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1 **A study of the formation mechanism of multilayer emulsions by**
2 **isothermal titration calorimetry and dynamic light scattering**

3

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11 **Abstract**

12 The formation of multilayered interfaces around oil droplets in oil-in-water (O/W)
13 systems has great advantages in delivering lipid-soluble active compounds. Until now,
14 few thermodynamic analyses have been performed on the preparation process of
15 multi-layer emulsions. In this study, isothermal titration calorimetry (ITC) and
16 dynamic light scattering (DLS) were used to investigate the binding properties and
17 physical properties of low methoxyl pectin (LMP) and sodium caseinate (CAS) based
18 multilayer emulsions during the formation process. ITC titration confirmed LMP does
19 bind CAS-coated droplets through the electrostatic forces and it was a spontaneous
20 exothermic process ($\Delta G < 0$, $\Delta H < 0$). Furthermore, stable multilayer emulsions can
21 be only formulated by titrating the primary emulsion into LMP instead of
22 back-titrating. Finally, the effects of different mixing methods on the stability of
23 multilayer emulsions were evaluated. The DLS results suggest that titration rather
24 than mixing directly can lead to more uniform and smaller-sized droplets, which can
25 be attributed to the fact that slow titration can effectively avoid droplet aggregation.

26

27 **Keywords:** Multilayer emulsions; Isothermal titration calorimetry; Dynamic light
28 scattering; Electrostatic interactions; Bridging flocculation

29 1. Introduction

30 Oil in water (O/W) emulsion systems, produced by homogenizing oil and
31 aqueous phases together in the presence of one or more emulsifiers, have been
32 extensively applied in foods such as soft drinks, milk, mayonnaise, and vinaigrette,
33 etc. (Guzey & McClements, 2006). Along with the food safety concerns, there is a
34 growing interest trend in the food field to replace commercial synthetic emulsifiers
35 with natural “label-friendly” ones (Dammak et al., 2020). In this regard, several food
36 protein-based emulsifiers, from different sources like milk, whey, eggs, legume,
37 oilseed, and so on, have been extensively investigated as natural emulsifiers in recent
38 years due to their amphiphilic character, polymeric structure, and electrical charge
39 characteristics (Kim et al., 2020). Despite their favorable properties, protein-stabilized
40 emulsions have been reported to be highly sensitive to environmental stresses such as
41 pH, ionic strength, temperature, and oxygen (Bouyer et al., 2012). This instability of
42 protein-based emulsions under these conditions will limit their further application in
43 certain commercial products. In fact, the quality and stability of emulsions can be
44 improved by combining protein emulsifiers with other natural polymers to form
45 multilayered interfaces (Surh et al., 2006). For example, protein-coated droplets are
46 either negatively or positively charged when the pH is below or above their isoelectric
47 point (pI) (Wang et al., 2019), and then they could attract the oppositely charged
48 polysaccharides present in the continuous phase, thereby forming a thicker layer at
49 O/W interface. Zhang et al. (2020) developed multilayer emulsions by addition of
50 camellia saponin (CS) into almond protein isolate (API) based primary emulsions at
51 pH 3. Compared with primary emulsions, droplets with multi-layered interfaces have
52 the ability to significantly inhibit lipid and protein oxidation during storage. Compare
53 with emulsions with single-layer interfaces, emulsions with multilayered interfaces
54 were also found to have better stability against environmental stresses, such as pH
55 extremes, high ionic strengths, thermal processing, and freezing (Ogawa et al., 2003,
56 2004).

57 The basic mechanism for the preparation of multilayer interfaces is by adding
58 oppositely charged polymers to a "primary" emulsion prepared by conventional

59 homogenization techniques (Ogawa et al., 2003). As a result, a “secondary” emulsion
60 with a two-layer interface could be formulated due to electrostatic attraction. This
61 process can be repeated several times to produce three or more-layer emulsions with
62 desired functional properties. Previous studies (Guzey & McClements, 2006;
63 McClements, 2012) demonstrated that adsorption layers can be deposited around
64 droplets in two different ways: a one-step or two-step mixing procedure, depending
65 on the charge-pH properties of the used biopolymer. In the one-step mixing method, a
66 solution containing oppositely charged biopolymers is directly mixed with a primary
67 emulsion containing charged droplets. This approach tends to promote the tendency of
68 droplets to flocculate or coalesce during the process, and the aggregation is generally
69 irreversible (Guzey & McClements, 2006; Surh et al., 2006). In the two-step method,
70 the solution containing the charged biopolymer is mixed with the primary emulsion at
71 a pH where there is no strong electrostatic attraction between them. The pH of the
72 mixture is then adjusted to a pH where the droplets and the unadsorbed polymer have
73 opposite charges, so that the polymer can be adsorbed to the droplet surface. This
74 method is usually preferred for the production of multilayered interfaces at laboratory
75 scale, since the second layer of biopolymer is more uniformly distributed throughout
76 the aqueous phase prior to adsorption (Liu et al., 2022; Mundo et al., 2021; Shi et al.,
77 2021). Nonetheless, the two-step approach has several drawbacks: (i) Irreversible
78 aggregation occurs when the pH is adjusted to pass the pI of the protein emulsifier; (ii)
79 not suitable for the preparation of emulsions with more than two layers; (iii) relatively
80 complex process is not suitable for industrial mass production. For this reason, the
81 one-step approach was selected in our study and further optimized to avoid droplet
82 aggregation for preparing stable multilayer emulsions.

83 To minimize droplet aggregation in the one step method, it is necessary to ensure
84 that enough biopolymers for coatings is present in the system to surround the entire
85 surface of the added oil droplets. Otherwise, bridging flocculation may occur, as one
86 polyelectrolyte may adsorb to the surface of multiple droplets, causing them to link
87 together (McClements, 2012). Even with sufficient free polyelectrolyte concentration
88 in the aqueous phase, droplet aggregation cannot be completely avoided because

89 particle-particle collisions are sometimes faster than the adsorption of biopolymers to
90 the surface of the primary coating droplets. One strategy to avoid the droplet
91 aggregation during the process of forming multilayer emulsions is to control the ratio
92 of mixing by titrating primary emulsions into coating solutions (Zeeb et al., 2015).
93 Therefore, the concentration and mixing methods of biopolymers must be carefully
94 designed to avoid extensive droplet aggregation and massive free polyelectrolyte
95 remaining in the aqueous phase after the coating step.

96 The saturation concentration for a multilayer system has been mostly
97 characterized by dynamic light scattering (DLS), which provides the saturation
98 condition by identifying the ζ -potential plateau (Okubo & Suda, 2003). In this study,
99 we used isothermal titration calorimetry (ITC) and conventional DLS to investigate
100 the interaction and thermodynamic characteristics between sodium caseinate (CAS)
101 stabilized emulsion and low methoxyl pectin (LMP) during the preparation of
102 multilayer emulsions. In the first part, the thermodynamic parameters and surface
103 charge during the formation (titration and back-titration) of the multilayer emulsion
104 were evaluated to understand the stabilization mechanism of the multilayer emulsion
105 during the preparation. Meanwhile, the advantages of ITC and DLS in measuring the
106 system are described and compared. In the second part, the multilayer emulsions
107 prepared by titration and direct mixing methods were compared in terms of particle
108 size distribution, zeta potential (ζ -potential), creaming behavior, microstructure, and
109 fluorescence intensity. This research could provide more information for the rational
110 design of multilayer emulsions.

111

112 **2. Materials and methods**

113 **2.1. Materials**

114 The powder of CAS with 93.20% protein content (determined by the Kjeldahl
115 method, N=6.38) was purchased from Fisher Scientific (UK). The powder of LMP
116 (esterification degree from 22% to 28% and acetylation degree from 20% to 23%)
117 with 81.21% carbohydrate content was obtained from Cargill (Baupte, France). A
118 medium chain triacylglycerol oil (MCT) was purchased from Gattefossé (Saint-Priest,

119 France). Imidazole, acetic acid, sodium azide, sodium hydroxide, and hydrochloric
120 acid were purchased from Sigma-Aldrich Chimie (St Quentin Fallavier, France). The
121 water used in all preparations was deionized water.

122 **2.2. Sample preparation**

123 Imidazole-acetate buffer solutions (5 mM) containing 0.04 wt.% sodium azide (to
124 avoid microbial growth, (Chang et al., 2016)) was prepared by dispersing weighed
125 amounts of imidazole and acetic acid into deionized water. Biopolymer stock
126 solutions were prepared by slowly dispersing 0.53 wt.% CAS and 0.50 wt.% LMP
127 separately into 5 mM imidazole-acetate buffer and stirring overnight to ensure
128 complete hydration. The pH of both stock solutions was then adjusted to 3.0 by
129 adding HCl (1 M) 24 h prior their use.

130 **2.3. Preparation of primary emulsions and multilayer emulsions**

131 Oil-in-water coarse emulsions were prepared by mixing 5.0 g MCT oil and 95 g
132 emulsifier aqueous solution (0.53 wt.% CAS stock solution at pH 3.0) by high-speed
133 homogenization (IKA, Staufen, Germany) under 13,500 rpm for 5 minutes. The
134 resultant pre-emulsions was then passed through a microfluidizer (Microfluidics
135 LM20, Microfluidics Corp., Newton, MA, USA) 5 times at 5×10^7 Pa to obtain
136 nanoemulsions (Liao et al., 2021). The obtained nanoemulsions (containing 0.5 wt.%
137 CAS) were then adjusted to pH 3.0 and considered as “primary emulsions”.

138 Multilayer emulsions were prepared by titrating the primary emulsions into LMP
139 stock solution (0.5 wt.%) at a rate of 2.5 mL/min by using an automated procedure
140 with the TitroLine 5000 (TitroLine 5000, Xylem Analytics GmbH, Weilheim in
141 Oberbayern, Germany). During the titration, the samples were kept under agitation
142 and different ratios of primary emulsion/LMP were collected for subsequent analysis.
143 To investigate the effect of LMP on the formulation of multilayer emulsions when the
144 coating material was insufficient, back-titration was also performed on the same ratio
145 of CAS-based emulsion/LMP. Meanwhile, direct mixing was performed by slowly
146 pouring different volumes of primary emulsions in same ratios (w/w) into the LMP
147 stock solution under stirring to compare the effects of different mixing methods on the
148 multilayer emulsions.

149 **2.4. Isothermal titration calorimetry (ITC)**

150 The energetics of the interactions between primary emulsions and LMP was
151 measured using an isothermal titration calorimeter (VP-ITC) from MicroCal (Malvern
152 Instruments, Northampton, MA) in a reaction cell volume of 1.4214 mL. LMP and
153 CAS-based emulsions were diluted to the desired concentration (0.05 or 0.50 wt.%)
154 with the same buffer (5 mM imidazole-acetate buffer, pH 3.0). All solutions were
155 degassed under stirring for 5–10 min before being loaded. The pH of each solution
156 was checked again before measuring to ensure the same values in the cell and in the
157 syringe. The calorimetric cell was filled with LMP solution (0.5 wt.%) and the electric
158 injector was filled with CAS-based emulsion (0.05 wt.%), equilibrated at 25 °C. The
159 titration was performed by adding an initial 2 μ L injection and 28 consecutive 10 μ L
160 injections while continuously stirring the mixture at 307 rpm. Each injection lasted 20
161 s with a 200 s interval between consecutive injections. Back-titration was also
162 performed by placing the CAS-based emulsion (0.5 wt.%) into the cell and the LMP
163 solution (0.05 wt.%) into the syringe, separately. Controlled titrations were performed
164 by injecting the primary emulsion or LMP solution into imidazole-acetate buffer to
165 obtain the heat of dilution, so corrected data were obtained by subtracting the heat of
166 dilution data from the raw data. Data analysis was carried out by Origin 7.0 software.
167 The Gibbs free energy is calculated by the equation $\Delta G = \Delta H - T\Delta S$. Measurements
168 were performed in triplicate and results are reported as mean and standard deviation.

169 **2.5. Intrinsic fluorescence**

170 Intrinsic fluorescence intensity of multilayer emulsions with different ratios of
171 CAS-based emulsion/LMP was carried out at room temperature by a multimode
172 microplate reader equipped with a temperature-controlled 96-wells microplate
173 (Thermo Scientific™ Varioskan). The excitation wavelength was set at 280 nm and
174 the emission spectrum was recorded in the wavelength range of 298-550 nm with an
175 excitation and emission slit width of 5 nm. The multilayer emulsions formulated by
176 titration in different ratios was diluted with 5 mM imidazole acetate buffer (pH 3.0) to
177 a final concentration of 0.05 wt.% CAS. The diluted samples were then evenly
178 transferred to 96-well plates for analysis, with 6 measurements per sample. The

179 fluorescence intensity of acetate-imidazole buffer and pure LMP solution were used
180 as a blank and a control, separately.

181 **2.6. Zeta potential (ζ potential)**

182 The ζ potential was performed by electrophoretic mobility (Malvern Instruments,
183 Worcestershire, UK) under room temperature. To avoid multiple scattering effects,
184 each sample was diluted to a concentration of around 0.05 wt.% with the same buffer
185 (5 mM imidazole-acetate buffer, pH 3). Diluted samples were transferred to
186 disposable cells for electrophoretic mobility measurement. All measurements were
187 performed three times and the ζ -potential values were calculated by the software as
188 the average of three individual measurements.

189 **2.7. Mean particle size**

190 Mean particle size (nm), polydispersity index (PdI) and size distribution were
191 determined by dynamic light scattering using a Zetasizer Nano-ZS90 (Malvern
192 Instruments, Worcestershire, UK) under room temperature. To eliminate multiple
193 scattering effects, each sample was diluted to a droplet concentration around 0.1 wt.%
194 with corresponding pH buffers. After 90 s of equilibrium, each sample was analyzed
195 three times.

196 **2.8. Microscopic image**

197 Before analysis, each sample should be gently stirred in glass tubes to ensure they
198 were homogeneous. A drop of the emulsion was placed on a microscope slide and
199 covered with a coverslip. The microstructure of samples was then visualized at room
200 temperature using an Evos bright-field microscope (EVOS M5000,
201 Thermo-Invitrogen, USA).

202 **2.9. Statistical analysis**

203 All experiments were carried out at least three times with freshly prepared
204 samples. Data are reported as means \pm standard deviations.

205 **3. Results and discussions**

206 **3.1. Formation mechanism of multilayer emulsions**

207 **3.1.1. Zeta potential analysis**

208 The appropriate concentration of the biopolymer coating around the primary
209 emulsion can usually be assessed by measuring the zeta potential (Okubo & Suda,
210 2003). It has been reported that the ζ -potential of colloidal systems varies with the
211 continuous addition of adsorbable polymers, and the plateau of the ζ -potential as
212 function of ratio is related to the surface saturation of the polymer coating (Chang et
213 al., 2016).

214 Fig. 1 showed the ζ -potential of multilayer emulsions prepared by titration and
215 back-titration as function of CAS/LMP or LMP/CAS ratio. The ζ -potential of pure
216 LMP solution (Fig.1A) at pH 3.0 is negative (-17.0 mV) which is attributed to the
217 ionization of its carboxyl groups. When the ratio of CAS-based emulsion/LMP is
218 increased, the ζ -potential initially remain stable until the ratio reached 0.5, then
219 became increasingly less negative, with a point of zero charge in the ratio range
220 around 1.25 - 1.50, and finally reached a plateau for higher ratio (3.0) (ζ -potential
221 close to that of primary emulsion). This is the fact that the primary emulsion exhibited
222 positive charge at pH 3.0 because the protonation of the basic side chain (NH_3^+) of the
223 casein molecule. As a result, LMP molecules can be adsorbed on the droplet surface
224 by electrostatic force, and then the overall negative charge of the system gradually
225 increases until there are not enough LMPs in the system to cover the newly dropped
226 droplet. As shown in Fig. 1B, the ζ -potential of the primary emulsion is positive
227 (+32.6 mV) and this value could be considered enough to ensure high intermolecular
228 repulsions (Lieberman et al., 2020). When the LMP concentration was increased, the
229 ζ -potential initially remains stable until the ratio reaches 0.3, then becomes
230 increasingly less positive, with a point of zero charge in the ratio range around 0.6 -
231 0.8, and finally reached a plateau at a ratio of 1.5 (Fig.1B). According to previous
232 reports (Chang et al., 2016; Liu et al., 2019), the plateau of ζ -potential indicates that
233 the lipid droplet surface is saturated with coating polysaccharides at this level. It
234 should be mentioned that during the experiment, the titration of the primary emulsion

235 into the LMP solution resulted in a stable multilayer emulsion, while titration in the
236 opposite direction (LMP solution into the primary emulsion) showed obvious flocs
237 and these flocs did not disappear with the continuous addition of LMP (Data not
238 shown). Based on our results, the forward titration reached a plateau at a ratio 3.0 (Fig.
239 1A), which corresponds exactly to a ratio of 0.3 (Fig. 1B) for titration of the primary
240 emulsion into the LMP solution. After the ratio 0.3, the absolute value of the zeta
241 potential begins to decrease, indicating that the droplet saturation potentials
242 corresponding to the titrated and back-titrated samples are consistent. Therefore, this
243 finding suggests that the zeta potential can represent the overall distribution of
244 particles in solution but cannot reflect whether the multilayer emulsion formulation is
245 stable during preparation (the occurrence of particle aggregation).

246 Meanwhile, to find the exact concentration of coating polymer at which the
247 multilayer emulsions reach the plateau, a large number of samples in different ratios
248 should be prepared to measure for ζ -potential measurements, which are considered
249 very complex and time-consuming processes. In this regard, we employed ITC to
250 measure thermodynamic parameters to gain a deeper understanding of the
251 stoichiometry of layer-by-layer interactions and to analyze the energetics of
252 multilayered interfaces processes.

253 3.1.2. ITC analysis

254 ITC have been widely used to investigate molecular interactions in biological
255 systems (Jafari et al., 2007; Wang et al., 2019), but there are few reports on the
256 interaction between droplets and molecules (Fotticchia et al., 2014). Hence, ITC was
257 used to obtain more information about the evolution of deposition around the primary
258 emulsion, such as the equilibrium adsorption constant, enthalpy of adsorption, and
259 adsorption stoichiometry. CAS-based emulsions and LMP solutions at pH 3.0 were
260 chosen to perform ITC tests at 25 °C because CAS-coated droplets showed positive
261 charge and LMP molecules showed negative charges at pH 3.0 (Wang et al., 2019). In
262 this study, heat flow versus time curves (the upper of Fig. 2) were obtained by
263 gradually injecting 0.5 wt.% primary emulsion or 0.5 wt.% LMP solution into a
264 reaction cell containing 0.05 g/L LMP solution or 0.05 wt.% primary emulsion at

265 25 °C. The enthalpy changes (the lower of [Fig. 2](#)) as function of mass ratio were
266 obtained by the software. The mass unit was used to replace the molecular unit in this
267 study, because both CAS and LMP are high molecular weight polymers.

268 The ITC measurements ([Fig. 2A](#)) showed that the titrating process was exothermic
269 with a stepwise change in the exothermic value for each injection. When the ratio of
270 primary emulsion/LMP is lower than 1.0, the values of heat released per injection are
271 very close, which indicates that the injected CAS-coated droplets have a more stable
272 interaction with the LMP molecules in the reaction cell at this stage. This phase also
273 corresponds to that when the absolute value potential of zeta ([Fig. 1A](#)) gradually
274 approaching the "0" point from larger values (-17.0 mV to -8.42 mV), implying that
275 multiple emulsions may go from stable to relatively unstable from this point (Ratio
276 1.0). This was probably attributed to the fact that, at the initial stage, excess negative
277 LMP molecules in the cell can quickly adsorb on the surface of the positively charged
278 emulsion droplets due to electrostatic interactions. Our previous study ([Wang et al.,
279 2019](#)) reported the interaction between CAS and LMP when used at the same contents
280 in this study. Interestingly, the heat released by the interaction between CAS and
281 LMP decreases with regularity from the beginning. This difference may stem from
282 differences in binding between molecules and between molecules and droplets. The
283 CAS-encapsulated droplets prepared in this study have nanoscale diameters with an
284 average particle size of about 300 nm, so the CAS molecules adsorbed on the surface
285 of the oil droplets have a more ordered structure in the system than free casein
286 molecules in the aqueous phase. As a result, each casein-coated droplet entering the
287 cell has enough LMP molecules to cover the droplet surface through electrostatic
288 attraction rather than disordered droplet aggregation, thereby releasing a relatively
289 stable heat per injection at the initial stage. For ITC measurements between CAS and
290 LMP solutions, complexes were formed during each injection and these complexes
291 were confirmed to be insoluble at pH 3.0 ([Wang et al., 2019](#)). Therefore, it can be
292 hypothesized that the casein molecules may rapidly form disordered network fibers
293 after entering the reaction cell, and the degree of network structure is more extensive,
294 and the heat released is higher. When the ratio was above 1.0, the heat released per

295 injection stated to decrease regularly (Fig. 2A). This may be attributed to the gradual
296 depletion of LMP chains in the reaction cell. Above the ratio of 1.4, the enthalpy
297 becomes slightly positive and remains stable, indicating that none of the LMP
298 molecules in the cell can interact with the newly added droplet. However, the result of
299 zeta potential (Fig.1 A) showed that the plateau was obtained when the ratio of
300 CAS-based emulsion/LMP was around 3.0, which was not consist with ITC results. It
301 was reported that the ζ -potential is highly sensitive to the environmental conditions,
302 and slight differences in the ion concentration can affect measurements (Fotticchia et
303 al., 2014). For instance, sample should be diluted before measurements the ζ -potential
304 and the dilution may also influence the authenticity of the system. Fotticchia et al.
305 (2014) showed similar results by titration chitosan or poly-l-lysine into egg lecithin
306 based emulsions and they demonstrated that ITC could provide more accurate
307 information than DLS.

308 Fig. 2A showed that the enthalpy change (ΔH) in the titration was -0.534.40
309 kcal/mg. The Gibbs free energy (ΔG) was also negative (-8.96 kcal/mg) by fitting the
310 curve of " one set of sites ", indicating that the interaction between the primary
311 emulsion and LMP was a spontaneous reaction. Similar exothermic sequences were
312 also found in ovalbumin/chitosan (Xiong et al., 2016) and soy proteins/gum Arabic
313 (Dong et al., 2015). This result supports the generally accepted view that adsorption is
314 primarily driven by electrostatic interactions (Burgos-Díaz et al., 2016; Fotticchia et
315 al., 2014). Indeed, the interaction in the preparation of the multilayer emulsion is a
316 more complex interaction than just an electrostatic mechanism. It may also include
317 hydrophobic interactions, van-der-Waals forces, and hydrogen bonding. However, we
318 cannot say whether these forces are present, because ITC measures the sum of all
319 events that occur in the reaction cell. Furthermore, comparing the ΔG value (-8.13
320 kcal/mg) obtained from the interaction between CAS and LMP (Wang et al., 2019), it
321 was found that the absolute value of ΔG is higher in this study. This could be
322 attributed to the fact that the adsorbed casein (+32.6 mV) exhibited stronger surface
323 charge than free casein molecules (+20 mV) in the continuous phase at pH 3.0 (Wang
324 et al., 2018).

325 ITC measurements by titrating LMP solution into primary emulsions were also
326 performed to understand the heat flow (Fig. 2B) in the presence of insufficient
327 polysaccharides. Instead of decreasing regularly, the heat released of each injection
328 increased in the initial phase and the maximum heating was obtained at ratio 0.3.
329 Several possibilities may explain this behavior: (i) insoluble flocs were clearly
330 observed by titration of LMP into the primary emulsion at the initial stage, and these
331 flocs did not disappear with the continuous addition of LMP (Data not shown). This
332 can be attributed to the bridging flocculation caused by negatively charged pectin
333 molecules and positively charged droplets. The bridging flocculation process may be
334 accompanied by more complex reactions than electrostatic adsorption, such as some
335 endothermic effects (unfavorable conformational changes, hydrophobic interactions,
336 etc. (Bou-Abdallah & Terpstra, 2012)) and, (ii) when the pH of stock solution was
337 adjusted to 3.0 by HCl, some ions remained in the solution may inhibit binding
338 interactions (Xiong et al., 2017) due to the formation of an ionic screen that prevents
339 the interaction between the two biopolymers. Interestingly, the ratio 0.3 also
340 corresponds to the starting decrease of ζ -potential (Fig. 1B), suggesting that the
341 overall droplet surface charge in this system changed. After this ratio, the heat
342 released after each injection started to decrease regularly and reaches the "0" point at
343 ratio 0.7. However, Fig. 1B showed that the ζ -potential reached a plateau until the
344 ratio 1.5, while ITC data indicated that there was no obvious heat changed at ratios
345 higher than 0.7. This finding suggested that the continued decreased in potential
346 caused by the addition of LMP is probably not accompanied by a significant thermal
347 change.

348 **3.1.3. Schematic representation of the formation of multilayer emulsions by** 349 **titration**

350 Based on the above results, a schematic diagram was established to summarize the
351 main events occurring as function of the ratio in the titration and back-titration
352 systems (Fig. 3). For the primary emulsion titration by LMP solution, stable
353 multilayer emulsions are successfully formed at the initial stage, which were stable to
354 droplet flocculation. This is because each injection contains enough LMP chains to

355 completely cover oil droplets and gives them a negative surface charge, thereby
356 avoiding droplet aggregation due to electrostatic repulsion. Theoretically, as the LMP
357 in the system is gradually depleted, the injected droplets will interact with other
358 LMP-coated droplets, causing large droplets to aggregate. In general, bridging
359 flocculation tends to occur earlier (before the biopolymer in the continuous phase is
360 completely depleted), which are mainly depending on the mixing methods (mixing
361 [\(Liu et al., 2019\)](#) or titration [\(Zeeb et al., 2015\)](#)) and biopolymer properties (e.g.,
362 charge density, molecular weight, and conformation [\(Gharsallaoui et al., 2010\)](#)). For
363 back-titration, bridging flocculation occurs at the initial stage, as the injected
364 negatively charged LMP molecules will adsorb to the surface of multiple droplets and
365 link them together. Although negatively charged pectin continued to be added to the
366 system, making the surface of the entire system negatively charged ([Fig. 1B](#)), the
367 flocs that had produced did not disappear. This finding is consistent with some other
368 reports [\(Liu et al., 2019; Surh et al., 2006\)](#). Clusters of droplets held together by
369 protein bridges is generally not reversible, which can be disrupted only by strong
370 shear forces (e.g. a second-stage of homogenization) [\(Dickinson, 2019\)](#). For instance,
371 [F. Liu et al \(2016\)](#) mixed primary emulsions and lactoferrin and then used a blender
372 and high pressure homogenizer to redisperse droplets to obtain stable multilayer
373 emulsions. However, [Fotticchia et al. \(2014\)](#) indicated that stable multilayer
374 emulsions can be prepared by titrating chitosan or poly-L-lysine into the primary
375 emulsion, although bridging flocculation occurs at the initial stage of titration. [Tang et](#)
376 [al. \(2021\)](#) successfully prepared multilayer emulsions by direct mixing relatively high
377 content of sodium alginate with chitosan based picking emulsions. These differences
378 may originate from the properties of different biopolymers (e.g., charge density,
379 molecular weight and conformation), the primary layer thickness of emulsion droplets,
380 as well as solution conditions (e.g., pH, ionic strength, temperature, and stirring)
381 [\(Burgos-Díaz et al., 2016\)](#).

382 **3.1.4. Analysis of fluorescence intensity**

383 Several studies have reported [\(Acharya et al., 2013; Qiu et al., 2015\)](#) that CAS or
384 CAS-based droplets exhibited an obvious emission band around 330 nm when the

385 extraction band was 280 nm due to the presence of tryptophan. For this reason,
386 measurements of tryptophan fluorescence spectroscopy during the formation of
387 CAS-LMP based multilayer emulsion were used to study CAS conformational
388 changes, owing to the fact that the intrinsic fluorescence of tryptophan residues is
389 highly sensitive to the polarity of microenvironments (Li et al., 2021).

390 Fig. 4A shows the fluorescence spectra of multilayer emulsions prepared by
391 titrating the primary emulsion into LMP solution with different ratios and all samples
392 maintained the same CAS content. All samples emulsion showed strong fluorescence
393 emission upon excitation at 280 nm, with a peak near 330 nm. Interestingly, when the
394 ratio ≤ 0.6 , there was no obvious shift in the maximum emission wavelength of
395 LMP-CAS based emulsion; however, when the ratio was above 0.6, the wavelength
396 corresponding to the maximum showed a slight red shift compared to the primary
397 emulsion. It has been reported that the red or blue shift in the emission band of CAS
398 was usually related to changes in the tryptophan environment (Li et al., 2019).
399 Therefore, no shift was observed at ratio ≤ 0.6 which suggest that binding of LMP and
400 CAS-coated droplets in this stage did not change the tryptophan environment.

401 The quenching of the fluorescence intensity has been reported as an indicator of
402 the interaction of the components in the solution (Wang et al., 2015). Therefore, the
403 adsorption force of LMP to the surface droplets of the primary emulsion can be
404 deduced from the test results. In this regard, the maximum fluorescence intensities of
405 these emulsions prepared by titration as a function of ratio are plotted in Fig. 4B. In
406 the ratio range of 0 to 0.6, titration-prepared samples showed similar maximum
407 fluorescence intensities; however, when the ratio is greater than 0.6, the maximum
408 intensity of samples decreases obviously with the increase of the ratio until it reaches
409 1.75. The decrease in entropy intensity indicates that some of the tryptophan residues
410 of CAS adsorbed on the oil droplets were transferred from the polar surface to the
411 hydrophobic interior, which may be due to the interaction of tryptophan located in the
412 hydrophobic region of the protein with pectin through hydrophobic bonds. The
413 decrease in intensity suggests that some of the tryptophan residues of CAS adsorbed
414 on the oil droplet are transferred from the polar surface to the hydrophobic interior,

415 which was probably attributed to the droplet aggregation. Similar behavior was
416 reported for aggregation of soybean protein isolate/pectin complexes (Li et al., 2021).
417 This result also indicates that formation mechanism of multilayer emulsions by LMP
418 and CAS coated droplet is not only electrostatic but also accompanied by a certain
419 degree of hydrophobic interactions (especially when the droplets are extensively
420 aggregated), which was supported by the data of ITC results. At the same time, the
421 maximum fluorescence intensity of these samples increased as the ratio increased
422 from 1.75 to 2.5. This can be attributed to the presence of some individual
423 CAS-coated droplets in the system due to the relatively high CAS-based
424 emulsion/LMP ratio.

425 **3.2. Effect of titration or mixing on the properties of multilayer emulsions:** 426 **analysis of droplet size and flocculation**

427 It should be noted that many papers demonstrated the successful preparation of
428 multilayer emulsions by adding primary emulsions to other coating materials
429 (Burgos-Díaz et al., 2016; Liu et al., 2019; Shi et al., 2021). However, there are no
430 details on how to define "add". In fact, there are many ways of addition, such as
431 slow/quick addition, titration or direct pouring, and these different ways of addition
432 seem to have a significant effect on droplet aggregation in the preparation of
433 multilayer emulsions. In this section, the direct mixing and titration under stirring
434 were used to prepare multilayer emulsions in different ratios of CAS-based
435 emulsion/LMP. Then, dynamic light scattering (DLS) (Fig. 5A-D), creaming index
436 (Fig. 6A), and optical microscopy (Fig.6 B) will be presented to evaluate the surface
437 charge, mean particle size and droplet aggregation of these emulsions.

438 Fig.5A exhibited the zeta potential of emulsions in both preparation methods.
439 Interestingly, the multilayer emulsions in both methods showed similar trends in
440 surface charge as a function of ratio, although distinct flocs were observed earlier in
441 the emulsions prepared by direct mixing. This might be the limitation of
442 microelectrophoresis mobility method: samples need to be highly diluted and this
443 process may have changed the surface charge of the system (Hu et al., 2017;
444 Salvia-Trujillo et al., 2013). For example, droplet aggregation in highly concentrated

445 emulsions may be partially redispersed to some extent after dilution and agitation.
446 Primary emulsions exhibited nanoscale diameter (~300 nm, [Fig. 5B](#)) with relatively
447 lower polydispersity index (PdI) values ([Fig. 5C](#)). As the ratio increases, all emulsions
448 showed increased droplet size and PdI values, which can be attributed to the fact that
449 negatively charged LMP adsorbed on the surface of CAS-coated droplets or droplet
450 aggregates. A similar result was found in the study of chitosan/pectin-stabilized
451 multilayer emulsions ([Liu et al., 2019](#)). It is worth noting that the particle size and PdI
452 values of samples prepared by mixing method are higher than those prepared by
453 titration one. Until the ratio reached 0.8, the titration-prepared multilayer emulsions
454 still exhibited nanoscale average droplet diameter (~600 nm) based on DLS analysis;
455 however, when the ratio was higher than 0.5, the size of sample prepared by mixing
456 was outside the measurement range of DLS. This was probably attributed the fact that
457 directly mixing provides more droplets in the same time than titration, and
458 consequently droplet–droplet collisions may occur more rapidly than polyelectrolyte
459 molecules adsorb to the droplet surfaces, thereby caused larger droplets ([Guzey &](#)
460 [McClements, 2006](#)). However, during the titration process, a small number of droplets
461 added each time can be quickly encapsulated by the polyelectrolyte at a lower
462 concentration ratio. As a result, once the surface charge reached a certain value, there
463 will be a strong electrostatic repulsion between the surface and similarly charged
464 polyelectrolytes in the aqueous phase, which limits further adsorption of the
465 polyelectrolyte, thereby reducing the probability of droplet-droplet collision and
466 retarding the occurrence of particle aggregation. This result was also confirmed by the
467 droplet size distribution. The titration-prepared samples did not show distinct peaks in
468 the larger size range until ratio reached 0.5, while even with a ratio of only 0.1 ([Fig.](#)
469 [5D](#)), distinct peaks in the larger size range were detected in the sample formed by
470 mixing ([Fig. 5E](#)). This result proved that droplet flocculation is readily to occur in the
471 mixing directly methods, indicating that different adding methods have a significant
472 effect on the droplet flocculation during the formulation of multilayer emulsions.

473 [Fig. 6 A](#) showed the visual appearance of multilayer emulsions prepared by
474 directly mixing and titration methods. As expected, those prepared by mixing showed

475 a cream layer when the ratio increased. Even at a ratio of 0.1, a phase separation can
476 be observed where an opaque cream layer at the top and a strongly turbid
477 droplet-depleted layer at the bottom, which was consistent with result of particle size
478 (Fig. 5E). This behavior can be attributed to bridging flocculation promotion leading
479 to an increase in particle size, which facilitates gravitational separation (Grundy et al.,
480 2018). However, when the ratio was increased to 0.5, there was a slight creaming at
481 the bottom of the emulsion prepared by mixing, which may be due to an increase in
482 viscosity (polymer entanglement or viscoelastic network formation) (Grundy et al.,
483 2018). As a result, the increased viscosity restricts the upward movement of the
484 aggregated droplets. Notably, the multilayer emulsion prepared by titration showed
485 much better stable creaming stability than that prepared by mixing, since no obvious
486 phase separation occurred until the ratio reached 1.0. This could be attributed to
487 relatively low PDI value (Fig. 5C) in these emulsions.

488 For further analysis, the microstructure of the primary emulsion (control) and
489 several selected multilayer emulsions prepared by titration in different ratios (0.5:1;
490 0.8:1; 1:1; 1.5:1; 2:1) were observed by optic microscopy. These samples represent
491 different states of droplets during the preparation of multilayer emulsions. Compared
492 to the primary emulsion, the multilayer emulsion also exhibits a very homogeneous
493 microstructure in the ratio ≤ 0.5 , which supports the results of the size measurements.
494 As the ratio increased, droplet aggregation was clearly observed at higher chitosan
495 concentrations, especially when the ratio was higher than 1.0. This is because there
496 are not enough LMP molecules in the system to coat the droplets, and then two or
497 more droplets share a polysaccharide chain to form aggregates. Remarkably, both
498 extensive droplet aggregation and significantly increased droplet size (Fig. 5B)
499 occurred around the ratio 1.0, which corresponds to the ratio where the heat released
500 by titration (Fig. 2A) began to decrease. These findings suggest that ITC could
501 provide accurate information on when flocculation occurs, leading to optimal
502 conditions for the preparation of O/W multilayer emulsions.

503 **4. Conclusion**

504 This study tried to explain the mechanism of the formation of multilayer

505 emulsions by titrating primary emulsions into coating materials. According to the
506 obtained results, ITC measurements by titration of the primary emulsion into the
507 coating material allow the acquisition of thermodynamic characteristics of the studied
508 system, which are fast, accurate and informative compared to the information
509 obtained from zeta potential measurements. ITC titration confirmed that LMP does
510 bind CAS-coated droplets through the electrostatic forces and it was a spontaneous
511 exothermic process ($\Delta G < 0$, $\Delta H < 0$). The binding is also accompanied by
512 hydrophobic forces. Titration of the primary emulsion into the LMP solution resulted
513 in a stable multilayer emulsion, while titration in the opposite direction showed
514 obvious flocs due to bridging flocculation. Meanwhile, more uniform and
515 smaller-sized droplets can be prepared by titrating the primary emulsion into LMP
516 solution rather than mixing them directly, which can be attributed to the fact that slow
517 addition can effectively avoid the aggregation of droplets. Based on emulsion stability
518 and microscopy observations, ITC appears to have the ability to define when
519 flocculation begins, thus providing optimal conditions for the preparation of O/W
520 multilayer emulsions. In this way, the remaining coating materials in the continuous
521 phase of the prepared double-layer emulsion can be reduced, so as to further prepare
522 emulsions with three or four interfaces..., thus providing more possibilities for O/W
523 food emulsion products.

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526 **CONFLICTS OF INTEREST**

527 The author declares that there is no conflict of interest that could be perceived as
528 prejudicing the impartiality of the research reported.

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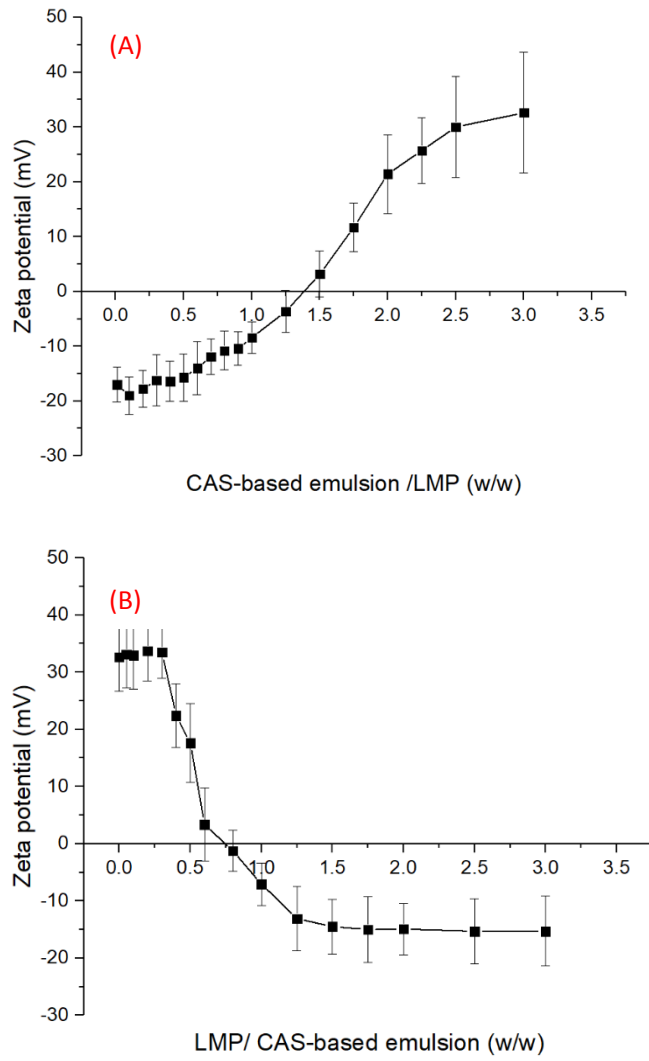
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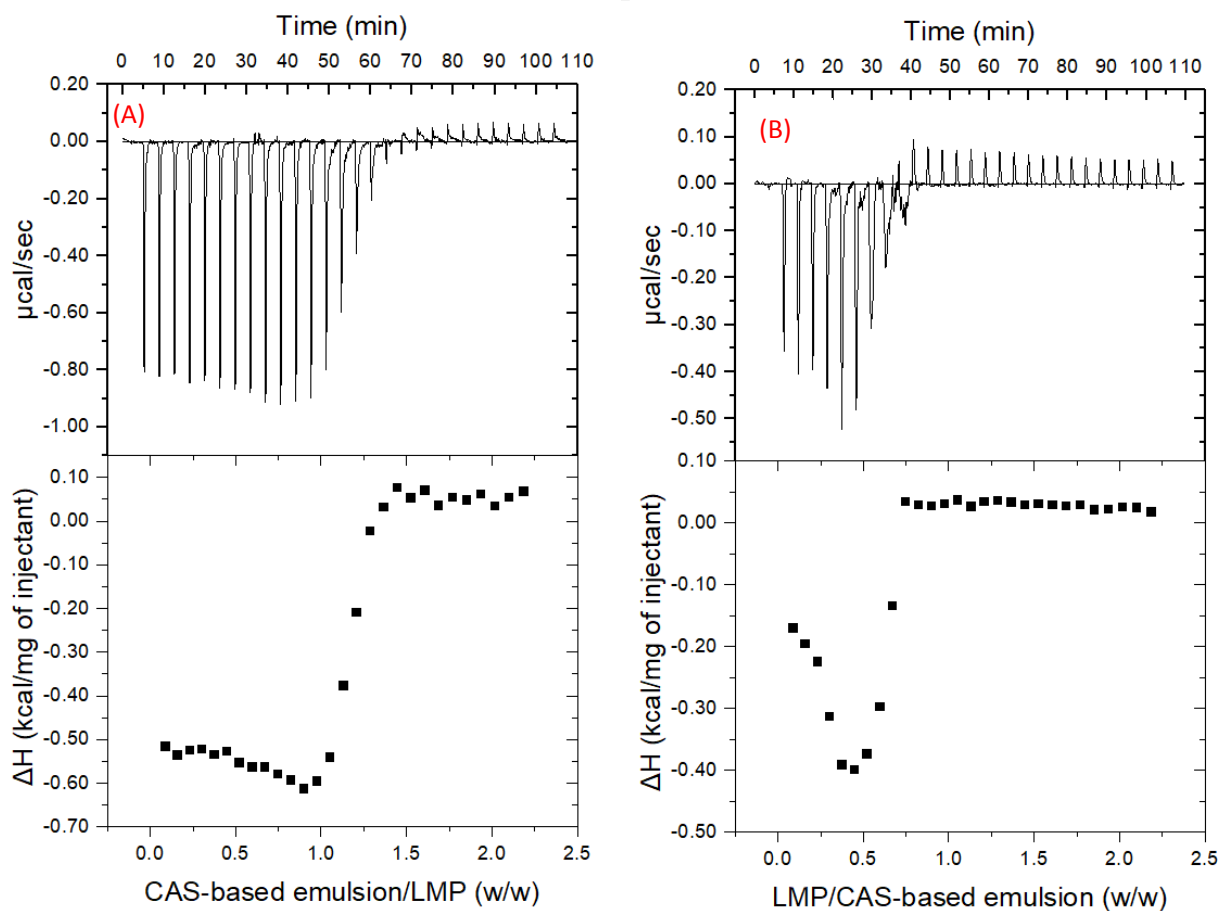
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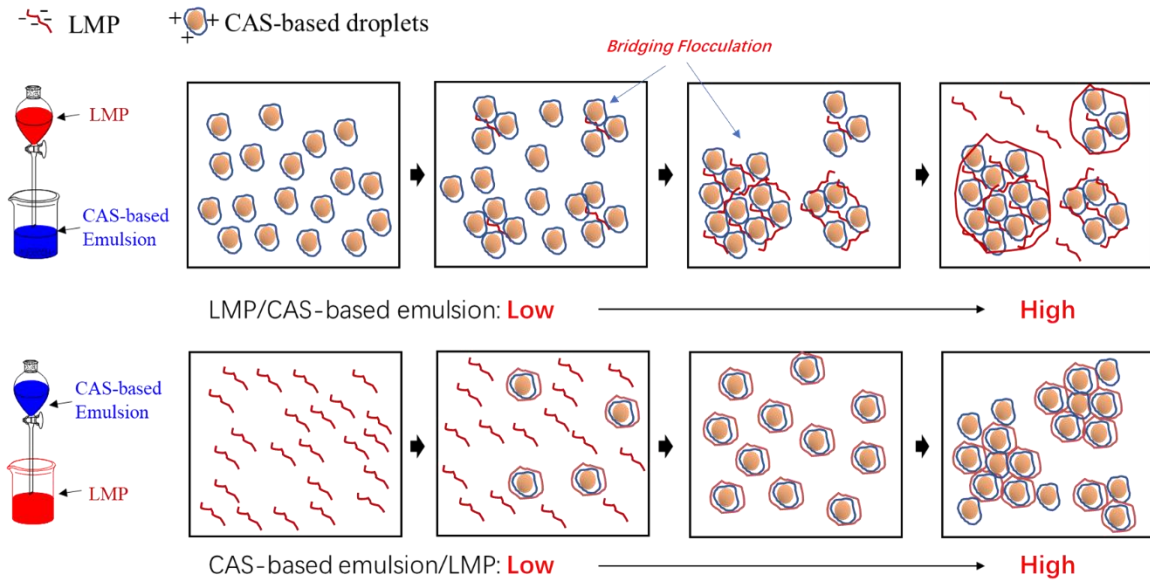
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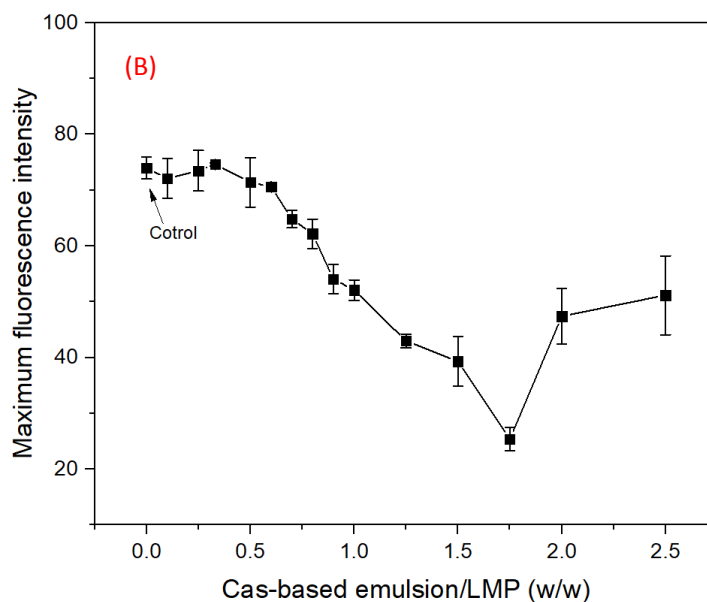
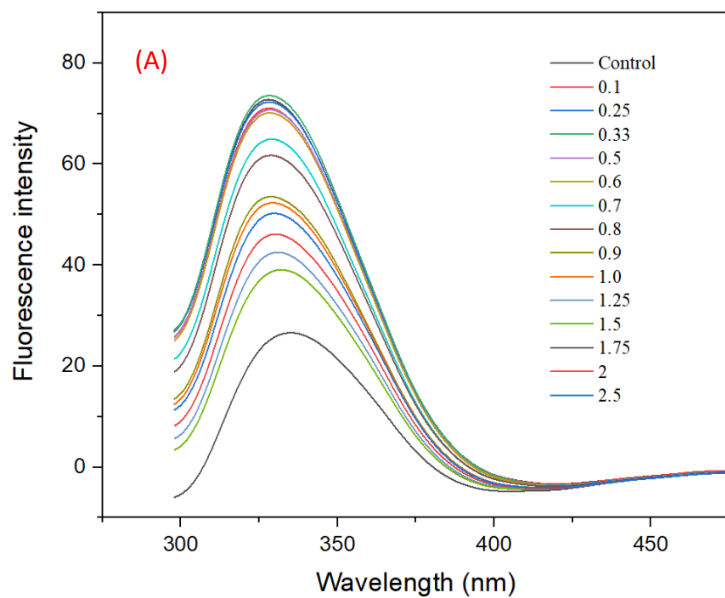
664 **Fig. 1.** ζ -potential of multilayer emulsions at pH 3.0 as a function of ratio: (A) titrating the
 665 primary emulsion into LMP solution or (B) titrating LMP solution into the primary emulsion.
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668 **Fig. 2.** ITC titration graphs (Upper) and enthalpy change (Lower) of (A) CAS-based
 669 emulsion/LMP and (B) LMP/CAS-based emulsion at pH 3.0. The calorimetric cell and the syringe
 670 were filled with 0.05 wt.% CAS-based emulsion and 0.5 wt.% LMP solution or 0.05 wt.% LMP
 671 and 0.5 wt.% CAS-based emulsion.
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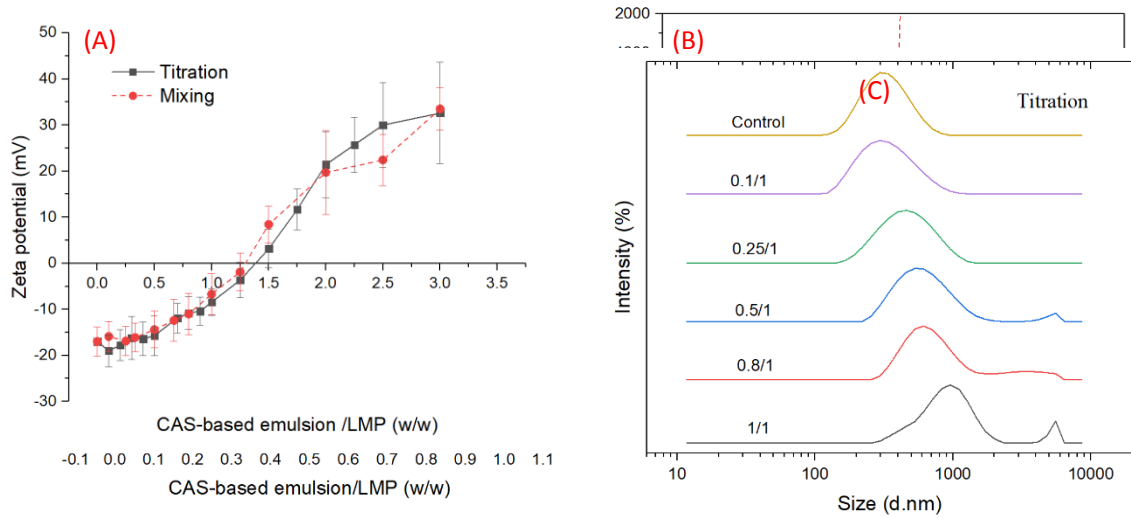


673 **Fig. 3.** Schematic representation of the formation of multilayer emulsions by titration based on
 674 CAS-coated emulsion and LMP at pH 3.0.
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676 **Fig. 4.** (A) Fluorescence spectra and (B) maximum intensity of multilayer emulsions as a function
 677 of ratio by titrating the primary emulsion into LMP solution. The excitation wavelength was set to
 678 280 nm and the emission slit width was 5 nm.
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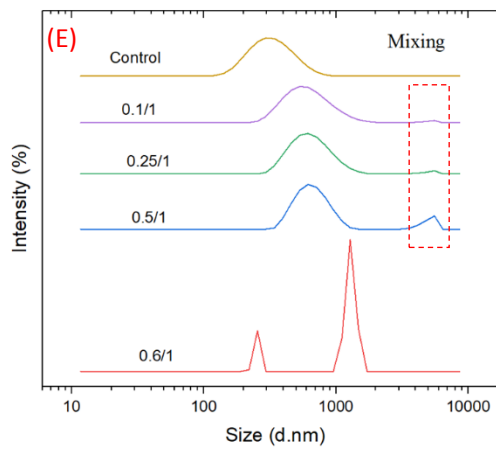
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706 **Fig. 5.** (A) ζ -potential, (B) Mean particle size, and (C) Polydispersity index as a function of ratio

707 by titrating or mixing directly based on CAS-based emulsion and LMP; The particle size

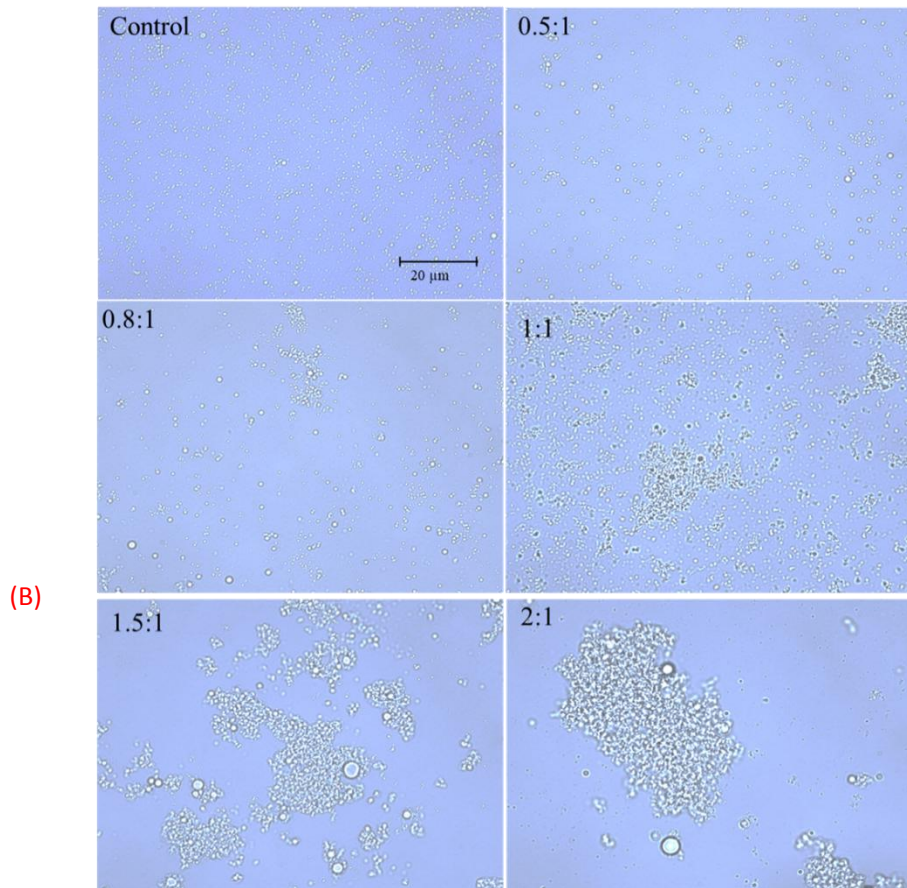
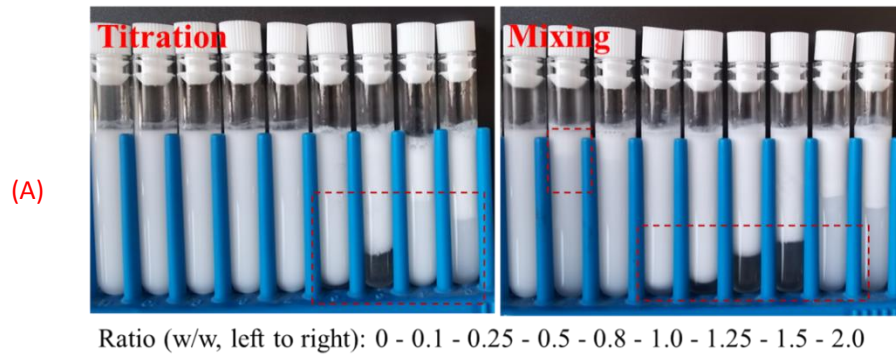
708 distributions as function of ratio prepared by (D) titration or (E) mixing.

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713 **Fig. 6.** (A) Visual appearance of multilayer emulsions prepared by titration or mixing as function
 714 of ratio and (B) Photomicrographs of multilayer emulsions prepared by titration. The scale bar is
 715 20 μm in length.

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