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Exploring the effect of experimental conditions on the synthesis and stability of alginate–gelatin coacervates

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Abstract

Alginate–gelatin coacervation has been studied by considering different experimental parameters, such as gelatin preheating, pH, alginate–gelatin ratio and their respective concentrations, and salt effect. Results were assessed in terms of size and polydispersion via dynamic light scattering, electrostatic charge in the surface by zeta potential measurements, electrostatic interaction forces by static light scattering, stability by turbidimetry and viscoelastic and pseudoplastic behavior by rheology (oscillatory and statistical analysis). According to the results, gelatin structure has to be previously modified to induce the proper interactions with a subsequent pH reduction. Specifically, stable coacervates (according to turbidimetry and dynamic light scattering) with a size of 300–600 nm and a polydispersion lower than 0.25 were obtained after preheating the gelatin at 37°C and with a subsequent pH reduction until 4–5 for an alginate–gelatin ratio between 1:4 and 1:6. However, different experimental conditions promote an unsuccessful coacervation, obtaining always precipitates and/or coacervates with a wider particle size distribution. Furthermore, in order to study the effect of the temperature on the coacervates, different cooling–heating cycles were applied on them over a week, showing the stability of the thermo-reversible coacervates for almost 5 days. Also, the interactions were characterized via static light scattering, analyzing the second virial coefficient. Moreover, rheological oscillatory results can be used to identify a proper coacervation due to the increase of the storage modulus. However, no significant changes were observed with statistical analysis due to the highly diluted character of the precursor solutions. These results highlighted how a proper combination of different experimental conditions, mainly temperature to promote a partial gelatin unraveling as well as pH reduction, is required to successfully produce coacervates. Finally, salt effect was proven to induce precipitation when NaCl was increasingly added to solutions of stable coacervates.

KEYWORDS
alginate, coacervates, gelatin, rheology

1 | INTRODUCTION

Complex coacervates are liquid–liquid separation systems held together mainly by electrostatic interactions between polyelectrolytes. They present as spherical droplets and resemble the form of dispersant emulsion with a size range from nanometers to micrometers.[1–4] The formation of these structures consists of a fundamental physico-chemical phenomenon, presented in many biological processes like protein transcription, antigen–antibody reactions, or enzymatic channeling.[5,6] In fact, this phenomenon has different mechanisms to assemble the condensed polymeric molecules into a liquid. The first processes observed occurred in solutions of oppositely charged biopolymers and solutions of proteins with polyphenols.[7,8] This coacervation mechanism is complex coacervation, which consists of the complexation of oppositely charged molecules. Alternative mechanisms of complexation include the simple coacervation, which involves single self-associating molecule, and the autocomplex coacervation, which associates groups of ampholytic polymers.[9,10]

Coacervates have a potential use in biomedicine, concerning stimuli-responsive drug delivery systems (mainly pH and temperature) because its formation is strongly dependent on the different protein conformations, polymer networks, modifications in its surface charge, etc.[11–14] As an example, they were used in tests of insulin release for diabetes treatment with pH-response (stable coacervates between pH 7.4 and 9.5) of histidine-rich beak proteins (DgHBP-s peptide). Coacervates were synthesized to encapsulate insulin and glucose oxidase.[15] The glucose oxidase reacts with glucose to produce gluconic acid, so the pH will decrease and will trigger the insulin release. Another work proposed a temperature-responsive coacervate system between elastin-like polypeptides and hyaluronic acid. According to the results, a successful coacervation was produced at different temperatures (ranging from 32 to 42°C) depending on the ratio polypeptide/hyaluronic acid, forming particles between 400 and 600 nm.[16] In this context, several reviews have been published concerning the potential of the coacervates for similar biomedical applications.[17–24]

One of the most promising coacervate is the one formed between the protein gelatin A and the polysaccharide alginate. Gelatin A is a collagen derivative, which properties are defined by its extraction source. Its peculiar conformation can be modified with the temperature.[25–27] If a gelatin solution is made at ambient temperature (25°C), the protein coils assemble into triple helices.[28] Alginate, additionally, is an anionic polysaccharide, constituted by β-D-mannuronate and α-L-guluronate residues.[29] Alginate is usually obtained from brown algae and due to its biocompatibility, it is proposed as a polymer for biomedical applications.[30–35] Therefore, coacervates alginate/gelatin A have different advantages, such as biocompatibility, biodegradability, as well as the use of economic and accessible polymers that do not require previous chemical modifications. This is prevented because the coacervation is performed by changing pH, volume ratio, or concentrations to modify gelatin A structure and alginate density charge.[36,37] All of these physicochemical properties enable this system to be used in proteins and factors delivery and release for different chronic wound treatments.[38]

As a consequence, several works have studied the coacervation between alginate and gelatin A. This system also shows different bioapplications suitable for tissue engineering or drug delivery systems, as gelatin A and alginate can form hydrogel matrices. Several studies shown in references comment on this issues. For instance, Serafin et al.[39] characterize these hydrogel matrices combined with hyaluronic acid (HA) as well. The combination of gelatin and HA resulted to provide the longest term swelling properties with a minimal swelling after 168 h, with controlled morphology and mechanical properties, potentially useful for tissue engineering treatments.

Zamboni et al.,[40] on the other hand, used a gelatin–alginate hydrogel to implement polylactic acid loaded with curcumin. This caused an improvement to the compressive strength and mechanical performance of the hydrogels, without influencing the swellability or degradation capacities of these. Also, these gels showed a positive effect on cell growth of fibroblast as well as preserving the viability of monocytes.

Lau et al.[38] reported, based in turbidity and dynamic light scattering (DLS) studies, that the best experimental conditions for this coacervation were a gelatin A/alginate volume ratio of 4:1 at ambient temperature, a pH around 5, and concentrations of 0.5% w/w for both polymers. This means the coacervation was performed with a gelatin A structure in form of coils, without its partially unraveling. Therefore, the coacervation was mainly based on the interactions between the coils and the alginate chains due to the change of pH.

The results reported particle size always around 1 micron, and with high values of polydispersity index (PDI). Nevertheless, that work was focused on the encapsulation of bovine serum albumin in the coacervates, which was performed successfully with a great encapsulation yield and a proper release. Also, Kim and co-workers[41] encapsulated fibroblast growth factor in this coacervate. They studied different parameters, such as gelatin molecular weight, different ratios gelatin/alginate (from 0.5 to 2.0% w/w) and pH, on the coacervates formations and encapsulation yield. Again, in spite of the high encapsulation yield and the positive results of in vitro assays for a potential wound healing application, the particles obtained reported size of around 1 micron and with a wide range of PDI (higher than 0.4).

Finally, Saravanan and Rao[42] encapsulated three different drugs in alginate–gelatin and pectin–gelatin coacervates, modifying the pH with different acids. They obtained proper values of encapsulation for diclofenac sodium and indomethacin, but both systems were not adequate to encapsulate metronidazole hydrochloride. This might be caused from the escape of drug molecules from the coacervation phase, since this drug is highly soluble in water. Supporting previous results, pH values higher than 5 were most likely to induce precipitation, and the ones lower promoted the electrostatic interactions between the polymers to form the coacervates.[38]

Moreover, when reducing pH to optimize coacervates synthesis, authors found that some acids, such as chlorhydric acid, do not only induce coacervation, but also cause partial precipitation of free alginic acid from sodium alginate solution when adding such strong acid. In this context, acetic acid is the preferred compound for this purpose.[42] Besides, this article highlighted that alginate–gelatin coacervates are

smaller and form less aggregates than the pectin–gelatin. However, the size was always higher than 50 microns and with a high value of PDI.[42]

Previous articles were mainly focused in the potential of the coacervation to encapsulate different compounds, without performing a comprehensive study of the interactions alginate–gelatin A. These interactions in aqueous solutions were studied in more detail for alginate with fish gelatin,[43] and the results confirmed that the pH is a key parameter to set a boundary (pH around 5.4) between the proper interactions to induce coacervation and to form insoluble complexes. Moreover, these interactions between these compounds are pH-reversible and can be modified with the addition of NaCl.[43] Salt concentration is also another important parameter to take into account when studying this electrostatic interaction between gelatin A and alginate.

Based on the previous results, it can be concluded that it is difficult to control the alginate–gelatin interactions to promote the formation of stable and monomodal distributed coacervates with a low value of PDI, as well as to obtain particles smaller than 800 nm. Moreover, previous studies did not consider the effect of the temperature, and all the coacervates were synthesized at 25°C. At this temperature, gelatin is organized in a coiled structure with triple helices in fibrous form. But, after increasing the temperature, this conformation changes[44] and therefore, the available charges to promote interactions with alginate are different. In fact, this strategy has been used in several occasions to obtain thermo-reversible and thermo-responsive nanogels for different applications.[43-45] It is these thermo-sensitive and thermo-reversible property of the gelatin A that can contribute to optimize the coacervate synthesis.[46,47] This study of the gelatin A structure, and its modification by temperature, has not been used yet to obtain a monodisperse and smaller coacervates system.

Besides, to the best of our knowledge, no rheological studies have been performed for this system in aqueous solutions. This study might be useful to elucidate if the interactions are strong to form a solution with solid-like behavior, or even to change its pseudoplasticity. This rheological study has been previously performed for different systems of mixtures of polysaccharide-gelatin.[48–52] As an example, rheology measurements of a mixture gelatin/HA indicated that, although gelatin can produce an entanglement effect and increases viscoelasticity, the behavior of HA predominates in a solution of both components.[48]

Additionally, the interactions between gelatin A and alginate can be further characterized through a static light scattering (SLS) measurement. This technique has not been used in references to report the characterization of coacervates, or the experimental condition effects on their synthesis. This might be due to the broad distribution and polydispersion index they have in particle size for the referenced results. This is another approach to understand coacervation mechanism in this study.

Therefore, the aim of this work is to perform a comprehensive study of the complex coacervation of alginate–gelatin A, based on DLS, SLS, optical microscopy turbidity, and rheology to identify the best conditions in terms of particle size, PDI, stability, salt concentration effect, viscoelastic behavior, and force in electrostatic interactions. Gelatin A is the chosen gelatin due to its isoelectrical point (IEP), which is affected by the acid pH to have a positive net charge.[53] This positive charge interacts better with the anionic polymer in order to create stronger electrostatic interactions and therefore more stable coacervates. Parameters, such as pH between 4 and 6[38,40] (not lower than 4 to avoid alginate dissociation), modified with acetic acid, gelatin–alginate volume ratio, and concentration will be studied.

Moreover, it is proposed that the gelatin A conformation in the coacervation process determines the coacervate stability and size. Since its conformation is inherently related to temperature, it is important to study how this process variable affects the coacervation mechanism. Also, the influence of a cooling–heating cycle is considered to determine the thermo-reversibility of these interactions. Finally, rheology measurements, oscillatory, and steady are performed to elucidate if the coacervates behavior is mainly solid, viscous, or if there is crossover between both regimes.

2 | EXPERIMENTAL

2.1 | Materials

Alginic acid sodium salt from brown algae (CAS 9005-38-3) with an average molecular weight around 150 kDa and a mannuronic acid to guluronic acid ratio of approximately 1.56, and gelatin from porcine skin powder type A (CAS 9000-70-8) with an average molecular weight of 163 kDa, with a gel strength of approximately 300 g Bloom were purchased from Sigma–Aldrich, Madrid, Spain. Also, acetic acid, glacial, ACS, 99.7+% was purchased from Thermo Fisher Scientific, Kandel, Germany. Moreover, a heating and stirring plate purchased from Fisher, Madrid, Spain, and an orbital shaker incubator ES20 from Biosan, Madrid, Spain, were used in order to keep coacervates at 37°C when necessary. Finally, a pH-Meter BASIC 20+ model purchased from Crison, Barcelona, Spain.

2.2 | Coacervates formation

Coacervates were synthesized by mixing two different aqueous solutions of gelatin A and alginate stirred overnight. Two temperatures (25 and 37°C) were used to produce the coacervation phenomenon. In this context, when the temperature was 37°C (this temperature was chosen because it is the physiological temperature), the gelatin solution was previously heated at 37°C to partially unravel its structure, using the heating stirring plate and controlling the temperature with a thermometric probe. After that, this temperature was kept constant during the mixing process. In this context, Figure 1 illustrates the different gelatin conformations depending on the pH and temperature. In this case, the selected experimental conditions indicated that the gelatin was found in fibers or partially unraveled.

Once alginate and gelatin A solutions were mixed following the proposed volume ratio (1:4–1:6), the pH was adjusted to

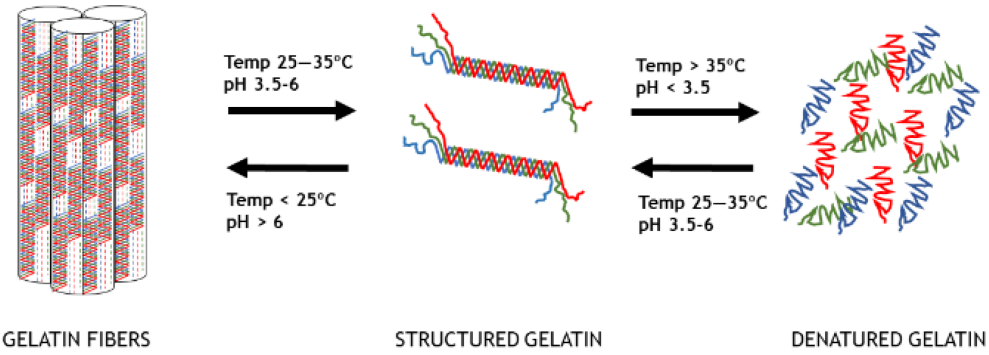

FIGURE 1 Gelatin structure configuration changes when controlling the temperature and pH-medium (adapted[42]).

TABLE 1 Dynamic light scattering results.

Run	Alginate conc. (% w/w)	Ratio alg–gel	pH	T (°C)	Z aver. (nm)	PDI
1	0.5	1:4	4	25	PF(*)	-
2				37	612 ± 205	0.21
3			5	25	402 ± 215	0.68
4				37	553 ± 182	0.17
5		1:5	4	25	PF(*)	-
6				37	750 ± 175	0.24
7			5	25	797 ± 224	0.63
8				37	561 ± 159	0.14
9		1:6	4	25	565 ± 214	0.69
10				37	685 ± 187	0.25
11			5	25	642 ± 234	0.59
12				37	360 ± 154	0.25
13	1.0	1:5	5	25	PF(*)	-
14				37	PF(*)	-
15	2.0	1:5	5	25	PF(*)	-
16				37	PF(*)	-

Note: (*) being PF precipitate formation.

the desired value with 0.5, 0.1, and 0.01 M solutions of acetic acid in order to prevent dilution problems while adjusting the desired pH values. This pH was selected between 4 and 6 due to the isoelectric point of the gelatin A, and the alginate pKa value, as stated previously. Coacervates samples were put under stirring with continuous pH measurement mode on pH-meter probe (previously calibrated with tampon solutions). Using this continuous measurement mode, it was possible to slowly add drops of the acetic acid solutions until the desired pH value was reached. Once it was adjusted, the coacervates samples were left under orbital shaking for 30 min at 25 or 37°C using an incubator.

In this context, the concentration of the gelatin A was always 0.5% w/w because a higher concentration of the gelatin did not produce a successful coacervation according to references.[38–40] Also, for a higher concentration of alginate (1.0% w/w and 2.0% w/w), only an alginate–gelatin A ratio of 1:5, since it was the optimal, as shown in Section 3. Finally, in order to study the thermo-reversibility of the heated coacervates at 37°C, the solution was cooled until 25°C, and then heated again until 37°C, keeping the pH constant. Table 1 (in results and discussion section) summarizes the experimental conditions of coacervation.

2.3 | Characterization in terms of optical microscopy, dynamic and SLS, and zeta potential measurements

Particle size and the aggregation of the coacervates are determined by DLS technique with a Zetasizer Nano (Malvern Instruments). Automatic attenuation was selected, and results were interpreted by number of particles per measurement. The peak integration method used by the equipment was the standard one. Moreover, the measurement angle was set at 173°. This same equipment was used to measure the zeta potential of the coacervates.

Moreover, coacervate samples synthesized at both temperatures (25 and 37°C) were taken to an optical microscope (Leica Optical compound light microscope DM1000) and were observed using the objective 100× and immersion oil. Single drops were poured in slides and adjusted first with the 40× objective. Afterwards, this objective

was removed and a drop of immersion oil was poured on the slide in order to create a film between it and the 100× objective that enables the extended zoom of the sample due to the change of the refractive index of the light in the medium (1.33 in oil and 1.00 in air). Once light and lenses were re-adjusted for the new objective, pictures were taken in IC Capture 2.4 software and further measured by IC Measure software, using a DFK MKU130 microscope camera of 13 megapixel.

SLS experiments were performed with the same Zetasizer Nano mentioned previously. This time the second virial coefficient (A2) was calculated. This parameter will characterize the electrostatic interactions that hold the coacervate system.

A2 is a thermodynamic parameter, which describes colloids stability as a balance between attraction and repulsion forces between the particles chains and the solvent. It is determined by the slope in the Debye equation, shown below:

$$\frac{K \cdot C}{R_\theta} = \frac{1}{MW} + 2 \cdot A2 \cdot C, \quad (1)$$

where K the Debye constant, C the sample concentration (mg/mL), R_θ the Rayleigh ratio (cm^{-1}), MW the average molecular weight (kDa), and $A2$ the second virial coefficient (mL mol/g).

Two series of coacervates were synthesized: One of them at 25°C while the other one was synthesized at 37°C, with pre-heated (partially unraveled) gelatin A. Experiments were done with coacervate solutions (1:5 and pH 5, for being optimal parameters) with the following concentration range: 1.5–6.5 mg/mL. This range allows to characterize the promotion of electrostatic interaction between gelatin A and alginate with increasing concentrations, until visible precipitation (higher than 6.5 mg/mL). The solutions were made following the previous procedure.

2.4 | Turbidity

Coacervates turbidity was measured by transmittance using a TurbiScan MA Classic model (Formulaction Scientific Instruments, France). The TurbiScan measures a 7.0 mL sample in the specific transparent lab tube inserted with its lid on. The backscatter sweep was also taken with every analysis of simple turbidity, determining whether the measurement was accurate or unstable with noisy signals.

This instrument was also used to characterize the salt concentration effect on coacervates synthesis. Coacervates at both synthesis temperatures (25 and 37°C) were added a small concentration of salt NaCl (50, 100, 150, and 200 mM) and their turbidity was measured to characterize the salt addition effect.

2.5 | Rheological analysis

The rheological properties of the coacervate samples were studied with a Rheometer AR 1500ex (TA Instruments, Madrid, Spain), equipped with an aluminum plate of 4-cm diameter and 1-mm gap. Both statistical and oscillatory flow analysis were performed. Coacervates were loaded on the Peltier and the temperature was set to the desired value, depending on the cold or heated formulation, at 25 and 37°C, respectively.

Statistical flow analysis consisted of a shear rate ramp from 0.1 to 200 s^{-1}. The controlled variable was the strain percentage at 2.0%, that was previously determined with a strain sweep at a frequency of 1 Hz. Afterwards, the results were fitted with the Cross model (Equation 1) obtaining as a result the different parameters of the equation.

$$\eta = \eta_\infty + \frac{\eta_0 - \eta_\infty}{1 + (K \times \dot{\gamma})^m}, \quad (2)$$

where η_0 the zero-shear-rate viscosity (Pa·s), η_∞ the infinity shear viscosity (Pa·s), K the consistency (s), and m the rate constant (dimensionless). All of these terms in the equation provide information about the rheological behavior of the solution, and therefore, the interactions within the system and its physico-chemical properties. Specifically, m is related to the power law relation between shear rate and shear stress in the shear thinning region, being $m = 0$ characteristic of Newtonian solutions, and K indicating the relaxation times, being its reverse the critical strain rate ($\dot{\gamma}_c$).

Oscillatory analysis was also performed on the samples with frequency sweeps, determining the evolution of the storage/elastic modulus (G'), and the loss/viscous modulus (G'') throughout the range of angular frequency from 0.5 to 500 s^{-1}. This evolution will indicate if the behavior of the coacervates is elastic- or viscous-like.

3 | RESULTS AND DISCUSSION

3.1 | Dynamic and SLS results

First results are concerning the effect of the different parameters on particle size, PDI, and stability of the coacervates and their electrostatic interactions. In this case, although not included in Table 1 (experimental conditions and DLS results), first experiments were performed with different ratios alginate–gelatin at 25°C with a pH of 7.

As was expected, precipitates were observed (Figure S1) due to the lack of required interactions alginate–gelatin A.

The coacervation was only produced after reducing the pH until acidic values (4–6) at 25°C, obtaining Tyndall effect (Figure S2). However, the PDI was always higher than 0.6 (as shown in Table 1), indicating the existence of at least a bimodal distribution (and sometimes even precipitates were observed). This is illustrated in Figure 2A,B for runs 7 and 11, respectively. This PDI, in spite of the successful coacervation, was always very high and strongly independent of the pH values (ranging from 4 to 5) and of the alginate–gelatin volume ratio. This phenomenon indicates an incomplete coacervation.

These results agree with the literature, since it is required an acidic pH around 4–6 to obtain coacervates.[42] The existence of a

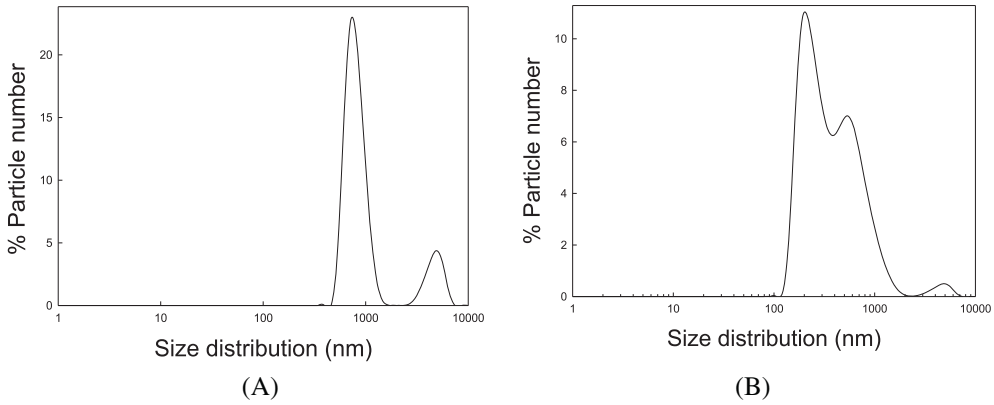

FIGURE 2 Bimodal particle distribution of (a) 1:5 and (b) 1:6 alginate: Gelatin ratio at pH 5 and 25°C.

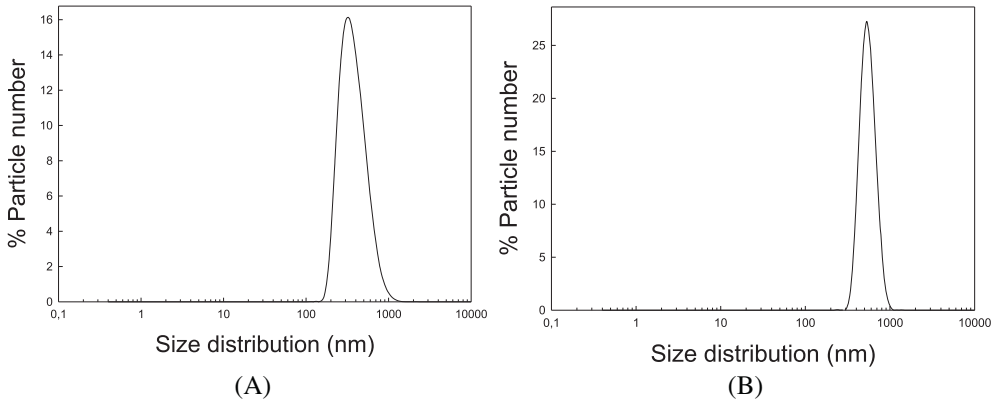

FIGURE 3 Unimodal size distribution for (a) 1:5 and (b) 1:6 alginate: Gelatin ratio at pH 5 and temperature 37°C with pre-heated gelatin at 37°C.

multimodal particle size distribution is explained due to the structure of the gelatin A at 25°C (fibers), which can partially prevent the interactions between all the available charges.

In order to test if the cause of the high PDI is the gelatin structure, same experiments were performed after heating the gelatin A solution until 37°C. This promotes its conformation change from fibers to a more unraveled structure, and a better mixing with the alginate, stablishing interactions at 37°C and pH reduction, with more functional groups available. This change induced the coacervation with reduced PDI values until 0.25 and lower (also shown in Table 1). This indicated the existence of a monodisperse particle size distribution, as illustrated in Figure 3A,B for the runs 8 and 12, respectively. In fact, the obtained size and PDIs were reduced in comparison with other results in literature.[38–40]

Moreover, the effect of the temperature was also proven to give better results when studying the autocorrelation function of the zeta sizer measurements (Figure S3).

Therefore, controlling the temperature is crucial to promote a monomodal coacervate system. In fact, some changes observed were also macroscopically visible, due to the correct synthesis in certain coacervates runs from precipitation at 25°C to a homogeneous sample at 37°C showing Tyndall effect (Figure S4).

Also, reaching a pH lower than 6 is a key parameter due to the isoelectric point (IEP) of the gelatin A. Values of pH under the IEP make the net charge of the protein (gelatin A) positive. In contrast with the negatively charged behavior of the alginate functional groups in aqueous solutions, this promotes the electrostatic interaction between both components, inducing coacervation.

Consequently, the higher the pH value is, the less coacervates will be formed, and the more unstable the system will be. Although the best results were obtained at a pH 5, there is not a clear effect of the pH in the particle size detected by DLS, since particle sizes are always between 400 and 700 nm in number measurements. Let it be noted that intensity and volume measurements did show similar results that the ones in number, and therefore, no added commentary on these measurements is made (see Figure S5). These particle distributions are explained by the gelatin A structure mentioned in the introduction. A solution with a homogeneous distribution of unraveled gelatin with more functional groups available for electrostatic interactions promotes the formation of similar size coacervates with the alginate.

A similar phenomenon was observed for the alginate–gelatin A ratio as long as the ratios are from 1:4 to 1:6 (particles always between 400 and 700 nm). Results indicated that ratios alginate–gelatin A below 1:4 and higher than 1:6 are not suitable to induce coacervation (Figure S3). On the one hand, high values of PDI were obtained for a ratio lower than 1:4 because there are not enough gelatin A functional groups available to interact with the alginate, and this allows the water to interfere with the coacervation phenomenon.[38]

On the other hand, due to the opposite effect, aggregates were found for a ratio higher than 1:6. The concentration of gelatin A at

this point is high, inducting these gelatin structures to bond amongst them, reorganizing the configuration to a more complex, non-soluble structure.[38] This phenomenon can be observed in the Figure S6.

Furthermore, additional experiments were performed to induce the coacervation with higher concentrations of alginate and gelatin A. Runs 13–16 indicate the formation of precipitates at these experimental conditions. This was attributed to the number of electrostatic interactions between both polymer–protein and protein–protein, promoting the formation of a high number of coacervates with a great difference in size.

Observing the improvements in results when pH and temperature act separately, it is clear that both parameters must be considered to induce a monomodal and stable coacervate system, heating up the solutions to partially unravel the gelatin structure, and lowering the pH values. These conditions will promote the electrostatic interactions between the functional groups of the partially unraveled protein and the alginate. Therefore, it can be concluded that it is possible to synthesize coacervates in a size range between 350 and 700 nm approximately with low PDI values, as long as temperature and pH are both controlled simultaneously.

Concluding, the best volume ratios are 1:4 or 1:5, due to the lower PDI values. Heating should be always applied (37°C), and pH values should be lowered to a value that ranges from 4 to 6 to start the coacervation. In order to further prove this point and study the temperature effect, optical microscopy was used to characterize two samples of the coacervates: 1:5 volume ratio, pH 5 and both synthesis temperatures (25 and 37°C).

Both of these samples were taken to an optical microscope and clear differences between their structures were observed. These images can be found in the Figures S7 and S8. Coacervates at 25°C showed a more polydisperse conformation in Figure S7a, with various size ranges of particles and even aggregates in certain zones, which are part of the gelatin A structure that has not been unraveled and therefore has not been able of stablish electrostatic interactions with alginate groups.

However, 37°C sample showed a more monodisperse particle distribution in Figure S7b, pointing that temperature effect is, once again, essential in order to obtain a homogeneous particle distribution due to a more complete coacervation process between the partially unraveled gelatin A structure and alginate. Nevertheless, it can also be observed that the image of coacervates synthesized at 25°C shows a higher definition of the particles than the 37°C synthesized coacervates sample. This is due to the difference in particle size slightly interfering with the detection limit of the optical microscope, and it is further proven in Figure S8. This figure shows the particles detected in both samples at 25°C (Figure S8a) and 37°C (Figure S8b) measured by the software mentioned previously. In this, image is verified that the samples do have the same size particle distribution detected by the DLS, and also further proves the monodispersity of those coacervates synthesized at 37°C, with a high percentage of the measurements entering the particle size 400–600 nm.

All of these results also confirm that we have achieved smaller coacervates droplets that the ones reported in literature by the same technique.[54] However, it was observed that after cooling, PDI and size of the coacervates were wider. Therefore, the effect of the temperature on the coacervates was also tested by carrying out daily repetitive cooling–heating cycles from 25 to 37°C during 7 days at a constant pH 5. The results showed that PDI values were kept still throughout the days after the heating stage. The size, however, slowly increased until approximately 1 µm. This size was constant for 5–7 days.

This can be explained due to the hysteresis cycle of the gelatin A structure that can be the reason why new interactions occur when heating the solution. Consequently, this transition between the different gelatin conformations (from helices fibers to a more disentangled linear structure) applies to the aggregation of the coacervates.

Results shown below, in Figure 4A,B, determine 5 days as a stability cycle for coacervates. Results for the sixth day showed particles determined at both too low and high size distribution (less than 100 nm and more than 5 µm, respectively), and a great PDI increase.

SLS experiments were also performed to characterize the electrostatic interactions between gelatin A and alginate. Following the methodology procedure, a Debye plot was made to show the results on two coacervates series.

Both series were synthesized coacervates at 1:5 volume ratio and pH 5, for the concentration range proposed, due to previous results that show these as optimal values for stability and particle distribution. Let it also be noted that the concentration reported in references is 5.0 mg/mL.[38–40] The Debye plot obtained for these experiments is shown in Figure 5 below:

The Debye plot represents the term $K \cdot C/R_\theta$ (1/kDa) versus C (mg/mL) from Equation 1. Therefore, the inverse of the intercept is the molecular weight (MW) of the coacervates and the slope will determine the second virial coefficient ($A2$).

Interactions particle–particle will be stronger than particle-solvent if this virial coefficient is negative. However, the interactions particle-solvent will prevail if the coefficient is positive. The tendency line equations provide this information.

First, both tendency lines show a coefficient of determination (R^2) of 0.85, which implies a good and valid correlation of the mathematical adjustment. These equation adjustments include the concentration range of our experimental values tested with DLS, confirming once again interactions in the concentration of 0.5% w/w.

The second virial coefficient is positive for the 25°C synthesized coacervates ($4.16 \times 10^{-4} \pm 6.62 \times 10^{-5}$ mL mol/g^2) meanwhile the run for 37°C synthesized coacervates shows a negative one ($-1.69 \times 10^{-4} \pm 2.88 \times 10^{-5}$ mL mol/g^2). This implies a change in the interaction forces between particles when the temperature increases. The heated coacervates have stronger electrostatic interactions between the compounds, meanwhile the cold ones have a stronger interaction with the solvent. This change contributes to a more stable coacervate system provided by the only difference of the pre-heated gelatin A and coacervation temperature rise to 37°C. Therefore, the temperature effect is proven once again. To conclude, another technique characterizes the electrostatic interactions that hold the coacervate system, and proves the benefits of the heated synthesis.

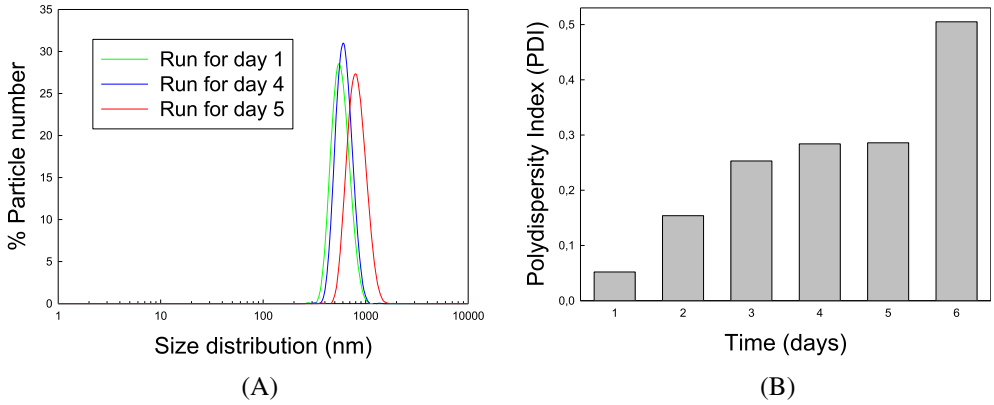

FIGURE 4 (a) Size distribution and (b) PDI values throughout thermo-reversibility conditions for 5 days.

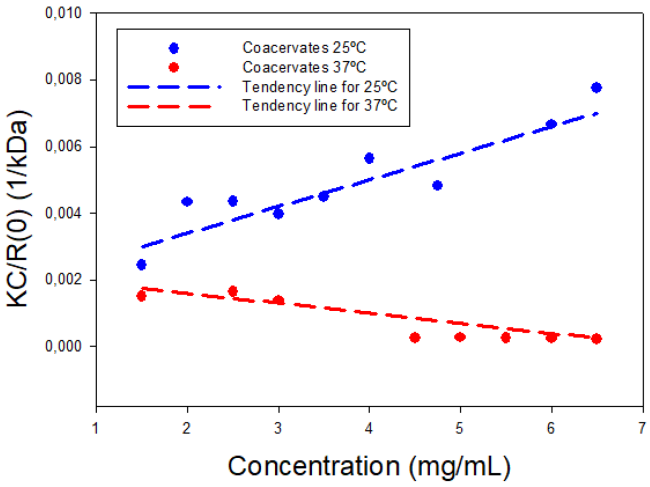

FIGURE 5 Debye plot performed by static light scattering on coacervates 1:5 and pH 5 at 25°C (blue) and 37°C (red), and their line tendencies.

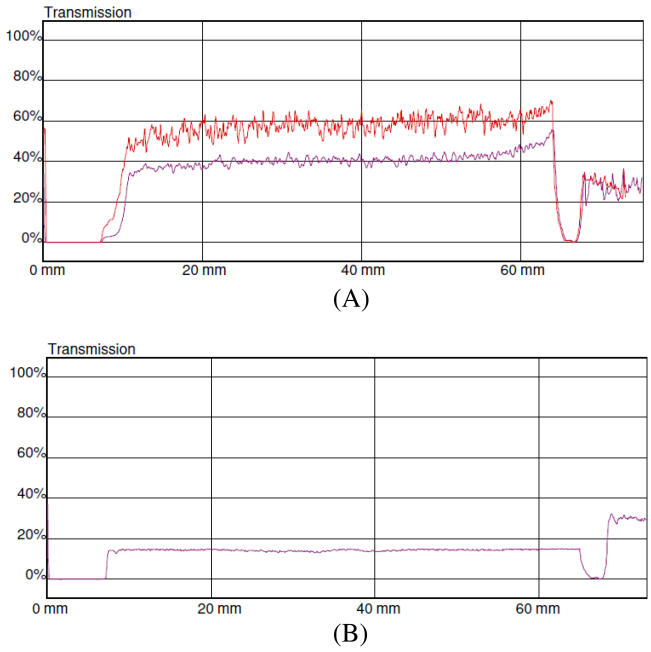

FIGURE 6 Transmission measured through samples of coacervate (a) 1:5 at 25°C (pH 4 [red] and pH 5 [purple]) and (b) 1:5 at 37°C and pH 5. This figure represents the light transmission percentage in the *y*-axis versus the height of the sample tube in the *x*-axis.

TABLE 2 Zeta potential results for coacervates with 1:5 alginate: Gelatin A volume ratio.

25°C		37°C	
pH 6	−25.66 ± 1.20 mV	pH 6	−24.00 ± 1.15 mV
pH 5	−13.76 ± 0.72 mV	pH 5	−17.90 ± 0.44 mV
pH 4	−13.76 ± 0.70 mV	pH 4	−16.73 ± 0.58 mV

Finally, zeta potential measurements were performed in order to characterize the systems surface net charge and further prove the temperature and pH effect on coacervate synthesis. The results are presented in Table 2.

These results in Table 2 confirm the protonation of the gelatin A when pH values decrease. This protonation of all the amino functional groups, whether they are in a full closed (25°C) or partially unraveled (37°C) coil. As proposed the isoelectric point of gelatin A is found between the values of 6 and 4, more concretely between 5 and 6. When protonation occurs, the net charge of the molecules will be more positive (thus, less negative).

Moreover, it has been stated that for several colloidal dispersions, a generalized value of less than −15 mV or more than 15 mV provides stability throughout time.[4] For this measurements, it is once again confirmed that a heating stage implementation to the synthesis process develops a more stable colloidal system. Table 2 results show that zeta potential is less than 15 mV for coacervates synthetized at pH 6 at both temperatures. These values suggest that there are enough electrostatic forces to provide stability to the colloidal system. For 25°C synthesized coacervates this could be the reason why occasionally precipitates were observed and certainly why the polydispersity index reported are higher, since aggregation amongst them could happen, as shown in the microscope images (Figures S7 and S8). However, it is important to note that not only this forces determine the stability

and aggregation in the colloidal dispersion of the samples. The interaction particle–particle or particle–medium is also important to analyze, and this is shown and commented in following results in this section.

3.2 | Turbidimetry results

Figure 6 shows the light transmitance throughout the cell used for tubidity measurement. *Y*-axis represents the values of transmittance along the tube that contains the coacervates sample where the height of tube is represented in *x*-axis in mm. Big steps are observed at the first measurements because it corresponds to bottom plug (0–10 mm) and another one is observed around 60 mm because it corresponds to the top of the sample (interphase gas–liquid).

This figure illustrates the results for the coacervate samples at 25°C (1:5 at pH 4 and 5) and 37°C (1:5 at pH 5). As it can be seen, the transmittance for coacervates at 25°C (60%) is far higher than the transmittance at 37°C (less than 20%), indicating how the temperature is a key parameter to obtain coacervates causing more turbidity. According to different studies, coacervates that showed more turbidity tend to be more stable.[1,4,38–40] This is due to the Tyndall effect provoked by the coacervates formed, which are in suspension in the sample bulk. The concentration of these coacervates promotes the turbidity of the sample, being able to absorb more light emitted by the turbidimeter. This is translated as a lower transmittance percentage, due to the lower percentage of light received in the turbidimeter detector. This light absorbance is caused by stable coacervates and their concentration in the bulk. Therefore, the more turbidity a sample shows, the more stable their coacervates will be.

Furthermore, the straight line in Figure 6B shows a high stability of the 37°C synthesized coacervates, meaning, a measurement with little to no noise, and a backscatter straight line. This phenomenon is not found in Figure 6A showing an unstable solution. Once again, this is translated into a homogeneous turbidity throughout the sample height in the sample tube, meaning the coacervates are more likely to be stable, monodisperse, and the concentration of these is balanced for the whole sample bulk.

Tyndall effect was more pronounced in the systems with no precipitates and where more coacervates were synthesized. The promotion of electrostatic interactions due to the gelatin A partially unraveled structure provokes a higher concentration of particles that presents a higher turbidity. This explains why the more stable systems present more turbidity. It is also important to indicate that a similar trend was observed for the different runs at different pHs and ratios (always a higher stability at 37°C than 25°C). Adding to these conclusions, the different turbidity of the samples agrees with the results that were obtained with dynamic and SLS.

3.3 | Salt effect on coacervates

As previously stated, the salt effect was also studied by turbidimetry. Small amounts of NaCl (50, 100, 150, and 200 mM) were added to the coacervates previously shown (Figure 6) for turbidimetry results (1:5 volume ratio, pH 5, and temperatures of 25 and 37°C). The salt effect was clearly visible at a macroscopic scale, since aggregates were progressively formed when the salt concentration increased in both temperatures. This is shown in Figure S9.

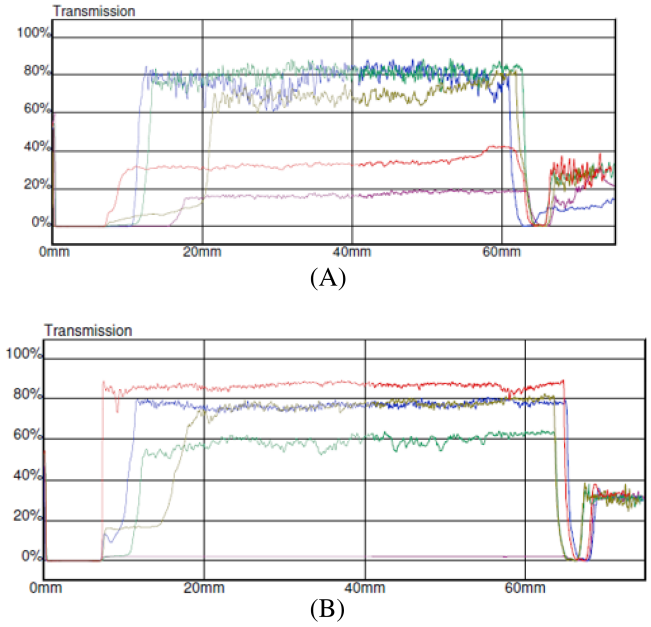

FIGURE 7 Transmission measurements of turbidity analysis of coacervates 1:5 and pH 5 and (a) 25°C synthesis with NaCl (mM): 0 (purple), 50 (red), 100 (gray), 150 (green), and 200 (blue); (b) 37°C synthesis with NaCl (mM): 0 (purple), 50 (green), 100 (gray), 150 (blue), and 200 (red). This figure represents the light transmission percentage in the *y*-axis versus the height of the sample tube in the *x*-axis.

According to the turbidimetry results, coacervates at 25°C showed precipitates at all salt concentrations, being the one without salt the only stable one. As salt concentration increased, so did the transmittance, indicating less stability of the coacervates. Also let it be noted that back scattering measurements went higher and more unstable as salt concentration increased. These backscatter measurements are shown in Figure S10a. This explains all the noise in the measurements signals reported in Figure 6A,B for the NaCl added samples. The precipitates formation causes a disparity in the transmittance of the sample throughout the tube height, since the concentration of the reagents will no longer be balanced. Thus, the more unstable is the sample, the more unstable will be the turbidity measurement. Moreover, the precipitates formation reduces the stable coacervates concentration, implying there will be less Tyndall effect, therefore less turbidity, more transmittance percentage, and less stability.

Coacervates (synthesized at 37°C) showed the same results when it came to salt effect, since the only visibly stable formulation was the one with no salt added. This is shown in transmittance measurements being more stable than the 25°C synthesized coacervates ones, and the backscatter measurements, shown in Figure S10b. The main results obtained by turbidimetry are shown in Figure 7 below.

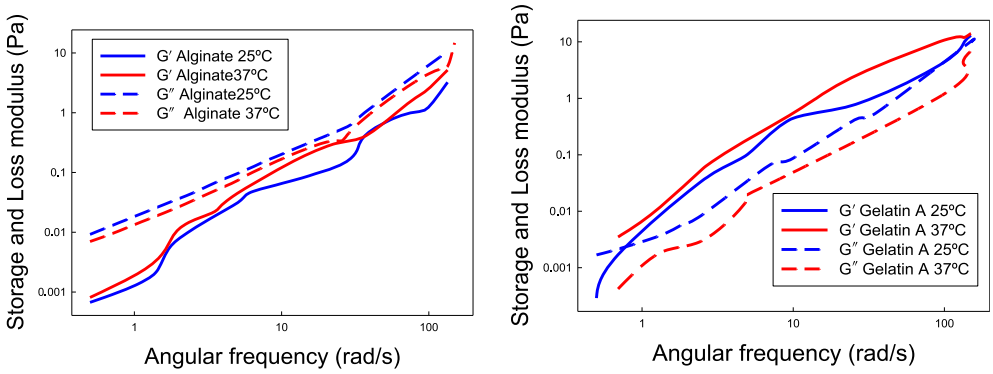

FIGURE 8 Oscillatory rheological analysis on (a) alginate and (b) gelatin A, both cold (25°C) and heated (37°C).

Salt effect on coacervates samples has been previously reported as a Debye length reducer, filtering repulsive interactions between the oppositely charged compounds in a system.[43] This can sometimes be a beneficial effect, like promoting fish gelatin and alginate coacervates formation as reported by Derkach et al., as long as salt concentration was lower than 50 ppm.[43] However, fish gelatin is a gelatin characterized to be significantly more monodisperse and smaller (13–15 nm) than the gelatin A that is used in this study. Gelatin A was characterized to have multiple peaks of bigger size distributions. Therefore, filtering interactions between gelatin A and alginate will promote the aggregates formations that will eventually precipitate, rather than coacervation process. Also, this effect has been reported as coacervation inhibitor when using inorganic acids to regulate pH. HCl would not induce correctly coacervation whereas acetic acid did because of the effect of diluted ions in water, preventing electrostatic interactions to happen.[42]

Nonetheless, it can be concluded that, once again, temperature must be controlled for an optimized synthesis. This is due to the presence of more stable solutions, even with precipitates, at 37 than 25°C. This was also proven by DLS measurements of these samples, being the PDI increased as the salt concentrations were. PDI values for 0, 50, 100, 150, and 200 mM NaCl added were: 0.63, 0.65, 0.76, 0.91, and 1.00 (at 25°C), and 0.14, 0.48, 0.58, 0.71, and 1.00 (at 37°C).

3.4 | Rheology characterization

Rheological oscillatory and statistical flow tests were performed on the samples to study the influence of the different experimental parameters on their elastic and viscous behavior.

3.4.1 | Oscillatory analysis

First, oscillatory analysis was performed on solutions of alginate and gelatin A (0.5% w/w both), at 25 and 37°C. Results are illustrated in Figure 8A,B. On the one hand, it can be seen how the storage modulus (G') of the gelatin increases with the temperature whereas the viscous one decreases.

Furthermore, for both conditions the storage modulus is always higher, indicating the slightly elastic nature of the solutions. The change on the spectrum of the gelatin is caused by the change on the conformation of the gelatin (from organized fibers to a partially unraveled structure). In this unraveled conformation, there are more available charges that can interact between them, increasing therefore the storage modulus. This correlation of G' values increasing when aqueous gelatin solutions are heated is supported by References 26,44.

On the other hand, alginate samples show a viscous-like behavior, since its loss modulus (G'') prevails over its storage modulus. This nature is not changed but restricted when heating due to an elastic modulus growth as well as viscous modulus decrease. Overall, it is demonstrated that heating these samples promotes their solid-nature, regardless of their natural behavior in aqueous solutions. This increase of the storage modulus is what probably induce a better coacervation process when mixing these solutions under heat.

Besides, the fact that the gelatin volume exceeds by 4–6 times the alginate volume, it is likely to predict the coacervates solid behavior.

Following these conclusions, the different moduli of the coacervates at different pH and temperature conditions were also determined. The results obtained for these experiences are illustrated in Figure 9A,B. It is possible to observe how the storage modulus increases with the temperature since the coacervate was formed due to the interactions alginate–gelatin A. As previously stated, the volume ratio of the solutions predicts their elastic nature.

Furthermore, storage modulus is always higher than the viscous modulus for all the experimental conditions, indicating the existence of interactions between both compounds being influenced mainly by the gelatin A presence. Figure 9B also highlights how the pH did not have an important influence on the storage modulus, since the values for these are similar for the different experimental. Nevertheless, there are slightly better results for pH 5.

However, in Figure 9A, the temperature is again proven to promote elasticity and stabilize the coacervate solutions, thus, pointing this parameter out as a fundamental one in the synthesis process. Finally, these results also indicated that the interactions are not strong enough to form a strong gel structure, since the storage modulus depends on the frequency.

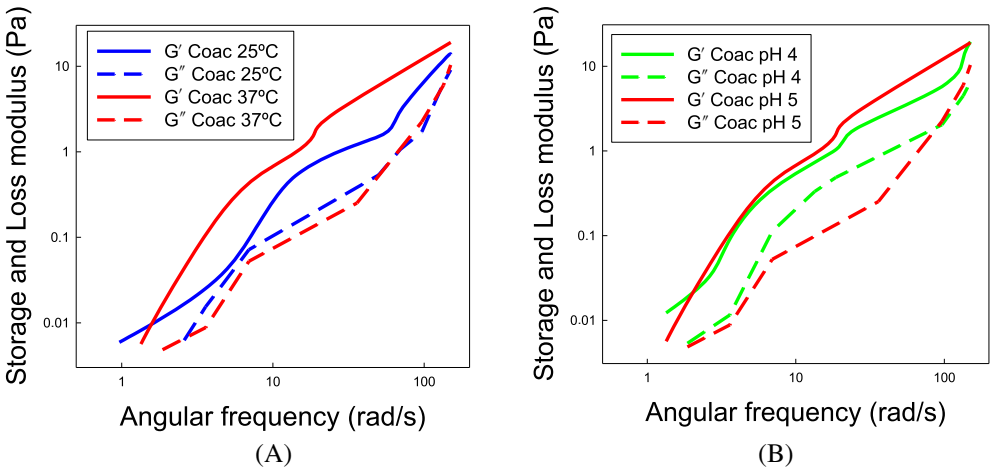

FIGURE 9 Oscillatory rheological analysis on coacervates with different (a) temperatures (cold at 25°C and heated at 37°C) and pH 5 and (b) pH values (at 4 and 5) and 37°C.

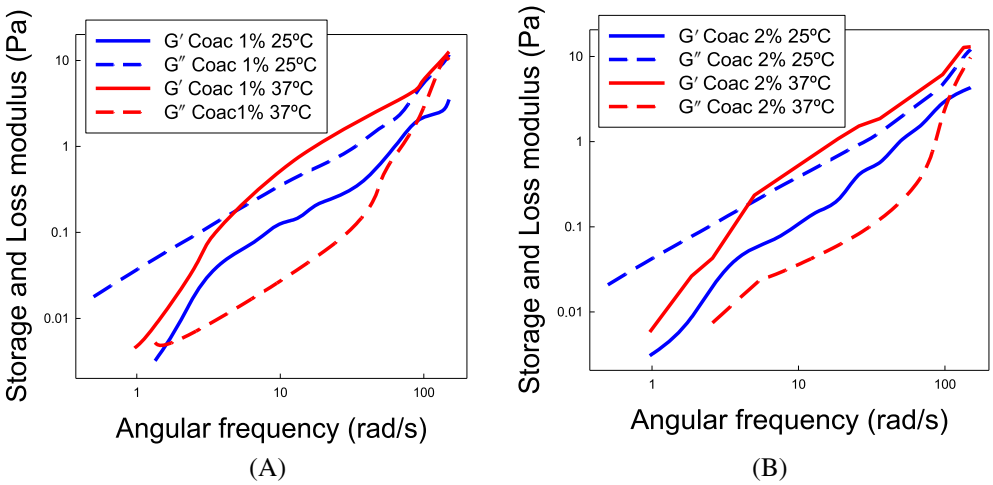

FIGURE 10 Oscillatory rheological analysis on concentrated coacervates of (a) 1% w/w and (b) 2% w/w, both cold (25°C) and heated (37°C) at pH 5.

Results with higher concentrations of gelatin and alginate (and coacervates) were also performed in order to study tendencies among the interactions further. This is motivated by the weak interactions detected in the results, due to the diluted character of the solutions. The results can be observed in Figure 10A,B, choosing a pH value of 5 due to present the best results. First, the solutions of gelatin change their tendency to a liquid-like behavior in the cold samples (25°C) as it is shown in Figure S11. However, this change in viscoelastic nature did not happen when studying alginate solutions. In this case, the viscous behavior was maintained, regardless the increasing concentration. Nevertheless, the temperature effect does not change, promoting the solid nature of the gelatin solutions until switching it back to a solid-like behavior (*G′* values prevail, again, over *G″* values). These results also predict the coacervates behavior, same as explained for lower concentrations.[49,50]

When the coacervation was induced on 1.0% and 2.0% w/w solutions at high temperature after reducing the pH to 5, due to previous results, the storage moduli also increased, highlighting that interactions occurred. In spite of this fact, precipitates and high values of PDI at 1.0% and 2.0% w/w concentrations were obtained. This phenomenon can be explained, taking into account that inducing interactions can increase the storage modulus, but also can promote precipitation and multimodal particle size.

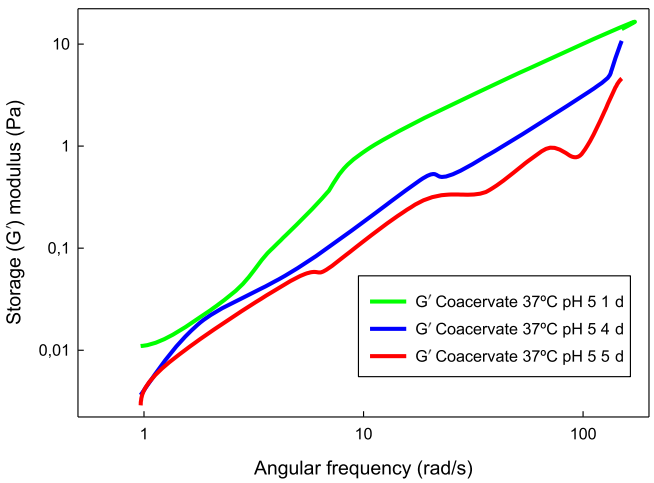

FIGURE 11 Oscillatory rheological analysis on storage *G′* modulus from heated (37°C) coacervate at pH 5 throughout the days (stability).

In fact, storage moduli of the systems with concentrations of 0.5% w/w are similar to the storage moduli for 1.0% and 2.0% w/w, indicating that both systems have a similar number of interactions, in spite of the concentration. However, the remaining solids in 1.0% and

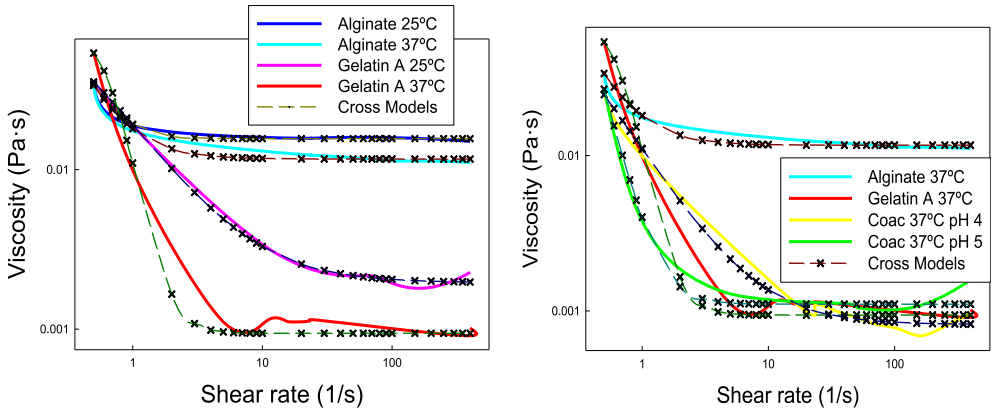

FIGURE 12 Static flow rheological analysis on (a) alginate and gelatin A cold (25°C) and heated (37°C), with their adjusted cross models (Equation 1) and (b) heated (37°C) alginate, gelatin A and coacervates solutions at 1:5 volume ratio and pH 4 and 5, with their adjusted cross models (Equation 1).

TABLE 3 Cross model parameters.

Solution	Temp (T, °C)	Zero-rate viscosity (η_0, Pa·s)	Consistency (K, s)	Critical strain ($\dot{\gamma}_c$, s^{-1})
Alg (0.5% w/w)	25	0.08	2.83	0.351
Alg (0.5% w/w)	37	0.34	7.16	0.139
Gelatin (0.5% w/w)	25	0.15	5.94	0.168
Gelatin (0.5% w/w)	37	0.07	1.58	0.632
Coacer (run 10)	37	0.28	3.25	0.306
Coacer (run 12)	37	0.41	1.28	0.781

2.0% w/w solutions cannot interact, resulting in the precipitation of small amounts of solids.

Oscillatory analyses were also performed on coacervates after the cooling–heating cycles. Figure 11 shows the change of the mechanical spectrum of the coacervates solutions after the respective cycles (1, 4, and 5 days). These results confirm the conclusions mentioned in the DLS section previously. This means, the properties for coacervates are lost during cold cycles (25°C), but recovered after heating (37°C). In this rheological study, the property is the elastic behavior (*G*′ values).

As it shows, the first day kept the initial rheological behavior of the solution (high storage modulus). However, this slightly predominant solid nature decreases after 4 days (4 cooling–heating cycles). This means the solution has a stronger tendency towards a liquid-like behavior at 25°C. Figure 10 shows how the elastic properties gradually decreases as more cycles are performed. This reflects the interactions between gelatin A and alginate decrease with time, due to possible re-assembly of the gelatin structure during the cooling stages.

The last mechanical spectrum is attributed to the hysteresis cycle of the cooling–heating of gelatin A that can induce different interactions (the transition between the different conformation of gelatin A structure).

It can be concluded that oscillatory results showed an increase of the elastic modulus when the synthesized coacervates were formed with a narrow PDI and proper values of turbidity. This phenomenon is due to the induced interactions, controlled by the temperature effect and the partial unraveling of gelatin A structure. Oscillatory analyses also indicate how the coacervates stability cannot be extended for more than 4–5 days.

Adding to previous DLS results, the *G*′ measurements for the sixth day presented significant unstability and value drops from the previous day. This characterized the end of the hysteresis cycle, since the coacervates properties are not recovered after the sixth heating cycle.

3.4.2 | Statistical flow analysis

Figure 12A,B and Table 3 show different viscosity-shear rate diagrams, as well as some of the obtained parameters after fitting the experimental results with Cross model, corresponding with Equation 2.

In Table 3, cold solutions (25°C) have a similar behavior in terms of pseudoplasticity, since their critical strain rates and zero-rate viscosities are almost identical. For all the solutions, a Newtonian plateau was obtained at large velocities of deformation (infinity shear viscosity close to 0). Moreover, a pronounced shear-thinning region was found at the beginning of the measurements for all the solutions due to the alginate and gelatin A structures. Table 3 also indicates how the temperature effect slightly modifies the values of the Cross model's parameters.

The formation of the coacervates, although increasing the zero-rate viscosity, did not modify significantly the pseudoplastic behavior of the polymers alone, showing a similar behavior in both of them. Zero-rate viscosities and critical strain rates are again similar, and are mainly independent of the temperature. Although the temperature is

determined as a factor that can affect the synthesized coacervates, statistical analyses did not provide a significant information concerning coacervates formation.

Furthermore, it is possible to observe the difference the pH makes in the coacervation process. Figure 12B highlights that the curve at pH 5, (best experimental conditions according to size and PDI), has an intermediate shape between the alginate and gelatin A behaviors. This phenomenon does not occur for pH 4 (shape of the curve similar to the gelatin one due to its excess).

4 | CONCLUSIONS

Alginate–gelatin A complex coacervation has been studied with dynamic and SLS, turbidimetry, and rheology. Different experimental parameters, such as alginate–gelatin A volume ratio, pH, and a previous gelatin A solutions heating (37°C) have been considered.

Experimental results indicated that a gelatin preheating with a pH reduction is required to form unimodal coacervate solutions. This preheating is mandatory to change gelatin A structure in order to facilitate the subsequent interactions between gelatin A and alginate at a lower pH, inducing coacervation.

Moreover, the alginate–gelatin A volume ratio and their respective concentrations must be controlled to reduce the PDI of the coacervates as well as the formation of precipitates. In fact, a particle size between 200 and 600 nm with a PDI of around 0.20 can be obtained depending on the experimental conditions (alginate–gelatin A volume ratio of 1:5 with a pH of 5 and a gelatin preheating at 37°C were the best experimental conditions).

Not only size distribution and PDI were proven to better with temperature, but also the electrostatic interactions between the compounds, even observing differences by optical microscopy. SLS measurements showed a negative second viral coefficient for the coacervate system when heated at 37°C, which highlighted stronger forces among particles than with the solvent.

It was also highlighted that the coacervates have to be heated again to recover their properties at 37°C (showing thermo-reversibility) because cooling stages increased their size and PDI. However, this cycle can be also performed for 4–5 days since the size increased every day due to the hysteresis cycle of the gelatin A structure, modifying the interactions. Results were confirmed also with rheology and its storage modulus.

Oscillatory analysis also highlighted the gelatin A pre-heating importance in coacervates synthesis, even changing the solutions from a liquid-nature to a solid-nature. Furthermore, these analyses confirmed the temperature to be an elasticity enhancer for the coacervates. However, statistical analysis, and also the Cross model, did not indicate significant changes amongst the different solutions, as presented in Table 3.

Besides, turbidimetry was also used to characterize the importance of the temperature effect and the disadvantages of the salt effect on this coacervates, resulting in precipitates when NaCl was added.

Therefore, a correct combination of pH, gelatin A preheating, as well as alginate–gelatin volume ratio, and their concentrations must be taken into account to synthesize in a reproducible way stable coacervates with a proper particle size and PDI, strong electrostatical forces, and elastic behavior.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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