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1     **Associative properties of rapeseed napin and pectin: competition between**  
2                     **liquid-liquid and liquid-solid phase separation.**

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7

8     **Abstract:** We investigated the assembly of a plant protein, rapeseed napin (NAP), mixed with a plant  
9     polysaccharide, highly methylated pectin (PEC). The optimum pH for NAP/PEC interactions was found  
10    at pH 4.0 for which ~~the charge difference between the two biopolymers is the highest, corresponding~~  
11    ~~to the highest electrostatic contribution between the two biopolymers.~~ Two types of phase transition  
12    were observed depending on pH and mixing ratios: liquid-solid and liquid-liquid phase separation.  
13    We showed that liquid-solid transition was favored by strong electrostatic attraction whereas liquid-  
14    liquid phase separation was promoted by weaker attraction. In addition, we highlighted a solid-to-  
15    liquid phase transition overtime for ratios with excess of proteins. We showed that **polysaccharide**  
16    **charge neutralization was a requisite for the transition as no rearrangement was observed when**  
17    **residual charges remained. We discuss the role of protein flexibility in this phenomenon as napin is**  
18    ~~predicted to be partially disordered.~~ **The underlying mechanism leading to this transition remains to**  
19    **be explored.** To the best of our knowledge, such solid-to-liquid transition has never been reported  
20    for protein-polysaccharide mixtures.

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26    **Keywords.** Complex coacervation. Napin. Pectin. Phase transition. Droplets millifluidic.

## 27 I. Introduction

28 Complex coacervation is an associative liquid-liquid phase separation primarily driven by attractive  
29 electrostatics interactions between two oppositely charged polyelectrolytes. There have been  
30 intense research efforts over the years to better understand and control complex coacervation with  
31 various applications in pharmaceutical, biomedical, cosmetic or food industry (Schmitt & Turgeon,  
32 2011; Turgeon, Schmitt, & Sanchez, 2007). In case of strong attraction, liquid-solid phase separation,  
33 also called precipitation, may occur (Comert, Malanowski, Azarikia, & Dubin, 2016). It is usually  
34 associated with undesirable impact during processes such as inhomogeneity and irreproducible  
35 kinetics (Comert & Dubin, 2017). Understanding the competition between the two mechanisms and  
36 identifying parameters leading to one or the other mechanism is essential in the further  
37 development of coacervation applications.

38 Liquid-liquid phase separation is a reversible mechanism leading to the formation of spherical  
39 droplets in equilibrium with a diluted phase. The liquid-like state of the dense phase is inferred from  
40 the spherical shape of phase separated domains as well as their ability to fuse into larger round-  
41 shaped objects. On contrary, liquid-solid transition is generally an irreversible phenomenon leading  
42 to amorphous solid phases of irregular shape. Depending on the scientific community, several  
43 designations are found for such solid phases: "aggregates" (Nigen, Croguennec, Renard, & Bouhallab,  
44 2007; Tavares, Croguennec, Hamon, Carvalho, & Bouhallab, 2015), "flocs" (Anema & de Kruif, 2014;  
45 Thongkaew, Hinrichs, Gibis, & Weiss, 2015) or "precipitates" (Comert et al., 2016; Obermeyer, Mills,  
46 Dong, Flores, & Olsen, 2016). In the literature, the differentiation between the two mechanisms is  
47 often obscured by the common use of turbidity to detect such transitions (Comert & Dubin, 2017).  
48 Both mechanisms induce an increase in turbidity but microscopy is needed to know whether the  
49 turbidity arises from liquid-liquid or liquid-solid phase separation. Looking into turbidity kinetics may  
50 also help ~~in~~ to discriminate between the two phenomena (Chapeau et al., 2016). It is generally  
51 accepted that strong attractive properties favor liquid-solid phase separation. In a recent review,  
52 Comert and Dubin discussed the influence of several parameters favoring liquid-solid phase  
53 transition (Comert & Dubin, 2017). Charge anisotropy, due to "positive patches" on protein surface  
54 or a blockwise distribution of charged groups along the polyelectrolyte, is thought to promote liquid-  
55 solid phase separation. It induces high local charge density leading to local strong attraction despite  
56 low net charge (Mattison, Dubin, & Brittain, 1998). The polyelectrolyte  $pK_a$  also play a role on the  
57 formation of solid precipitates in protein-polyelectrolyte systems. In case of low  $pK_a$ , lowering the pH  
58 results in an increase in protein charge density while the charge density of the polyelectrolyte  
59 remains maximal due to complete charge dissociation. In this case, the electrostatic interaction are  
60 strong and may lead to liquid-solid phase transition as observed for polyvinyl sulfonic acid and

61 lysozyme (Romanini, Braia, Angarten, Loh, & Picó, 2007). The structure of the polyelectrolyte also  
62 modulates the type of phase separation observed. Coacervation is favored by branched  
63 polyelectrolyte (Comert et al., 2016) and flexible ones (Cousin, Gummel, Combet, & Boué, 2011;  
64 Kizilay, Kayitmazer, & Dubin, 2011).

65 Here, we aimed at investigating the interplay between liquid-liquid and liquid-solid phase separation  
66 in protein-polysaccharide mixtures. We investigated the electrostatic assembly of a basic protein,  
67 rapeseed napin (NAP), and an acidic polysaccharide, highly methylated pectin (PEC) known to form  
68 electrostatic complexes at pH 7 (Schmidt, 2004). **Turbidity measurements, phase compositions**  
69 **determination and microscopy observations were performed.** The assembly of NAP/PEC mixtures  
70 was assessed as a function of pH to tune NAP/PEC binding affinity and as a function of NAP/PEC ratio  
71 to tune complex charge. ~~We did so using turbidity measurement, phase compositions determination~~  
72 ~~and microscopy.~~ We showed that liquid-solid phase separation is favored at low pH when the  
73 electrostatic attraction was the strongest. On contrary, liquid-liquid phase separation **was** favored at  
74 intermediate pH. More interestingly, a transition from solid-to-liquid was observed overtime at low  
75 pH in case of excess of protein suggesting that the liquid-solid phase separation is not always  
76 irreversible. We discuss the potential role of charge re-arrangement and protein **intrinsic**  
77 **disorderflexibility** in this behaviour.

78

## 79 **II. Materials and Methods**

### 80 *II.1. Materials*

81 Napin was purified from defatted rapeseed meal using an adapted protocol already published  
82 (Schmidt et al., 2004). Defatted rapeseed meals were obtained at the pilot scale using CETIOM  
83 facilities (Pessac, France). Proteins were extracted from the meal in an extraction buffer (50 mM Tris,  
84 1M NaCl, 15 mM sodium disulfite, 5 mM EDTA, pH 8.5) during 1 hour at room temperature. After  
85 centrifugation at 17 000 g during 20 minutes at 20°C, a second extraction was performed to recover  
86 most of soluble proteins. Supernatant were combined and filtered on a sintered filter (N°0). Pigments  
87 were removed by a size-exclusion chromatography on a Cellufine GH 25 column (Amicon, 100 x  
88 887 mm, 7.5 L) equilibrated in buffer A (50 mM Tris, 5 mM EDTA, pH 8.5). Proteins were eluted with  
89 buffer A. Napins were then fractionated by ionic exchange chromatography using a SPsepharose fast  
90 flow column (Amersham XK50/15, 300 ml). The elution of napin was performed using an increasing  
91 gradient of buffer B (buffer A + 1 M NaCl). The eluted fractions were then extensively dialysed  
92 against water at 4°C and freeze dried. Napin was further purified by gel filtration on a sephadex G50

93 medium (Pharmacia , 1.8L) equilibrated in buffer C (50mM Tris, 0.75M NaCl, pH 8.5). Finally, the  
94 samples were dialyzed against water before freeze-drying.

95 The protein content of purified powder was higher than 95% on a dry basis, using the Dumas Method  
96 with a corrective factor of 5.5 (Mosse, 1990). Ash content was about 8.5 % using thermogravimetric  
97 analysis under nitrogen atmosphere. Napin powder was stored under vacuum at -20 °C until use. It  
98 was placed at 20 °C in a desiccator containing K<sub>2</sub>CO<sub>3</sub> saturated salt during whole time of experiments  
99 to ensure constant moisture content. **The purity of napin was checked by SDS-PAGE electrophoresis  
100 and mass spectrometry as presented in Supplementary data. Two major isoforms of napin were  
101 identified ascribed to 2SS3 and 2SS2, respectively. These results were in agreement with those  
102 previously described by Schmidt et al. (2004).**

103 Highly methoxylated Pectin (UniPectin QC-100, batch number 47572901) provided by Cargill (France)  
104 **was used with a degree of methylation of 71 % and a mean molecular weight of 227 kg mol<sup>-1</sup>. A  
105 detailed characterisation is given in Supplementary data.** Pectin powder was stored at room  
106 temperature during the whole time of experiments. A characterization study performed on individual  
107 biopolymer is given in Supplementary data (Figure S1).

108 Hydrochloric acid (HCl), sodium hydroxide (NaOH) and sunflower oil were supplied from Sigma.  
109 Bodipy TR Cadaverin dye ( $\lambda_{exc}$  588 nm,  $\lambda_{em}$  616 nm, D6251) and Alexa Fluor 350 NHS ester dye ( $\lambda_{exc}$   
110 346 nm,  $\lambda_{em}$  442 nm, A10168) were provided by ThermoScientific. N-(3-Dimethylamino-propyl)-N'-  
111 ethyl-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) used for pectin activation  
112 came from Sigma. Milli-Q water was used in all sample preparations.

113

## 114 *II.2. Biopolymers stocks dispersions*

115 Napin and Pectin powders were solubilized overnight at 20°C. The following buffers were used  
116 according to the pH: acetate buffer (10 mM) for pH 4.0 and pH 5.0; MES buffer (10 mM) and  
117 phosphate buffer (10 mM) for pH 6.0 and pH 7.0, respectively. The ionic strength (I) was adjusted to  
118 25, 50, 75, or 150 mM using NaCl. The resulted biopolymers dispersions were then filtered on a  
119 0.2  $\mu$ m cellulose acetate membrane (Sartorius, France). The protein concentration was determined  
120 by UV absorption spectrophotometry at a wavelength of 278 nm using  $\epsilon_{Napin} = 0.57 \text{ L.g}^{-1}.\text{cm}^{-1}$   
121 (Schmidt, Renard, Rondeau, Richomme, Popineau, & Axelos, 2004) whereas polysaccharide  
122 concentration was obtained by dry matter analysis. Concentrations were adjusted in a range  
123 between 0.1 to 1 wt% by dilution in the buffer.

## 124 ***II.3. Biopolymers charge determination***

### 125 ***II.3.1. Electrophoretic mobility***

126 Electrophoretic mobility measurements were performed on biopolymers stock dispersions as a  
127 function of pH using a zetasizer Nano Series (Nano-ZS, malvern instrument). Experiments were  
128 conducted at 20°C in triplicate. Biopolymers were solubilised overnight at 0.25 wt% in Milli-Q water  
129 under magnetic stirring before being filtered on 0.2 µm cellulose acetate membrane (Sartorius). The  
130 pH of the dispersions was adjusted in a range between 3.0 and 11.0 with HCl or NaOH 0.2M before  
131 experiments.. Similarly, measurements were performed on biopolymers mixtures at pH 4.0, 0.5 wt%  
132 total concentrations for several NAP/PEC mixing ratios ranging from 1:8 to 16:1.

### 133 ***II.3.2. Potentiometric titration***

134 Biopolymers surface charge evolution with pH was obtained by potentiometric titration as described  
135 previously (Salis et al., 2011) using an automated titranto 905 (Methrom, France). Biopolymer  
136 solutions (0.1 wt%) in Milli-Q water, filtered through 0.2 µm (cellulose acetate, Sartorius) was pre-  
137 titrated by HCl 10<sup>-2</sup> M to reach a pH equal to 2.5. The resulted acidic dispersions was then titrated to  
138 pH 11 using NaOH 10<sup>-2</sup> M. Pre-titration and titrations were performed in the same conditions as a  
139 blank solution made of milli-Q water. The biopolymer surface charge Z<sub>p</sub> was finally calculated as a  
140 function of pH using the following equation:

$$141 \quad Z_p = \frac{[HCl](V_{HCl} - V_{HCl\ blank}) - [NaOH](V_{NaOH} - V_{NaOH\ blank})}{m_{biop}/M_{biop}}$$

142 where V<sub>HCl</sub> is the volume (mL) of HCl used in the pre-titration to reach pH 2.5 for the sample and the  
143 blank. V<sub>NaOH</sub> is the volume of NaOH used in the titration of both sample and blank. m<sub>biop</sub> (g) is the  
144 mass of biopolymer introduced in the sample and M<sub>biop</sub> (g/mol) is the molecular weight of the  
145 biopolymers. [HCl] and [NaOH] are the molar concentration (mol/L) of hydrochloric acid and sodium  
146 hydroxide used during pre-titration and titration experiments.

## 147 ***II.4. Screening of NAP/PEC interactions using microplates***

148 The screening of NAP/PEC interactions was performed at 0.1 wt% total biopolymers concentration  
149 using an Automated Laboratory Microplates handling (Biomek 3 000, France) combined with a shaker  
150 (Variomag Teleshake, Thermofischer). Experiments were conducted in a 20°C regulated room. The  
151 impact of pH (4.0, 5.0, 6.0 and 7.0) and NAP/PEC mixing ratio (8:1, 4:1, 3:2, 2:3, 1:4 and 1:8) was  
152 investigated. The equivalence between initial NAP/PEC mixing ratio and the percentage of napin in  
153 the mixture was given in Table 1. Note that ionic strength was set to 25 mM for all pH values.

154 Mixtures were generated directly in a polystyrene 96 well microplates with flat bottom (651101,  
155 Greiner bio one, France). The volume of each well was set at 200  $\mu\text{L}$ . The filling of microplates was  
156 performed as follows. First, wells were filled with **napi**n stock dispersion prepared at 0.5 wt%. Then,  
157 protein was diluted by addition of buffer. Finally, pectin solution at 0.5 wt% was added. Shaking steps  
158 after buffer and pectin addition ensured the mixing. The time required for one 96 well microplate  
159 preparation was about 100 min. The time  $t_0$  used in the following will referred to the time required to  
160 generate the 96 biopolymers mixtures, *i.e.*, after pectin was added in the first well: this time was  
161 about 60 min. The impact of pH and mixing ratio on NAP/PEC interactions was assessed through  
162 turbidity measurements: the absorbance was recorded at a wavelength of 600 nm using a  
163 Microplates spectrophotometer (Biotek Epoch Microplate Spectrophotometer, France).  
164 Measurements were performed immediately after microplate generation ( $t_0$ ) and once a day during  
165 one week. Microplates were stored at 20  $^{\circ}\text{C}$  during experimental time. Absorbance was converted  
166 into turbidity ( $\tau$ ,  $\text{cm}^{-1}$ ) using the following equation:  $\tau = (2.303 \times A_{600}) / l$ , where  $l$  is the light path  
167 length (cm).  $l$ , equal to 0.6 cm, was calculated according to the following equation:  $l = (4V/\pi d^2)$ . After  
168 one week, NAP/PEC assemblies were also observed directly within the microplate using an inverted  
169 phase contrast optical microscope (BX51, Olympus, Germany) set at the magnification  $\times 40$ .

## 170 *II.5. Droplets based millifluidic for NAP/PEC screening of interactions*

### 171 **II.5.1. Millifluidic set-up**

172 Millifluidic was shown to be an efficient and low material consuming approach to screen biopolymers  
173 interactions at total concentrations higher than 0.1 wt% (Amine, Boire, Davy, Marquis, & Renard,  
174 2017). The developed experimental set-up, presented in Figure 1A is made of 4 modular parts. First,  
175 droplets generation in a sunflower oil continuous phase was ensured by a co-flow geometry  
176 comprising three inlets: two for the biopolymers dispersed phases and one for the oil continuous  
177 phase (Figure 1B). Two 2.5 mL glass syringes (Hamilton) respectively filled with NAP and PEC  
178 dispersions were placed on a syringe pump (Harvard Apparatus PHD 2000, France). A T-junction (ID  
179 0.25 mm, Upchurch Scientific<sup>®</sup>) connected to the initial stocks dispersions through FEP tubes (interior  
180 diameter (ID) 0.51 mm, outside diameter (OD) 1.57 mm, length (L) 40 cm) ensured the mixing of the  
181 two components. The resulting mixture went through a fused silica capillary tube (ID 0.7 mm, OD  
182 0.85 mm, L 12.5 cm) at a total flow rate of 3 mL / h. The continuous oil phase was pumped through a  
183 FEP tube (ID 0.51 mm, OD 1.57 mm L 40 cm) at a flow rate of 10 mL / h. Droplets of about 1.9  $\mu\text{L}$ ,  
184 displaying same size, shape and compositions, were finally generated into a transparent Tygon<sup>®</sup> tube  
185 (ID 1.42 mm, OD 3mm, L 55 cm). After generation, droplets went through a winding Tygon<sup>®</sup> tube  
186 (L 30 cm) which ensured the complete mixing within droplets based on chaotic advection (Song, Tice,

187 & Ismagilov, 2003) (Figure 1C). Droplets then reached a straight Tygon® tube (L 15 cm) placed on a  
188 PDMS support filled with ethylene glycol. This allowed on-line droplets observations 5 min after  
189 mixture generation (Figure 1D). Finally, an additional detachable module for long time droplets  
190 storage was connected to the device. The Tygon® tube containing droplets was connected to a multi  
191 junction made of PDMS. This allowed a convenient and rapid storage of 9 mixing conditions in a  
192 second PDMS support filled with ethylene glycol (Figure 1E). The use of slide clamps placed on the  
193 tubes (Word Precision Instruments, France) allowed a successive filling of each tube. Tubes were  
194 finally disconnected from the millifluidic device for longer storage and observed as a function of time  
195 directly within the PDMS support containing ethylene glycol.

196 Preliminary interfacial tension measurements confirmed that in the chosen range of concentrations,  
197 the percentage of adsorbed protein at the interface was negligible compared to the initial bulk  
198 concentration (see Supplementary data, Figure S2).

199

### 200 ***II.5.2. Optical set-up for droplets observations***

201 Biopolymers droplets observations were carried out under continuous flow, directly within the  
202 Tygon® tube immersed in an ethylene glycol bath designed with PDMS. The light source was  
203 composed of 12 green LED (Radiospares, France) and arranged to obtain a dark field image on the  
204 camera. Pictures were taken at a distance of 155 mm from the Tygon tube using an objective with a  
205 fixed 1.5x magnification (OPT-043-216310, Alliance Vision). The objective was connected to a 7x  
206 manual zoom (OPT-043-300098, Alliance Vision), a TV-tube (OPT-001 0.67x, D 35 mm, L 117.7 mm,  
207 Alliance Vision) and a camera (SVS-ECO674MTLGEC, GigE, 2/3" C", 1920x2460, Alliance Vision,  
208 France) (Figure 1A). 10 pictures (1 droplet per pictures) were taken. A MatLab procedure was finally  
209 used to determine the average grey level. The analysis was done on a circular section of each droplet  
210 corresponding to about 75-80 % of the total area. Grey levels were converted into turbidity using a  
211 calibration based on TiO<sub>2</sub> dispersions as detailed previously (Amine et al., 2017).

### 212 ***II.5.3. Screening of NAP/PEC interactions by droplets-based millifluidic***

213 Experiments were performed at pH 4.0 in acetate buffer (10mM, ionic strength of 25 mM) and room  
214 temperature. The impact of total biopolymers concentrations from 0.1 to 1 wt %, and Pr:Ps mixing  
215 ratio from 1:8 to 8:1 on droplets grey level were investigated. Mixing ratio was tuned by flow rates  
216 variation. Between two ratios, an equilibrium time of 80 s was applied to reach the desired  
217 composition. Droplets at the desired composition were finally generated during 35 s, analysed on-



218 line, and stored over two days in the PDMS support. Observations were also performed after 24 h  
219 and 48 h.

## 220 *II.6. Determination of phases composition at thermodynamic equilibrium*

221 Phases composition of biopolymers mixtures was determined for the following mixing ratios: 2:3, 1:1,  
222 3:2, 4:1 and 8:1 at 0.5 wt% in 10 mM acetate buffer (pH 4.0, ionic strength of 25 mM). NAP/PEC  
223 mixtures were prepared into 1 mL conical bottom-sealed pipette tip and the volume was set at  
224 300  $\mu$ L for each condition. The amounts of **napin** and pectin used for mixtures preparation were  
225 accurately weighted to deduce the exact initial compositions. Mixtures were equilibrated 48 h in a  
226 20 °C regulated room, before being centrifuged 1 h at 10 000 g. The supernatant, corresponding to  
227 the dilute phase was then collected using micropipette. **Napin** content in the dilute phase was  
228 obtained by UV absorption spectrophotometry at a wavelength  $\lambda$  of 278 nm. Pectin content was  
229 determined by the automated MHDP method (Thibault, 1979). Conical bottom-sealed pipette tips  
230 containing the coacervate phase were weighted and coacervate phase composition was deduced  
231 using mass conservation equation. **This gravimetric approach gave an estimation of NAP and PEC**  
232 **contents in pellets with an accuracy of about +/-5 %.**

## 233 *II.7. Microscopy*

### 234 *II.7.1. Phase contrast microscopy*

235 Biopolymers mixtures were observed as a function of time using a phase contrast optical microscope  
236 (BX51, Olympus, Germany) set at the magnification x 40 to evidence the presence or absence of  
237 coacervates. Observations were carried out on a glass microscope slide with a geneframe<sup>®</sup> (Thermo  
238 scientific).

## 239 **III. Results & discussion**

### 240 *III.1. Napin and pectin carry opposite charges in a wide range of pH*

241 Electrophoretic mobility was determined as a function of pH to identify conditions where napin and  
242 pectin carry opposite charges. Under acidic conditions, napin electrophoretic mobility is positive and  
243 decreases to reach 0 at a pH of 9.5 as reported in Figure 2. This pH value corresponds to the  
244 isoelectric point (IEP) of napin in agreement with literature data (Ericson et al., 1986; Gehrig,  
245 Krzyzaniak, Barciszewski, & Biemann, 1996; Josefsson, Lenman, Ericson, & Rask, 1987; Monsalve &  
246 Rodriguez, 1990). For higher pH, electrophoretic mobility is negative. Electrophoretic mobilities of

247 pectin are negative over the entire range of pH and reach a plateau value above pH 6.0 meaning all  
248 carboxylic functions are deprotonated. These negative values are in accordance with the  $pK_a$  of 2.8-3  
249 previously determined for pectin (Ralet, Dronnet, Buchholt, & Thibault, 2001). These results suggest  
250 that electrostatic assembly between napin and pectin can take place over a wide pH range between  
251 3.0 and 9.5.

252

### 253 *III.2 NAP/PEC assemblies are maximal for the highest charge difference*

254 We intended to identify NAP/PEC assembly conditions using turbidity measurements as a function of  
255 pH, in a range where biopolymers carry opposite charges. Turbidity increases for decreasing pH,  
256 being the highest at pH 4.0 (Figure 3A). This pH corresponds to a point where the overall charge  
257 difference between biopolymers is maximal as seen in Figure 2. Increasing pH from 4.0 to 7.0 induces  
258 a decrease in the overall charge difference between biopolymers. The electrostatic attraction  
259 contribution between napin and pectin is therefore expected to be maximal at pH 4.0. pH 4.0 also  
260 corresponds to the pH of electrical equivalence (EEP) defined as the pH where biopolymers carried  
261 equal and opposite charges (Burgess & Carless, 1984). In this case, the overall charge balance reaches  
262 0 as plotted in Figure 3B. The formation of NAP/PEC assemblies, maximal at pH 4.0 and characterized  
263 by high turbidity, is therefore primarily driven by long-range electrostatics interactions which is  
264 consistent with protein-polysaccharide complex coacervation (de Kruijff, Weinbreck, & de Vries, 2004).

265 Protein:Polysaccharide (Pr:Ps) mixing ratio is another important parameter in mixed biopolymers  
266 systems. It controls biopolymers charge balance while keeping the binding affinity constant  
267 (Antonov, Mazzawi, & Dubin, 2010; Xia & Dubin, 1994). Low NAP/PEC mixing ratio (protein content  
268 lower than 20%) is associated to low turbidity, suggesting no macroscopic phase separation in the  
269 entire pH range investigated (Figure 3A). In these cases, the number of protein molecules in the  
270 solution may not be sufficient to neutralise the negative charges of all pectin molecules. It is a  
271 common result already observed in the literature and has been ascribed to a negative residual net  
272 charge of the complexes which remain soluble (Zaitsev, Izumrudov, & Zezin, 1992). As NAP/PEC  
273 mixing ratio increases, the higher protein content may lead to the neutralization of polysaccharide  
274 charges inducing macroscopic phase separation. A sharp increase in turbidity is indeed observed at  
275 pH 4.0, with a maximum for a NAP/PEC mixing ratio of 3:2.

276 The optimum NAP/PEC mixing ratio for NAP/PEC assemblies, defined as the highest turbidity, is pH  
277 dependent and shifts towards higher NAP/PEC mixing ratio for increasing pH. At high pH values, close  
278 to napin IEP, a decrease in surface charge density and electrophoretic mobility is observed. On the  
279 contrary, pectin surface charge density and electrophoretic mobility remain constant, in view of

280 complete deprotonation of carboxylic groups. Therefore, more napin molecules are required to  
281 compensate pectin negative charges. As NAP/PEC assembly is maximal at pH 4, further experiments  
282 were conducted at this pH.

283

### 284 *III.2. NAP/PEC phase separates into associative assemblies independently of* 285 *total concentration.*

286 Associative phase separation and complex coacervation usually occurs in a restricted range of  
287 concentration (Veis, Bodor, & Mussell, 1967). We assessed the role of total biopolymers  
288 concentration on NAP/PEC assemblies using droplets based millifluidic to limit material consumption  
289 (Amine et al., 2017). Experiments were conducted at pH 4.0 for concentrations ranging from 0.1 to  
290 1 wt%. The high pectin viscosity as well as its high intrinsic turbidity prevented to work at higher  
291 pectin concentration. The evolution of droplets grey level obtained from image analysis is presented  
292 in Figure 4A as a function of NAP/PEC mixing ratio. In the **studied** range of concentrations, pure **napin**  
293 **and** pure **pectin** are limpid. Turbidity profiles follow a similar trend regardless of total biopolymers  
294 concentration. Droplets are limpid at low NAP/PEC mixing ratio (1:8, 1:4) meaning that no  
295 macroscopic phase separation occurred. This was confirmed by optical microscopy observations of  
296 millifluidic droplets, where no supramolecular assemblies were detected (data not shown). At these  
297 NAP/PEC ratio, small complexes probably form. Further experiments like dynamic light scattering  
298 should be performed to probe their size and kinetics of formation. The maximum of interaction is  
299 comprised between a ratio of 1:1 and 3:2 according to the total biopolymers concentration.  
300 However, for these two ratios, the turbidity of droplets is heterogeneous as shown in Figure 4B. The  
301 heterogeneity is more pronounced at high concentrations. It is not possible to conclude whether the  
302 difference in grey level between ratios 1:1 and 3:2 was significant or not. For total biopolymers  
303 concentrations ranging from 0.1 to 1 wt%, NAP/PEC interaction profile is thus similar. In this range of  
304 concentration, phase separation profile does not depend on concentration. The increase in grey level  
305 with total biopolymers concentration at fixed Pr:Ps mixing ratio is assigned to an increase in dense  
306 lower phase volume fraction. This behaviour was previously evidenced on BSA/PDMDAAC mixtures  
307 where no effect of total solute concentration on the phase boundary was detected (Mattison,  
308 Brittain, & Dubin, 1995). Considering pectin/gelatin mixtures, McMullen et al. highlighted an  
309 optimum total biopolymers concentration of 2% for coacervation (McMullen, Newton, & Becker,  
310 1982). Above this value, coacervation yield decreased. Self-suppression of NAP/PEC interactions may  
311 also occur but high viscosity of pectin above 1 wt% prevented to perform efficient mixing of the  
312 biopolymers using a T-junction in droplets millifluidic. In addition, turbidity assigned to pectin self-  
313 aggregation at high pectin concentration excluded a clear interpretation of any increase of turbidity.

314 Phase composition was determined at thermodynamic equilibrium at a total concentration of 0.5  
315 wt%. It provides additional information as compared to turbidity measurement: the phase separation  
316 yield. The maximum of phase separation yield was obtained for a mixing ratio of 3:2, where 83% of  
317 the initial quantity of biopolymers was in the dense lower phase (Figure 4D). For a mixing ratio of 1:1,  
318 the yield of phase separation is equal to 75%. These results are well correlated with the high turbidity  
319 probed using droplets based millifluidic for these two mixing ratios. From these complementary  
320 results, NAP/PEC phase diagram at pH 4.0 is plotted in Figure 4C. Note that additional conditions  
321 close to the initiation of phase separation should be investigated to have a more precise idea of the  
322 position of the coexistence curve. In addition, the detailed napin and pectin compositions at  
323 thermodynamic equilibrium are given in supplementary data (see Supplementary data, Figure S3).  
324 Both biopolymers are enriched in the dense phase confirming an associative phase separation. The  
325 co-localization of both biopolymers within assemblies was further confirmed using confocal laser  
326 scanning microscopy (see Supplementary data, Figure S4).

327

### 328 *III.3. Charge neutralisation controls the structure of NAP/PEC assembly*

329 The structure of NAP/PEC assemblies was assessed using phase contrast microscopy. Observations  
330 were performed over time (1h) on mixtures with different mixing ratios inducing macroscopic phase  
331 separation (8:1, 4:1, 3:2, 1:1, 2:3). Two types of structures are evidenced by phase contrast  
332 microscopy according to observation time and mixing ratio: coacervates and solid-like structures as  
333 shown in Figure 5. When protein is in excess in the initial solution, *i.e* mixing ratio of 8:1, 4:1 and 3:2,  
334 a coexistence of these two structures is observed immediately after biopolymers mixing (2min).  
335 Coacervates droplets size and number increase over time (15 and 60 min after biopolymers mixing).  
336 The size of the unshaped solid-like structures, which displayed high similarities with solid precipitates  
337 (Comert & Dubin, 2017; Kayitmazer, Koksai, & Iyilik, 2015), increases as mixing ratio decreased.  
338 Interestingly, the solid-like structures rearrange over time and form after 1 h large liquid-like  
339 structures. It suggests a solid-to-liquid transition occurring over time at these three mixing ratios.  
340 Microscopy experiments performed on several supports, glass slide, microplate and low binding  
341 microplate, led to similar observations, confirming that solid-to-liquid transition was not due to  
342 surface effects (data not shown). When protein content within mixtures is further decreased  
343 (NAP/PEC mixing ratio of 1:1 and 2:3), only solid-like structures are visible. Despite an increase in  
344 structures density with time, the characteristic size does not increase. We therefore evidenced two  
345 types of NAP/PEC assembly depending on both mixing ratio and time: liquid droplets and solid-like  
346 structures being predominant in the system.

347 To better understand factors underlying these structural differences according to Pr:Ps mixing ratio,  
348 the charge of mixtures was determined by measuring the electrophoretic mobility  $\mu_E$ . **Two regimes of**  
349 **mobility can be observed as a function of the initial mixing ratio as displayed in Figure 6.** In conditions  
350 where solid-like structures are formed (NAP/PEC ratio from 1:8 to 1:1) a highly negative  $\mu_E$  **is**  
351 **obtained which depends weakly on initial mixing ratio and is very similar to pectin mobility. This**  
352 **suggests that pectin is the main contributor to the electrophoretic mobility. ~~An excess of negatively~~**  
353 **~~charged pectin macromolecules might stabilize the assemblies and prevent coalescence to occur.~~**  
354 **~~This could explain the smaller size of NAP/PEC assemblies observed at these ratios.~~** In contrast, a  $\mu_E$   
355 close to 0 is obtained in conditions where rearrangement over time is observed (NAP/PEC ratio from  
356 3:2 to 8:1). **~~Charge neutralisation is a requisite for spherical coacervate droplets formation that can~~**  
357 **~~be subjected to coalescence phenomenon.~~** The measurement of an electrophoretic mobility on  
358 multiphasic dispersions is however questionable as it gives an average value of multiple species  
359 present in dispersions such as free biopolymers, soluble and insoluble complexes and coacervates.

360 An alternative method can be used to evaluate the charge of NAP/PEC assemblies. It consists in  
361 determining each biopolymer charge using potentiometric titration and calculating theoretical  
362 charges of the assemblies based on composition. The evolution of the **number of charge per mol of**  
363 **biopolymer**,  $Z_p/M_w$ , as a function of pH was determined for both napin and pectin as shown on Figure  
364 7A. Highly similar profiles were obtained when compared to electrophoretic mobility measurements  
365 presented on Figure 2. The point where napin carried 0 charges is around pH 9.5 whereas pectin is  
366 completely deprotonated above pH 6 as evidenced by the plateau value. Additionally, experimental  
367 data obtained for napin are very similar to those calculated using the Uniprot calculator software  
368 with napin sequence 2SS3. At pH 4, one napin molecule carries 14 positive charges whereas each  
369 pectin molecule carries 160 negative charges. From the knowledge of each biopolymer charge at pH  
370 4 and from phase compositions obtained at thermodynamic equilibrium, Pr/Ps [+]/[-] charge ratio in  
371 the dense lower phase was evaluated as a function of initial mixture composition (Figure 7B). Pr/Ps  
372 [+]/[-] charge ratio in the dense lower phase is highly dependent on the initial weight mixing ratio:  
373 the higher the initial Pr:Ps weight mixing ratio is, the higher the Pr/Ps [+]/[-] charge ratio in the dense  
374 lower phase is. This indicates an increase in protein content within the lower phase. This trend has  
375 been previously reported for other protein-polysaccharide mixtures (Comert et al., 2016; de Kruif et  
376 al., 2004; Schmitt, Sanchez, Thomas, & Hardy, 1999; Sanchez et al., 2002; Pathak, Priyadarshini,  
377 Rawat, & Bohidar, 2017; Niu et al., 2015; Sperber, Schols, Cohen Stuart, Norde, & Voragen, 2009;  
378 Wee et al., 2014; Niu et al., 2014; Vinayahan, Williams, & Phillips, 2010) and was ascribed to a mass  
379 action phenomenon. As the initial protein concentration increases, more protein molecules are  
380 involved in phase separation. At high Pr:Ps weight mixing ratios (4:1 and 8:1), where solid to liquid  
381 transition **is** observed, charge compensation is not reached (Pr/Ps [+]/[-] charge ratio not equal to 1)

382 and corresponds to the highest charge asymmetry. This result contradicts the measured  $\mu_E$  which  
383 equals 0 for these Pr:Ps ratios (see Fig. 6). It is therefore difficult to conclude whether coacervation  
384 occurs at charge neutrality. The composition of the complexes could be different from the weight  
385 mixing and charge ratios due to charge regularization. This assumption could therefore justify the  
386 discrepancies observed between mobility measurements on complexes and calculated charge ratio  
387 from phase composition. Similar discrepancies were observed in the complex coacervation process  
388 between lactotransferrin and  $\beta$ -lactoglobulin (Anema & de Kruif, 2014). The calculated mixing charge  
389 ratio was systematically lower than the mixing ratio at zero zeta potential whatever the pH. In  
390 another study dealing with the complex coacervation between lactoferrin and caseins, the same  
391 authors also highlighted the existence of charged coacervates for mixing ratios far away from the  
392 optimum mixing fraction (Anema & de Kruif, 2016). In another study dealing with polysaccharide  
393 (hyaluronic acid) - polysaccharide (chitosan) complex coacervation, coacervate suspensions were also  
394 still observed with zeta potential values much higher than zero (Kayitmazer et al., 2015). This non-  
395 stoichiometric coacervation was explained by the fact that some interpolymer complexes assumed  
396 net charges further from neutrality in order for others to attain it (disproportionation theory),  $pK_a$   
397 shifts within the system due to charge-charge interactions, partial loss of configurational entropy  
398 arising from the flexibility of polysaccharides, and “mismatch” (inequivalence) in charge spacing of  
399 the polysaccharide chains.

400

#### 401 *III.4. NAP/PEC aggregation is promoted by high electrostatic attraction*

402 It has been previously suggested that liquid-solid phase separation is favoured for strong  
403 electrostatic attraction (Comert & Dubin, 2017). To check whether the liquid-solid phase separation  
404 of NAP/PEC was controlled by the charge difference between biopolymers, mixtures at a fixed  
405 NAP/PEC ratio of 8:1 were prepared at pH ranging from 4.0 to 9.0. Increasing pH from 4.0 to 9.0 leads  
406 to a decrease in napin positive charges as it approached its IEP (9.5) whereas pectin negative charges  
407 reaches a plateau value above pH 6.0 (Figure 2A). Increasing pH from 4.0 to 9.0 leads therefore to a  
408 decrease in initial charge ratio  $[+]/[-]$  between biopolymers from 11.9 to 1.4. Micrographs obtained  
409 after 1h by phase contrast microscopy are presented in Figure 8. Regardless of pH, a mixing ratio of  
410 8:1 leads to the formation of coacervates droplets. However, an increase in pH induces a decrease in  
411 droplets size which appears also much more homogeneous in size. The suppression of large solid-like  
412 structures above pH 5 is attributed to the decrease in initial NAP/PEC charge ratio and consequently  
413 attractive electrostatic forces. Liquid-solid phase separation in NAP/PEC mixtures is indeed driven by  
414 strong electrostatic attraction.

415 Strong electrostatic attraction is however not sufficient to promote liquid-solid transition.  
416 The flexibility of biopolymers also plays a crucial role (Pathak et al., 2017). We investigated  
417 the assembly of napin with the major fraction of Acacia gum isolated by hydrophobic  
418 interaction chromatography, HIC-F1, and characterized elsewhere (Renard et al. 2006;  
419 Sanchez et al. 2008). HIC-F1 is a hyperbranched arabinogalactan-peptide characterized by a  
420 molecular weight and an electrophoretic mobility similar to HM-pectin in our buffer  
421 conditions. Their persistence length is however different: about 3 nm for HIC-F1 (Sanchez et  
422 al., 2008) and 8 nm for pectin (Schmidt et al., 2009). At pH 4.0, high turbidity was observed  
423 in NAP/HIC-F1 mixtures for mixing ratios comprised between 2:3 and 8:1 (Figure 9A). This  
424 high turbidity suggests strong associative properties and it was always associated with  
425 spherical droplets as displayed in Figure 9B. It can therefore be concluded that only liquid-  
426 liquid phase separation occurs when napin is mixed with a more flexible biopolymer such as  
427 HIC-F1.

428

### 429 *III.VI. Deciphering the driving force of solid-to-liquid transition*

430 For specific conditions where protein was in large excess in the initial mixture (ratio 8:1 to 3:2) a  
431 transition from solid-like to liquid-like droplets phase was observed in time. In these conditions,  
432 polysaccharide charges are probably neutralised by the excess of proteins. Residual interactions  
433 between NAP/PEC may play a key role in the re-arrangement over time. Time-dependent  
434 coacervation has been observed for gelatin A – gelatin B mixtures. The size of soluble intermolecular  
435 solid-like structures in the supernatant increased with time and was ascribed to residual electrostatic  
436 interactions (Tiwari, Bindal, & Bohidar, 2009). However, no microscopy observations clearly  
437 identified solid-to-liquid transition for gelatin A – gelatin B. A solid-to-liquid transition has been  
438 previously evidenced for apo  $\alpha$ -lactalbumin/lysozyme mixture upon increasing temperature. It has  
439 been ascribed to an increased flexibility of apo  $\alpha$ -lactalbumin due to its thermal denaturation (Nigen  
440 et al., 2007). Interestingly, previous NMR study reported the existence of several disordered loops in  
441 the tertiary structure of napin (Rico et al., 1996). The resulting 3D-structure displays disordered  
442 regions as highlighted by arrows in Figure 10A. In addition, napin disordered regions are hydrophilic  
443 as given by the hydropathy index in Figure 10B. As flexibility and hydration are interconnected  
444 parameters, they could contribute to the solid-to-liquid transition experimentally observed. The  
445 underlying mechanism is however not clear yet and deserve further investigations. As the solid-to-  
446 liquid transition occurs in excess of protein, a competition between protein-protein and protein-  
447 polysaccharide interactions may also occur. We checked the aggregation state of napin in our

448 experimental conditions using dynamic light scattering and found an hydrodynamic radius of 2.0 nm  
449 (data not shown), value in agreement with previously reported data (Schmidt et al., 2004). The initial  
450 aggregation state of napin is therefore not involved in the re-arrangement. However, it cannot be  
451 excluded that protein-protein interactions occur in the condensed coacervate-like phase as  
452 concentrations are higher than 200 g/L. This issue would need to be investigated in a future work.

453

454

## 455 **Conclusion**

456 In the current study, the electrostatic assembly of NAP/PEC was investigated in a pH range where  
457 biopolymers carry opposite net charges. Conditions of interactions were screened as a function of pH  
458 and mixing ratio to tune biopolymer binding affinity and complexes charge. The formation of  
459 NAP/PEC electrostatic assemblies was found to be maximal at pH 4.0. Up to 1 wt%, no impact of total  
460 biopolymers concentration was evidenced on phase separation conditions. However, the type of  
461 mechanism involved, liquid-liquid and liquid-solid phase separation, was strongly dependent on pH  
462 and initial NAP/PEC mixing ratio. Strong electrostatic interactions promoted by low pH led to liquid-  
463 solid phase separation whereas liquid-liquid phase separation was favoured for weaker attraction.  
464 However, for a given interaction potential, we showed that the NAP/PEC ratio also affected the type  
465 of phase separation mechanism. In case of excess of proteins, a reorganization of solid-like  
466 amorphous structures into liquid-like droplets phase was observed in time. This kinetically-driven  
467 phase transition could be promoted by residual interactions between biopolymers and/or protein  
468 conformation.

469

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478



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## Figure captions

**Figure 1.** Droplets-based millifluidic for the screening of biopolymers interactions A. Droplets-based millifluidic experimental set-up B. Millifluidic droplets production using a co-flow geometry. Mixing between napin (NAP) and pectin (PEC) biopolymers was ensured by a T-junction using sunflower oil (OIL) as continuous phase. C. Droplets flowed through a winding tube to optimize the mixing within droplets based on chaotic advection. D. Polydimethylsiloxane (PDMS) bath designed for droplets observation and limitation of reflexion. Observations were performed using a camera and an objective to get dark field images. E. Detachable module used for long term storage of 9 mixing conditions. Flow was guided in the desired tube using slide clamps.

**Figure 2.** Electrophoretic mobility of napin (circles) and pectin (squares) as a function of pH. Measurements were performed in triplicates at 0.25 wt% in water. Dashed and dotted lines are guide to the eye.

**Figure 3.** Turbidity of NAP/PEC mixtures as a function of the initial mixing ratios at pH 4, 5, 6 and 7 at a total biopolymers concentration of 0.1 wt% and  $I = 25$  mM.

**Figure 4.** Impact of biopolymers total concentration (from 0.1 to 1 wt%) at pH 4 on NAP/PEC interactions investigated through droplets-based millifluidic. A. Evolution of the droplet grey level as a function of mixing ratio and total concentration. B. Examples of millifluidic droplets observed on-line containing NAP/PEC mixtures at various mixing ratio and total concentration. C. NAP/PEC phase diagram obtained from droplets-based millifluidic experiments and highlighting monophasic (empty circles) and biphasic conditions (filled circles). D. Phase separation yield (%) as a function of mixing ratio at pH 4 and total biopolymer concentration of 0.5 wt%.

**Figure 5.** Phase contrast microscopy observations in time of NAP/PEC mixtures at a total biopolymer concentration of 0.5 wt%, pH 4,  $I = 25$  mM and mixing ratios of 8:1, 4:1, 3:2, 1:1 and 2:3.

**Figure 6.** Evolution of the electrophoretic mobility of NAP/PEC mixtures prepared at various mixing ratio. Experiments were conducted at  $I = 25$  mM, pH = 4 and 0.5 wt% total biopolymer concentration.

**Figure 7.** A. Surface charge density  $Z_p/M_w$  of biopolymers obtained by potentiometric titration of 0.1wt% biopolymers dispersions in water. B. Evolution of Pr/Ps charge ratio in the initial mixture and in the lower dense phase as a function of the initial napin concentration. Pr/Ps charge ratios were obtained from potentiometric titrations and phase compositions at thermodynamic equilibrium. Red dashed line represents a Pr/Ps charge ratio equal to 1.

**Figure 8.** Structures obtained by phase contrast microscopy for a Pr:Ps weight mixing ratio of 8:1 at pH 4, 5, and 9. Total biopolymers concentration was set at 0.5 wt% and  $I = 25$  mM.

**Figure 9.** A. Turbidity measured at 600 nm of Napin/HIC-F1 mixtures as a function of napin content at a total biopolymer concentration of 0.5 wt% in 25 mM acetate buffer (pH = 4.0,  $I = 25$  mM). B. Phase contrast microscopy of Napin/ HIC-F1 mixtures for mixing ratio of 8:1, 4:1, 3:2 and 2:3. Scale bars stand for 50  $\mu$ m.

**Figure 10.** A. 3D-structure of Napin resolved by NMR (Rico, Bruix, González, Monsalve, & Rodríguez, 1996) Arrows indicate disordered loops. B. Hydrophathy index calculated using Expasy Webserver according to Kyle & Doolittle. In blue, the two polypeptide chains linked by disulphide bonds in native napin are reported. In blue, disordered domains are reported.

Figure 1

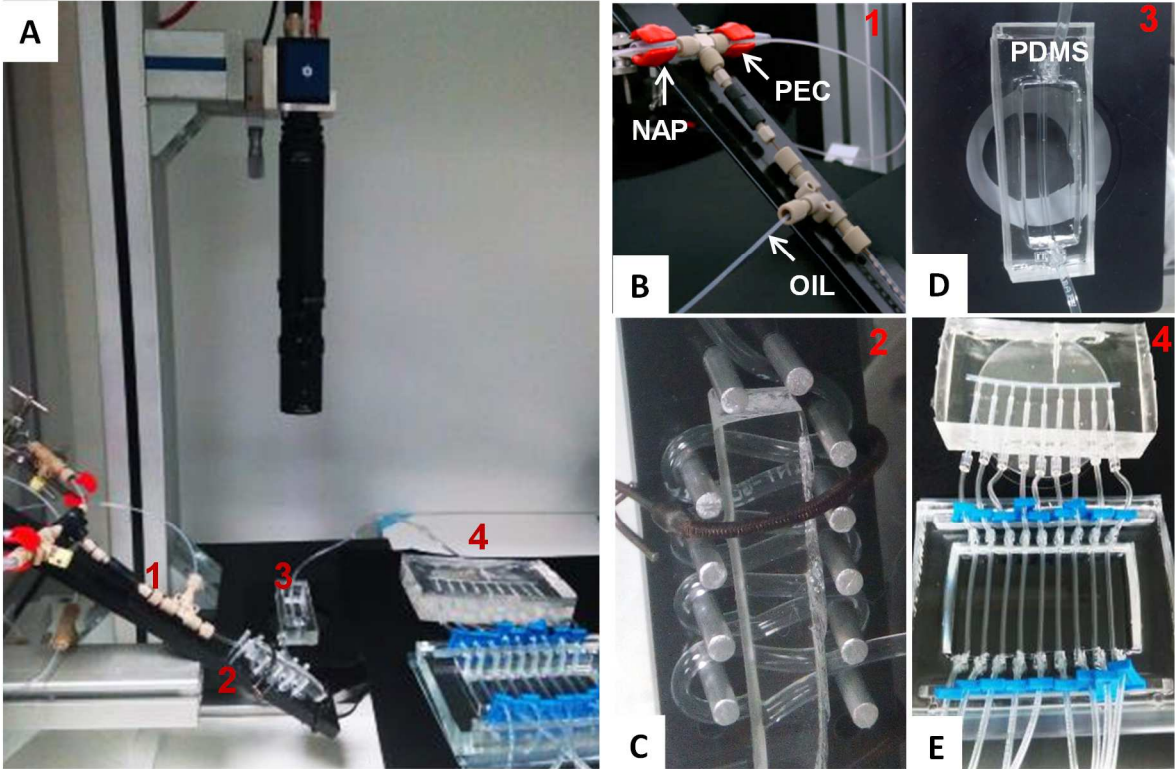




Figure 2

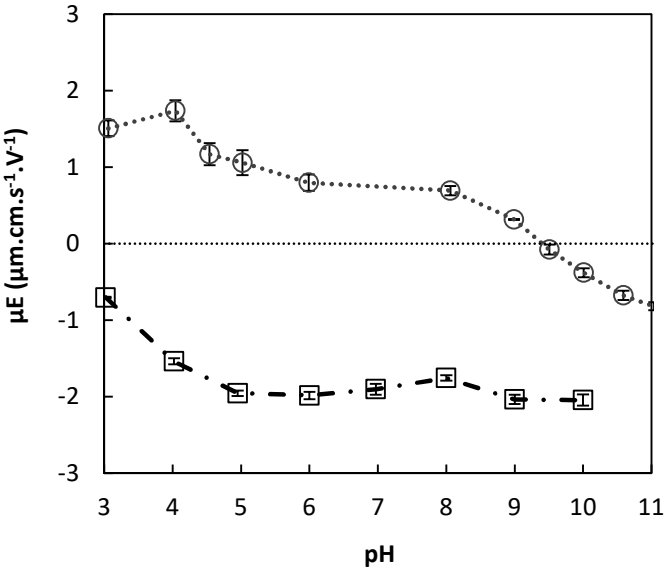


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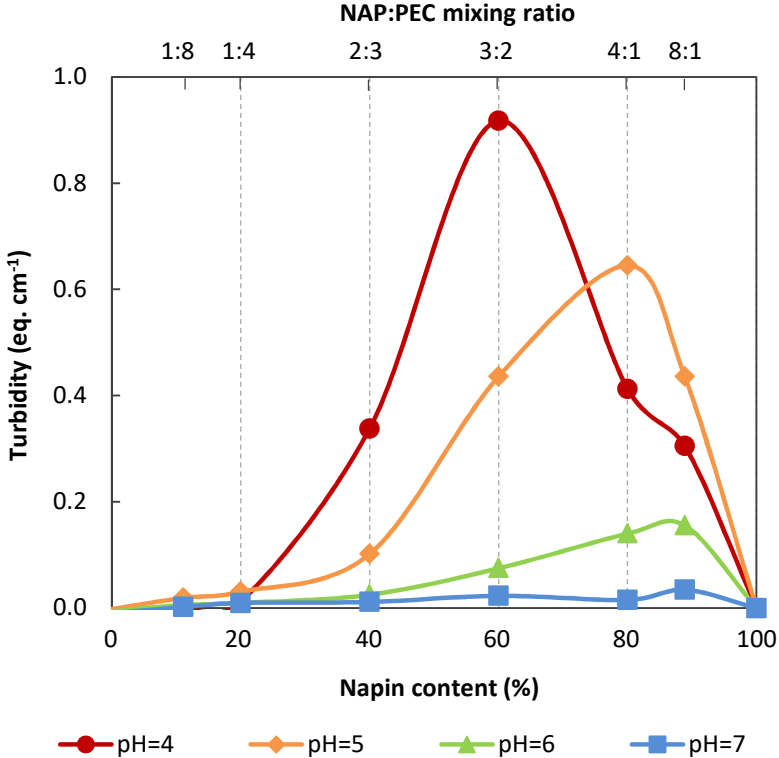


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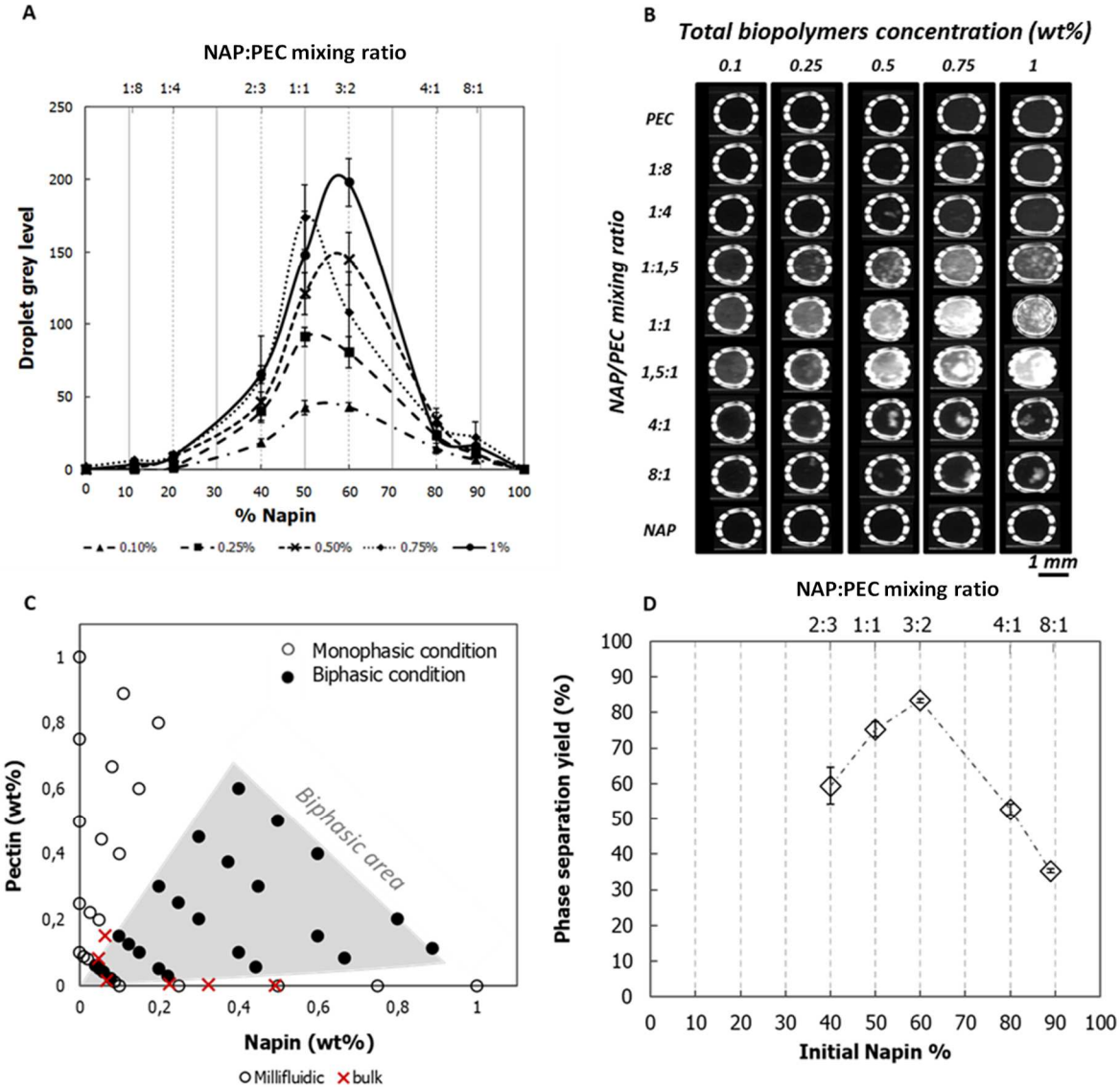


Figure 5

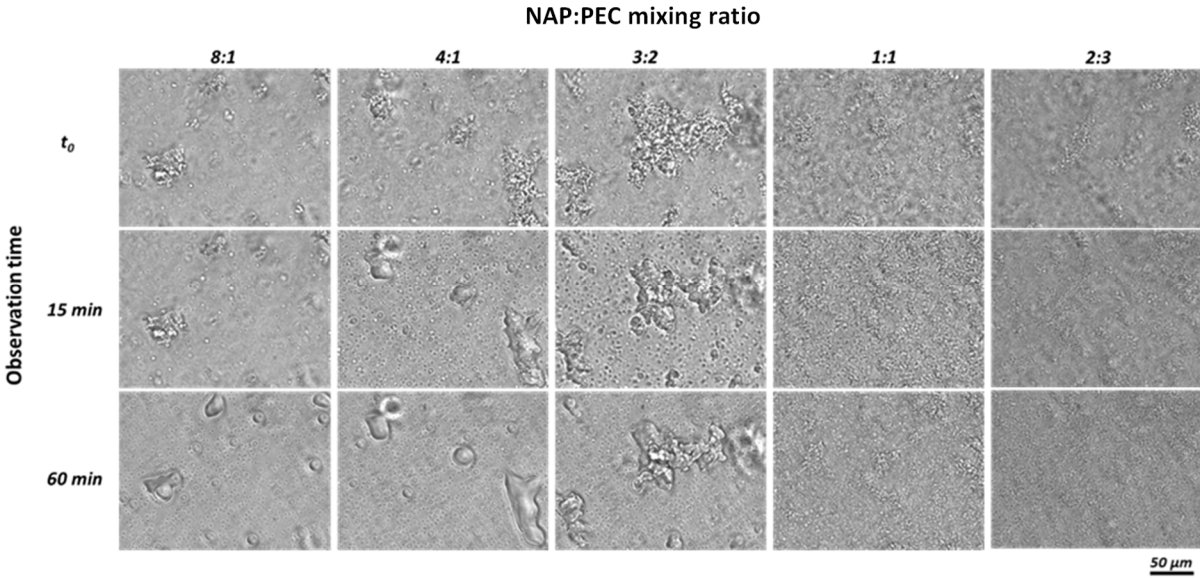


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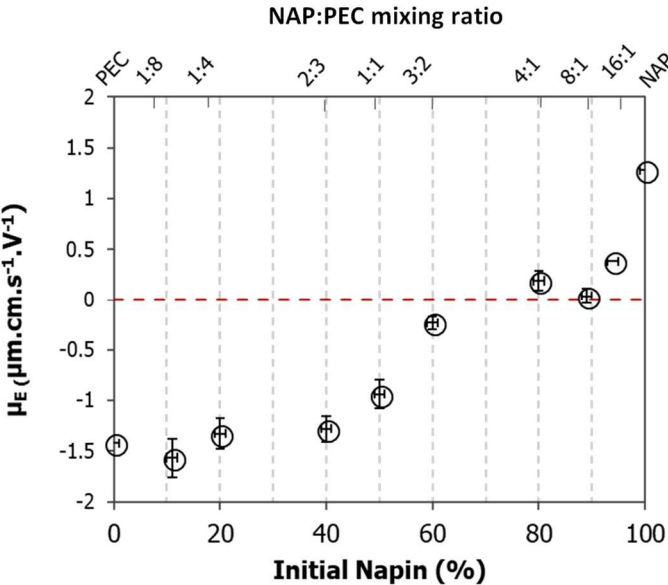


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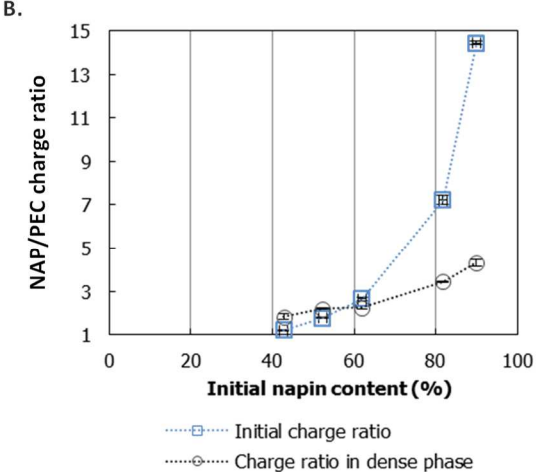
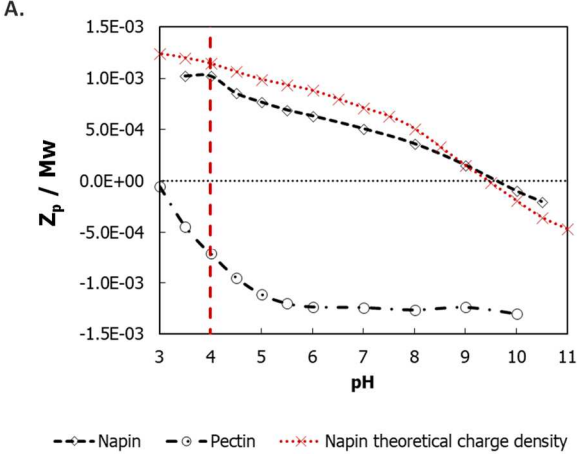


Figure 8

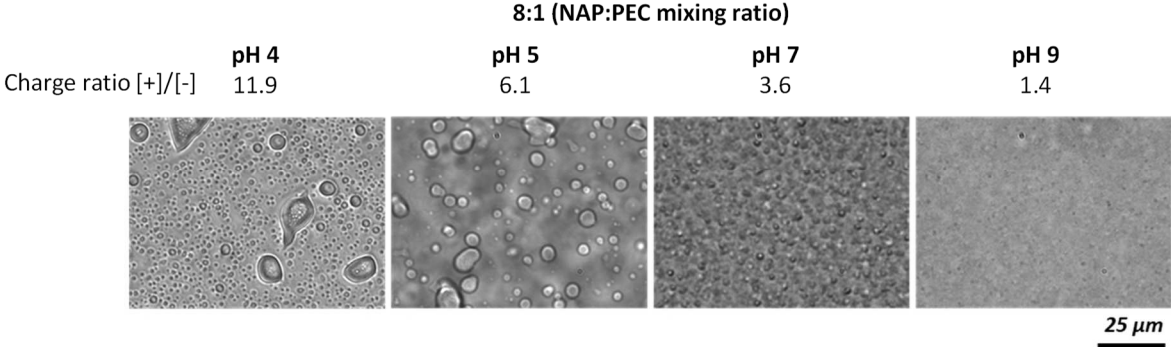


Figure 9

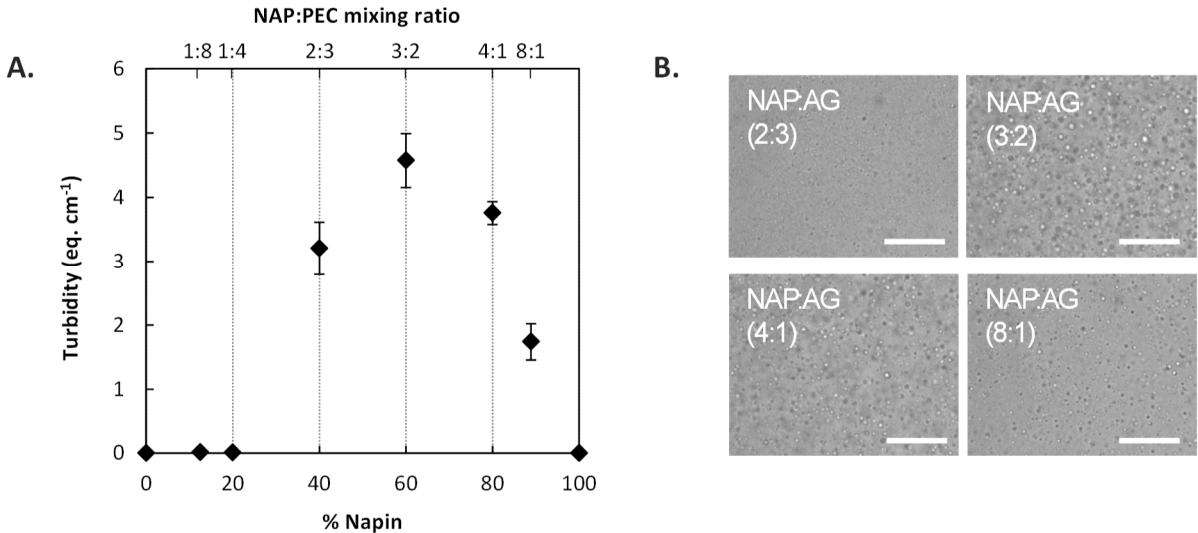
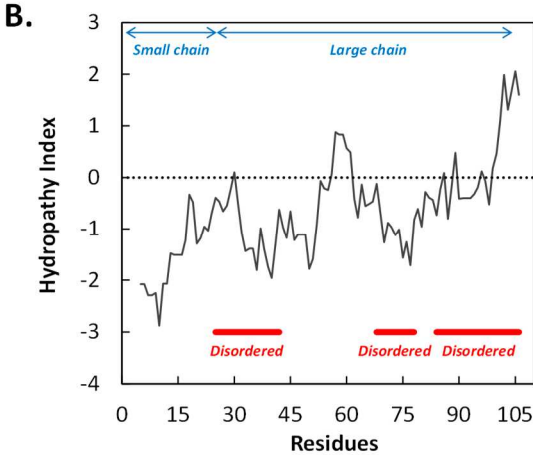




Figure 10



**Table 1.** Equivalence between protein:polysaccharide (Pr:Ps) ratio and proportion of napin (NAP) (%) in the initial mixture

<i>Pr:Ps mass ratio</i>	<b>0:1</b>	<b>1:8</b>	<b>1:4</b>	<b>2:3</b>	<b>1:1</b>	<b>3:2</b>	<b>4:1</b>	<b>8:1</b>	<b>1:0</b>
<i>% of NAP in initial mixture</i>	0	11	20	40	50	60	80	89	100

Graphical abstract

