Gum Arabic and chitosan self-assembly: thermodynamic and mechanism aspects

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Abstract

The interactions between gum Arabic (GA) and chitosan (CN) in solution have been investigated upon pH (4.5, 5.0 and 5.5) and temperature (5, 25 and 45 °C). Isothermal titration calorimetry (ITC) has been used to determine the thermodynamic parameters involved in the complex coacervation process between these two polymers. Zeta potential, turbidity measurements and optical microscopy were used as complementary methods to have a better understanding of the phenomenon at the origin of the self-assembly. Unlike data already published by authors, concerning the GA-CN coacervation model, a raise in temperature seemed to favor electrostatic interactions. The enthalpy became more favorable upon temperature, a phenomenon probably related to the availability of CN charges. A raise in temperature would lead to a compaction of CN, decreasing its steric hindrance during self-assembly. According to all experiments conducted in this study, the GA/CN interaction optimal molar ratio would be 4:1. However, this optimal ratio changed upon pH-temperature combinations. The results presented in this study should lead to a better understanding of the interactions between charged polymers and in the end, help in their application in the food, pharmaceutical and cosmetic industries.

Keywords: Complex coacervation; interactions; Isothermal titration calorimetry; gum Arabic; chitosan; self-assembly

1. Introduction

Complex coacervation is a phenomenon in which polyelectrolytes interact to form a liquid phase rich in polymer called coacervate and an equilibrium phase poor in polymer called the diluted phase (Kizilay, Kayitmazer, & Dubin, 2011). It is used in the industry to encapsulate several molecules of interest such as vitamins, plants oils and flavors (Bhatia, Khattak, & Roberts, 2005; Lankalapalli & Kolapalli, 2009). This bottom-up approach is a construction of complexes by self-assembly of molecules due to interactions between two polymers, which depend on changes in environmental conditions, such as pH, ionic strength, temperature, or concentration (Joye & McClements, 2014; Kim, Yang, Park, Lim, & Cha, 2017; Schmitt & Turgeon, 2011). This eco-friendly approach does not need an external energy contribution and is performed under soft conditions (in aqueous medium). Several polyelectrolytes are suitable for this approach such as proteins and polysaccharides. Polysaccharides are good candidates for complex coacervation because they can be charged in solution, they are not expensive, readily available, bio-compatible and have low toxicity (Anwunobi & Emeje, 2011). Complex coacervation of polyelectrolytes has been studied by several authors (Kayitmazer, 2017; Priftis, Laugel, & Tirrell, 2012; Veis, 2011) who agreed that different parameters drive the formation of complexes. Early studies (Overbeek & Voorn, 1957) suggested that coacervation results from electrostatic attractions. Most of the time, the two polyelectrolytes attract each other because they are oppositely charged, forming complexes prior to coacervation (Veis & Aranyi, 1960). When the two oppositely-charged polymers form a complex driven by electrostatic interaction, counterions and water molecules are released in the bulk, contributing to an entropy gain of the system.

In this study, complex coacervation between chitosan (CN) and gum Arabic (GA), two polysaccharides of opposite charges, has been studied through thermodynamic and mechanistic aspects. CN is a β (1-4)-linked glucosamine which is found in the cellular wall of zygomycetes, bacteria and yeast. The main source of CN comes from the deacetylation under alkaline conditions of the chitin of crustaceans shells. This biopolymer is film-forming, biodegradable and biocompatible. It exhibits antimicrobial and antioxidant activities (Fathi, Martín, & McClements, 2014; Soni, Mahmoud, Chang, El-Giar, & Hassan, 2018; Sonia & Sharma, 2012). Furthermore, CN is well-known for presenting a large density of positive charges (Fathi et al., 2014). In order to have a coacervation phenomenon with CN, GA was chosen as polyanion. This polymer comes from an exudate from the stems and branches of acacia trees, which grow across the Sahelian belt of Africa (*Acacia senegal* and *Acacia seyal*) (Lopez-Torrez, Costalat, Williams, Doco, & Sanchez, 2015). It is an effective encapsulation agent due to its high water solubility, the low viscosity of concentrated solutions and its ability to act as an oil-in-water emulsifier. Furthermore, GA is often used as polyanion in several studies on complex coacervation (Aberkane, Jasniewski, Gaiani, Scher, & Sanchez, 2010; Butstraen & Salaün, 2014; Eratte, Wang, Dowling, Barrow, & Adhikari, 2014; Shaddel et al., 2018). GA is essentially made of polysaccharides and salts. It contains three different fractions: 85-90 wt. % of arabinogalactan, 10 wt. % of arabinogalactan-protein complex and 2 wt. % of one or two glycoproteins (Lopez-Torrez et al., 2015). The fractions containing proteins, responsible for the surfactant activity of the GA, are found in higher amount in *Acacia senegal* exudate than in the one of *Acacia seyal* (1.8% w/w vs. 0.9% w/w, respectively) (Lopez-Torrez et al., 2015). In this study, the dialyzed total gum was used. Ionized CN (pKa from 6.3 to 7.0 (Beppu & Santana, 2002)) has an extended conformation in acetic acid solution, so its charges are accessible (Espinosa-Andrews, Báez-González, Cruz-Sosa, & Vernon-Carter, 2007; Schatz et al., 2004). Ionized GA (pKa close to 3.6 (Aberkane et al., 2010)) has a globular structure (Moschakis,

Murray, & Biliaderis, 2010; Renard, Garnier, Lapp, Schmitt, & Sanchez, 2012) in aqueous solution. The influence of pH, ionic strength, temperature, or polyelectrolytes concentration on GA and CN coacervation has already been studied by several authors (Avadi et al., 2010a, 2010b; Butstraen & Salaün, 2014; Espinosa-Andrews et al., 2007; Espinosa-Andrews, Sandoval-Castilla, Vázquez-Torres, Vernon-Carter, & Lobato-Calleros, 2010; Maqbool, Ali, & Alderson, 2010; Roldan-Cruz, Carmona-Ascencio, Vernon-Carter, & Alvarez-Ramirez, 2016; Sakloetsakun, Preechagoon, Bernkop-Schnürch, & Pongjanyakul, 2015; Tan, Xie, Zhang, Cai, & Xia, 2016). However, the comprehension of the coacervates formation mechanism is still an issue to be tackled. This study aimed to better understand this mechanism by determining the corresponding thermodynamic parameters (stoichiometry, entropy and enthalpy) using Isothermal Titration Calorimetry to understand what drove the complex coacervation between these two polymers. Another aim of this work was to understand the part played by the different parameters to shed some light on the mechanism of this phenomenon.

2. Materials and Methods:

2.1. Materials

Chitosan (CN) MMW (medium molecular weight, Mw 310 kDa, deacetylation degree of 75%) was purchased from Sigma-Aldrich (France). Gum Arabic (GA) Instantgum AA from *Acacia senegal* (Mw 400 kDa (Renard, Lavenant-Gourgeon, Ralet, & Sanchez, 2006)) was a gift from Nexira (France). Acetic acid, NaOH and ultrapure water were of analytical grade.

2.2. Methods

2.2.1. Polymers purification

CN and GA were dialyzed against acetic acid (1% v/v) and water, respectively, with a high grade regenerated membrane (MWCO 10 000 Da from Membrane Filtration Products Inc.) to eliminate excess salts. Dialysis was stopped when the conductivity outside the membrane was equal to the one of acetic acid (1% v/v) or ultrapure water, respectively. Samples were centrifuged then for 30 min at 12 000 g to remove insoluble material and air bubbles and then freeze-dried and stored at 4 °C. The molecular weights of both polysaccharides were verified by SEC-MALLS measurement post-dialysis and were very close to the ones provided by the suppliers.

2.2.2. Polymers stock solutions preparation

CN 0.02% w/v (6.45×10^{-4} mM) and GA 1.00% w/v (2.50×10^{-2} mM) stock solutions were prepared by dissolving purified CN or GA in acetic acid (1% v/v in ultrapure water). The solutions were stirred on a magnetic plate at 400 rpm overnight at 4 °C to make sure the polymers were completely dissolved. Though GA is soluble in water, the mixtures were made by adding GA solubilized in acetic acid, so the dilution of acetic acid in water signal would not interfere with the polymers interaction signal, during ITC measurements when GA solution was added to CN solution. The CN solution was prepared at 0.02% w/v (6.45×10^{-4} mM), concentration for which its viscosity was low enough.

2.2.3. Polymers mixtures preparation

Polymers mixtures were prepared at different temperatures (5, 25 and 45 °C), at different pH (4.5, 5.0 and 5.5 by adding either acetic acid (1 N) or NaOH (5 N) as required), and different

polymers molar ratio. After pH adjustment, the required amount of polymer solutions recovered from stocks solutions were placed 15 min in a thermostatic degassing chamber to reach the targeted temperature and remove bubbles. GA solutions were then added to CN solutions and then analyzed after 5 min to compare samples in identical conditions.

2.2.4. Isothermal titration calorimetry (ITC)

ITC measurements were carried out with an Affinity ITC equipped with a standard volume (965 μL) gold cell (TA instruments, Waters SAS, France) to measure the dissociation constant (K_d) and the enthalpic variation due to interaction between CN and GA. The entropy and the Gibbs free energy were obtained from the following equations:

$$\Delta G = - R.T.\ln(1/K_d) \quad (\text{Eq. 1})$$

$$T\Delta S = \Delta H - \Delta G \quad (\text{Eq. 2})$$

With ΔG : Gibbs free energy variation (kJ.mol^{-1}), ΔH : enthalpy variation (kJ.mol^{-1}), ΔS : entropy variation ($\text{kJ.mol}^{-1}.\text{K}^{-1}$), T : temperature (K) and R is the specific gas constant ($8.3144598 \text{ J.mol}^{-1}.\text{K}^{-1}$).

Ultrapure water was used in the reference cell. All solutions were degassed under vacuum for 15 min using the associated degassing station (TA instruments, Waters SAS, France) at the temperature of titration (5, 25 or 45 $^{\circ}\text{C}$). The injector microsyringe (320 μL), was loaded with stock solution of GA (1.00% w/v GA ($2.50 \times 10^{-2} \text{ mM}$) in 1.00% v/v acid acetic solution). 5 μL were sequentially injected 50 times each 300 s to the titration cell (volume = 965 μL) filled with CN solution (0.02% w/v CN ($6.45 \times 10^{-4} \text{ mM}$) in 1.00% v/v acid acetic solution). The polymers concentrations were chosen based on preliminary measurements to optimize concentrations in order to have complete titrations without signal saturation. For each measurement, the pH, the

temperature and the acetic acid solvent were the same in the cell and in the syringe to limit the dilution effect. The heat of dilution from the blank titration was subtracted from the raw data in all experiments. Data acquisition was performed using the ITC Run software and data were analyzed with the NanoAnalyse software (TA instruments, Waters SAS, France). The “Independent” model was chosen as the best model from the plot residual values to fit the obtained isotherms. All measurements were made in triplicate.

2.2.5. Approximate Zeta potential

The approximate Zeta potential of the polymers alone (0.02 w/v for CN (6.45×10^{-4} mM) and 1.00% w/v for GA (2.50×10^{-2} mM)) and of the mixtures were calculated from measurement of the electrophoretic mobility at controlled temperatures (5, 25 and 45 °C) using a Zetasizer Nano-ZS instrument (Malvern Panalytical, United Kingdom) equipped with a He/Ne ion laser ($\lambda = 532$ nm). Measurements were collected on a detector at 173°. An automatic titrator (Malvern Multipurpose Titrator MPT2) was also used for the automation of Zeta potential measurements upon the GA/CN molar ratio (0.02 w/v for CN (6.45×10^{-4} mM) and 1.00% w/v for GA (2.50×10^{-2} mM)). 25 injections of 124 μ L of GA solution were made in 12 mL of CN solution. Investigated solution was filled into a Zeta potential folded capillary cell (DTS1070, Malvern Panalytical). The instrument determined the Zeta potential with the Smoluchowski equation (Jayme, Dunstan, & Gee, 1999). All measurements were made in triplicate.

2.2.6. Turbidity measurements

Turbidities of the GA/CN mixtures were measured with a UV-visible spectrophotometer (Shimadzu 1280, Japan) at 660 nm. The transmittance (T) of the spectrophotometer was calibrated with 0.1 N acetic acid to 100% and the turbidity of the samples was reported as 100-T%. The relative turbidity (τ) was calculated from the equation 3:

$$\tau = -\frac{1}{L} \ln \frac{I_t}{I_0} \quad (\text{Eq. 3})$$

Where L was the optical path length (1 cm) and I_t and I_0 were the transmitted light intensity and the incident light intensity, respectively. All measurements were made in triplicate.

2.2.7. Optical microscopy

The mixtures were observed by optical microscopy (Olympus BH2, Japan) at x40 magnification. The images were recorded with a CMOS 5 MPixel camera (Olympus) under the control of TouPView software. The micrographs presented are representative of the samples. They were analyzed by using an image analysis software (ImageJ) in order to obtain the particles size and number. Polydispersity index of the sample was obtained using equation 4:

$$\text{Polydispersity index} = \left(\frac{\text{Standard deviation}}{\text{Average size}} \right)^2 \quad (\text{Eq. 4})$$

2.2.8. Statistical analysis

The values were expressed in mean $\pm$ standard deviation. Differences between mean values were assessed using the one-way analysis of variance (ANOVA) which was performed using Excel for Statistical Analysis and XLSTAT. Differences were considered statistically significant when p_{value} was below 0.05.

3. Results and discussion:

3.1. pH influence over the approximate Zeta potentials of GA and CN

Complex coacervation depends on a few parameters, such as: polymer ratio, total polymer concentration, polymer backbone flexibility, polymer charges, ionic strength and pH (de Kruif,

Weinbreck, & de Vries, 2004; Espinosa-Andrews et al., 2007; Sanchez, Mekhloufi, & Renard, 2006; Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998; Turgeon, Schmitt, & Sanchez, 2007). Since coacervation is supposed to be mostly driven by electrostatic interactions, the influence of the pH on the coacervate formation was first investigated. The chosen pH range was 4.5 to 5.5. Below pH 3.5, carboxylic groups of GA were protonated and contraction of its molecular backbone could suppress the coacervation phenomenon (Espinosa-Andrews et al., 2007). For pH values above 6.0, close to CN pKa, its degree of ionization and its solubility decreased (Espinosa-Andrews et al., 2007; Sonia & Sharma, 2012).

From **Table 1** CN and GA were charged in the chosen pH range. The pH strongly modified the CN surface charge but not the one of GA. The approximate Zeta potential of CN in acetic acid solution was thus higher at pH 4.5 (37.2 ± 3.1 mV) than at pH 5.5 (24.5 ± 1.9 mV) at 5 °C and twice higher at 5 °C (37.2 ± 3.1 mV) than at 45 °C (17.8 ± 0.8 mV) at pH 4.5. The CN Zeta potential decreased upon pH and temperature, which was consistent with the literature (Fan, Yan, Xu, & Ni, 2012; Fillion, Lavertu, & Buschmann, 2007). GA charge in acetic solution did not change upon pH. The pKa of CN was in a range from 6.3 to 7.0 (Beppu & Santana, 2002) and the pKa of GA was close to 3.6 (Aberkane et al., 2010) which could explain the changes of charges upon pH. In the studied conditions, when the pH value was distant to the pKa of CN, the positive value of Zeta potential increased, suggesting an increase of the cationic charge onto the polymer. Similar results were observed with GA in ultrapure water but not in acetic acid (data not shown). Normally, GA was supposed to be more charged when pH increased beyond its pKa. Nevertheless, in acetic acid solution it was not the case. This may be explained by an increase of the ionic strength when the pH increased due to the addition of NaOH to obtain the targeted pH; indeed it is known that when the ionic strength increases along with the pH, the net charge of the polymer decreases, as it has been shown on pectin, CN and alginate (Abodinar, Smith, & Morris, 2014).

It was chosen to focus on the pH 4.5, 5.0 and 5.5, so the pH would be far enough from the pKa of both polysaccharides. As many authors already reported (Butstraen & Salaün, 2014; Espinosa-Andrews et al., 2013), the data from the biopolymers approximate Zeta potentials led to presume that more GA than CN in the mixtures was necessary in order to reach stoichiometry (in other words, the optimal molar ratio where all CN charges were neutralized by GA charges), at any pH (between four and five times more GA than CN in the literature (Butstraen & Salaün, 2014; Espinosa-Andrews et al., 2007, 2010)). The results from **Table 1** suggested that the higher the pH, the fewer GA would be needed to neutralize the CN positive charges ($|ZP_{CN}/ZP_{GA}|$ of 2.7 for pH 4.5, 2.1 for pH 5.0 and 2.2 for pH 5.5 at 25 °C). Therefore, it seemed that twice the amount of GA compared to CN would be needed to obtain neutral complexes if all the charges were involved in the interaction, for all experimental conditions. The obtained results have also shown that the temperature had no influence over the Zeta potentials of GA. On the contrary, the CN Zeta potentials decreased with the raise of temperature in acetic acid solutions. A raise in temperature would lead to a compaction of CN, decreasing its steric hindrance during self-assembly so the charges would be less available for the interaction.

Since this study aimed to understand what drives complex coacervation between GA and CN, ITC measurements were performed in order to elucidate the thermodynamic pathway related to the complexation between GA and CN. Complex coacervation of GA and CN upon molar ratio was thus first investigated.

3.2. Thermodynamic parameters and energies involved in the coacervation process

The thermodynamic parameters involved in complex coacervation between GA and CN were determined using ITC (Aberkane et al., 2010; Xiong et al., 2016) at different pH and

different temperatures (**Figure 1**). This method leads to determine the stoichiometry (n) of the interaction, if the coacervation is enthalpy-driven or entropy-driven and the related thermodynamic values as ΔG , ΔH , $T\Delta S$ and dissociation constant K_d .

Since corrected heat rates were negative in all cases, the interaction major contribution was mostly exothermic (**Figure 1.A and B**) related to a spontaneous phenomenon. The peak areas corresponded to the energy released or absorbed by the cell containing the CN solution after each GA solution injection. A sequence of successive exothermic peaks of decreasing intensity was initially observed in most cases. The binding energy decreased upon GA addition until it stabilized (around the 20th injection). At the end of the experiment, a stabilized sequence of weaker exothermic peaks was always observed, related to a dilution phenomenon. This heat of dilution was nevertheless temperature-dependent (at pH 5.0, -0.2, -0.3 and -0.5 $\mu\text{J}\cdot\text{s}^{-1}$ at 5 °C, 25 °C and 45 °C, respectively) and pH-dependent (at 45 °C, -0.3, -0.5 and -0.75 $\mu\text{J}\cdot\text{s}^{-1}$ for pH 4.5, 5.0 and 5.5, respectively). Moreover, constant endothermic peaks were observed at the end of titration, when GA was in excess. Peaks intensities varied upon studied conditions, which were more important at 45 °C (between 0.1 to 0.2 $\mu\text{J}\cdot\text{s}^{-1}$) than at 5 °C (0.005 to 0.008 $\mu\text{J}\cdot\text{s}^{-1}$). A switch from an exothermic phenomenon to an endothermic one indicated two successive molecular events (Aberkane et al., 2010; Kayitmazer, 2017). The first one might be attributed to the electrostatic interaction between macromolecules (formation of soluble complexes or coacervates) while the second one could be attributed to aggregation, coalescence of the complexes or to a reorganization of the complexes. In some cases, as for pH 5.0 at 45 °C, at the beginning of titration an increase of the binding energy were observed before titration, which is often encountered in ITC measurements for the first 20 μL injected. It could be explained either by a diffusion of GA into the cell during the temperature equilibration or because the two polymers were forming complexes prior to coacervation, opening the chitosan structure in some conditions. Both pH and temperature influenced the intensity of the exothermic signals of the

interaction. At a given pH (**Figure 1.A**), the intensity of the signal increased upon temperature. The same behavior has been observed upon pH at a given temperature (**Figure 1.B**). This was observed at all studied conditions (data not shown).

The binding isotherms were then fitted using the “Independent” model in order to obtain the corresponding thermodynamic parameters (**Figure 1.C and D**). At 5 °C, due to weak signals, the repeatability was not optimal. The first five enthalpy values were not taken into account while modeling the raw data (grey dots **Figure 1.C and D**). According to raw data, enthalpy values were increasing in absolute value when the temperature and the pH increased, corresponding to more electrostatic interactions.

The enthalpy upon molar ratio followed a typical titration curve with the center of the linear segment of the sigmoid corresponding to the stoichiometry of the interaction (the molar ratio where all the charges of the CN were neutralized by the charges of the GA). Since the inflection point was not always at the same molar ratio, the stoichiometry appeared to be slightly different upon studied conditions. The thermodynamic parameters obtained from the fit and from Eq. 1 and Eq. 2 were given in **Table 2** and are represented on **Figure 2**.

For all experimental conditions, ΔG was negative and close to -40 kJ.mol^{-1} which confirmed that the interactions between GA and CN were always spontaneous. ΔH , corresponding to electrostatic interactions (Aberkane et al., 2010; Duhoranimana et al., 2018; Kayitmazer, 2017; Priftis et al., 2012), was always negative (from -37.6 ± 11.6 to $-854.1 \pm 45.7 \text{ kJ.mol}^{-1}$), so favorable to the interaction (negative contribution to ΔG). $T\Delta S$ was almost always negative, so unfavorable to the interaction (positive contribution to ΔG). The K_d values were always very small (from 1.7×10^{-8} to 26.8×10^{-8}) which meant that the interactions between the two polymers were quite strong because complexes are considered to have high-affinity when their K_d value is smaller than 10^{-6} (Qin & Gronenborn, 2014).

The entropic and enthalpic contributions were strongly influenced by temperature and pH. When temperature increased, the entropy significantly ($p\text{-value} = 2.5 \times 10^{-5}$) decreased (from $+0.6 \pm 14.2 \text{ kJ.mol}^{-1}$ at 5°C to $-519.8 \pm 2.0 \text{ kJ.mol}^{-1}$ at 45°C , at pH 5.0) and the enthalpy significantly ($p\text{-value} = 3.4 \times 10^{-5}$) increased (from $-37.6 \pm 11.6 \text{ kJ.mol}^{-1}$ at 5°C to $-561.6 \pm 2.5 \text{ kJ.mol}^{-1}$ at 45°C , at pH 5.0). The same phenomenon was observed when pH increased (entropy decreased significantly from $-23.3 \pm 2.3 \text{ kJ.mol}^{-1}$ at pH 4.5 to $-283.0 \pm 11.8 \text{ kJ.mol}^{-1}$ at pH 5.5 at 25°C with a $p\text{-value}$ of 0.0001, and enthalpy decreased from $-67.7 \pm 2.7 \text{ kJ.mol}^{-1}$ at pH 4.5 to $-322.3 \pm 10.8 \text{ kJ.mol}^{-1}$ at pH 5.5, at 25°C with a $p\text{-value}$ of 0.0009). The enthalpy became thus more favorable to the interaction when pH and temperature increased, whereas the entropy did not. Enthalpy depended on pH for most systems because it was related to the ionization of the polymers (Feng, Leduc, & Pelton, 2008; Kayitmazer, 2017). Authors have reported that complex coacervation between weak polyelectrolytes was mostly driven by a negative enthalpy due to the electrostatic interactions between two oppositely charged components. However, it was unexpected that the raise in temperature would decrease the entropic contribution to the interaction (Li, Kelkar, & Wagner, 2012; Priftis et al., 2012). This result was unexpected because the opposite is generally observed in the literature (Aberkane et al., 2010; Li et al., 2012; Priftis et al., 2012). Indeed, hydrophobic interactions are enhanced by increasing the temperature due to conformational changes of polysaccharide structure and release of binding water and counterions in the bulk favoring entropic contribution (Kelly, Gudo, Mitchell, & Harding, 1994; Schmitt et al., 1998). In the cases of biopolymer-based complexes, the temperature-induced conformational changes play dominant roles (Dickinson et al., 2001; Priftis et al., 2012; Schmitt et al., 1998). It has also been described that CN hydrodynamic diameter should decrease upon temperature and pH (Chen & Tsaih, 1998; Fan et al., 2012). Furthermore, the CN was degassed and incubated at the temperature of experiment for 20

minutes before ITC measurements. At 45 °C, the structure of CN was more compact and binding water was already released before titration. Due to the thermodynamic equilibrium of the sample, the corresponding entropic contribution was not measured. At 45 °C or at pH 5.5, fewer charges onto CN would be available for the GA charges, making the whole system less random, disfavoring entropic contribution. The counterions release entropy only played a minor role, which might be explained by the fact that the two polymers were dialyzed. The unfavorable entropic effects could be due to the loss of polymer conformation freedom after complexation (Dickinson, 2008). When all charges were involved in the interactions, the system became less random and more ordered, which could explain why the entropy was more unfavorable for higher temperatures. Moreover, a more compacted structure lead to a better interaction between CN and GA, since the steric hindrance of CN decreased.

The stoichiometry (n) slightly changed upon temperature or pH. For example, it went from 3.2 ± 0.3 at pH 4.5 to 2.7 ± 0.1 at pH 5.5, at 45 °C (with a p-value of 0.02) and to 4.0 ± 0.1 at 5 °C to 2.7 ± 0.1 at 45 °C, at pH 5.5, with a p-value of 2.2×10^{-6}). The Zeta potential of CN evolved with temperature contrary to the one of GA. The theoretical values of the Zeta potential of the mixtures suggested that the stoichiometry should decrease a little with the raise from pH 4.5 to 5.5 (**Table 1**). However, as mentioned previously, pH and temperature had a small influence over the stoichiometry consistently with the ZP values. The higher the pH, the lower the stoichiometry; at 25 °C the stoichiometry got from 4.1 ± 0.8 at pH 4.5 to 3.3 ± 0.2 at pH 5.5 (with a p-value of 0.07, so the values were not statistically different but the tendency was strong). The higher the temperature, the lower the stoichiometry; at pH 5.5, the stoichiometry got from 4.0 ± 0.1 at 5 °C to 2.7 ± 0.1 at 45 °C (with a p-value of 2.2×10^{-6}). A raise in temperature also meant that the backbones of the polymers were more flexible and more likely to encounter each other since the solvent should be less viscous (Qiao, Di, Ma, Ma, & Xia, 2010; Roy, Ghosh, & Sarkar, 2010). Even if the tendencies were consistent with the

expectations regarding the Zeta potential values of both polymers (**Table 1**), the values were not the same (at 25 °C the stoichiometry was expected to decrease from 2.7 ± 0.2 at pH 4.5 to 2.2 ± 0.1 at pH 5.5). Furthermore, though the GA Zeta potential did not evolve upon temperature, the CN Zeta potential decreased upon temperature (Filion et al., 2007), so the ratio of charges between the two polymers became smaller.

It can also be noted that the thermodynamic parameters and stoichiometry were really close between pH 4.5 at 25 °C and pH 5.5 at 5 °C (**Table 2**), and between pH 4.5 at 45 °C and pH 5.5 at 25 °C (**Table 2**). This suggested that a raise of pH could be tempered by a decrease in temperature (**Table 2 and Figure 2**). The optical micrographs of these conditions were shown in **Figure 3**. Between pH 4.5 at 45 °C and pH 5.5 at 25 °C and between pH 4.5 at 25 °C and pH 5.5 at 5 °C, the objects on the micrographs looked almost the same (same number of coacervates and same average diameter of the coacervates for each combinations). Table of analysis of these micrographs are provided in supplementary material.

Even if the enthalpy and entropy varied upon pH and temperature, the Gibbs energy was found to be almost constant whatever the physicochemical conditions (around -40 kJ.mol^{-1}). This could mean that the formation of the complexes were not driven by the same forces and that the complexes formed did probably not have the same shape. The Zeta potential of the mixtures in the different conditions were measured to appreciate how it coincided with the ITC measurements. The macroscopic aspect of the samples, the turbidity of the mixtures and the shape of the complexes observed from optical microscopy were studied to corroborate ITC data.

3.3. Turbidimetric and Zeta potential measurements and optical microscopy upon molar ratio of gum Arabic and chitosan complex coacervation

The influence of the GA/CN molar ratio on the coacervate formation was investigated (**Figure 4**). These experiments were performed at pH 5.0 so the pH would be far enough from the pKa of both polymers, in order for them to be charged. For CN and GA mixtures, Zeta potential and turbidity were measured.

When the two polymers solutions were mixed together the sample became turbid, which meant new objects were formed (**Figure 4**). Moreover, after 24 hours, two different phases appeared (data not shown). The upper phase was transparent, the phase below was turbid: it was a liquid/liquid associative phase separation (complex coacervation). It is known that coacervates are not stable and that they coalesce upon time. Knowing this, it is important to characterize the samples in the same conditions, so all measurements were made 5 minutes after mixing the solutions. The turbidity of the mixture increased from $0.283 \pm 0.088 \text{ cm}^{-1}$ at the ratio 1:1 to $2.744 \pm 0.392 \text{ cm}^{-1}$ at the ratio 4:1. Ratio 4:1 to ratio 10:1 were not statistically different from each other ($p_0 > 0.05$). However the turbidity seemed to have a tendency to decrease with the increase of the GA proportion in the mixtures (**Figure 4**). This decrease can either be related to a change of structure of the objects, or to a dilution of the system. The volumes of GA added excluded the dilution hypothesis. In addition, a change of structure of the complexes would be consistent with the endothermic peaks at the end of ITC titration (**Figure 1.A and B**).

This result was consistent with published data from other authors and with the ITC stoichiometry (**Table 2** and **Figure 2**): at 25 °C pH 5.0, the stoichiometry was 4.0 ± 0.9 and in the literature, in the same conditions, optimal molar ratios were in a range from 4 to 5 (Butstraen

& Salaün, 2014; Espinosa-Andrews et al., 2007). Objects were formed when the molar ratio was sufficient, in other words when there was enough GA to neutralize the charges of CN. When the molar ratio GA/CN was higher than 4:1 the turbidity decreased. This phenomenon could be explained by a reorganization of the coacervates due to an excess of GA in order to obtain thermodynamic equilibrium in the system. These results were corroborated with optical microscopy discussed farther (**Figure 5**) and with the sequence of endothermic peaks obtained at the end of titration by ITC measurements (**Figure 1**).

The Zeta potential of the objects has also been investigated. For molar ratio below 1:1, CN was in excess so the mixture Zeta potential was close to the one of CN (21.6 ± 0.8 mV). When more gum was added, the Zeta potential got closer to neutrality (around ratio 3:1). Finally, when GA was in excess, the Zeta potential of the mixture tended to the one of GA (-10.2 ± 0.2 mV, **Table 1**). Similar results with mixtures GA/CN at different pH were already published by Butstraen *et al.*, 2014 and Espinosa-Andrews *et al.*, 2007 (Butstraen & Salaün, 2014; Espinosa-Andrews et al., 2007). The maximum turbidity (2.744 ± 0.362 cm⁻¹) was observed when the Zeta potential value was close to zero at a molar ratio of 4:1, which was consistent with the ITC stoichiometry in these given conditions (4.0 ± 0.9) (Coelho et al., 2011). Optical micrographs of the mixtures were collected to study the shape of the objects and to confirm if the loss of turbidity was due to dilution or to a change of shape of the coacervates. In the ITC measurements (**Figure 1**) when the GA was in excess, there was an exothermic/endothermic sequence which would support the theory of complexes reorganization.

For molar ratios below 3:1, no object was observed. From ratio 3:1, some coacervates appeared. When more GA was added (from molar ratios 4:1) more coacervates were formed (**Figure 5**), from 88 ± 8 coacervates in the optical field for 3:1 to 747 ± 82 for 4:1. Table of results from image analyses is provided in supplementary material. These molar ratios were

consistent with the ITC molar ratio at 25 °C, pH 5.0 (4.0 ± 0.9). For molar ratios above 6:1 (245 ± 59 coacervates in the optical field for 6:1), the coacervates size and number decreased and then disappeared (molar ratio 10:1). These results were consistent with the turbidity behavior of the mixtures and their macroscopic aspects (**Figure 4 and Figure 5**). For extreme ratios (1:1 and 10:1), due to low turbidity, no object was observed by optical microscopy. It was possible that complexes were formed at these ratios but that they were too small to be observed by optical microscopy. A change of the size and shape of the objects, observed when the molar ratio increased, was consistent with the endothermic sequences observed on the ITC measurements (**Figure 1**). Moreover, a change of the shape of the complexes observed by optical micrographs confirmed the absence of dilution phenomenon.

From the microscopy observations, one can note that the polydispersity of size of the objects was quite high. CN had indeed non-charged polar residues (Coelho et al., 2011) because its deacetylation degree was around 75%, so 25% of its N-acetylglucosamine residues were not charged and GA has large non-charged segments. Indeed, GA contains only 23% of glucuronic acid, which are the GA residues charged when deprotonated (Coelho et al., 2011; Renard et al., 2006). The charges were heterogeneously distributed on polydisperse polymers, which can explain the irregularity of the sizes of the coacervates.

The optimal molar ratio for the formation of coacervate determined by optical microscopy (between to 4:1 and 5:1) was consistent with the stoichiometry determined from ITC measurements (4.0 ± 0.9 for pH 5.0 at 25 °C) (**Figure 1**) and the optimal ratio obtained by Zeta potential measurements (3.2 ± 0.2), when the value was close to zero and with the maximum turbidity of the mixture (4.0 ± 0.4). However, the first complexes were formed for a ratio 3:1, but other phenomenon occurred for higher molar ratio. On the **Figure 5** from molar ratio 4:1 to 5:1 the coacervates were different, which could be explained by a reorganization of the system.

The loss in turbidity along with the increase of the ratio could be explained by a reorganization of the complexes to achieve thermodynamic equilibrium. These results were also observed on the optical micrographs and were correlated by the ITC measurements.

These interactions between the polymers were indeed partially driven by the pH-related net charge of the two polymers. The influence of the pH on the interactions between GA and CN had thus been investigated.

3.4. Effect of the pH of the solutions on the interactions between gum Arabic and chitosan

From ITC results, it seemed that the major interaction that drove complex coacervation was the electrostatic interactions related to the enthalpy (**Table 2** and **Figure 2**). Since the charges of CN varied upon pH (**Table 1**), the influence of pH on the mixtures was investigated. These experiments were conducted at 25 °C. The approximate Zeta potential of the mixtures was measured at pH 4.5, 5.0 and 5.5 for different molar ratios (**Figure 6.A**). For low GA/CN molar ratio (below 1:1), the approximate Zeta potential was close to the one of CN (21.6 ± 0.8 mV). The more GA was added, the lower the Zeta potential of the mixture got until it almost reached the GA Zeta potential (-10.2 ± 0.2 mV, **Table 1**). At pH 4.5, 5.0 and 5.5, the Zeta potential reached zero for a molar ratio of 3.0:1; 3.3:1 and 2.4:1, respectively (**Figure 6A**). Turbidity measurements were conducted at low molar ratio (1:1) and at optimal molar ratio for pH 5.0 (4:1) determined by ITC (**Figure 6B**).

For the molar ratio 1:1, the turbidity was low all along the pH range (0.089 ± 0.005 and 0.164 ± 0.007 cm⁻¹ at pH 4.5 and 5.0, respectively) but it was higher at pH 5.5 (0.293 ± 0.011 cm⁻¹) (**Figure 6.B**). From the results obtained by optical micrographs and ITC (**Figure 5** and **Table 2**), the molar ratio 4:1 was supposed to be almost optimal at pH 5.0. At this ratio, when

the pH increased, the turbidity decreased from $2.953 \pm 0.044 \text{ cm}^{-1}$ at pH 4.5 to $2.554 \pm 0.033 \text{ cm}^{-1}$ at pH 5.5 (**Figure 6**). Indeed, the higher the pH, the smaller the difference of charge between GA and CN (**Table 1**) and the lower the stoichiometry, as the tendencies have been seen previously in the ITC results ($n = 4.1 \pm 0.8$ at pH 4.5 and $n = 3.3 \pm 0.2$ at pH 5.5) and with the Zeta potential data (the Zeta potential reached zero for a molar ratio of 3.0:1 at pH 4.5 and 2.4:1 at pH 5.5). There should be one optimal molar ratio for each pH.

From the optical micrographs (**Figure 7**) coacervates were formed for all pH for a ratio of 4:1. At ratio 1:1, no visible coacervate was formed at pH 4.5 and 5.0, whereas some coacervates were formed at pH 5.5. This result was consistent with the Zeta potential and turbidity of the mixtures (**Figure 6**). For a molar ratio of 4:1, though pH had no influence over the size or polydispersity of the coacervates formed, it impacted the number of coacervates (264 ± 18 coacervates in the optical field for pH 4.5, 747 ± 82 for pH 5.0 and 353 ± 17 for pH 5.5). Table of results from image analyses is provided in supplementary material.

At different pH, the complexes between GA and CN had different shapes and were driven by different enthalpy and entropy contributions. According to ITC results (**Figure 1**) at 25 °C the stoichiometry at pH 4.5 would be 4.1 ± 0.8 , at pH 5.0: 4.0 ± 0.9 and at pH 5.5: 3.3 ± 0.2 . Ever for low molar ratios, it was possible to obtain coacervates by decreasing pH. ITC measurement also revealed that the interactions were also different upon temperature. At pH 5.0, the complex coacervation of GA and CN was then investigated at different temperatures (5, 25 and 45 °C).

3.5. Effect of the temperature of the solutions on the interactions between gum Arabic and chitosan

At the beginning of titration, the Zeta potential (**Figure 8**) corresponded to the one of the CN (21.6 ± 0.8 mV), and at the end of the titration, the Zeta of the mixture corresponded to the one of GA alone (-10.2 ± 0.2 mV). Between the beginning and the end of the titration, for all temperatures the Zeta potential decreased. However, it differed according to the temperature. The stoichiometry decreased when temperature increased (**Figure 8**) as observed by ITC. Indeed the molar ratio for which the approximate Zeta potential was close to 0 mV varied with temperature from 4.2:1 at 5 °C to 3.4:1 at 25 °C and 2.5:1 at 45 °C at pH 5.0 (against $3.8:1 \pm 0.2$, $4.0:1 \pm 0.9$ and $2.9:1 \pm 0.2$ with the ITC, respectively). These results were strongly consistent with the ones obtained from ITC (**Table 2** and **Figure 2**).

These results were consistent with the previous hypothesis: this could mean that the CN had different conformation at different temperatures and that some of its charges were not accessible at high temperatures (the stoichiometry being reached for lower molar ratio at 45 °C than at 5 °C). At 5 °C, more GA was needed to counter the charges of the CN because more CN charges were accessible, so the enthalpy was less favorable than at 45 ° (**Table 2** and **Figure 2**). The aspect of the sample has been investigated by optical microscopy (**Figure 9**).

At molar ratio 1:1, whatever the temperature, no object was formed. At molar ratio 4:1, coacervates were observed for all temperatures and the complexes formed were strongly different. However, the number and shape of the coacervates varied upon temperature. At 5 °C, the coacervates were less numerous than at 25 °C (**Figure 9**) (63 ± 9 coacervates in the optical field at 5 °C against 747 ± 82 at 25 °C). Table of results from image analyses is provided in supplementary material. Temperature seemed to have an impact on the size of the coacervates,

as reported previously in this study. At 45 °C for a ratio of 4:1, the coacervates were bigger and less numerous (the average diameter of the coacervates was $7.3 \pm 0.8 \mu\text{m}$ at 45 °C against 3.6 ± 0.5 at 25 °C and the number of coacervates was 173 ± 36 at 45 °C), which could be explained by a coalescence phenomenon of the complexes at higher temperature. Even though the coacervates were bigger, no settling was observed 5 minutes after mixing.

3.6. Possible mechanism of the interaction between gum Arabic and chitosan

From previous experiments, it was observed that pH, temperature and molar ratio strongly influenced the coacervation phenomenon, in terms of thermodynamic pathway and structural aspects of the objects formed. The obtained results allowed us to propose a mechanism involved in the formation of GA/CN complexes (**Figure 10**).

The complex coacervation between GA and CN were investigated. When mixed in solution, those two oppositely charged polysaccharides formed a turbid dispersion which led to assume they were forming complexes (**Figure 4**). The optical micrographs of the mixtures in different conditions of pH and temperature showed that these parameters had a significant influence over the complexes formed (**Figure 10**). The different thermodynamic parameters obtained for each condition confirmed this influence (**Table 2** and **Figure 2**).

In order to study the influence of the molar ratio, the interactions between GA and CN were investigated at a given pH and a given temperature. At a ratio of 1:1, no complexe was visible (pH 5.0, 25 °C, **Figure 10.A**). However, it was possible that objects were formed (GA charges would interact with CN charges), but that they were too small to be seen by optical microscopy. When more GA was added to the CN, more charges of the polymers interacted. The stoichiometry value determined by ITC was confirmed from optical microscopy (ratio 4:1),

turbidity (maximum turbidity between ratios 3:1 to 4:1) and Zeta potential (close to 0 between 3:1 and 4:1). Upon molar ratio, visible complexes were indeed formed (ratio 3:1) and all GA charges interacted with all CN charges, obtaining neutral visible coacervates. At a ratio 6:1, when GA was in excess, the complexes got smaller. In order to reach an equilibrium in the system, the GA could desorb and the coacervates could change. This hypothesis is consistent with the small endothermic event at the end of titration (ITC results, **Figure 1**). This could be explained by this reorganization within the system.

To study the influence of the pH, the interactions between GA and CN were investigated at a given temperature and a given molar ratio (25 °C, ratio 1:1, **Figure 10.B**). The complexes were different upon pH. At pH 4.5 and 5.0, no complex was observed. At pH 5.5, visible coacervates were formed. Even at a small molar ratio, it was possible to have a coacervation phenomenon by tempering with the pH, since pH affected the Zeta potential of CN (**Table 1**), so less GA would be needed to achieve stoichiometry. The optimal molar ratio was smaller at higher pH ($2.2:1 \pm 0.1$ at pH 5.5 against $2.7:1 \pm 0.2$ at pH 4.5). Since when the pH raised, the Zeta potential of CN decreased, there were fewer free charges available for the interaction in the system which might explain that the entropy was less favorable when the pH increased (the system was more ordered).

To study the influence of the temperature, the interactions between GA and CN were investigated at a given pH and a given molar ratio (pH 5.0, ratio 4:1, **Figure 10.C**). The complexes between GA and CN changed upon temperature in the same way that they did upon pH. When GA was in excess (more GA than was necessary to achieve stoichiometry), at 25 °C, more coacervates were formed. At 45 °C, the coacervates were bigger, suggesting a coalescence of the coacervate because of enhanced thermal motion at this temperature. At a given pH, there was an optimal molar ratio for each temperature (**Figure 10.C**). This was inherent to the shape

of the polymers at different temperature, especially the one of CN. Indeed, CN was compacted at 45 °C, so less charges were available for the interaction, needing less GA to achieve stoichiometry. This was consistent with the stoichiometry determined by ITC. The fewer GA involved in the interaction, the smaller the coacervates.

For each pH-temperature combination, there was an optimal molar ratio for coacervation. For example, at a pH 5.0, at 5 °C the optimal molar ratio was 4:1, and at 25 and 45 °C the stoichiometry was around 3:1 and 2:1, respectively (**Figure 10**). The size and shape of the coacervates changed upon pH, temperature and molar ratio.

4. Conclusions

The interactions of gum Arabic (GA) and chitosan (CN) in solution have been investigated upon pH (4.5, 5.0 and 5.5) and temperature (5, 25 and 45 °C). The stoichiometry and the thermodynamic parameters of the interaction in these conditions were determined using ITC. The approximate Zeta potential and the turbidity were confirmed to be strong methods to approximate the stoichiometry of the interaction. For each pH-temperature combination, there was a given optimal molar ratio. The interaction was always spontaneous (ΔG negative) and it was always enthalpy-driven (negative enthalpy). Unlike what can be found in literature, a raise in temperature also favored electrostatic interactions between GA and CN, which was related to the conformation and charges of the polymers. A raise in temperature lead to a compaction of CN and to a diminution of its steric hindrance and to the accessibility of its charges, forming coacervates for lower molar ratios. The same phenomenon happened upon a raise of pH. High temperatures favored coalescence leading to a more ordered system, disfavoring entropy (negative entropy parameter). It was also shown that a raise of pH could be tempered by a decrease in temperature in order to obtain the same objects, using a close thermodynamic

pathway. Even for low molar ratios, by choosing a fitted pH/temperature combination, it was possible to form coacervates. Based on these results, it should be possible to control the formation of complexes by controlling the pH-temperature combination.

In the different conditions studied, different shaped-complexes were formed. The way they were formed and the shape of the objects might strongly influence their capacity to encapsulate and to deliver a molecule of interest. This study showed that by knowing the charges of polymers upon different parameters, it could be possible to predict the effect of these parameters on the coacervation of these polymers. It would also be interesting to find the better pH/temperature combination to limit coalescence. Moreover, it would be interesting to study the influence of ionic strength on the coacervates formation, due to the presence of counterions masking the charges. Now that the mechanism and driving forces of the interactions are better understood, it should be used to investigate the relation between the shape of the complexes and their ability to encapsulate a molecule and to study the impact of the molecule on the coacervate added during the coacervates formation. Hopefully the different forms of the complexes could lead to encapsulation in different medium and to deliver the molecule to a chosen target. Other shapes of complexes could be obtained by taking other polymers or by modifying GA or CN for example.

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575 References

- 576 Aberkane, L., Jasniewski, J., Gaiani, C., Scher, J., & Sanchez, C. (2010). Thermodynamic
577 characterization of acacia gum-beta-lactoglobulin complex coacervation. *Langmuir: The ACS*
578 *Journal of Surfaces and Colloids*, 26(15), 12523–12533. <https://doi.org/10.1021/la100705d>
- 579 Abodinar, A., Smith, A. M., & Morris, G. A. (2014). A novel method to estimate the stiffness
580 of carbohydrate polyelectrolyte polymers based on the ionic strength dependence of zeta
581 potential. *Carbohydrate Polymers*, 112, 6–9. <https://doi.org/10.1016/j.carbpol.2014.05.063>
- 582 Anwunobi, A. P., Emeje, M. O. (2011). Recent applications of natural polymers in nanodrug
583 delivery. *Journal of Nanomedicine & Nanotechnology*, s4(1). [https://doi.org/10.4172/2157-](https://doi.org/10.4172/2157-7439.S4-002)
584 7439.S4-002
- 585 Avadi, M. R., Sadeghi, A. M. M., Mohammadpour, N., Abedin, S., Atyabi, F., Dinarvand, R.,
586 & Rafiee-Tehrani, M. (2010a). Preparation and characterization of insulin nanoparticles using
587 chitosan and Arabic gum with ionic gelation method. *Nanomedicine: Nanotechnology, Biology*
588 *and Medicine*, 6(1), 58–63. <https://doi.org/10.1016/j.nano.2009.04.007>
- 589 Beppu, M. M., & Santana, C. C. (2002). Influence of calcification solution on in vitro chitosan
590 mineralization. *Materials Research*, 5(1), 47–50. [https://doi.org/10.1590/S1516-](https://doi.org/10.1590/S1516-14392002000100008)
591 14392002000100008
- 592 Bhatia, S. R., Khattak, S. F., & Roberts, S. C. (2005). Polyelectrolytes for cell encapsulation.
593 *Current Opinion in Colloid & Interface Science*, 10(1), 45–51.
594 <https://doi.org/10.1016/j.cocis.2005.05.004>
- 595 Butstraen, C., & Salaün, F. (2014). Preparation of microcapsules by complex coacervation of
596 gum Arabic and chitosan. *Carbohydrate Polymers*, 99, 608–616.
597 <https://doi.org/10.1016/j.carbpol.2013.09.006>
- 598 Chen, R. H., & Tsaih, M. L. (1998). Effect of temperature on the intrinsic viscosity and
599 conformation of chitosans in dilute HCl solution. *International Journal of Biological*
600 *Macromolecules*, 23(2), 135–141. [https://doi.org/10.1016/S0141-8130\(98\)00036-1](https://doi.org/10.1016/S0141-8130(98)00036-1)
- 601 Coelho, S., Moreno-Flores, S., Toca-Herrera, J. L., Coelho, M. A. N., Carmo Pereira, M., &
602 Rocha, S. (2011). Nanostructure of polysaccharide complexes. *Journal of Colloid and Interface*
603 *Science*, 363(2), 450–455. <https://doi.org/10.1016/j.jcis.2011.07.098>
- 604 de Kruif, C. G., Weinbreck, F., & de Vries, R. (2004). Complex coacervation of proteins and
605 anionic polysaccharides. *Current Opinion in Colloid & Interface Science*, 9(5), 340–349.
606 <https://doi.org/10.1016/j.cocis.2004.09.006>
- 607 Dickinson, E. (2008). Interfacial structure and stability of food emulsions as affected by protein
608 –polysaccharide interactions. *Soft Matter*, 4(5), 932–942. <https://doi.org/10.1039/B718319D>

609 Dickinson, E., Miller, R., Sanchez, C., Despond, S., Schmitt, C., & Hardy, J. (2001). Effect of
610 heat and shear on β -lactoglobulin-acacia gum complex coacervation. *Food Colloids*, 332–342.
611 <https://doi.org/10.1039/9781847550842-00332>

612 Duhoranimana, E., Yu, J., Mukeshimana, O., Habinshuti, I., Karangwa, E., Xu, X. Muhoza, B.,
613 Xia, S., Zhang, X. (2018). Thermodynamic characterization of Gelatin–Sodium carboxymethyl
614 cellulose complex coacervation encapsulating Conjugated Linoleic Acid (CLA). *Food*
615 *Hydrocolloids*, 80, 149–159. <https://doi.org/10.1016/j.foodhyd.2018.02.011>

616 Eratte, D., Wang, B., Dowling, K., J. Barrow, C., & P. Adhikari, B. (2014). Complex
617 coacervation with whey protein isolate and gum arabic for the microencapsulation of omega-3
618 rich tuna oil. *Food & Function*, 5(11), 2743–2750. <https://doi.org/10.1039/C4FO00296B>

619 Espinosa-Andrews, H., Báez-González, J. G., Cruz-Sosa, F., & Vernon-Carter, E. J. (2007).
620 Gum Arabic–chitosan complex coacervation. *Biomacromolecules*, 8(4), 1313–1318.
621 <https://doi.org/10.1021/bm0611634>

622 Espinosa-Andrews, H., Enríquez-Ramírez, K. E., García-Márquez, E., Ramírez-Santiago, C.,
623 Lobato-Calleros, C., & Vernon-Carter, J. (2013). Interrelationship between the zeta potential
624 and viscoelastic properties in coacervates complexes. *Carbohydrate Polymers*, 95(1), 161–166.
625 <https://doi.org/10.1016/j.carbpol.2013.02.053>

626 Espinosa-Andrews, H., Sandoval-Castilla, O., Vázquez-Torres, H., Vernon-Carter, E. J., &
627 Lobato-Calleros, C. (2010). Determination of the gum Arabic–chitosan interactions by Fourier
628 Transform Infrared Spectroscopy and characterization of the microstructure and rheological
629 features of their coacervates. *Carbohydrate Polymers*, 79(3), 541–546.
630 <https://doi.org/10.1016/j.carbpol.2009.08.040>

631 Fan, W., Yan, W., Xu, Z., & Ni, H. (2012). Formation mechanism of monodisperse, low
632 molecular weight chitosan nanoparticles by ionic gelation technique. *Colloids and Surfaces B:*
633 *Biointerfaces*, 90, 21–27. <https://doi.org/10.1016/j.colsurfb.2011.09.042>

634 Fathi, M., Martín, Á., & McClements, D. J. (2014). Nanoencapsulation of food ingredients
635 using carbohydrate based delivery systems. *Trends in Food Science & Technology*, 39(1), 18–
636 39. <https://doi.org/10.1016/j.tifs.2014.06.007>

637 Feng, X., Leduc, M., & Pelton, R. (2008). Polyelectrolyte complex characterization with
638 isothermal titration calorimetry and colloid titration. *Colloids and Surfaces A: Physicochemical*
639 *and Engineering Aspects*, 317(1), 535–542. <https://doi.org/10.1016/j.colsurfa.2007.11.053>

640 Filion, D., Lavertu, M., & D. Buschmann, M. (2007). Ionization and solubility of chitosan
641 solutions related to thermosensitive chitosan/glycerol-phosphate systems. *Biomacromolecules*,
642 8(10), 3224–3234. <https://doi.org/10.1021/bm700520m>

643 Jayme, M. L., Dunstan, D. E., & Gee, M. L. (1999). Zeta potentials of gum arabic stabilised oil
644 in water emulsions. *Food Hydrocolloids*, 13(6), 459–465. [https://doi.org/10.1016/S0268-](https://doi.org/10.1016/S0268-005X(99)00029-6)
645 [005X\(99\)00029-6](https://doi.org/10.1016/S0268-005X(99)00029-6)

646 Joye, I. J., & McClements, D. J. (2014). Biopolymer-based nanoparticles and microparticles:
 647 Fabrication, characterization, and application. *Current Opinion in Colloid & Interface Science*,
 648 19(5), 417–427. <https://doi.org/10.1016/j.cocis.2014.07.002>

649 Kayitmazer, A. B. (2017). Thermodynamics of complex coacervation. *Advances in Colloid and*
 650 *Interface Science*, 239, 169–177. <https://doi.org/10.1016/j.cis.2016.07.006>

651 Kelly, R., Gudo, E. S., Mitchell, J. R., & Harding, S. E. (1994). Some observations on the nature
 652 of heated mixtures of bovine serum albumin with an alginate and a pectin. *Carbohydrate*
 653 *Polymers*, 23(2), 115–120. [https://doi.org/10.1016/0144-8617\(94\)90035-3](https://doi.org/10.1016/0144-8617(94)90035-3)

654 Kim, H. J., Yang, B., Park, T. Y., Lim, S., & Cha, H. J. (2017). Complex coacervates based on
 655 recombinant mussel adhesive proteins: their characterization and applications. *Soft Matter*,
 656 13(42), 7704–7716. <https://doi.org/10.1039/C7SM01735A>

657 Kizilay, E., Kayitmazer, A. B., & Dubin, P. L. (2011). Complexation and coacervation of
 658 polyelectrolytes with oppositely charged colloids. *Advances in Colloid and Interface Science*,
 659 167(1–2), 24–37. <https://doi.org/10.1016/j.cis.2011.06.006>

660 Lankalapalli, S., & Kolapalli, V. R. M. (2009). Polyelectrolyte complexes: a review of their
 661 applicability in drug delivery technology. *Indian Journal of Pharmaceutical Sciences*, 71(5),
 662 481–487. <https://doi.org/10.4103/0250-474X.58165>

663 Li, D., Kelkar, M. S., & Wagner, N. J. (2012). Phase behavior and molecular thermodynamics
 664 of coacervation in oppositely charged polyelectrolyte/surfactant systems: a cationic polymer JR
 665 400 and anionic surfactant SDS mixture. *Langmuir*, 28(28), 10348–10362.
 666 <https://doi.org/10.1021/la301475s>

667 Lopez-Torrez, L., Costalat, M., Williams, P., Doco, T., & Sanchez, C. (2015). Acacia senegal
 668 vs. Acacia seyal gums – Part 1: Composition and structure of hyperbranched plant exudates.
 669 *Food Hydrocolloids*, 51, 41–53. <https://doi.org/10.1016/j.foodhyd.2015.04.019>

670 Maqbool, M., Ali, A., & Alderson, P. G. (2010). A combination of gum arabic and chitosan can
 671 control anthracnose caused by *Colletotrichum musae* and enhance the shelf-life of banana fruit.
 672 *Journal of Horticultural Science & Biotechnology*, 85(5), 432–436.
 673 <https://doi.org/https://doi.org/10.1080/14620316.2010.11512693>

674 Moschakis, T., Murray, B. S., & Biliaderis, C. G. (2010). Modifications in stability and
 675 structure of whey protein-coated o/w emulsions by interacting chitosan and gum arabic mixed
 676 dispersions. *Food Hydrocolloids*, 24(1), 8–17. <https://doi.org/10.1016/j.foodhyd.2009.07.001>

677 Overbeek, J. T. G., & Voorn, M. J. (1957). Phase separation in polyelectrolyte solutions. Theory
 678 of complex coacervation. *Journal of Cellular and Comparative Physiology*, 49(S1), 7–26.
 679 <https://doi.org/10.1002/jcp.1030490404>

680 Priftis, D., Laugel, N., & Tirrell, M. (2012). Thermodynamic characterization of polypeptide
 681 complex coacervation. *Langmuir*, 28(45), 15947–15957. <https://doi.org/10.1021/la302729r>

- Qiao, Y., Di, Z., Ma, Y., Ma, P., & Xia, S. (2010). Viscosities of pure water, acetic acid + water, and p-Xylene + acetic acid + water at different temperature and pressure. *Chinese Journal of Chemical Engineering*, 18(3), 446–454. [https://doi.org/10.1016/S1004-9541\(10\)60242-X](https://doi.org/10.1016/S1004-9541(10)60242-X)
- Qin, J., & Gronenborn, A. M. (2014). Weak protein complexes: challenging to study but essential for life. *The FEBS Journal*, 1948–1949. <https://doi.org/10.1111/febs.12744>
- Renard, D., Garnier, C., Lapp, A., Schmitt, C., & Sanchez, C. (2012). Structure of arabinogalactan-protein from Acacia gum: from porous ellipsoids to supramolecular architectures. *Carbohydrate Polymers*, 90(1), 322–332. <https://doi.org/10.1016/j.carbpol.2012.05.046>
- Renard, D., Lavenant-Gourgeon, L., Ralet, M.-C., & Sanchez, C. (2006). Acacia senegal Gum: continuum of molecular species differing by their protein to sugar ratio, molecular weight, and charges. *Biomacromolecules*, 7(9), 2637–2649. <https://doi.org/10.1021/bm060145j>
- Roldan-Cruz, C., Carmona-Ascencio, J., Vernon-Carter, E. J., & Alvarez-Ramirez, J. (2016). Electrical impedance spectroscopy for monitoring the gum Arabic–chitosan complexation process in bulk solution. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 495, 125–135. <https://doi.org/10.1016/j.colsurfa.2016.02.004>
- Roy, M. N., Ghosh, G., & Sarkar, L. (2010). Viscous synergic behaviour and solvent–solvent interactions occurring in liquid mixtures of aqueous alkanols with some alkanoic acids. *Physics and Chemistry of Liquids*, 48(5), 564–579. <https://doi.org/10.1080/00319100903161507>
- Sakloetsakun, D., Preechagoon, D., Bernkop-Schnürch, A., & Pongjanyakul, T. (2015). Chitosan–gum arabic polyelectrolyte complex films: physicochemical, mechanical and mucoadhesive properties. *Pharmaceutical Development and Technology*, 1–10. <https://doi.org/10.3109/10837450.2015.1035727>
- Sanchez, C., Mekhloufi, G., & Renard, D. (2006). Complex coacervation between β -lactoglobulin and Acacia gum: A nucleation and growth mechanism. *Journal of Colloid and Interface Science*, 299(2), 867–873. <https://doi.org/10.1016/j.jcis.2006.02.031>
- Schatz, C., Lucas, J.-M., Viton, C., Domard, A., Pichot, C., & Delair, T. (2004). Formation and properties of positively charged colloids based on polyelectrolyte complexes of biopolymers. *Langmuir*, 20(18), 7766–7778. <https://doi.org/10.1021/la049460m>
- Schmitt, C., Sanchez, C., Desobry-Banon, S., & Hardy, J. (1998). Structure and technofunctional properties of protein-polysaccharide complexes: a review. *Critical Reviews in Food Science and Nutrition*, 38(8), 689–753. <https://doi.org/10.1080/10408699891274354>
- Schmitt, C., & Turgeon, S. L. (2011). Protein/polysaccharide complexes and coacervates in food systems. *Advances in Colloid and Interface Science*, 167(1), 63–70. <https://doi.org/10.1016/j.cis.2010.10.001>
- Shaddel, R., Hesari, J., Azadmard-Damirchi, S., Hamishehkar, H., Fathi-Achachlouei, B., & Huang, Q. (2018). Use of gelatin and gum Arabic for encapsulation of black raspberry

720 anthocyanins by complex coacervation. *International Journal of Biological Macromolecules*,
721 107, 1800–1810. <https://doi.org/10.1016/j.ijbiomac.2017.10.044>

722 Soni, B., Mahmoud, B., Chang, S., El-Giar, E. M., & Hassan, E. B. (2018). Physicochemical,
723 antimicrobial and antioxidant properties of chitosan/TEMPO biocomposite packaging films.
724 *Food Packaging and Shelf Life*, 17, 73–79. <https://doi.org/10.1016/j.fpsl.2018.06.001>

725 Sonia, T. A., & Sharma, C. P. (2012). An overview of natural polymers for oral insulin delivery.
726 *Drug Discovery Today*, 17(13–14), 784–792. <https://doi.org/10.1016/j.drudis.2012.03.019>

727 Tan, C., Xie, J., Zhang, X., Cai, J., & Xia, S. (2016). Polysaccharide-based nanoparticles by
728 chitosan and gum arabic polyelectrolyte complexation as carriers for curcumin. *Food*
729 *Hydrocolloids*, 57, 236–245. <https://doi.org/10.1016/j.foodhyd.2016.01.021>

730 Turgeon, S. L., Schmitt, C., & Sanchez, C. (2007). Protein–polysaccharide complexes and
731 coacervates. *Current Opinion in Colloid & Interface Science*, 12(4–5), 166–178.
732 <https://doi.org/10.1016/j.cocis.2007.07.007>

733 Veis, A. (2011). A review of the early development of the thermodynamics of the complex
734 coacervation phase separation. *Advances in Colloid and Interface Science*, 167(1–2), 2–11.
735 <https://doi.org/10.1016/j.cis.2011.01.007>

736 Veis, A., & Aranyi, C. (1960). Phase separation in polyelectrolyte systems. I. Complex
737 coacervates of gelatin. *The Journal of Physical Chemistry*, 64(9), 1203–1210.
738 <https://doi.org/10.1021/j100838a022>

739 Xiong, W., Ren, C., Jin, W., Tian, J., Wang, Y., Shah, B. R., Li, J., Li, B. (2016). Ovalbumin-
740 chitosan complex coacervation: phase behavior, thermodynamic and rheological properties.
741 *Food Hydrocolloids*, 61, 895–902. <https://doi.org/10.1016/j.foodhyd.2016.07.018>

Figure captions

Figure 1. (A) Corrected heat rate upon time of mixtures of **gum Arabic** and chitosan at pH 5.0 at 5, 25 and 45 °C (A) and at 45 °C at pH 4.5, 5.0 and 5.5, (B) Enthalpy and fit upon **GA/CN** molar ratio of mixtures of **gum Arabic** and chitosan at pH 5.0 at 5, 25 and 45 °C, (C) and at 45 °C at pH 4.5, 5.0 and 5.5 (D)

Figure 2. Influence of pH and temperature on (A) the optimal molar ratio (n), (B) the enthalpic (ΔH) and (C) and the entropic ($T\Delta S$) contributions of gum Arabic and chitosan interactions.

Figure 3. Optical micrographs of **gum Arabic** and chitosan mixtures (A) at pH 4.5 at 25 °C and pH 5.5 at 5 °C and (B) at pH 4.5 at 45 °C and pH 5.5 at 25 °C at 5°C, 5 min after mixing **for a** **GA/CN** molar ratio of 4:1

Figure 4. Turbidity (cm^{-1}) and approximate Zeta potential (mV), of **gum Arabic** and chitosan mixtures upon **GA/CN** molar ratio at pH 5.0 and 25 °C, 5 min after mixing

Figure 5. Optical micrographs of **gum Arabic** and chitosan mixtures upon different **GA/CN** molar ratios 5 min after mixing at 25 °C, pH 5.0

Figure 6. (A) Approximate Zeta potential (mV) of the **gum Arabic** and chitosan mixtures upon **GA/CN** molar ratios at different pH, at 25 °C and (B) turbidity (cm^{-1}) of **gum Arabic** and chitosan mixtures for **GA/CN** molar ratios of 1:1 and 4:1 at different pH, 5 min after mixing

Figure 7. Optical micrographs of **gum Arabic** and chitosan mixtures upon pH for **GA/CN** molar ratios 4:1 and 1:1 at 25 °C

Figure 8. Approximate Zeta potential (mV) of the **gum Arabic** and chitosan mixtures upon molar ratio at different temperatures, at pH 5.0

Figure 9. Optical micrographs of **gum Arabic** and chitosan mixtures upon different temperatures for **GA/CN** molar ratios 4:1 and 1:1 at pH 5.0

Figure 10. Optical micrographs and schematic representation of the influence of (A) the molar ratio at pH 5.0, 25 °C, (B) the pH at 25 °C, **GA/CN** molar ratio 1:1 and (C) the temperature at pH 5.0, **GA/CN** molar ratio 4:1 on the coacervation of **gum Arabic** and chitosan

Figure 1

Figure 2

Figure 3

Figure 4

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Figure 9

Figure 10

Table captions

Table 1. Approximate Zeta potential of chitosan (0.02% w/v) and **gum Arabic** (1.00% w/v) solutions in acetic acid 1.00% v/v upon pH and temperature and comparative Zeta potential ratios ($|ZP_{CN}/ZP_{GA}|$). “a, b, c, d, e and f” mean that the statistical analysis showed no significant differences between the results

Table 2. Thermodynamic parameters (stoichiometry (n), enthalpic contribution (ΔH), entropic contribution ($T\Delta S$), Gibbs free energy (ΔG) and dissociation constant (K_d)) of binding between **gum Arabic** and chitosan upon pH and temperature

Table 1

Temperature (°C)	pH	Approximate Zeta Potential (mV)		$ ZP_{CN}/ZP_{GA} $
		Chitosan 0.02% w/v in acetic acid	Gum Arabic 1.00% w/v in acetic acid	
5	4.5	37.2 ± 3.1^a	-9.9 ± 0.4^f	3.1 ± 1.5
	5	22.9 ± 0.7^b	-9.8 ± 1.0^f	2.3 ± 0.2
	5.5	24.5 ± 1.9^b	-9.4 ± 0.2^f	1.6 ± 1.1
25	4.5	28.0 ± 0.9^c	-10.5 ± 0.8^f	2.7 ± 0.2
	5	21.6 ± 0.8^{bd}	-10.2 ± 0.2^f	2.1 ± 0.1
	5.5	20.3 ± 1.0^d	-9.1 ± 0.3^f	2.2 ± 0.1
45	4.5	17.8 ± 0.8^e	-10.3 ± 0.2^f	2.0 ± 0.5
	5	23.0 ± 0.4^b	-9.8 ± 1.2^f	2.2 ± 0.1
	5.5	15.6 ± 1.5^e	-9.1 ± 0.3^f	1.8 ± 0.4

Table 2

T (°C)	pH	n	ΔH (kJ.mol ⁻¹)	$T\Delta S$ (kJ.mol ⁻¹)	ΔG (kJ.mol ⁻¹)	K_d (10 ⁻⁸)
5	4.5	3.0 ± 0.5	-59.6 ± 14.2	-21.3 ± 14.0	-38.3 ± 0.2	6.6 ± 0.7
	5.0	3.8 ± 0.2	-37.6 ± 11.6	+0.6 ± 14.2	-38.2 ± 2.7	13.3 ± 1.8
	5.5	4.0 ± 0.1	-217.1 ± 18.8	-183.3 ± 19.4	-33.8 ± 0.6	26.8 ± 16.9
25	4.5	4.1 ± 0.8	-67.7 ± 2.7	-23.3 ± 2.3	-44.3 ± 0.5	1.7 ± 0.3
	5.0	4.0 ± 0.9	-135.2 ± 0.2	-95.4 ± 2.3	-39.7 ± 2.0	12.9 ± 9.5
	5.5	3.3 ± 0.2	-322.3 ± 10.8	-283.0 ± 11.8	-39.3 ± 0.9	13.5 ± 5.0
45	4.5	3.2 ± 0.3	-293.9 ± 70.6	-249.0 ± 70.7	-44.9 ± 0.1	4.4 ± 0.1
	5.0	2.9 ± 0.2	-561.6 ± 2.5	-519.8 ± 2.0	-41.8 ± 0.5	16.0 ± 2.6
	5.5	2.7 ± 0.1	-854.1 ± 45.7	-812.5 ± 46.0	-41.5 ± 0.3	15.2 ± 1.6

Gum Arabic/Chitosan complex coacervation

Coacervates formation

Isothermal Titration Calorimetry

Temperature favored enthalpy