1 \text{ mg/mL}, C_L \leq 0.00625\% \text{ w/v}) \) where soluble complexes was formed during interaction between oppositely charged zein and Laponite; Region II, the charge neutralization region \( (C_Z = 1 \text{ mg/mL}, 0.00625 < C_L \leq 0.05\% \text{ w/v}) \) where zein–Laponite complexes form neutral coacervates; and Region III, the interesting overcharged coacervates region \( (C_Z = 1 \text{ mg/mL}, C_L > 0.05\% \text{ w/v}) \). Investigation of coacervates using a fluorescence imaging technique showed that the size of neutral coacervates in region II was large (mean size = 1223.7 nm) owing to aggregation as compared to the small size of coacervates (mean size = 464.7 nm) in region III owing to repulsion between overcharged coacervates. Differential scanning calorimeter, DSC, revealed the presence of an ample amount of bound water in region III. The presence of bound water was evident from the presence of an additional peak at 107 °C in region III apart from normal enthalpy of evaporation of water from coacervates.

INTRODUCTION

Compartmentalization has long been postulated as an important means of concentrating the substances crucial for the origin of life.1 This is driven by a spontaneous and self-assembly process by concentrating the substances like RNA, DNA and proteins. Spatial localization and concentration of a substance were very crucial during origin of life to start any biochemical reaction.2 One of the possible known route to achieve this was described as the coacervation phenomenon, which is a complex process of liquid–liquid phase separation. In the process, generally, two oppositely charged polymers like nucleotides, polypeptides, or lipids interact to give a polymer-rich phase called coacervates.3–6 Moreover, being stable over a wide range of physiochemical conditions, coacervates provide a suitable compartment to accumulate and up-concentrate different molecules.7,8 The role of coacervation was found to take place in the extracellular matrix (ECM) during the process of elastogenesis.9 During elastogenesis, tropoelastin undergo a coacervation process where they self-aggregate to give a concentrated and ordered structure.10–13 The coacervate phase was also found to occur during the interaction between polynucleotides and polypeptide.3,14 Coacervation also imparts its importance in many organisms for their survival. In the case of sandcastle worms and mussels, the coacervation phenomenon was found to play an important role in the formation of adhesive in a wet environment.15–18 The study of the beak of the humboldt squid (Dosidicus gigas) revealed that the gradient of a soft base to an exceptionally hard tip (rostrum) of the beak comes from the self-coacervation of the beak proteins.19

Apart from the role of coacervates in natural phenomena of biological system, coacervates showed a promising role in various applications, such as biomedicine,20 small molecule uptake,21 nanobioreactors,22 pharmaceutical and food industries,23 encapsulation of molecules,24 gene delivery,25 cartilage mimics,26 tissue culture scaffolds,27 and drug delivery vehicle.28 Researchers have focused a lot to explore the application of coacervates in the field of drug delivery. Li et al.29 showed the use of zein–chitosan complex coacervate particles in the slow release of curcumin. Zein–chitosan complex coacervation was...
studied by Ren et al. to investigate the effect of ultrasound frequency in the encapsulation of resveratrol. Thermodynamics and wetting kinetics of zein coacervate was studied by Li et al. Their study also revealed the formation of zein coacervate in a water/propanol glycol solvent and its ability to encapsulate limonene. Injectable hydrogel coacervate was used by Lee et al. for the delivery of anticancer drug bortezomib. Hieu et al. have reported iron cross-linked carboxymethyl cellulose complex coacervate beads for the sustained release of ibuprofen drug. Chenglong et al. reported a dextran-based coacervate nanodroplet as potential gene carriers for efficient cancer therapy. A water-soluble starch derivative anionic and cationic polymer that undergoes nanoparticle formation via coacervation was reported by Barthold et al. The group discussed the potential use of the nanoparticles in pulmonary delivery of protein/peptides. While exploring the efficiency of coacervates in drug delivery, a very interesting work was carried by Lim et al. They showed that a Humboldt squid beak-derived biomimetic peptide coacervate can be used for encapsulating insulin with high efficiency along with its controlled release. Chitosan-based coacervates for propolis encapsulation and its release and cytotoxic effect was reported by Sato et al.

The above literature suggests that coacervates can be potentially used as drug delivery systems. As far as zein is concerned, it has been used in adhesive, food industry, biodegradable plastic, delivery of small molecules, encapsulation of molecules, etc. The complex coacervation of zein with polymer-like chitosan, ds-DNA, etc. has been studied; however, complex coacervation of zein with a clay nanomaterial has not been studied and explored for its potential application as a drug delivery system. Future prospect of the zein-Laponite coacervates laid in the fact that both cationic and hydrophobic drugs could be loaded to these coacervates. In the coacervates, the cationic drug can be attached to the negatively charged surface of Laponite, while the hydrophobic drug can be loaded to zein via electrostatic and hydrophobic interactions, respectively. The coacervates thus will have the potential to carry cationic and hydrophobic drugs simultaneously and thus possibly can be used as a dual drug delivery system.

In this paper, we have studied the interaction between a nanoclay disc, Laponite, and a hydrophobic protein, zein. The interaction was studied at pH 4 where Laponite and zein exhibits negative and positive charges, respectively. The paper reports the formation of neutral coacervates and an interesting overcharged coacervates phase during the interaction between zein and Laponite.

### RESULTS AND DISCUSSION

Zein is a positively charged hydrophobic protein soluble in aqueous ethanol at pH 4, whereas Laponite is a hydrophilic particle soluble in water having a negatively charged surface at all pH. This property of zein and Laponite led to the complex formation between zein and Laponite in a 60% v/v aqueous ethanol solution at pH 4 via electrostatic interaction. The complex formation between the fixed concentration of zein, $C_Z$ (1 mg/mL) and varying concentrations of Laponite, $C_L$ was investigated using viscosity measurement, the dynamic light-scattering (DLS) technique, zeta potential, turbidity, imaging, and differential scanning calorimetric data.

**Viscosity Measurements.** Viscosity of zein (1 mg/mL) in a 60% v/v ethanol solution at pH 4 was observed to be 2.93 mPa s. The viscosity of Laponite in deionized water at various concentrations remained almost constant as shown in Figure 1. However, the viscosity of the samples having fixed zein concentration, $C_Z$ (1 mg/mL), and varying Laponite concentrations, $C_L$, showed a rise in viscosity. As the concentration of the Laponite increases in samples, we saw a rise in the relative viscosity values, which indicated the formation of large zein-Laponite complexes. The relative viscosity profile (Figure 1) for zein-Laponite complexes was fitted linearly, which gave two distinct regions. The two regions were demarcated by the change in the slope of the viscosity profile. In the first region ($C_Z = 1$ mg/mL, $C_L = 0.00078–0.0188$ w/v), the viscosity remained almost constant indicating the formation of small-sized zein-Laponite complexes. Beyond $C_L = 0.0188$ w/v, we saw a second region where linear fitting of viscosity data points had a positive slope. The positive slope indicated the formation of larger zein-Laponite complexes in samples having higher Laponite concentrations.

**Dynamic Light Scattering.** Dynamic light scattering, DLS, is used to calculate the size of the particle undergoing Brownian motion. For a dilute system of spherical and monodisperse particles undergoing Brownian motion, a field autocorrelation function, $g_1(\tau)$, in dynamic light scattering can be fitted with single exponential as given in eq 1.

$$g_1(\tau) = \exp(-q^2\tau)$$

where $D$ is the translational diffusion coefficient, $\tau$ is the delay time, and $q$ is the magnitude of scattering vector as given in eq 2.

$$q = \frac{4\pi n}{\lambda} \sin \frac{\theta}{2}$$

where, $n$ is the refractive index of the solution, $\lambda$ is the wavelength of laser, and $\theta$ is the scattering angle.

However, for polydisperse samples, the CONTIN method was used where the field autocorrelation function is given as

$$g_1(\tau) = \int_0^\infty G(\Gamma) \exp(-\Gamma \tau) \, d\Gamma$$

where $\Gamma$ is the decay constant and $G(\Gamma)$ is the decay rate distribution function obtained by performing inverse Laplace transformation on eq 3.

The mean size and polydispersity index (PDI) of the complexes formed during the interaction between zein and Laponite were calculated using the CONTIN method by the software in the dynamic light-scattering experiment. The mean
hydrodynamic diameter and PDI values of zein–Laponite complexes at various ĈZ were plotted and tabulated in Figure 2 and Table 1, respectively. The inset of Figure 2 showed the variation of mean hydrodynamic diameter of the complexes with their standard deviation.

![Figure 2. Variation of hydrodynamic diameter, d, of the zein–Laponite complex at different concentrations of Laponite, ĈL. The concentration of zein, ĈZ was fixed at 1 mg/mL. The Arrow indicates the concentration after which a drastic change in the hydrodynamic diameter was observed. Inset shows variation of d with their standard deviation.](image)

| ĈL (% w/v) | d (nm) | standard deviation/nm | PDI  |
|------------|--------|------------------------|------|
| 0          | 395.3  | 8.9                    | 0.548|
| 0.00078    | 439    | 16.69                  | 0.694|
| 0.00156    | 427.4  | 26.82                  | 0.713|
| 0.00312    | 559.8  | 78.9                   | 0.905|
| 0.00625    | 662.5  | 41.32                  | 0.717|

The variation of mean hydrodynamic diameter for zein–Laponite complexes at a fixed concentration of zein, ĈZ, and varying concentrations of Laponite, ĈL, was plotted in Figure 2. The figure suggested that the hydrodynamic diameter (d) of the complexes increased slowly up to ĈL = 0.00625% w/v, and then it rose drastically after ĈL > 0.00625% w/v. The drastic rise in complex size revealed the formation of larger complexes. Initially, up to five data points (Figure 2), the solution was visibly clear; therefore, we could be able to measure size using DLS. However, at the sixth data point (ĈL = 0.00625% w/v) and after that, the solution becomes turbid. The turbid sample suffers multiple scattering and does not give the correct size and so measuring the size using a turbid solution does not make any sense. However, we have taken the sixth data point just to demarcate region I (clear solution region) and region II (turbid solution region).

**Visual Inspection of the Zein–Laponite Complexes.** Zein–Laponite complexes for fixed ĈZ (1 mg/mL) and varying ĈL (7.8 × 10⁻⁴ to 0.25% w/v) formed in the 60% v/v aqueous ethanol solution at pH 4 was stored in sample vials at 25 °C. Figure 3 shows the picture of the sample vials that was taken after 24 h.

![Figure 3. Zein–Laponite complexes in 60% v/v aqueous ethanol solution at pH 4 after 24 h. Different regions based on visual inspection was classified as (a) Region I, ĈZ = 1 mg/mL, ĈL = 0.00078−0.00625% w/v, having smaller soluble complexes; (b) Region II, ĈZ = 1 mg/mL, ĈL = 0.0125−0.05% w/v, having a visually dense coacervate phase; and (c) Region III, ĈZ = 1 mg/mL, ĈL = 0.1−0.25% w/v, having a visually sparse coacervate phase.](image)

**Turbidity Measurements.** Figure 4 depicted the turbidity of the samples immediately (t = 0) after adding varying concentrations of Laponite, ĈL (7.8 × 10⁻⁴ to 0.15% w/v), to the fixed concentration of zein, ĈZ (1 mg/mL), in the 60% v/v ethanol solution. Stable soluble complexes with a slow rise in turbidity was observed for ĈL = 0.00078–0.00625% w/v, while a considerable increase in the turbidity was observed for ĈL = 0.025 and 0.05% w/v. At a further higher concentration of Laponite (ĈL = 0.1 and 0.15% w/v), the turbidity decreased drastically. Therefore, on the basis of variation of turbidity, different regions were classified as (i) Region I (ĈZ = 1 mg/mL, ĈL ≤ 0.00625% w/v), (ii) Region II (ĈZ = 1 mg/mL, 0.00625 < ĈL ≤ 0.05% w/v), and (iii) Region III (ĈZ = 1 mg/mL, ĈL > 0.05% w/v).

The study of turbidity as a function of time was done to further get insights on the complex formation. The change of turbidity with time gave information about the liquid–liquid phase separation phenomenon as depicted in Figure 5.

It was evident from Figure 5 that time-dependent turbidity for zein–Laponite complexes remained almost constant for region I (ĈZ = 1 mg/mL, ĈL ≤ 0.00625% w/v). In region II (ĈZ = 1 mg/
mL, 0.00625 < C_L ≤ 0.05% w/v), the turbidity grew much larger at the initial time followed by a decrease in turbidity with the passage of time. The reason for this behavior owes its explanation from the fact that at the initial time, the high values of turbidity indicated the formation of large-sized zein–Laponite complexes. However, with the passage of time, these complexes due to large size became unstable in the solution phase and undergo liquid–liquid phase separation (coacervation) indicated by the drop in the turbidity values. The phase-separated state in region II could be seen in Figure 3. In region III (C_Z = 1 mg/mL, C_L > 0.05% w/v), the initial value of turbidity decreased as compared to region II. Nevertheless, the turbidity remained almost constant for each C_L in this region for almost an hour, and we could not see a drop in turbidity till that time to indicate liquid–liquid phase separation phenomena. Instead of the above fact, liquid–liquid phase separation was observed in region III at a much longer waiting time with visually sparse density of coacervates (Figure 3). The reason for the large waiting time for the liquid–liquid phase separation (coacervation) and visually sparse density of coacervates in region III as compared to region II has been discussed in the Zeta Potential section.

Zeta, ξ, Potential. Zeta potential experiment was done to ascertain that zein–Laponite complexes were formed owing to electrostatic interaction between positively charged zein and a negatively charged Laponite surface in the 60% v/v aqueous ethanol solution at pH 4. Moreover, zeta potential data was also used to understand the reason for the fast coacervation process and the visually dense coacervate phase in region II as compared to the long waiting time for coacervation and visually sparse density of coacervates in region III.

The size of pure zein calculated from DLS measurement was nearly 400 nm, and the size of the zein–Laponite complex grew large, i.e., more than 7000 nm for C_L = 0.0125% w/v. For C_L > 0.0125% w/v, the samples grew turbid indicating the further large size of the complexes. Therefore, the Smoluchowski equation was used to convert electrophoretic mobility to zeta potential because this equation is used when κ_a ≫ 1, where a is the radius of the particle and κ^−1 is the Debye length. Zeta potential and the standard deviation for varying C_L in deionized water at pH 7 and zein–Laponite complexes having fixed C_Z (1 mg/mL) and varying C_L in the 60% v/v ethanol solution at pH 4 have been plotted and tabulated in Figure 6 and Table 2, respectively.

Figure 6 depicts that the ξ potential of zein was +23 mV at pH = 4 in the 60% v/v ethanol solution. With the increase in the concentration of Laponite, C_L, the zeta potential tends to decrease, which is further followed by charge reversal (over-charging). The plot of the zeta potential as a function of C_L suggested that Figure 6 could be segregated in three regions based on the zeta potential of the complexes. Region I (C_Z = 1 mg/mL, C_L ≤ 0.00625% w/v) consists of complexes that are not fully charge-neutralized and forms soluble and stable complexes. The solution phase in this region looked a little turbid due to
formation of complexes. The region II ($C_Z = 1$ mg/mL, $0.00625 < C_L \leq 0.05\%$ w/v) consists of complexes that are charge-neutralized, and therefore they tend to aggregate. The aggregates are unstable in the solution phase and undergo liquid–liquid phase separation to give coacervates. The coacervates in region II were dense as seen from naked eyes.

Beyond the charge neutralization point, region III ($C_Z = 1$ mg/mL, $C_L > 0.05\%$ w/v) was observed. This is the region where overcharging was observed. It was expected that beyond the charge neutralization point, if more complementary particles are added, further binding is not favored and the zeta potential would remain same. However, we saw charge reversal in zein–Laponite complexes when the excess of Laponite was added beyond the charge neutralization point. Many theoretical predictions and experimental data revealed that complementary particles can bind to the charge-neutralized complexes to give overcharged complexes, and this overcharging phenomenon is energetically favored. Various other studies revealed that non-DLVO (Derjaguin, Landau, Verwey, and Overbeek) interaction, such as charge patch interaction, was responsible for the overcharging phenomenon. The overcharged complexes in solution try to repel each other and therefore inhibit fast coacervation. Accordingly, zein–Laponite complexes before and after the charge-neutralized region was stabilized by electrostatic and charge patch repulsion, respectively. On the other hand, in the charge-neutralized region, electrostatic repulsion between complexes vanishes and van der Waals interactions dominate, which causes unstable dispersion and therefore rapid aggregation and coacervation. Nevertheless, overcharging that inhibited fast coacervation resulted in the visually sparse coacervate phase due to charge patch repulsion between complexes in region III as compared to the visually dense coacervate phase in region II due to aggregation of neutral zein–Laponite complexes.

**Imaging.** SEM images of complexes having different $C_L$ were shown in Figure 7. Figure 7a–c corresponds to $C_L = 0, 0.00156, \text{ and } 0.00312\%$ w/v, respectively, and belongs to region I. It could be seen that for $C_L = 0\%$ w/v, i.e., pure zein ($C_Z = 1$ mg/mL), the sample is polydisperse, and the size of zein varies from 94 to 360 nm. At higher Laponite concentrations ($0.00156$ and $0.0032\%$ w/v), samples remain polydisperse and the size of zein–Laponite complexes increased with the increase in Laponite
concentration. Figure 7d–f corresponds to $C_L = 0.025, 0.05,$ and $0.2\% \text{ w/v}$, respectively. Figure 7d,e belongs to region II where neutral coacervates were formed, while Figure 7f belongs to region III where overcharged coacervates were formed. The coacervate phase is a liquid phase with densely packed zein–Laponite complexes in a mobile state, and therefore, the dehydrated SEM images of the coacervates will appear as aggregates. The SEM image of aggregates in region III (Figure 7f) looked sparse with voids in the aggregate phase as compared to densely packed aggregates in region II (Figure 7d,e). The possible reason for this may be attributed to electrostatic repulsion between the overcharged complexes in the coacervate phase of region III.

It should be noted that SEM images were taken after dehydrating the samples, and therefore the coacervate phase looked like aggregates. The image of coacervates in the hydrated state was thus obtained using phase contrast imaging. Figure 8 showed the phase contrast image of coacervates in region II and region III.

Figure 8. Phase contrast image of coacervates and its size distribution for (a, d) $C_L = 0.025\% \text{ w/v}$, (b, e) $C_L = 0.05\% \text{ w/v}$, and (c, f) $C_L = 0.15\% \text{ w/v}$, respectively.

The image of bigger size coacervates in region II due to aggregation and smaller size coacervates in region III due to repulsion between overcharged complexes can be seen in Figure 8a–c, respectively. The average size of coacervates along with its size distribution for region II and region III was shown in Figure 8d–f, respectively. It is pretty clear from Figure 8d that exactly at the charge-neutralized concentration (Region II, $C_Z = 1 \text{ mg/mL}, C_L = 0.025\% \text{ w/v}$), we saw large coacervate particles with an average size of 1223.7 nm due to aggregation of neutral coacervates. However, if we slightly deviate from the charge-neutralized concentration but remained in region II (Region II, $C_Z = 1 \text{ mg/mL}, C_L = 0.05\% \text{ w/v}$), we saw lesser aggregation with an average coacervate size of 699.2 nm (Figure 8e). Nevertheless, in the overcharged region (Region III, $C_Z = 1 \text{ mg/mL}, C_L = 0.15\% \text{ w/v}$), aggregation was inhibited, and we saw smaller coacervates with an average particle size of 464.7 nm (Figure 8f).

**Differential Scanning Calorimeter.** Hydration of polymers was driven by interaction between polymer–water and water–water interactions. Water molecules that are not in the vicinity of the polymers interact with each other to give
water–water interaction. The water–water interaction between water molecules gives rise to bulk water. However, water molecules that are in the close vicinity to polymers render polymer–water interaction, and we call these water molecules as bound water. It was therefore felt important to understand the hydration behavior of coacervates in terms of bulk and bound water. The hydration behavior of coacervates in the two regions (region II and region III) was therefore studied using differential scanning calorimeter, DSC, as shown in Figure 9.

![Figure 9](https://pubs.acs.org/doi/10.1021/acs.omega.9b04647)

Figure 9. Differential scanning calorimetry (DSC) thermogram of zein–Laponite complex coacervates obtained from region II (C_{Z} = 1 mg/mL, 0.00625 &lt; C_{L} ≤ 0.05% w/v) and region III (C_{Z} = 1 mg/mL, C_{L} &gt; 0.05% w/v).

In region II, the neutral complexes form tight and close-packed aggregates of coacervates, and therefore a small area would be available for the water molecule to hydrate the densely packed aggregates of coacervates. We believe that because of this reason, the water–water interaction will be favored to give bulk water. The enthalpy for evaporation of bulk water in this region was observed between 90–100 °C as depicted in Figure 9. However, in the region III, the overcharged complexes in the coacervates repel each other. This repulsion will create voids and facilitate a large amount of water molecules to interact with the coacervate phase. The interaction will enrich polymer–water interaction to give a sufficient amount of bound water in region III. We believe that enthalpy of evaporation of these bound water gave an extra peak at 107 °C in region III.

**DLVO Theory.** Stability of charged particles and colloids in the solution phase has been well described by DLVO theory.\(^{65–70}\) According to DLVO theory, the stability of charged colloids or particles was governed by the sum of two forces, i.e., the electrostatic force and van der Waals force.

\[
F_T = F_E + F_V
\]

(4)

where \(F_T\) represents the total interaction force, \(F_E\) corresponds to the electrostatic force, and \(F_V\) is the van der Waals force.

For highly charged colloids or particles, the electrostatic repulsive force is more than van der Waals attractive force, and so the colloids/particles will remain stable in the solution phase. However, if some ions were added to screen the charged particles, then the van der Waals attractive force will dominate and particles will aggregate.

Nevertheless, some non-DLVO terms, such as hydrophobic and charge patch interactions, may exist in some colloidal systems, and so the total interactions in eq 4 should be modified. The modified interactions given by eq 5 gives extended DLVO theory.

\[
F_T = F_E + F_V + F_N
\]

(5)

where \(F_N\) represents forces arising due to non-DLVO terms.

It is to be noted that zein is a hydrophobic protein, and at a high laponite concentration, the zein–Laponite complexes acquire the charge reversal phenomenon (overcharged phenomenon) probably because of charge patch interactions. Thus, as far as zein–Laponite complexes are concerned, we believe that these complexes should follow extended DLVO theory to give liquid–liquid phase separation.

Nevertheless, the stability ratio \(W\) is often calculated to understand the aggregation process predicted using DLVO or extended DLVO theory.\(^{55,66,71}\) For \(W = 1\), the aggregation is diffusion limited; therefore, fast aggregation occurs, while values of \(W\) between 1 and 100 corresponds to the slow aggregation process. The stability ratio \(W\) using dynamic light-scattering (DLS) and static light-scattering (SLS) experiments was calculated using eq 6.

\[
W = \frac{k_{DLS}^{fast}}{k_{DLS}^{DLS}} = \left. \frac{\frac{d\theta(t)}{dt}}{\frac{d\theta(t)}{dt}} \right|_{t \to 0} \quad (fast)
\]

(6)

\(\theta(t)\) where, \(t\) is the time, \(k_{DLS}^{fast}\) refers to the rate constant of actual measurement, \(k_{DLS}^{DLS}\) refers to the fast rate constant, and \(R_0(t)\) is the hydrodynamic radius of the particle at time \(t\).

Literature\(^{5,46–49}\) suggests that the rise in turbidity can be hypothesized as the aggregation of inter- and intrapolymeric complexes in a cooperative manner. Thus, size can be directly related to turbidity, and therefore we can redefine our stability factor using turbidity data as eq 7.

\[
W = \frac{k_{DLS}^{fast}}{k_{DLS}^{DLS}} = \left. \frac{\frac{d\theta(t)}{dt}}{\frac{d\theta(t)}{dt}} \right|_{t \to 0} \quad (fast)
\]

(7)

\(A(t)\) where \(A(t) = 100\% - \theta(t)\), and the values of \(A(t)\) were obtained from Figure 5 at different \(t\), \(\frac{d\theta(t)}{dt} \left|_{t \to 0} \right. \) and \(\frac{d\theta(t)}{dt} \left|_{t \to 0} \right. \) refers to as the rate of change of turbidity of the actual measurement and the fast rate of change of turbidity, respectively, as \(t\) approaches zero. The values of \(\frac{d\theta(t)}{dt} \left|_{t \to 0} \right. \) for zein–Laponite complexes at different \(C_L\) was obtained from the slope of the straight line by fitting few initial data points of Figure 5.

Figure 10 depicted the plot of zeta potential and \(W\) for different zein–Laponite complexes, which was obtained from Figure 6 and eq 7, respectively. The plot indicated that at \(C_L = 0.025\%\) w/v, the value of \(W = 1\), which means that at this \(C_L\), the aggregation is diffusion limited and the aggregation process is fast. It should be noted that at this concentration, i.e., \(C_L = 0.025\%\) w/v, the zeta potential of zein–Laponite complexes goes down to almost zero. Figure 10 also suggests that \(C_L = 0.025\%\) w/v is the critical coagulation concentration, because at this concentration, we saw a transition between the slow (\(W\) between 1 and 100) and fast (\(W = 1\)) aggregation regime. Before and beyond \(C_L = 0.025\%\) w/v, the aggregation rate is slow because of the positively charged and negatively charged (overcharged) zein–Laponite complexes, respectively. The restabilization or slow aggregation process of particles in the presence of excess ionic liquids, polymers, surfactants, etc. has been associated with charge reversal or the overcharging phenomenon as reported in various studies.\(^{56–59}\)
Overcharging Phenomena in Coacervates. Coacervates formed due to zein and Laponite interactions can be broadly divided into two regions. These regions can be identified as at and after the charge neutralization point of zein–Laponite complexes, i.e., region II and region III, respectively. At the charge neutralization region, neutral complexes aggregate to form larger complexes. These large complexes become unstable in the solution to give a neutral coacervate phase via the liquid–liquid phase separation mechanism. Beyond the neutralization point, interesting overcharging behavior of the complexes was noticed. The overcharged complexes played an important role in suppressing the dynamics of coacervation due to electrostatic repulsion; however, at a sufficiently long time, we get overcharged coacervates. Thus, it felt important to compare different systems in Refs. [43,72–75] where complex coacervation and overcharging were observed due to intermolecular binding (Table 3). Various other studies are available in which layered double hydroxide [55,56] (diameter, 334 nm), latex [56] (diameter, 220 nm), Laponite [57] (diameter, 30 nm), halloysite [58] (length, 200–500 nm), and hematite [59] (diameter, 140 nm) particles have shown an overcharging effect in the presence of polyelectrolyte, ionic liquid, polymer, protamine, and surfactant, respectively. As mentioned in studies in Refs. [35–39,67] various reasons, such as hydrophobicity, charge patch interaction, chain length, etc., were responsible for the overcharging phenomenon. Interestingly, we may notice that in all the above cases, overcharging was observed when one molecule is stiffer than the counter molecule. Therefore, we believe that apart from various reasons cited above, relatively high stiffness of one molecule as compared to its partner molecule could be a possible reason for getting the overcharging phenomenon.

Future Prospect of Coacervates as Dual Drug Delivery System. The idea of encapsulating drugs in coacervates, Refs. [33,70–72] making films of coacervates Refs. [20,78,79] and loading cationic drugs in zein–polymer hydrogels Refs. [80,81] for the release of drug has been studied in many cases. The same idea could be used in our zein–Laponite coacervates for using it as a drug delivery system. Moreover, a combinatorial drug delivery system provides a therapeutic effect to overcome drug resistance along with lower toxicity and improved efficacy. Therefore, designing new vehicles to carry more than one drug at a time seems quite reasonable and promising. Zein–Laponite coacervates could be a possible solution for such a dual drug delivery carrier. Future prospect of zein–Laponite coacervates as a dual drug delivery system laid in the fact that both cationic and hydrophobic drugs could be loaded to these coacervates. In the coacervates, the cationic drug could be attached to the negatively charged surface of Laponite, while the hydrophobic drug could be attached to zein via electrostatic and hydrophobic interactions, respectively.

An important factor that determines the role of particles to be used as a drug carrier is related to its size. Refs. [81–83] Interestingly, we have seen that the size of our coacervates could vary over a large range (464–1223 nm) based on zein–Laponite interactions. Nevertheless, the size of zein–Laponite coacervates could further be tuned if we can vary the size of zein or the sample preparation parameter for Laponite. Different sizes of zein which depends upon the fabrication parameter, such as pH, solvent, and temperature, Refs. [84] and the sensitivity of Laponite towards sample preparation, Refs. [85] could affect the size of zein–Laponite complexes.

CONCLUSION

For the first time, the paper reports complexation between the hydrophobic corn protein, zein, and a negatively charged nanodisc, Laponite in a 60% v/v ethanol solution at pH 4. It was observed that electrostatic interaction between zein and Laponite at pH 4 was responsible for the complexation. The complexation between the fixed concentration of zein and varying concentrations of Laponite led to various phase states. It was observed that at a low concentration of Laponite, $C_L < 0.00625\%$ w/v, soluble and stable zein–Laponite complexes were formed. However, for the concentration range of 0.00625 < $C_L$ ≤ 0.05% w/v and $C_L > 0.05\%$ w/v, neutral charged and overcharged complex coacervates were formed, respectively. The neutral coacervates tend to aggregate to give large-sized coacervates, whereas overcharged coacervates have relatively smaller sizes due to repulsion between the coacervates. It was also revealed that in overcharged coacervates, bound water was responsible for giving an extra peak for the enthalpy of evaporation at 107 °C.

EXPERIMENTAL SECTION

Materials. Zein was purchased from TCI chemicals (CAS no. 9010-66-6), India and used as received. The specification sheet for zein reports that it has been obtained from corn with a

Table 3. Comparison of Binding Leading to Complex Coacervation in Diverse Systems

| S. no. | properties | GA + GB | GA + L | GA + DNA | zein + Laponite (this work) |
|-------|------------|---------|--------|---------|---------------------------|
| 1     | binding type | protein–protein | protein–colloid | protein–nucleic acid | protein–colloid |
| 2     | persistence length | 10 nm (GA) | 10 nm (GA) | 10 nm (GA) | 2 nm (Z) |
| 3     | zeta pot. ratio | 2 (GB:GA) | 5 (L:GA) | 16 (DNA:GA) | 1 (Z:L) |
| 4     | overcharging | absent | absent | present | present |
| 5     | pH | 6.5 | 7.2 | 6.0 | 4.0 |

GA, GB, Z, and L represents Gelatin A, Gelatin B, Zein, and Laponite, respectively.
total nitrogen content of 14% and 0.2% drying loss. LAPONITE RD (Laponite) (Lot no. 0001603378, BYK-Additives and Instruments) was received from Aroma Chemical Agencies (India) Pvt. Ltd., New Delhi, as a gift. As indicated by the BYK datasheet, Laponite appeared as free flowing white powder having a bulk density of 1000 kg/m$^3$ and a surface area of 370 m$^2$/g. The technical datasheet from BYK additives and Instruments states that it is a synthetic clay having an empirical formula of Na$_{0.5}$[Si$_4$Mg$_6$Li$_3$O$_{20}$(OH)$_4$]$_{0.5}$ and is in the form of disc-shaped crystals with a diameter of 25 nm and thickness of 1 nm. Absolute ethanol was purchased in Labogen Pvt. Ltd., India.

**Preparation of Zein–Laponite Complexes.** Stock solution of zein was prepared by dissolving a known amount of zein in 80% v/v ethanol solution. The stock solution of zein was maintained at pH = 4 using 0.1 M HCl. The obtained stock solution of zein, which appeared clear, was filtered with a 0.2 μm syringe filter. Laponite powder was dried in an oven for 4 h to remove moisture. The dried Laponite was then stirred in a known amount of deionized water (pH = 7) to get a clear stock solution. The stock solution of Laponite was also filtered with a 0.2 μm syringe filter. Finally, series of samples were prepared by mixing stock solution of zein and stock solution of Laponite in a known volume while maintaining the pH of the solution at 4 using 0.1 M HCl. The series of samples thus prepared should have a fixed concentration of zein, C$_z = 1$ mg/mL, and varying concentrations of Laponite, C$_l$ (7.8 × 10$^{-4}$ – 0.25% w/v) in a 60% v/v ethanol solution at pH 4. All the samples were prepared at room temperature, 25 °C.

**Instrumentation and Characterization.** Samples were analyzed with a Zetasizer Nano-ZS instrument (Malvern Instruments Ltd., India) for the mean particle size and for the zeta potential. Dynamic light scattering measurements were analyzed with a Zetasizer Nano-ZS instrument (Malvern Instruments Ltd., India) for the mean particle size and for the zeta potential. Slow condense matter laboratory, Centre for Interdisciplinary Research In Basic Sciences, Jamia Millia Islamia, New Delhi 110025, India; orcid:0000-0002-8646-4724; Phone: +91 8130792962; Email: narfin@jmi.ac.in

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**Notes**

The authors declare no competing financial interest.

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