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► **To cite this version:**

Nannan Chen, Taco Nicolaï, Christophe Chassenieux, Yong Wang. pH and ionic strength responsive core-shell protein microgels fabricated via simple coacervation of soy globulins. *Food Hydrocolloids*, 2020, 105, pp.105853. 10.1016/j.foodhyd.2020.105853 . hal-03026648

HAL Id: hal-03026648

<https://hal.science/hal-03026648v1>

Submitted on 3 Dec 2020

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1 **pH and ionic strength responsive core-shell protein microgels**
2 **fabricated via simple coacervation of soy globulins**

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8 **Abstract**

9 The influence of the salt concentration, pH, protein concentration and temperature was
10 investigated on spontaneous reversible coacervation in soy protein isolate (SPI) solutions.
11 Coacervation was characterized by formation of protein enriched micro-domains and was most
12 significant at 0.1 M NaCl. It was predominant below 40 °C and favored by lowering the
13 temperature. Decreasing the pH caused the spherical coacervates to form irregular flocs.
14 Increasing the protein concentration led to the formation of larger coacervates, but lowered the
15 rates of coacervation. Irreversibly crosslinked microgels were fabricated by heating suspensions
16 of coacervates at 80 °C. The effects of the pH and the salt concentration on the morphology and
17 the protein composition of the microgels were analyzed. The microgels swelled and displayed
18 a core-shell structure when the electrostatic repulsion between proteins was increased by
19 removing salt or adjusting the pH away from the isoelectric point. Reducing the electrostatic
20 repulsion induced de-swelling of the microgels and disappearance of the core-shell structure.
21 By tuning the electrostatic interaction, the SPI microgels could bind or release positively
22 charged lysozyme. These results show that SPI microgels are promising bioactive compound
23 carriers.

24 **Keywords:** Soy protein isolate; coacervation; phase separation; microgel; swelling; release

25 **1. Introduction**

26 Microgels are microparticles consisting of a network of crosslinked macromolecules
27 (Plamper & Richtering, 2017; Wang, Guo, Dong, Cui, & Hao, 2019). Due to their swelling and
28 de-swelling behavior upon changes of the environmental conditions, they can be used to
29 encapsulate bioactive compounds and control their release (Bysell, Mansson, Hansson, &
30 Malmsten, 2011). They have also been used to modify the rheology properties of food matrices
31 (Inthavong, Chassenieux, & Nicolai, 2019; Sağlam, Venema, de Vries, Shi, & van der Linden,
32 2013) and to stabilize emulsions and foams (Dickinson, 2015). Protein is one of the promising
33 materials to fabricate microgels as it is biocompatible and environmental friendly. The swelling
34 and de-swelling of protein gels can be modulated by the pH and the ionic strength, due to the
35 presence of charged acidic (e.g. carboxylic) and basic (e.g. ammonium) groups in the
36 polypeptide chains (Sağlam, Venema, de Vries, & van der Linden, 2013). Stable protein gels
37 can be formed either by heating or by cold-gelation through hydrophobic interactions, disulfide
38 bonds or hydrogen bonds without the need to add chemical crosslinkers (Chen, Remondetto,
39 & Subirade, 2006).

40 Several approaches have been developed to fabricate protein microgels. Protein
41 microgels with sizes of several hundred nanometers have been obtained by mechanical breakup
42 of bulk protein gels (Jiao, Shi, Wang, & Binks, 2018; Murray & Phisarnchananan, 2016). Whey
43 protein/ β -lactoglobulin microgels with sizes around 200 nm have been fabricated by heating
44 solutions in a narrow pH range close to the isoelectric point (Schmitt et al., 2010) or in the
45 presence of CaCl_2 (Phan-Xuan et al., 2014). Creating micro-domains of a protein dense phase
46 followed by heating is another efficient method to make whey protein microgels which can be

47 achieved with water in oil emulsions (Sağlam, Venema, de Vries, Sagis, & van der Linden,
48 2011) or by segregative phase separation in mixtures with polysaccharides (Aymard, Williams,
49 Clark, & Norton, 2000). During heating, the dense protein domains crosslink into microgels
50 with sizes larger than a micrometer. Much work has been done to fabricate milk protein based
51 microgels, but fabrication of microgels based on plant proteins is much less common.
52 Compared to animal protein, plant protein is more abundant and economical. There is currently
53 an increasing effort to explore the possibility to replace animal proteins with plant proteins in
54 food products, in view of the shortage of animal proteins for an increasing world population
55 and the environmental problems caused by animal husbandry (Alves & Tavares, 2019).

56 Variation of the solvent conditions, such as temperature, pH and ionic strength can
57 induce simple coacervation (self-coacervation) of protein in solution leading to liquid-liquid
58 microphase separation (LLPS) with spherical protein-rich domains (coacervates) embedded
59 within a continuous protein poor phase. The coacervates can coalesce and deform under shear
60 stress (Boire et al., 2019; Ivanov et al., 2019). Recently, protein condensation initiated by
61 coacervation of proteins in cells has attracted much attention as it is related to the stability and
62 function of membraneless organelles and associated with several protein aggregation diseases
63 including neurodegenerative disorders such as Alzheimer's disease and amyotrophic lateral
64 sclerosis (Mitrea et al., 2018; Mitrea & Kriwacki, 2016). Outside cells, simple coacervation of
65 protein has found applications in microencapsulation for lipophilic compounds (Lazko,
66 Popineau, & Legrand, 2004), novel adhesive production (Mohammadi, Beaune, Stokke,
67 Timonen, & Linder, 2018) and delivery of bioactive proteins (Lim, Ping, & Miserez, 2018).
68 Simple coacervation has been observed for plant proteins, including soy glycinin (Chen, Zhao,

69 Nicolai, & Chassenieux, 2017b), pea protein (Cochereau, Nicolai, Chassenieux, & Silva, 2019),
70 broadbean protein (Popello, Suchkov, Grinberg, & Tolstoguzov, 1992), gliadin (Boire, Menut,
71 Morel, & Sanchez, 2013), zein (Muthuselvi & Dhathathreyan, 2006) and kafirin (Oguntoyinbo,
72 Taylor, & Taylor, 2018). However, compared to the complex coacervation that usually occurs
73 between oppositely charged proteins and polysaccharides (Schmitt & Turgeon, 2011), the
74 investigation of the simple coacervation of protein is still quite limited.

75 Soy protein is widely used as an ingredient in the food industry for its good functional
76 and nutritional properties. It contains two main components: glycinin and β -conglycinin with
77 molar masses of about 3.6×10^5 and 2.0×10^5 g/mol, respectively (Nishinari, Fang, Guo, &
78 Phillips, 2014). Soy glycinin has the lowest solubility at intermediate NaCl concentrations 0.1–
79 0.2 M (Wolf, 1958). The solubility of glycinin is further decreased by lowering the temperature
80 (Lakemond, de Jongh, Hessing, Gruppen, & Voragen, 2000; Wolf & Sly, 1967). In a recent
81 study, Chen et al. (2017b) show that the low solubility of glycinin at 0.1–0.2 M is related to the
82 coacervation of glycinin. Initially, spherical protein rich domains are formed that with time
83 coalesce into a continuous layer. Interestingly, β -conglycinin can partially inhibit the
84 coacervation of soy glycinin (Chen et al., 2017b). Normally, depending on the type of the
85 protein, the coacervates are destroyed upon the temperature change above or below a critical
86 value and the turbid suspension becomes transparent at the same time (Raut & Kalonia, 2016).
87 However, by heating glycinin coacervates, hollow protein microcapsules were formed (Chen
88 et al., 2017b; Zhao, Guo, Ding, Ye, & Liu, 2019). Recently, similar phenomena have been
89 reported also for pea protein (Cochereau et al., 2019). Further investigation showed that the
90 soy glycinin microcapsules has pH and ionic strength responsive permeability (Chen, Zhang,

91 Mei, & Wang, 2018). Interestingly, in the presence of β -conglycinin with the glycinin/ β -
92 conglycinin ratio of 0.5–5, homogeneous microgels instead of hollow capsules are formed
93 when the glycinin coacervates are heated (Chen et al., 2017b).

94 As mentioned above, in our previous work, we isolated the two components of soy
95 protein, and mainly focused on the hollow microcapsules formed through the coacervation of
96 glycinin under heating (Chen et al. 2017b; Chen et al. 2018). We also found that in the presence
97 of a proper ratio of β -conglycinin, the coacervates could change into microgels instead of
98 hollow microcapsules during heating (Chen et al., 2017b). However, the properties (e.g.,
99 swelling, loading capacity and mechanical properties) that relate to the application of these
100 microgels are as yet unknown. Besides, the influence of the solvent conditions (salt
101 concentration, pH, and temperature) on the glycinin coacervation in the presence β -conglycinin
102 have not yet been investigated. SPI contains both glycinin and β -conglycinin at a ratio (1.27 –
103 3.6) that falls in the range (0.5–5) where microgels can be formed (Blazek, & Caldwell, 2009;
104 Chen et al., 2017b). Therefore, the objective of the work presented here was to fabricate soy
105 protein microgels by triggering the coacervation of glycinin in soy protein isolates (SPI)
106 solutions and to investigate the properties of the microgels that are formed. We will first show
107 the influence of the pH, the ionic strength, the protein concentration (C) and the temperature
108 (T) on the coacervation of glycinin in SPI suspensions. Then we will demonstrate that SPI
109 microgels can be fabricated by heating the SPI suspensions containing glycinin coacervates.
110 The swelling behavior of the microgels upon changes in the pH or the ionic strength will be
111 investigated. We will show that swollen microgels have a core-shell structure and discuss the
112 possible formation mechanism of it. Finally, the interaction between the microgels and

113 macromolecules (dextran and lysozyme) was investigated in order to evaluate their potential
114 as bioactive compound carriers. The current study is also different from our another previous
115 work in which we focus on the nano-scale fractal aggregates and gel formation of SPI as
116 influenced by salt. That work was conducted on homogenous SPI solutions in the presence of
117 salt, but in the absence of coacervation (Chen, Zhao, Chassenieux, & Nicolai, 2017a).

118 **2. Materials and Methods**

119 **2.1. Materials**

120 Defatted soybean flour (7B) was kindly provided by Archer Daniels Midland Company,
121 U.S. Rhodamine B and fluorescein isothiocyanate labeled dextran (FITC-dextran, 150 kDa)
122 were purchased from Sigma-Aldrich (St. Louis, MO, U.S.). FITC-lysozyme was purchased
123 from Angfei Biotechnology Co., Ltd. (Guangzhou, China). Bicinchoninic acid (BCA) protein
124 assay kits were purchased from Dingguo (Beijing, China). The water used in the research is
125 deionized water. All other chemicals were of analytical reagent grade and obtained in China.

126 **2.2. Soy protein isolate (SPI) preparation**

127 SPI was prepared by dispersing the soybean flour in water at pH 7.5. The suspension
128 was stirred at room temperature (23 ± 2 °C) for 1 h and then centrifuged at 10^4g for 30 min.
129 The supernatant was collected and centrifuged again at 10^4g for 20 min after adjusting the pH
130 to 4.5 to induce precipitation of the soy globulins. Deionised water was added to the collected
131 precipitate at a ratio of 1:5 and the pH was adjusted to 7. The suspension was stirred until it
132 became homogenous and centrifuged at 10^4g for 30 min to remove insoluble material. The
133 purified SPI solution was stored at 4 °C before use. The protein concentration was determined

134 using a BCA protein assay kit. SDS-PAGE showed that the purified SPI contained 64%
135 glycinin and 32% β -conglycinin.

136 **2.3. Preparation of protein solutions**

137 SPI solutions with different concentrations were prepared from a stock solution at $C =$
138 95 g/L and pH 6.7. The NaCl concentration of the protein solutions was set by adding aliquots
139 of a concentrated NaCl solution (2 or 4 M) and the pH was set by adding 0.1 M HCl or NaOH.
140 The SPI solutions were put in water baths at different temperatures to evaluate the influence of
141 temperature on the coacervation. The turbidity and microstructure of the suspensions was
142 probed with UV-visible spectrometry and confocal laser scanning microscopy (CLSM).

143 **2.4. Fabrication of SPI microgels via coacervation**

144 The pH of a SPI solution (20 g/L) was adjusted to pH 6.7. Then the ionic strength was
145 adjusted to 0.1 M NaCl to induce coacervation and kept at 23 °C for 18 h while mildly stirring
146 (200 rpm) during which the pH decreased to 6.25. Subsequently, the temperature of the
147 suspension was increased to 80 °C, kept at 80 °C for 30 min in a water bath while stirring and
148 then cooled back to room temperature. The microgels were isolated from the suspension by
149 centrifugation at 5000g for 10 min and re-dispersed in water. The pH and ionic strength of the
150 microgel suspension were adjusted by adding aliquots of 0.1 M NaOH, 0.1 M HCl or 2 M NaCl
151 solutions to evaluate their swelling at different solvent conditions. The isolated microgels that
152 dispersed in water were mixed with FITC-dextran (1 mg/mL) or FITC-lysozyme to investigate
153 the diffusion of biomacromolecules into the microgels. The microstructure of each suspension
154 was determined by CLSM.

155 **2.5. Determination of the protein composition of the coacervates and the microgels**

156 Microgel suspensions were centrifuged at 5000g for 10 min. The isolated microgels were
157 solubilized by adding 1% sodium dodecyl sulfate (SDS). The protein concentration of the
158 supernatant and the solubilized precipitate were determined using a BCA protein assay kit. The
159 percentage of free protein released from the microgels was calculated by dividing the protein
160 concentration in the supernatant by the total protein concentration. SDS-PAGE was used to
161 determine the protein composition of the supernatant and the precipitate. The same procedure
162 was used to determine the composition of the coacervate suspensions except that in this case
163 the precipitates were solubilized in water.

164 **2.6. Turbidity**

165 The turbidity of the SPI solutions was determined by measuring the absorbance of the
166 suspension at 500 nm using a UV-visible spectrometer E201 (Thermo Scientific, United States).
167 Cuvettes with path lengths of 0.5 cm were used.

168 **2.7. Confocal laser scanning microscopy (CLSM)**

169 The soy proteins were physically labeled with the fluorochrome rhodamine B by adding
170 a small amount of a concentrated rhodamine solution to the SPI suspensions to obtain a final
171 concentration of 5 ppm. Samples were inserted between a concave slide and a coverslip. The
172 microstructure of the suspensions at different solvent conditions were obtained at 20 °C with a
173 Zeiss LSM 880 Confocal Microscope (Zeiss, Germany). A water immersion objective lens
174 (63×/1.2NA) was used. The diffusion of FITC-dextran or FITC-lysozyme into the microgel
175 was determined by measuring the fluorescence intensity of the FITC signal in the microgels.

176 The incident light was emitted by a laser beam at 561 nm for rhodamine B and at 488 nm for
177 FITC. Transmitted light differential interference contrast (DIC) images were taken at the same
178 time by the other channel. The particle size distribution (180 particles were counted) and
179 fluorescence intensity profiles were obtained using the Image J software.

180 **2.8. Zeta-potential and hydrodynamic diameter measurements**

181 The surface charge of microgels at different pH or ionic strength and the diameter of the
182 microgels dispersed in denaturing agents (dithiothreitol (DTT), urea or SDS) was determined
183 by a commercial dynamic light scattering and microelectrophoresis device (Nano-ZS, Malvern
184 Instruments, Worcestershire, UK). The device is equipped with a 633 nm laser and operates at
185 a fixed angle of 173°. Notice that the value of the diameter is influenced by polydispersity and
186 non-sphericity of the particles if they are larger than the inverse of the scattering wave vector,
187 i.e. about 20 nm at 173°. This causes the measured apparent diameter to be less than the true
188 z-average diameter.

189 **2.9. Electrophoresis**

190 Reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was
191 performed on a discontinuous buffered system, using 12% separating gel and 5% stacking gel.
192 The protein sample was mixed with an equal volume of 2× reducing sample buffer which
193 contained β-mercaptoethanol and then heated at 90 °C for 10 min. After the electrophoresis,
194 the gel was stained using the Coomassie brilliant blue R-250 stain solution for 1 h and then
195 soaked in de-staining solution (methanol/acetic acid/water, 1:1:8, v/v/v) for 24 h with two or

196 three changes of solution. The protein composition was analyzed by measuring the relative
197 brightness of the bands using the Image J software.

198 **3. Results and Discussion**

199 **3.1. Influence of adding NaCl on coacervation of glycinin in SPI solution**

200 The ionic strength of SPI solutions at $C = 10$ g/L and pH 7.0 was adjusted by adding 2 or
201 4 M NaCl. After 1 h of preparation, solutions at 0.1, 0.15 and 0.2 M NaCl became turbid, while
202 at 0.05 and 0.5 M, the solutions remained clear (**Figure 1A**). The microstructure of the
203 solutions was probed with CLSM (**Figure 1B**) which showed that after 1 h, SPI phase separated
204 on microscopic length scales leading to the formation of well-defined spherical dense protein
205 domains with diameters of approximately 1 μm . After 24 h, phase separation also occurred at
206 0.05 M, but it was less important than at 0.1 M. Clusters of domains appeared at 0.2 M. The
207 suspensions containing 0.1 M NaCl were centrifuged after standing for 24 h and the precipitates
208 were collected. The protein composition of the precipitates was analyzed by SDS-PAGE and
209 compared with that of the SPI solution without phase separation. It showed that the proteins in
210 the homogeneous SPI solution (lane 1) were composed of 64% glycinin and 32% β -conglycinin,
211 whereas the precipitate from the phase separated SPI suspension (lane 2) contained 87%
212 glycinin and 11% β -conglycinin (**Figure 1C**).

213 These observations show that simple coacervation of protein occurred in SPI solutions by
214 adding salt which was caused by the association of glycinin. The low solubility of SPI at 0.1–
215 0.2 M NaCl caused by association of glycinin has been reported before, but was not related to
216 coacervation (Wolf, 1958). Adding salt screened the electrostatic repulsion between protein

217 molecules (**Figure S1**) which promotes aggregation of the proteins, but it fails to explain why
218 the solubility increased after adding more than 0.15 M NaCl even though the surface charge
219 remained low. Jiang et al. (2015) has investigated in detail the influence of salt on the protein
220 hydrophobicity and structure of SPI in aqueous solution. They showed that the solubility of
221 SPI is negatively correlated with its hydrophobicity. The hydrophobicity was highest at 0.1 M
222 NaCl which corresponded to the lowest solubility. In addition, at 0.1 M NaCl, the α -helix
223 content in both SPI and glycinin solutions was lowest (Jiang, et al., 2015; Kim, Kim, Yang, &
224 Kwon, 2004). Lakemond et al. (2000) have shown that the salt concentration influences the
225 relative exposure of basic and acidic polypeptides of glycinin. As the basic polypeptide is more
226 hydrophobic than the acidic one, the change of exposure will influence the surface
227 hydrophobicity of the proteins. The present results show that the insolubility is in fact caused
228 by coacervation which was reversible as the sediments obtained by centrifugation could be
229 completely solubilized in water.

230 **3.2. Influence of the pH on coacervation of glycinin in SPI solutions**

231 The effect of the pH on coacervation of glycinin in SPI solutions in the presence of 0.1 M
232 NaCl is shown in **Figure 2**. The pH was adjusted before adding salt. In salt free solutions, the
233 solution remained homogeneous on microscopic length scales above pH 5.8, but irregular large
234 flocs were observed below pH 5.8 (Chen et al., 2017b). After adding salt, see **Figure 2**, the
235 solutions with an initial pH of 7.5 remained homogenous. However, for $6.6 \leq \text{pH} \leq 7.2$, well-
236 defined spherical coacervates were observed. At pH 6.4, the coacervates tended to associate
237 and finally at $\text{pH} \leq 6.2$, large irregular flocs were observed.

238 The protein coacervates dissolved when diluted in water, but the irregular flocs
239 remained intact upon dilution. To form well defined spherical coacervates, molecular
240 interaction must be large enough to drive association but not so large so as to avoid formation
241 of strong bonds within and between coacervates (Amine, Boire, Kermarrec, & Renard, 2019;
242 Comert & Dubin, 2017). The isoelectric point of glycinin is around pH 5. Decreasing the pH
243 towards the isoelectric point reduces the charge density of the proteins, which decreases the
244 strength of repulsive interactions between proteins and favors therefore their coacervation.
245 However, at $\text{pH} \leq 6.4$, the interaction became too strong, leading to flocculation.

246 **3.3. Influence of the protein concentration on coacervation of glycinin in SPI solutions**

247 The SPI stock solution ($C = 95 \text{ g/L}$, $\text{pH} 6.7$) was diluted to different protein concentrations
248 followed by adjusting the NaCl concentration to 0.1 M. The microstructures of the suspensions
249 (1 h and 24 h after preparation) are shown in **Figure 3A**. One hour after preparation,
250 coacervation occurred at $C \leq 30 \text{ g/L}$, but not yet at $C = 50 \text{ g/L}$. The size of the coacervates
251 increased with increasing protein concentration. After one day, the coacervates had grown by
252 coalescence and large coacervates had appeared also at $C = 50 \text{ g/L}$. No phase separation was
253 observed for $C \geq 70 \text{ g/L}$ even after 3 days (**Figure S2**).

254 **Figure 3B** shows the evolution of the turbidity with time at 20 °C for SPI solutions at C
255 = 10, 20 and 50 g/L containing 0.1 M NaCl. The values were normalized by C so as to correct
256 for the trivial concentration dependence of the turbidity. For $C = 10 \text{ g/L}$, the turbidity increased
257 weakly at first followed by a strong increase after about 20 min. At $C = 20 \text{ g/L}$, the turbidity
258 remained low during 20 min and then increased more weakly than at $C = 10 \text{ g/L}$. At $C = 50 \text{ g/L}$,

259 the turbidity remained low up to at least 120 min. These results confirm that the rate of glycinin
260 coacervation decreases with increasing SPI concentration.

261 Above the denaturation temperature of the soy proteins, the inhibition effect of β -
262 conglycinin on the glycinin aggregation by forming soluble complexes has been studied in
263 detail in the past (Damodaran & Kinsella, 1982; Guo et al., 2012). In a recent study, it was
264 found that at temperature as low as 20 °C and in the presence of β -conglycinin, the coacervation
265 of glycinin was slowed down and the rate of coacervation decreased with increasing fraction
266 of β -conglycinin (Chen et al., 2017b). This indicates that the inhibition effect of β -conglycinin
267 exists even at low temperatures where the interactions between the two components are weaker
268 and reversible (Chen et al., 2017b). For the SPI investigated here, the ratio of glycinin/ β -
269 conglycinin was fixed, and we found that by increasing the total protein concentration, the rate
270 of coacervation decreased. We propose that two kinds of association may occur at the same
271 time. One is the coacervation of glycinin triggered by adding salt and the other is the association
272 between glycinin and β -conglycinin. At low protein concentrations, coacervation of glycinin is
273 predominant, but with increasing protein concentration, the interaction between glycinin and
274 β -conglycinin becomes more important, which decreases the tendency for glycinin to phase
275 separate.

276 **3.4. Influence of temperature on coacervation of glycinin in SPI solutions**

277 The salt concentration of SPI solutions at $C = 20$ g/L and pH 7.1 was adjusted to 0.1 M
278 NaCl. At these conditions, no phase separation occurred just after adding salt. The solution was
279 then immediately kept at different temperatures. **Figure 4A** shows the turbidity of SPI solutions

280 kept at different temperatures for 5 h. With increasing T , the turbidity decreased and reached a
281 minimum at 40 °C. When T was further increased, the turbidity gradually increased again. The
282 CLSM images show that only at $T < 40$ °C, spherical protein coacervates were observed
283 (**Figure 4B**). Above 40 °C, no phase separation occurred and the visual appearance of the
284 solution was semi-transparent (**Figure 4A**). These suspensions were then kept at room
285 temperature (23 °C) for 18 h. The turbidity of the suspensions that were initially kept at 40–
286 60 °C increased significantly compared to those before cooling (**Figure S3**) which was
287 accompanied by the appearance of protein coacervates (**Figure 4B**). The solution that was
288 initially kept at 80 °C still remained homogeneous without phase separation.

289 These results clearly show that two kinds of aggregation process occurred in the SPI
290 solutions with increasing temperature, when 0.1 M NaCl was added, as accounted for by the
291 presence of a characteristic temperature where the solutions showed lowest turbidity. At $T <$
292 40 °C, coacervation of glycinin was predominant and was favored by lowering the temperature.
293 At $T > 40$ °C, the coacervation was inhibited. Instead, thermal aggregation that leads to
294 irreversible aggregation of SPI was gradually enhanced with increasing temperature. It has
295 been shown previously that upon thermal denaturation in salt solutions, nanoparticles with radii
296 less than 100 nm are formed at low protein concentrations which associate into large fractal
297 aggregates at higher protein concentrations (Chen et al., 2017a). Furthermore, above a critical
298 gel concentration, the aggregates percolate and form a gel (Chen et al., 2017a). The presence
299 of protein coacervates after cooling was most likely due to the glycinin fraction that had not
300 yet denatured during the thermal treatment. The denaturation extent increased with increasing
301 temperature and was complete after keeping the sample at 80 °C for 5 h. Therefore, protein

302 coacervates were not formed in this case. The molecular interactions at play during thermal
303 aggregation have already been investigated in detail (Guo et al.,2012). It was shown that
304 hydrophobic interactions as well as disulfide bond formation are predominant, which makes
305 the structure of the aggregates formed upon heating stable and irreversible. The present results
306 show that, for protein coacervation, electrostatic interaction plays a more important role.
307 Furthermore, contrary to thermal aggregation, coacervation is reversible, i.e. the coacervates
308 can re-disperse when the solvent conditions are changed.

309 **3.5. Heat induced microgel formation from glycinin coacervates**

310 The pH of SPI solutions at 20 g/L was adjusted to pH 6.7 and the NaCl concentration of
311 the solutions was adjusted to 0.1 M. At these conditions, coacervation occurred immediately
312 after adding salt. The suspensions were kept stirring at 23 °C for 18 h in order to induce
313 formation of more protein coacervates and subsequently heated at 80 °C for 30 min to denature
314 the proteins. The microstructure of the suspensions as well as the particle size distribution and
315 the fluorescence intensity distribution of representative particles are shown in **Figure 5**. Before
316 heating, spherical protein coacervates with an average diameter of 1.9 µm were formed (**Figure**
317 **5A**). After heating, the coacervates transformed into microgels with a smaller average diameter
318 of approximately 1.4 µm (**Figure 5B**).

319 The microgels suspensions were centrifuged at 5000g for 10 min and the sedimented
320 microgels were re-dispersed in water. The pH of the microgel suspensions obtained in this
321 manner was 7. Interestingly, in water, the microgels swelled to a diameter of approximately
322 1.8 µm similar to that of the coacervates and showed a core-shell structure, which was attested

323 by the stronger fluorescence intensity at the edge, indicating a denser shell and a less dense
324 core (**Figure 5C**). The thickness of the shell was approximately 0.25 μm . By increasing the
325 salt concentration back to 0.1 M NaCl, the microgels de-swelled and the core-shell structure
326 disappeared (**Figure 5D**). The size of the microgels decreased back to around 1.3 μm .

327 The swelling and de-swelling behavior of the microgels could also be modulated by
328 changing the pH (**Figure 6**). The microgels swelled with increasing pH to an average diameter
329 of 2.3 μm at pH 10 and the shell thickness increased to 0.35 μm . When the pH was decreased
330 to pH 6, the microgels de-swelled to an average diameter of 1.2 μm and the core shell structure
331 was no longer visible. Below pH 6, i.e. closer to the isoelectric point, the microgels aggregated
332 into clusters (**Figure S4**). However, when the pH was further decreased below 4, the microgels
333 swelled again and the core-shell structure reappeared as shown in **Figure 6**. These results show
334 that pH and ionic strength responsive core-shell SPI microgels were formed through protein
335 coacervation. To our knowledge, fabricating protein microgels with a core-shell structure by
336 protein self-assembly has not been reported before.

337 The pH and ionic strength responsive swelling and de-swelling behavior of the SPI
338 microgels indicated that the electrostatic interaction plays an important role. The zeta-potential
339 of the microgels suspension at different pH was measured (**Figure S5**). Before adding salt, the
340 pH of the suspensions was 7 and the microgels were negatively charged with a zeta-potential
341 value of approximately -20 mV. Due to the strong electrostatic repulsion between the proteins,
342 the microgels were swollen. When the NaCl concentration was increased to 0.1 M, the zeta-
343 potential decreased to -9 mV. The decrease of electrostatic repulsion between the proteins

344 caused de-swelling of the microgels. With increasing pH above 7, the proteins carried more
345 negative charges as shown by the increase of the zeta potential, which led to increasing
346 electrostatic repulsion between the proteins. Therefore, the microgels swelled to a larger extent.
347 Upon lowering pH towards the isoelectric point (pH 5.1), the net charge of the proteins
348 decreased. Therefore, the electrostatic repulsion decreased, leading to de-swelling of the
349 microgel between pH 4.5 and 6. When the pH was decreased below 4, the positive charge
350 density of the proteins increased, causing an increase of the electrostatic repulsion and as a
351 consequence swelling of the microgels. Swelling and de-swelling of protein microgels
352 controlled by the charge density has earlier also been reported for whey protein microgels
353 (Sağlam et al., 2013).

354 **3.6. Protein composition of SPI microgels**

355 The SPI microgel suspensions at different pH were centrifuged and the protein
356 concentration in the supernatant was analyzed to determine the protein content that was
357 released from the microgels (**Figure 7A**). The amount of protein that was released from the
358 microgels was lowest (4%) between pH 4.5 and 6. With increasing pH above 6 or decreasing
359 pH below 4.5, the released protein fraction increased to 56% at pH 3 and 10. At pH 11, the
360 release of protein increased further to 68% and led to the partial dissociation of the microgels
361 (**Figure S4**). The protein composition of the supernatant and precipitates was analyzed by SDS-
362 PAGE (**Figure 7B**). Between pH 6 and 4.5, the protein content in the supernatant was too low
363 for a proper characterization of the composition. Below pH 4.5 or above 6, the main protein
364 component in the supernatant was glycinin with only small amounts of β -conglycinin. This

365 means that some free glycinin that was not incorporated into the network of the microgels was
366 released when they swelled. At pH 11, the microgels were partially dissociated (**Figure S4**)
367 and more β -conglycinin was present in the supernatant compared to other pH values, indicating
368 that β -conglycinin had been incorporated into the network of the microgel. SDS-PAGE
369 analysis of the precipitates is shown in **Figure 7B** and the corresponding protein fractions are
370 plotted as a function of the pH in **Figure S6**. Between pH 6.5 and 4.5, the main protein
371 component of the precipitate was glycinin, which accounted for around 78% of the total amount
372 of protein in the precipitate. With increasing pH above 6.5 or decreasing pH below 4.5, the
373 amount of glycinin in the precipitate decreased to 49% at pH 3 and to 54% at pH 10 while the
374 β -conglycinin content increased from 15% to 35% and 31%, respectively. This corroborates
375 the findings that during swelling, free glycinin has been released from the microgels.
376 Furthermore, SDS-PAGE analysis of the precipitates confirmed that both glycinin and β -
377 conglycinin were incorporated into the network of the microgel. However, before heating, the
378 main component of the coacervates was glycinin which accounted for 87% of the total protein
379 in the precipitate (**Figure 1C**). With decreasing pH below 4 or increasing pH above 6.5, the
380 content of basic polypeptide increased and became higher than that of the acid polypeptide,
381 while between pH 4 and 6.5, the amount of the acid polypeptide was larger than that of the
382 basic polypeptide (**Figure S6**). This indicates that more acid polypeptides were free and
383 released from the microgels during swelling, whereas more basic polypeptides were bound to
384 the microgels. Recently, Wu et al. have done a systematic investigation of the release of protein
385 from SPI bulk gels (Wu, Hua, Chen, Kong, & Zhang, 2015; Wu et al., 2018). They found that
386 the released proteins are mainly glycinin subunits and free acidic polypeptides while most of

387 β -conglycinin remains bound to the gel network, which is in accordance with our present results.
388 One possibility to explain this behavior is the different denaturation temperature of the different
389 subunits in glycinin, causing part of the glycinin to be not yet fully denatured and available to
390 bind to the microgel network (Prak et al., 2005).

391 In the absence of β -conglycinin, glycinin coacervates transformed into hollow
392 microcapsules (Chen et al., 2017b). Some free glycinin remained in the cavity of the
393 microcapsules, but no network was formed in the cavity (Chen et al., 2018; Chen et al., 2017b).
394 However, in the presence of β -conglycinin, a network was formed in the core of glycinin
395 coacervate. We suggest that during heating β -conglycinin diffused into the coacervates and
396 interacted with the glycinin which induced formation of a network in the core. This suggestion
397 is supported by the finding that β -conglycinin has a tendency to interact with glycinin during
398 heating (Damodaran & Kinsella, 1982; Guo et al., 2012).

399 In order to determine the interactions that stabilized the microgels, the microgel
400 suspensions were adjusted to pH 8.5 and then centrifuged to remove free protein. The
401 precipitates were subsequently dispersed in different solvents and the apparent hydrodynamic
402 diameter was measured (**Figure 7C**). After addition of 40 mM DTT, 6 M urea or 1% SDS, the
403 size of the microgels decreased, especially for those dispersed in 1% SDS. The size of the
404 microgels further decreased by adding 40 mM DTT in addition to SDS or Urea. DTT can break
405 disulfide bonds, while urea and SDS disrupt hydrophobic interactions. These results show that
406 hydrophobic interactions are important to stabilize the microgel, but that disulfides bonds are
407 also formed.

408 **3.7. Influence of the pH on the diffusion of macromolecules into SPI microgels**

409 Microgels have been widely applied as bioactive compound carriers (Bysell et al., 2011).
410 By swelling or de-swelling the microgels, diffusion of molecules into or out of the microgels
411 could be controlled. Here, FITC-dextran (150 kDa) was used as model bioactive compounds
412 to check the diffusion of molecules into the microgels as a function of the pH (**Figure 8**). At
413 pH 6.5, the dextran was excluded from the microgels resulting in low fluorescence intensity of
414 the microgels. When the pH was 3.5, the dextran concentration in the microgels was higher
415 than that in the bulk, causing stronger fluorescence by the microgels. At pH 10 and 3, the
416 fluorescence intensity of the core of the microgel was almost the same as that of the bulk.
417 However, at pH 3, the shell of the microgels showed stronger fluorescence while at pH 10 the
418 fluorescence was less. The implication is that at pH 3 the dextran chains preferred to locate in
419 the shell, whereas at pH 10 they were expelled from it.

420 The diffusion of molecules into the microgels depends on many factors, including the pore
421 size of the network and the interaction with proteins. At pH 6.5, microgels display a net
422 negative charge and FITC-dextran is also slightly negatively charged (Berth, Voigt,
423 Dautzenberg, Donath, & Möhwald, 2002). Electrostatic repulsion could therefore be a reason
424 for FITC-dextran to be excluded at pH 6.5. However, this does not explain the fluorescence of
425 the cores at pH 10. It is also possible that the FITC-dextran chains were larger than the pores
426 of the microgel at pH 6.5 and therefore could not easily penetrate into the microgels. The
427 increase of the fluorescence intensity of the microgels with increasing pH would in that case
428 be caused by an increase of the pore size caused by swelling. Another possible reason for the

429 penetration of dextran into the core at pH 10 is that at high pH, the structure of the protein
430 network is destabilized due to the cleavage of disulfide bonds and changes the protein structure
431 (Jiang, Xiong, Chen, 2011). At pH 3.5, the microgels were positively charged and swollen.
432 Due to the increased pore size of the network and the electrostatic attraction, the FITC-dextran
433 could penetrate and preferentially bind to the microgels. When the pH was readjusted back to
434 7, most of the FITC-dextran was released from the microgel (**Figure S7**). The decrease of the
435 contrast of the microgels at pH 3 compared to that at pH 3.5 was perhaps due to the increased
436 ionic strength caused by addition of more HCl. The fact that the diffusion of the molecules
437 could be controlled by the pH shows that the SPI microgels have the potential to be used as
438 bioactive compound carriers. Especially, electrostatic attraction between microgels and
439 molecules of opposite charges enhances the loading, which can be an efficient method for
440 encapsulating bioactive compounds such as proteins (Bysell et al., 2011). In the following
441 section, the interaction between microgels and oppositely charged proteins will be investigated.

442 **3.8. Binding and release of lysozyme to SPI microgels**

443 The microgel suspensions were incubated with lysozyme labeled with FITC at pH 9.
444 CLSM images of the mixtures are shown in **Figure 9A**. At low lysozyme concentrations, the
445 microgels showed a core-shell structure with stronger fluorescence displayed by the shell, i.e.
446 the shell contained a higher lysozyme content than the core. With increasing lysozyme
447 concentration, the fluorescence of the core increased while the microgels de-swelled slightly
448 and finally aggregated into clusters. At all concentrations, the microgels fluoresced strongly
449 implying binding of the lysozyme. Lysozyme, with an isoelectric point of pH 10.8, is positively

450 charged at pH 9 (Yi et al., 2017). It binds to the microgels that are negatively charged at pH >
451 5.1 due to the electrostatic attraction. With increasing lysozyme concentration, the amount of
452 lysozyme that bound to the microgels increased which reduced the negative charge density of
453 the microgels, leading to decreased electrostatic repulsion between proteins. Therefore, the
454 microgels slightly de-swelled and finally aggregated into clusters.

455 The electrostatic attraction between lysozyme and the microgels can be reduced by
456 adding salt or adjusting the pH to trigger the release of the lysozyme. **Figure 9B** shows the
457 DIC and fluorescence images as well as merged images of the two channels for the lysozyme
458 loaded microgel suspensions at different ionic strength. Profiles of the fluorescence intensity
459 of representative microgels are also shown. With increasing NaCl concentration, the
460 fluorescence intensity from FITC-lysozyme decreased implying that lysozyme was released.
461 When the ionic strength was increased to 0.5 M, most of the lysozyme was released. The DIC
462 images showed that the microgels remained intact in all cases. Salt screens the electrostatic
463 attraction between the positively charged lysozyme and the negatively charged microgels,
464 which explains why more lysozyme is released with increasing salt concentration. Release of
465 the lysozyme also occurred after decreasing the pH to 3.5 (**Figure S8**), because the microgels
466 became positively charged, causing electrostatic repulsion between microgels and lysozyme.

467 **4. Conclusions**

468 In summary, coacervation of glycinin in SPI solutions was influenced by the ionic
469 strength, pH, protein concentration and temperature. It was promoted by increasing the NaCl
470 concentration towards 0.1 M, decreasing the pH and lowering the temperature. Increasing the
471 protein concentration slowed down the glycinin coacervation. Two types of self-assembly

472 processes were observed as a function of the temperature. Below about 40 °C, coacervation of
473 glycinin was dominant and favored by decreasing temperature. Above 40 °C, thermal
474 aggregation was dominant and favored by increasing temperature, with both glycinin and β -
475 conglycinin participating. Heating the SPI suspensions containing glycinin coacervates led to
476 formation of stable microgels with a core-shell structures, which were composed of both
477 glycinin and β -conglycinin. The swelling and de-swelling of the microgels could be modulated
478 by changing the electrostatic interaction. The microgels can be loaded with oppositely charged
479 lysozymes and their release can be triggered by reducing the electrostatic interaction either by
480 adding salt or by changing the pH. This demonstrates that SPI microgels can be used as
481 bioactive compound carriers. Further research is needed to reveal the exact interaction between
482 glycinin coacervates and β -conglycinin and the influence of solvent conditions on the microgel
483 structure and their properties.

484 **Supplementary material**

485 Zeta-potential measurement of SPI solution in different NaCl concentration; Visual appearance
486 of the 70g/L protein solution with 0.1 M salt at different times; Visual appearance of the SPI
487 solution incubated at 40 and 60 °C with cooling to 23 °C; CLSM images of microgel
488 suspension at pH 5 and pH 11; zeta-potential measurement of the microgel suspension; CLSM
489 image of the microgel and FITC-dextran mixture under acidic condition with subsequent
490 neutralization; images of SPI microgels loaded with lysozyme at basic conditions with
491 subsequent adjustment of pH to 3.5.

492 **Funding sources**

493 This study was financially supported by Postdoctoral Innovation Talent Support Program,
494 China (BX20190145), China Postdoctoral Science Foundation (2019M663387) and Natural
495 Science Foundation of Guangdong Province, China (2019A1515011176).

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635 **Figure Captions**

636 **Figure 1.** Visual appearance **(A)** and CLSM images **(B)** of SPI solutions (10 g/L, pH 7)
637 at different NaCl concentrations. The images were taken 1 h (top row) and 24 h (bottom
638 row) after adding salt. The scale bar represents 5 μm . **(C)** Protein composition of the
639 SPI solution (lane 1) and the precipitates (lane 2).

640 **Figure 2.** CLSM images of SPI solutions ($C = 10\text{g/L}$) adjusted to different pH and
641 subsequently to $[\text{NaCl}] = 0.1\text{ M}$. Images were taken 2 h after preparation. The scale bar
642 represents 5 μm .

643 **Figure 3. (A)** CLSM images of SPI solutions at different protein concentrations at 0.1
644 M NaCl. Images were taken after 1 h (top row) and 24 h (bottom row). The scale bar
645 represents 5 μm . **(B)** Evolution of the turbidity with time at 20 $^{\circ}\text{C}$ for SPI solutions at
646 different protein concentrations in the presence of 0.1 M NaCl.

647 **Figure 4. (A)** Turbidity of SPI suspensions (20 g/L, 0.1 M NaCl) kept at different
648 temperatures for 5 h. The inserted picture shows the visual appearance of the
649 suspensions. **(B)** CLSM images of SPI solutions incubated at different temperatures for
650 5 h (top row) and subsequently kept at 23 $^{\circ}\text{C}$ for 18 h (bottom row). The scale bar
651 represents 10 μm .

652 **Figure 5.** CLSM images, particle size distributions and fluorescence intensity profiles
653 of representative particles for a SPI suspension with glycinin coacervates **(A)**; a SPI
654 suspension with glycinin coacervates after heating at 80 $^{\circ}\text{C}$ for 30 min **(B)**; microgels
655 dispersed in water **(C)**; microgels dispersed in 0.1 M NaCl **(D)**. The scale bar represents
656 5 μm .

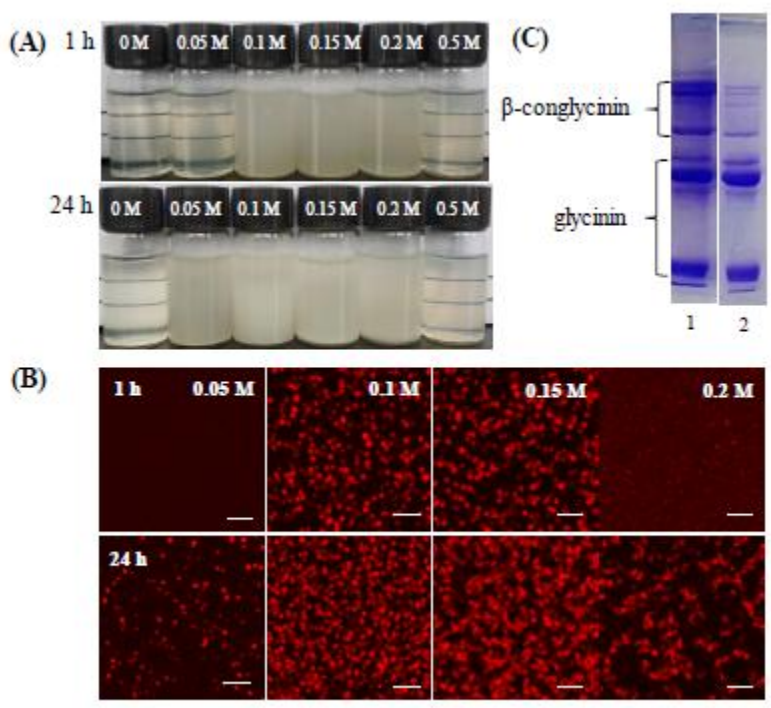
657 **Figure 6.** CLSM images, particle size distribution and fluorescence intensity profiles
658 of representative particles of SPI microgels at different pH. The scale bar represents 5
659 μm .

660 **Figure 7. (A)** Percentage of protein released from the microgels. **(B)** SDS-PAGE slabs
661 of the supernatant and precipitate of microgel suspensions at different pH. **(C)** Apparent
662 hydrodynamic diameter of microgels dispersed in different denaturing agents: 40 mM
663 DTT, 6 M Urea, 1% SDS, 6 M Urea + 40 mM DTT, 1% SDS + 40 mM DTT.

664 **Figure 8.** CLSM images of microgel suspensions in the presence of FITC-dextran at
665 different pH. The scale bar represents 5 μm .

666 **Figure 9. (A)** CLSM images of suspensions of microgels with FITC-lysozyme at
667 different concentrations indicated in the images. **(B)** DIC/Fluorescence CLSM images
668 and profiles of the fluorescence intensity of microgels loaded with lysozyme dispersed
669 in water with different NaCl concentrations. The scale bar represents 5 μm .

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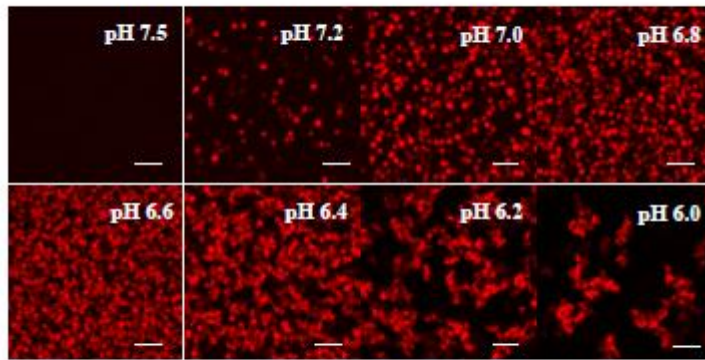


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2 Figure 1.

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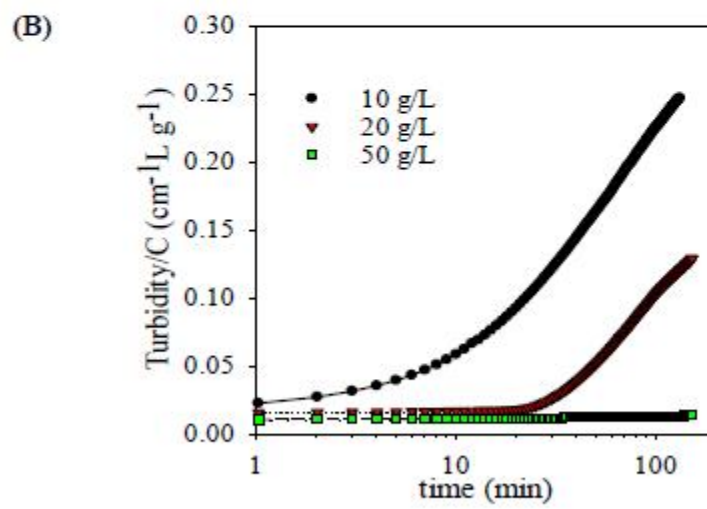
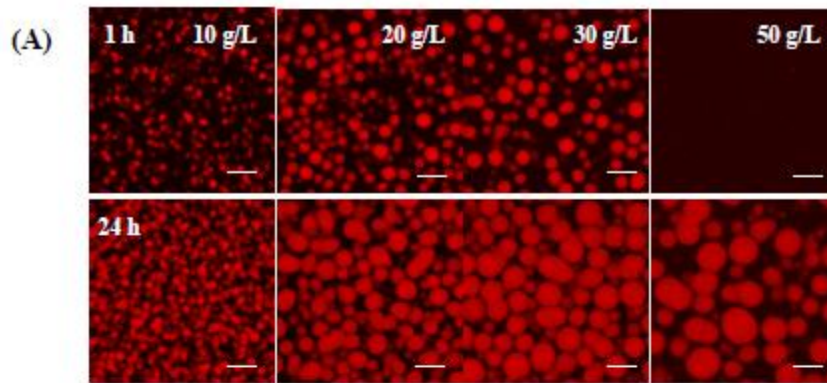


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4 **Figure 2.**

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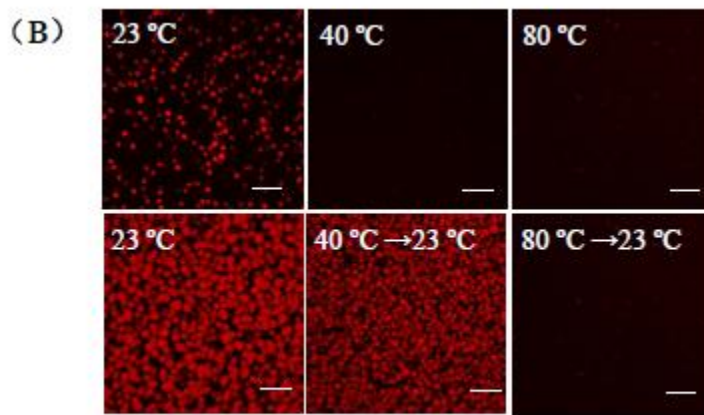
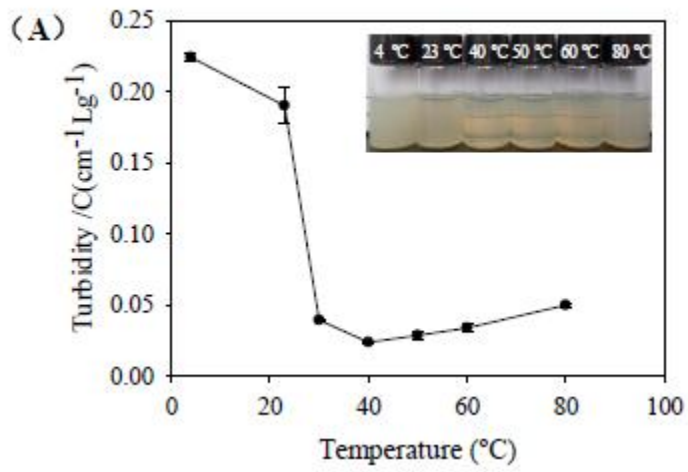


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6 Figure 3.

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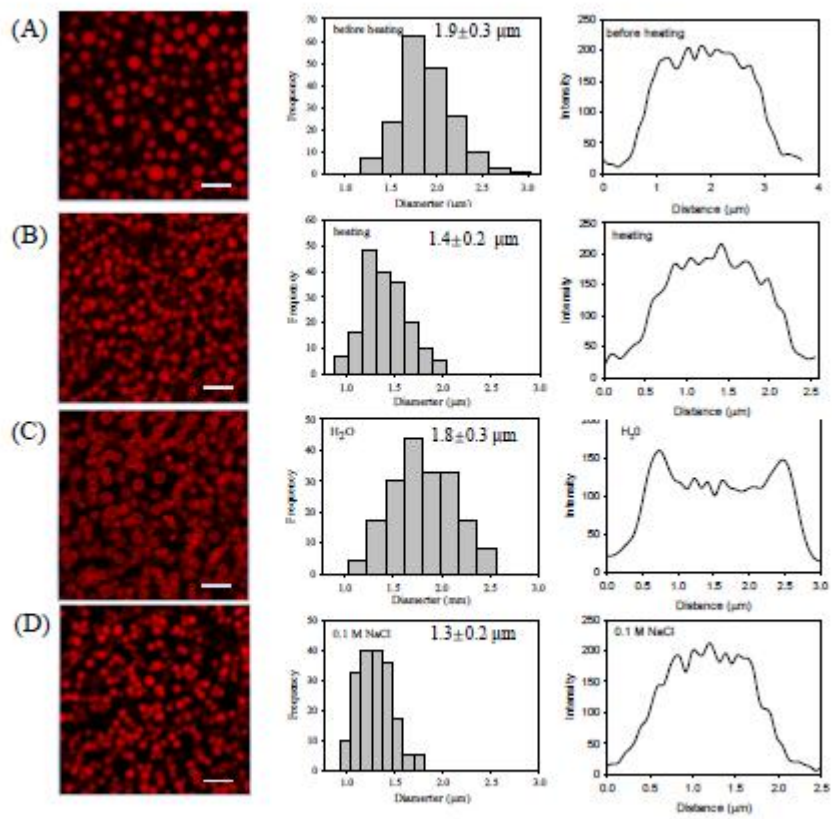


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8 Figure 4.

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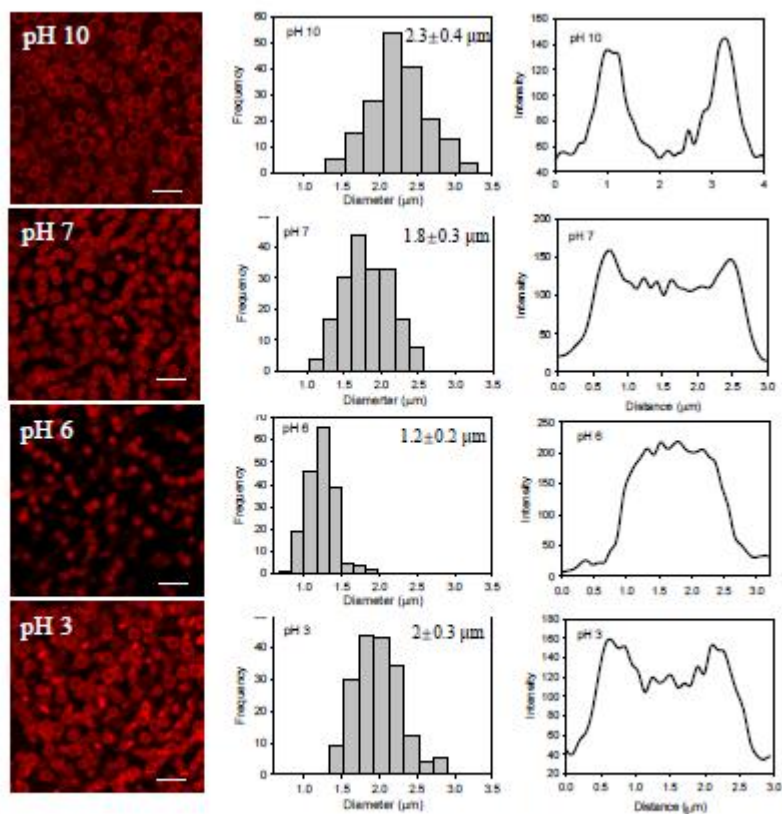


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10 Figure 5.

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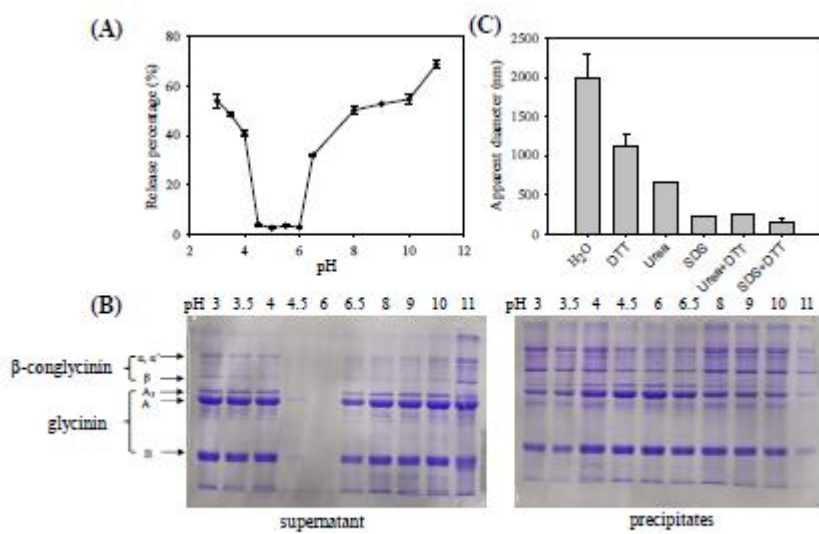


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12 Figure 6.

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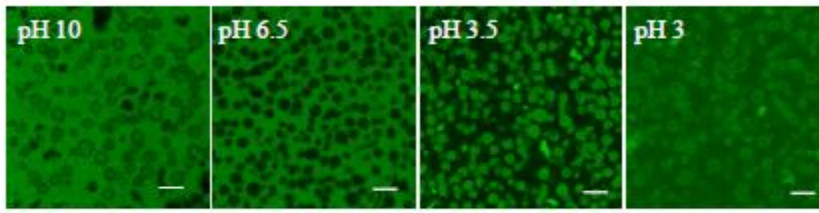


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14 Figure 7.

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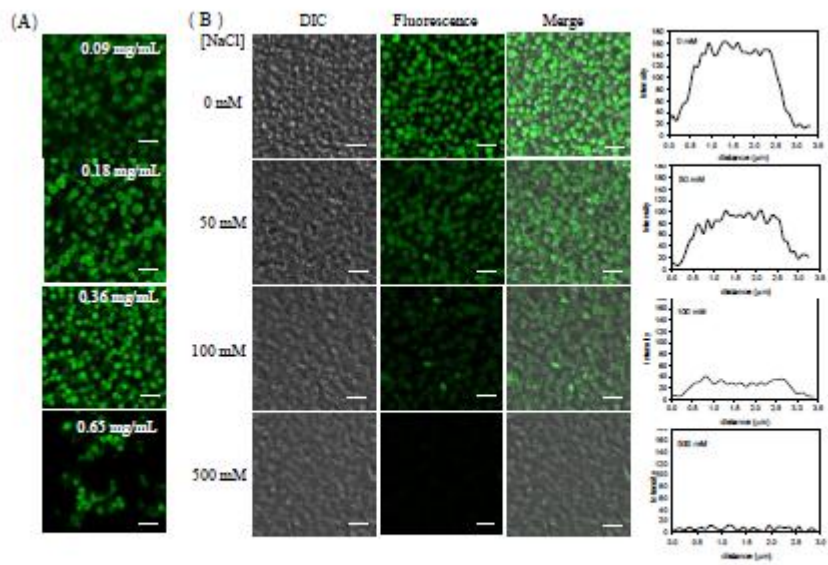


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16 **Figure 8.**

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18 **Figure 9.**

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