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1 **Complex coacervation of natural sophorolipid bolaamphiphile**
2 **micelles with cationic polyelectrolytes**

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19 **Abstract**

20 Complex coacervation of polyelectrolyte with surfactant micelles is a promising system for a
21 wide range of applications. However, the development of “green coacervates”, from bio-
22 based surfactant and biopolymers has not been explored, yet. Herein, complex coacervation of
23 natural micelles from a bolaform sophorolipid biosurfactant with oppositely charged cationic
24 polyelectrolytes (i.e., chitosan oligosaccharide lactate, poly (L-lysine) and poly(allylamine))
25 was investigated. Turbidity titration, light and scanning electron microscopy (SEM), dynamic
26 light scattering (DLS), cryogenic transmission electron microscopy (cryo-TEM) and Small
27 Angle X-ray Scattering (SAXS) were used to monitor the evolution of complex structures as
28 function of pH and polyelectrolyte concentration. Phase boundaries of the biosurfactant-
29 polyelectrolyte systems were obtained and revealed the feasibility of coacervation in water
30 over a broad pH range, from pH 5 to pH 9. The state of complexation was found to depend
31 primarily on pH and concentration and the used polyelectrolyte. Light microscopy and SEM
32 demonstrated the associative macrophase separation and cryo-TEM highlighted the influence
33 of the desolvation level on the coacervates arrangement where two main structures were
34 formed as function of the coacervation stage namely spherical particles and aggregates. The
35 SAXS data demonstrated that the sophorolipid micelles maintained their structure integrity
36 following their binding to the cationic polyelectrolyte.

37 **Keywords:** *Bolaform; Sophorolipid; Complex coacervation; Polyelectrolyte, Cryo-TEM.*

38

39 **1. Introduction**

40 Glycolipids, composed of a carbohydrate-based hydrophilic head covalently linked to a fatty
41 acid or a fatty alcohol,¹ are an interesting alternative to conventional surfactants because of
42 their biobased and renewable origin, good biocompatibility and biodegradability.²⁻⁴ Among
43 them, microbial glycolipids like sophorolipids are an attractive class of molecules which are
44 obtained from exclusively renewable agro-resources (rapeseed oil, oleic acid, carbohydrates)
45 through a fermentation process of the yeast *Starmerella bombicola* with remarkable
46 production rates (upper to 300 g.L⁻¹)⁵ and reduced environmental impact biosynthesis.^{6,7}
47 These molecules possess several potential applications⁸ in cosmetic⁹ and anticancer,¹⁰ but also
48 like a structuring agent for self-assembled nanomaterials,¹¹ or as antimicrobial agents.¹²
49 Nevertheless, the development of novel applications involving sophorolipids in particular, and
50 microbial glycolipids in general, may undergo through the investigation of their binding to
51 further colloidal species or macromolecules, as shown by Dubey *et al.*, who reported that the
52 gelation kinetic of silk fibroin can be triggered by sophorolipids¹³ or by Madsen *et al.*, who

53 demonstrated that the thermal stability of a lipase from *Thermomyces lanuginosus* can be
54 enhanced following binding to rhamnolipids and sophorolipids.¹⁴ In this context, the phase
55 behavior of polymer-surfactant mixtures is of a great importance for both scientific and
56 industrial fields since they are frequently used in various formulations for foods, detergents
57 and cosmetics.¹⁵ Their molecular association could lead to the formation of a wide range of
58 structures like gels, micelle-decorated network, aggregates, complexes and precipitates.^{15,16}
59 By controlling the interaction between surfactants and polymers, it is also possible to induce
60 complex coacervation, a process during which a homogeneous macromolecular aqueous
61 solution undergoes an associative liquid-liquid phase separation.¹⁷

62 Coacervation is considered as an eco-friendly process as it usually takes place in water
63 and at relatively mild conditions of pH and temperature. In addition, it is also a cost-effective
64 technique since neither a special device nor extensive production steps are required. The
65 obtained structures induced by demixing are considered among the more intriguing systems in
66 colloid chemistry.¹⁸⁻²⁰ Their exotic character attracted scientists even beyond the field of
67 colloidal chemistry like Oparin, a Russian biologist, who proposed that coacervates could be
68 the origin of life on earth²¹ and some recent studies are heading towards the same direction.²²⁻
69 ^{24,25,26} This process was initially reported by Tiebackx in 1911 without using the word,²⁷ he
70 found that the addition of an acid to a mixed solution of Arabic gum and gelatin results in
71 phase separation. It was almost two decades later that the term “coacervation” was coined by
72 Bungenberg de Jong and Kruyt who studied the phase behavior of several binary mixtures by
73 optical microscopy.¹⁷

74 Typically, this phenomenon can be divided into “simple” and “complex”. Simple
75 coacervation involves only one colloidal specie or macromolecule and can be achieved
76 through self-charge neutralization by the addition of dehydrating agents like alcohols²⁸ and
77 salts.²⁹ Complex coacervation, on the contrary, consists of more than one macromolecular
78 component^{30,31} and it can occur between polyelectrolytes and oppositely charged
79 polyelectrolytes,³²⁻³⁷ proteins,³⁸⁻⁴⁰ dendrimers⁴¹ or micelles.⁴²⁻⁴⁷ The preparation of
80 coacervates has gained a lot of interest due to their broad range of applications in food,³⁹
81 tissue engineering,^{48,49} drug delivery,⁵⁰ underwater adhesives,^{51,52} porous material⁵³ and water
82 treatment.^{54,55}

83 Commonly, surfactant-polymer coacervation can occur between nonionic surfactant and
84 nonionic polymer or polyelectrolyte but also between ionic surfactant and nonionic polymers,
85 or polyelectrolyte.⁵⁶ However complex coacervation between oppositely charged
86 polyelectrolytes and surfactants has drawn much more attention due to the broad applications

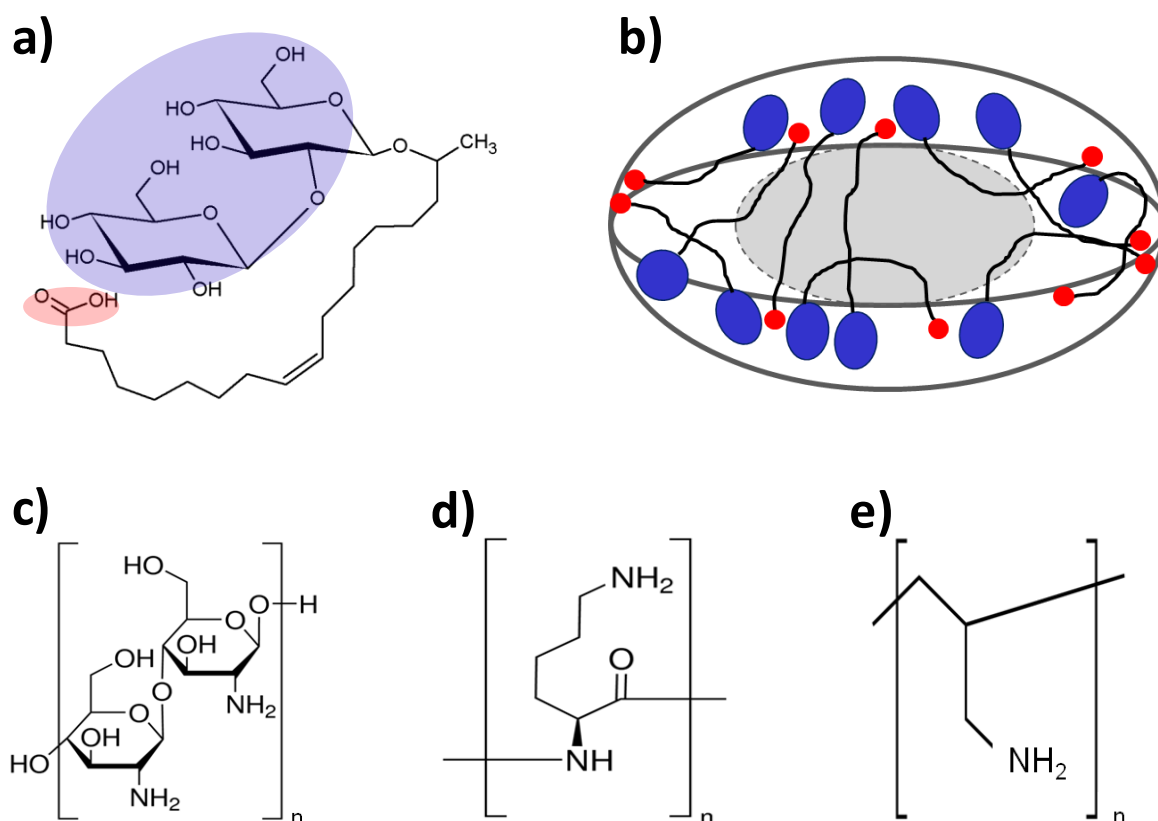
87 in technological areas.⁵⁷ To date, the surfactant types used to induce complex coacervation are
88 usually single chain or gemini type which are composed of a long hydrocarbon chain, an ionic
89 group, a rigid spacer, a second ionic group and another hydrocarbon tail.⁵⁶

90 In the context of complex coacervation between oppositely charged surfactant and
91 polymers, the electrostatic binding must be strong enough to induce coacervation, but not too
92 strong, otherwise precipitation occurs.⁵⁶ To overcome potential precipitation by excessive
93 electrostatic interaction, Dubin and co-workers, developed an interesting strategy based on the
94 preparation of mixed micelles from anionic (sodium dodecylsulfate), or cationic
95 (dodecyltrimethylammonium bromide), surfactant with a nonionic surfactant (Triton X-100).
96 By adjusting the mole fraction of the charged surfactant, they were able to induce and to
97 control the coacervation process with cationic (poly-(dimethyldiallylammonium chloride) or
98 anionic (poly(sodium styrenesulfonate) or poly(sodium acrylate)) polyelectrolytes.^{47,58,59}

99 This process depends generally on many physicochemical parameters like temperature,
100 pH, charge ratio of the macroions and colloid properties like molecular weight and chain
101 flexibility.^{60,61} However, it was found that a minimum salt addition is required to modulate the
102 interaction strength between micelles and cationic polyelectrolyte (PEC) by charge screening,
103 or to enhance the PEC chain flexibility by decreasing intra-chain electrostatic repulsion.⁴²

104 Considering the increasing restrictions in terms of using chemical surfactants, the
105 development of coacervates based on biobased amphiphiles and biomacromolecules becomes
106 an important challenge. Therein, Imura *et al.*, found that mannosylerythritol lipids, a
107 glycolipid biosurfactant, can form spontaneous coacervates in water.⁶² These structures are
108 obtained by simple coacervation and are induced by efficient dehydration in water.
109 Nonetheless, the complex coacervation, a phenomenon with great interest in industry,
110 between macromolecules and glycolipids has not been reported in this context, yet.

111 Herein, we develop green complex coacervates based on a microbial biosurfactant and a
112 set of three polymers, including one bio-derived polymer. We use a bolaform acidic
113 sophorolipid (SL) constituted of a sophorose (glucose $\beta(1,2)$) linked to the C17 atom of oleic
114 acid via an acetal bond (Figure 1a). The study was also motivated by the original micellar
115 structure reported for sophorolipids and to the fact that the carboxylic group is being free of
116 access at the opposite side of the molecule (Figure 1b).⁶³ Unlike conventional surfactants,
117 where the coacervation process is controlled by the surfactant molar fraction, salt or by the
118 addition of a nonionic surfactant using Dubin's strategy, SL has itself a tunable charge, for its
119 pH sensitivity ($pK_a = 5.8$); therefore, the global charge of the resulting micelles could be
120 easily handled just by pH without the need of a second further surfactant, or other additives.⁶³



122

123 **Figure 1 – (a) Chemical structure of acidic form of SL; (b) model of the structure of the micelle formed**
 124 **from SL alone at equilibrium in water (red dot and blue ellipse respectively schematize the COOH and**
 125 **sphingosine groups in the SL molecule in a)) and chemical structure of (c) CHL, (d) PLL and (e) PAA**
 126 **polyamines.**

127

128 In the field of complex coacervation, no studies between polymers and bolaamphiphile
 129 micelles have been reported yet. In the meanwhile, complex coacervation between polymers
 130 and surfactants has never been proposed in the context of green chemistry, where both the
 131 amphiphile and the polymer are biosourced. Here, the behavior of SL micelles was studied in
 132 the presence of three water-soluble polyamines,^{35–37} chitosan oligosaccharide lactate (CHL),
 133 poly(L-lysine) (PLL) and poly(allylamine) (PAA) (Figure 1c-e): CHL is a chitosan derivative,
 134 a cationic polysaccharide obtained by deacetylation of chitin which is a structural
 135 polysaccharide of insects and crustaceans shells. Chitosan is well-known for its wide
 136 availability, biocompatibility, biodegradability and poor toxicity.⁶⁴ PLL and PAA are
 137 synthetic polymers, although the former is based on peptide coupling of the amino acid lysine,
 138 while the latter is a classical petrochemical polymer based on allylamine.⁶⁵ These polymers
 139 have been chosen on the following basis: CHL is a semi-flexible biopolymer, while PLL and

140 PAA are flexible synthetic polymers.⁶⁵ The comparison between the three polyelectrolytes,
141 although of different chemical origin, shows the broad validity of the complex coacervation
142 using SL micelles.

143 In the current paper, we explore the complex coacervation of SL micelles with the PEC
144 mentioned above. The influence of pH, as well as PEC concentration was monitored by
145 turbidimetric titration and dynamic light scattering (DLS). The resulting structures were
146 examined by small angle X-ray scattering (SAXS), cryo-transmission electron microscopy
147 (cryo-TEM), scanning electron microscopy (SEM) and optical microscopy.

148

149 **2. Experimental Section**

150 **Chemicals**

151 SL were purchased from Soliance (Givaudan Active Beauty, France) and hydrolyzed in
152 alkaline medium and the pH was then decreased to around 4.5 to obtain the open acidic form
153 and finally recovered using method 1 as reported previously.⁶⁶ The purity is evaluated at
154 about 90% of both terminal and sub-terminal C18:1 congeners and their equilibrium state in
155 water is micellar, as described elsewhere.^{67,68,69} The critical micellization concentration (cmc)
156 is around 0.1 mg/mL at room temperature. CHL ($M_n \approx 5$ kDa, $pK_a \approx 6.5$)⁷⁰ with a
157 deacetylation degree $> 90\%$, PLL hydrobromide ($M_w \approx 1-5$ kDa, $pK_a \approx 10$)⁷¹ and PAA
158 hydrochloride ($M_w \approx 17.5$ kDa, $pK_a \approx 9.5$)⁷¹ were purchased from Sigma-Aldrich. All other
159 chemicals were of reagent grade and were used without further purification.

160

161 **Preparation of solutions**

162 SL (10 or 50 mg/mL), CHL (4 mg/mL), PLL (10 mg/mL) and PAA (4 mg/mL) stock
163 solutions were prepared by dispersing the former in Milli-Q-grade water. The solutions were
164 stirred until complete hydration. The pH of SL (10 mg/mL) and the cationic PEC solutions
165 was typically between 3 and 4 except for PLL solution ($pH > 5.5$), for which it was decreased
166 to 3.5 by adding 2 μ L of HCl (1M).

167

168 **Turbidimetric titration**

169 The influence of pH and cationic molecules concentration on the formation of coacervates
170 droplets was investigated by measuring the absorbance at a wavelength range of 400-700 nm.
171 The turbidity was then reported as $100 - \%T$ (where T is the transmittance and is equal to 10^{-A}
172 and A is the absorbance at 450 nm). Data were recorded at room temperature using a UV/Vis
173 spectrophotometer (UVIKON XL, BioTeK) and a UV-Vis-NIR spectrophotometer (Cary

174 5000, Agilent Technologies) for some experiments.

175 To study the influence of pH, equal volumes of SL and PEC solutions were mixed after
176 appropriate dilution of the stock solutions with varying final concentrations range for CHL
177 (0.5-2 mg/mL), PLL (1-5 mg/mL) and PAA (0.3-2 mg/mL) while the final concentration of
178 SL solution was kept constant (5 mg/mL). Next, the pH of 2 mL of each mixture was
179 increased progressively by the addition of small amounts (2, 5 or 10 μ L) of NaOH 0.1 M
180 under gentle stirring and the final turbidity curves were recorded only after complete
181 homogenization of the solution and pH stabilization. It is worth mentioning that the turbidity
182 of pure compound solutions was also measured as function of pH.

183 The coacervation dependence on SL concentration was measured at optimal pH-values for
184 CHL (pH 5.6, 1.4 mg/mL), PLL (pH 6.2, 2 mg/mL) and PAA (pH 6.3, 0.75 mg/mL) by step-
185 wise additions (2 or 5 μ L) of SL solution (50 mg/mL) with the corresponding pH under gentle
186 stirring. The final turbidity curves were recorded only after complete homogenization of the
187 mixture and the pH was regularly measured and adjusted if necessary with NaOH (1M).

188

189 **Dynamic Light Scattering measurements (DLS)**

190 Size distribution and electrophoretic mobility of SL free micelles and SL-PEC complexes as a
191 function of pH were measured by DLS using a Malvern Zetasizer Nano ZS90 (Malvern
192 Instruments Ltd, Worcestershire, UK) equipped with a 4 mW He-Ne laser at a wavelength of
193 633 nm. Measurements were made at 25 °C with a fixed angle of 90° and three acquisitions of
194 15 measurements per sample. Although zeta potential (ζ) calculated using Smoluchowski,
195 Hückel, or Henry equations is a more common way to quantify surface charge, no attempt has
196 been made to convert the electrophoretic mobility values (μ) into ζ because the complex
197 system composed of SL micelles, free polymer and SL-PEC coacervates cannot be described
198 by the usual, simple, hard-sphere model, hypothesized in standard theories relating μ to ζ .⁷²

199

200 **Small Angle X-ray Scattering (SAXS)**

201 SAXS experiments are performed at 25°C immediately after sample preparation on the
202 BioSAXS BM29 beamline at the ESRF synchrotron facility (Grenoble, France) using 12.5
203 KeV energy and a sample-to-detector distance of 2.867 m, the beamline standard
204 configuration. The energy is calibrated by measuring the L_I and L_{III} edges of platinum and the
205 sample-to-detector distance is determined using silver behenate ($d_{ref} = 58.38 \text{ \AA}$).
206 (http://www.esrf.eu/home/UsersAndScience/Experiments/MX/About_our_beamlines/bm29.ht

207 ml).⁷³ For this experiment, we employ the automatic sample changer for liquids using the 96-
208 well plates and about 100 μL of each sample.⁷⁴ The liquid sample is automatically loaded into
209 a 1.8 mm quartz glass capillary and ten acquisitions of 1 s each are taken as the sample passes
210 the beam. Individual frames are manually controlled for systematic changes and averaged for
211 better statistics if none are found. Eventual changes can be either due to intrinsic sample
212 heterogeneity or radiation damage. The signal of the Pilatus 1M 2D detector, used to record
213 the data, is integrated azimuthally with PyFAI to obtain the $I(q)$ vs. q spectrum ($q =$
214 $4\pi \sin \theta / \lambda$, where 2θ is the scattering angle) after masking systematically wrong pixels and
215 the beam stop shadow.⁷⁵ Absolute intensity units were determined by measuring the scattering
216 signal of water (0.0163 cm^{-1}).

217

218 **Coacervates imaging**

219 *Light Microscopy.* To highlight the coacervate droplets, images were acquired using a
220 Nikon DS-R11 optical microscope in Brightfield mode and a Zeiss AxioImager D1
221 microscope in differential interference contrast (DIC) mode.

222 *Scanning Electron Microscopy (SEM).* The coacervates solutions were freeze-dried during
223 48 hours and the obtained samples were observed using a Hitachi (S-3400N) electron
224 microscope operating at 3 kV.

225 *Cryogenic Transmission Electron Microscopy (Cryo-TEM).* These experiments were
226 carried out on an FEI Tecnai 120 twin microscope operating at 120 kV equipped with a Gatan
227 Orius CCD numeric camera. The sample holder was a Gatan Cryoholder (Gatan 626DH,
228 Gatan). On both microscopes, Digital Micrograph software was used for image acquisition.
229 Cryofixation was done on a homemade cryofixation device. The solutions were deposited on
230 a glow-discharged holey carbon coated TEM copper grid (Quantifoil R2/2, Germany). Excess
231 solution was removed and the grid was immediately plunged into liquid ethane at $-180 \text{ }^\circ\text{C}$
232 before transferring them into liquid nitrogen. All grids were kept at liquid nitrogen
233 temperature throughout all experimentation.

234

235 **Quantification of coacervation**

236 *Nuclear Magnetic Resonance (NMR):* solution NMR has been here used to quantify both the
237 extent of coacervation and the ratio between the COOH of SL and NH_2 of the polyelectrolytes
238 given in Figure 1. One should note that in the rest of the manuscript we will broadly refer to
239 them as C=O and NH_x , which respectively account for the carboxylic/carboxylate and

240 amino/ammonium groups. Solution NMR is a technique being sensitive to molecular species
241 with fast tumbling in solution. In this work, NMR is mainly sensitive to both SL and
242 polyelectrolyte in solution, not associated in complex coacervates: out of the coacervation
243 region, NMR is sensitive to the entire SL and polyelectrolyte population, while in the
244 coacervation region, NMR is only sensitive to SL and polyelectrolyte in equilibrium with the
245 complex coacervates. Quantification is probed with two different methods; in the first one,
246 solutions containing the coacervates are analysed directly by ^1H NMR. As controls, we have
247 analysed the SL-polyelectrolyte mixtures out of the coacervation region as well as the single
248 components within and out of the coacervation region. In the second approach, the
249 coacervates are centrifuged out of the solution. The supernatant is analysed as such while the
250 coacervates are redispersed in the same volume of water at a pH set out of the coacervation
251 region, so to detect the entire SL and polyelectrolyte population.

252 For the first set of experiments, we have studied each control solution individually (SL, CHL,
253 PLL, PAA) and their association (SL-CHL, SL-PLL, SL-PAA). All solutions are freshly
254 prepared in D_2O at pH (pD) values below and above the known pH of coacervation,
255 determined according to the turbidity data. One should note that we do not experience any
256 difference when using deuterated instead of hydrogenated water. 1 M NaOD and 1 M DCl
257 solutions have been employed to change pD. Controls: SL (5 mg/mL, pD: 4.36, 6.33), CHL
258 (1.4 mg/mL, pD: 3.80, 6.09), PLL (2 mg/mL, pD: 4.58, 7.14) and PAA (0.75 mg/mL, pD:
259 3.91, 6.23). For the study of the coacervates, we have used exactly the same concentration
260 values employed for the controls and the following pD values: SL-CHL, pD= 4.46, 6.12; SL-
261 PAA, pD= 4.00, 6.00; SL/PLL, pD= 4.35, 7.00. In the second set of experiments, we have
262 centrifuged the coacervate (SL-CHL, SL-PLL and SL-PAA solutions at pD > 6) at 3000 rpm
263 for 1 h, a condition which is known to separate efficiently the colloid-rich phase without
264 destructuring the coacervates.⁴³ The supernatant has been removed and analyzed as such
265 while the coacervate has been redispersed in 500 μL D_2O at pH below 6, out of the
266 coacervation region, as explained above.

267 All ^1H solution NMR experiments are acquired on a Bruker Avance III 300 spectrometer
268 using a 5 mm 1H-X BBFO probe. Number of transient is 16 with 2.3 s recycling delay,
269 acquisition time of 2.72 s and a receiver gain of 256. We have employed a 5 mm NMR tube
270 containing exactly 500 μL of solution. For quantitation purposes, these conditions have been
271 kept constant throughout all experiments.

272 The $\text{C}=\text{O}/\text{NH}_x$ ratio has been determined by the integral ratio between the CH_2 groups in α -
273 position for SL ($\text{R}-\text{CH}_2-\text{C}=\text{O}$, $\delta = 2.33$ ppm) and the polyelectrolyte (CHL: $\text{R}-\text{CH}-\text{NH}_x$, $\delta =$

274 3.16 ppm; PAA: R-CH₂-NH_x, δ = 3.10 ppm; PLL: R-CH₂-NH_x, δ = 3.00 ppm) at pH < 5. The
275 extent of coacervation is calculated by measuring the intensity loss between the SL-
276 polyelectrolyte mixture out of the coacervation region (pD < 5, all species detected) and in the
277 coacervation region (pD > 5, only free SL and polyelectrolyte detected) in the 3-4 ppm region.
278 The signal loss corresponds to the amount of sample in the coacervate phase. The latter is
279 compared with the direct measurement of the intensity of the coacervate rich-phase after
280 centrifugation and redispersion.

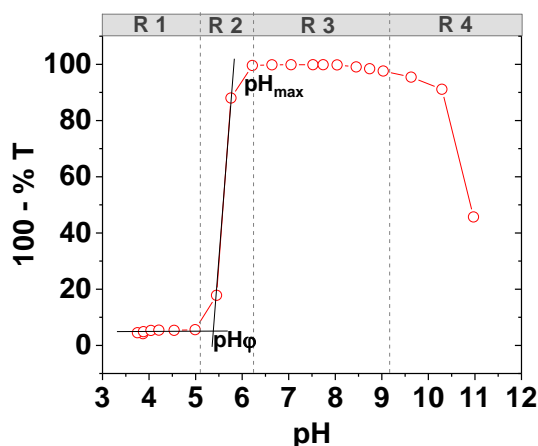
281

282 **3. Results and discussion**

283 **pH-induced complex coacervation**

284 Complex coacervation based on charge pairing occurs when positive and negative
285 charges in the micelles and macromolecules compensate. In pH-responsive systems,³² the
286 ionization rate depends on pH and the pKa of the acid, or base, in the given molecule. If the
287 M_w of SL is known, the number and weight average molecular weight (M_n and M_w) of the
288 commercial PEC used here are only estimated. For this reason, although one can estimate the
289 relative mass fraction values of SL and PEC needed to obtain a 1:1 molar ratio of their
290 ionizable groups (COOH and NH₂), a variation in the relative concentrations is necessary.
291 Similarly, the differences in the pKa values among all molecular partners suggest that if
292 complex coacervation occurs, that will be strongly pH-dependent. For these reasons, complex
293 coacervation was qualitatively determined using turbidity measurements as a function of pH
294 and PEC concentration for a given SL amount (5 mg/mL and 10 mg/mL, respectively 8 and
295 16 mM).

296 A typical turbidimetric titration curve of SL-PAA (C_{PAA}= 0.75 mg/mL) is shown in
297 Figure 2. The coacervation process as a function of pH can be described in terms of a set of
298 specific pH values corresponding to the limits of four different regions of phase behavior.
299 *Region 1*: at low pH-values, generally below pH 5, the solution is clear and the turbidity is
300 constant and close to zero; *Region 2*: an abrupt increase in turbidity from a starting pH,
301 designed as pH₀, characterizes this region and it reflects the cloudy aspect of the solution.
302 *Region 3*: this pH interval is characterized by a plateau from a starting pH_{max} and where the
303 turbidity is constant and maximum and where the solution shows an opalescent behavior.
304 *Region 4*: the turbidity decreases progressively until a transparent solution again. As a general
305 remark, transparency in *Region 1* and *Region 4* strongly depend on the solubility of each
306 component (micelle and polymer), an aspect which will be discussed later.



307

308 **Figure 2 - Turbidity (100-%T) as a function of pH for a SL-PAA mixture. [SL]= 5 mg/mL; [PAA] = 0.75**
 309 **mg/mL. *R* stands for Region.**

310 The turbidimetric titration of SL with CHL, PLL or PAA at various PEC concentrations
 311 are presented in Figure 3a-c, while Figure S 1 and Figure S 2 show the specific system SL at
 312 10 mg/mL and CHL in the concentration range between 0.25 and 2.0 mg/mL. Figure 3 shows
 313 that any SL-PEC mixture displays the same pH-dependent turbidity profile described in
 314 Figure 2, however the type and concentration of PEC have a strong impact on the phase
 315 transition, as discussed above, and analyzed in more detail hereafter. Figure 3a-c shows that
 316 the transition pH in *Region 2*, and the stability of *Region 3* as a function of pH, strongly
 317 depend on the SL-PEC ratio. This aspect will be discussed in more detail below. *Region 4* is
 318 identified for all systems by the sudden loss in turbidity, except for SL-CHL, the turbidity of
 319 which is still high due to the insolubility of CHL above pH 7. The phenomena observed by
 320 turbidity and described above are classically-observed in oppositely charged PEC, like
 321 chitosan with Arabic gum,⁷⁶ or with hyaluronic acid³² and for PEC-protein systems, like poly-
 322 (dimethyldiallylammonium chloride) with bovine serum albumin,⁷⁷ Arabic gum with whey
 323 protein⁷⁸ or with β -lactoglobulin.⁷⁹ Micelles-PEC systems show a similar phenomenon, such
 324 as partially described for polyacrylic acid with mixed micelles of *n*-hexadecyl trimethyl
 325 ammonium chloride and *n*-dodecyl hexaoxyethylene glycol monoether.⁸⁰ It should be noted
 326 that pH-induced coacervation is classically observed for oppositely charged PECs, or for
 327 PEC-protein systems, but less discussed for PEC-micelles systems. The main reason is, unlike
 328 the SL used in this work and which is pH-sensitive ($pK_a= 5.8$), the charge density of most
 329 conventional surfactants, such as sodium dodecyl sulfide (SDS), are practically not sensitive
 330 to pH variation in a standard pH range range ($3 < pH < 10$). Else, in a number of studies
 331 implying a model system of mixed micelles (SDS-Triton X-100), authors generally use the
 332 cationic poly(diallyldimethylammoniumchloride), which is a pH-independent polyion.⁸¹

333 One should also note that the global charge of the SL micelles is not only modulated by
334 the deionization degree of the carboxylic groups, but also by their spatial localization, which
335 is not clearly defined, as commonly found in standard micelles composed of ionic surfactants
336 (e.g., SDS). A combination of scattering techniques and Molecular Dynamics (MD)
337 simulations indicate that the carboxylic groups can be localized in a much broader and poorly-
338 defined shell volume around the hydrophobic core.⁶³

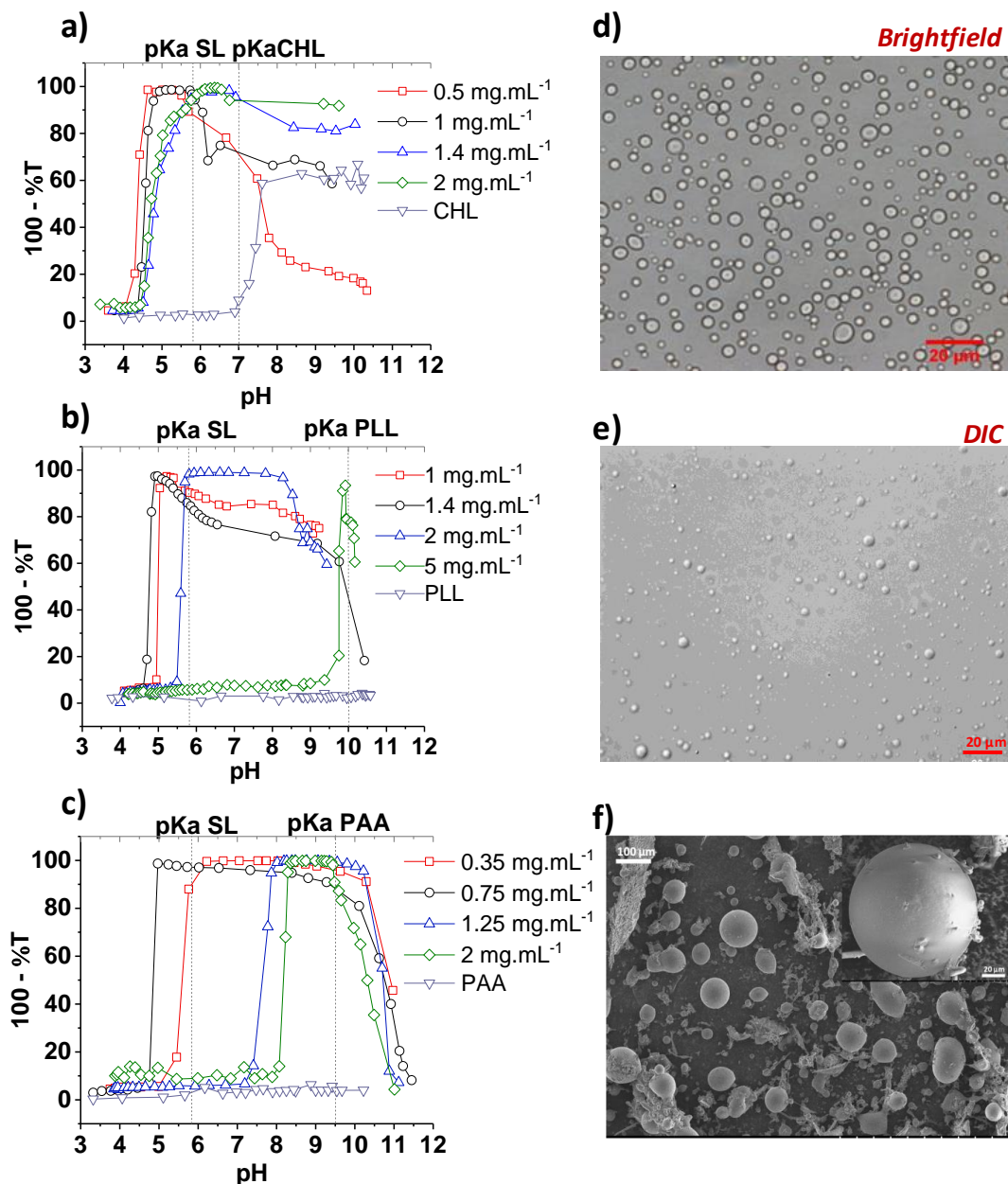
339 According to the wide literature data on complex coacervation involving oppositely
340 charged PEC-colloids, *Region 1* identifies the coexistence of two water-soluble, non- or
341 weakly-interacting species, in this work represented by SL micelles^{63,67,68} and PEC. *Region 2*
342 defines the point of initial SL-PEC interaction leading to insoluble complex formation. In
343 *Region 3* the complex SL-PEC coacervate is stable at any pH. The end of the plateau is
344 generally related to the appearance of spurious precipitates, which could coexist with
345 coacervates, since the turbidity remains relatively high. In *Region 4*, the coacervate is no
346 longer stable. These hypotheses are confirmed by additional experiments. Figure 3d-e show
347 the optical microscopy images recorded for SL-CHL and SL-PLL at the maximum turbidity
348 plateau (*Region 3*), and they show the widespread presence of spherical droplets, which
349 supports the idea of a liquid-liquid phase separation for all samples. Figure 3f and Figure S 3
350 confirm these assumptions for all systems using SEM on freeze-dried samples. Figure S 2
351 shows a series of crossed experiments performed on the SL-CHL system. pH-dependent
352 electrophoretic mobility data confirm the charge-dependency of the complex coacervate
353 formation, as the decrease in monotonic charge (at low pH the overall charge should be
354 positive, as expected for the presence of positively-charged ammonium groups on PEC and
355 neutral SL micelles) correlates with an increase in turbidity: *Region 3*, where charge matching
356 should occur, is effectively characterized by the lowest charge. This behavior is typical for
357 complex coacervates³². Macrophase separation is shown by the presence of large droplets in
358 Figure S 2d while meso- and microscale phase separation is demonstrated by a combination
359 of DLS and cryo-TEM, respectively indicating a colloidal dispersion having an average
360 hydrodynamic radius of ~300 nm (Figure S 2b) and an apparent radius in the order of 100 nm
361 (Figure S 2c).

362 Additional tests have been done by varying the overall SL concentration. Figure S 1
363 shows a SL solution at 10 mg/mL for various amounts of CHL as a function of pH. One can
364 easily determine the presence of Regions 1 through 4 between pH 4 and pH 10, whereas the
365 lower the amount of CHL, the lower the turbidity in *Region 4*, above pH 7, dependent on the
366 insoluble CHL at neutral-basic pH. Figure S 4 goes even further as it shows the evolution of

367 the turbidity as a function of the SL concentration for a constant PEC amount ($C_{\text{CHL}}= 1.4$
368 mg/mL , $C_{\text{PLL}}= 2 \text{ mg/mL}$, $C_{\text{PAA}}= 0.75 \text{ mg/mL}$) and pH values ($\text{pH}_{\text{SL-CHL}}= 5.6$; $\text{pH}_{\text{SL-PLL}}= 6.2$;
369 $\text{pH}_{\text{SL-PAA}}= 6.3$), which has been chosen to be in the stable complex coacervate phase, at the
370 top of the plateau (*Region 3*) for each system, from Figure 3a-c. Figure S 4 shows that the
371 turbidity appears after the addition of at least 1 mg/mL of SL, which is a value at which SL
372 are already in the micellar state (cmc of SL is at least ten times lower). However, SL is added
373 in an environment within positive charges, localized on the polymers. In this situation, the
374 interaction between SL and PEC could reflect the following three-step mechanism, generally
375 found in the literature:^{15,57,82} (a) at low surfactant concentration, the SL monomers starts to
376 interact with the PEC chain by electrostatic binding between the carboxylic groups and
377 cationic sites of SL and PEC, respectively (region where the turbidity remains minimal). (b)
378 When the SL critical aggregation concentration (cac) is reached, the SL monomers will be
379 integrated into the micelles (region where the turbidity starts to increase). (c) With further
380 increasing of SL molecules, the binding sites of PEC are gradually saturated which will lead
381 to complex coacervation (region of maximum turbidity). As shown in Figure S 4, the turbidity
382 increases abruptly for SL-PLL and SL-PAA compared to SL-CHL, for which the turbidity
383 increases gradually. This could be explained by the differences in the relative ionization
384 degrees of the each PEC and SL at the pH under study. At pH 6.2, the ionization degree
385 (please refer to the Electronic Supplementary Information for more details) of PLL and PAA
386 (β) is unitary ($\beta= 1$, as their $\text{pK}_a \sim 10$); the ionization degree of SL is also high ($\alpha \sim 0.7$). Under
387 these conditions, each SL molecule added to the PEC solution contributes to screen the PEC
388 charge. On the contrary, at pH 5.6, the ionization degree of CHL is ~ 0.9 , while the ionization
389 degree of SL is lower ($\alpha \sim 0.4$). Under these circumstances, the charge of CHL is compensated
390 by two SL molecules.

391 Even if the gradual addition of SL surfactant molecules to the PEC solution is may not
392 reflect the same scenario if compared to that of mixing preformed SL micelles upon adding a
393 PEC solution before pH titration, one can identify the SL concentration of 5 mg/mL (0.0080
394 M) as being a good compromise for the formation of a robust SL-PEC complex coacervate in
395 all systems. Considering the average molecular masses of each PEC used in this experiment
396 (refer to the experimental section), one can estimate the optimal charge ratio $[\text{COO}^-]:[\text{NH}_3^+]$
397 at which complex coacervation starts to occurs to be 0.32, 0.27 and 0.5 for, respectively, SL-
398 CHL, SL-PLL and SL-PAA systems. The detailed approach of the charge $[\text{COO}^-]:[\text{NH}_3^+]$
399 ratio determination is described in the Electronic Supplementary Information. Even if the
400 effective $[\text{COO}^-]:[\text{NH}_3^+]$ ratio is not unitary, our data are in line with literature, where non-

401 stoichiometric complex coacervation can easily be found, for instance in a chitosan and
402 hyaluronic acid mixture in a 50 mM NaCl solution and where the $[\text{COO}^-]:[\text{NH}_3^+]$ charge ratio
403 varies between 0.08 and 0.72.³² The authors attributed this observation to the chain semi-
404 flexibility and potential charge mismatch between the oppositely charge polyelectrolytes.³²
405 Non-stoichiometric charge ratio values were also reported for PEC-micelles⁵⁹ and it is
406 generally explained as a mismatch between a calculated “macro-scale stoichiometry” and a
407 “micro-scale stoichiometry”, which is related to the effective ratio within the coacervates.
408 Moreover, from a thermodynamic point of view, the most common way to describe complex
409 coacervation is based on the Flory-Huggins theory which considers the change of the Gibbs
410 free energy of mixing ($\Delta G_{mix} = \Delta H_{mix} - T\Delta S_{mix}$), where ΔH_{mix} and ΔS_{mix} are, respectively, the
411 variation of enthalpy and entropy and T is the temperature of the mixture.^{83,84} The
412 coacervation between polymers and micelles is thus driven by the enthalpy of complexation
413 and by entropy increase, due to the release of condensed counterions; in fact, the entropic
414 contribution is even thought to drive complex coacervation, as described by Rigsbee and
415 Dubin.⁸⁵ Using isothermal titration calorimetry, they demonstrated that both complexation
416 and coacervation of poly-(dimethyldiallylammonium chloride) with
417 dodecyltrimethylammonium bromide/ TritonX-100 micelles were mainly entropy-driven.
418 Since no salt was added (generally NaCl is classically used), the nature and amount of
419 counterions arising from the used PEC could impact significantly their condensation and
420 release which will increase the entropy (ΔS_{mix}) and affect the coacervation process. Even if it
421 is hard to estimate the entropic contribution to complex coacervation in the SL-PEC systems
422 studied here, one should not forget that any pH change involves the presence of counterions,
423 which could play an important role in determining the exact coacervation boundary. Further
424 experiments should be performed to confirm this hypothesis, although they are not the scope
425 of this work.



426
 427 **Figure 3 – Turbidity (100-%T) as function of pH for the systems (a) SL-CHL, (b) SL-PLL and (c) SL-**
 428 **PAA at different PEC concentrations and constant SL concentration (5mg/mL) and T = 23 °C. (d-e)**
 429 **Optical microscope images of SL-CHL at pH 5.62 and SL-PAA at pH 7.64. (f) SEM images of the freeze**
 430 **dried SL-PLL coacervates at pH 7.**

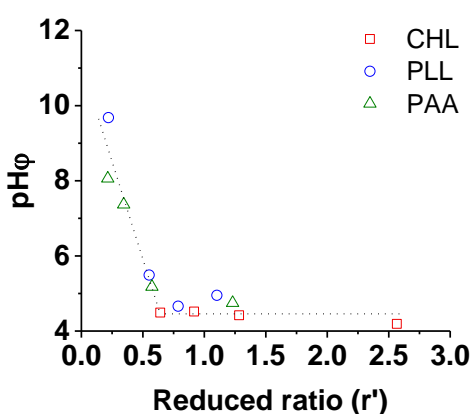
431

432 **Transition pH from a monophasic to a biphasic system**

433 Theoretically, the coacervation process should be favored in a condition of charge
 434 compensation between the negatively-charged SL and positively-charged PEC, that is above
 435 the pKa of SL and below the pKa of PEC. The PEC linear charge density and SL micelles
 436 surface charge density are controlled by the ionization degree of the terminal carboxylic and
 437 amino groups (Figure 1a). From the turbidimetric curves in Figure 3, it should be noticed that

438 the width of the pH domain for a stable complex coacervate (*Region 3*) considerably depends
 439 on the type of PEC, and it can be ranked in the following order: CHL < PLL < PAA. The
 440 important difference between CHL and the other PEC could be related to the interval width
 441 between the SL pKa, which is close to 5.8,⁶³ and the pKa of the PEC, that is 7, 10 and 9.5 for
 442 CHL, PLL and PAA, respectively. Figure S 5 illustrates the influence of the pKa of PEC on
 443 the optimal coacervation region width (Figure S 1). However, the variation between PLL and
 444 PAA, the pKa values of which are very close, could be related to the employed concentrations
 445 of PLL (1-5 mg/mL) and PAA (0.35-2 mg/mL), and therefore to the SL-PEC stoichiometry.
 446 Moreover, the molecular weight of PAA (Mw= 17.5 kDa) and PLL (Mw ≈ 1-5 kDa) could
 447 also affect the coacervation. In fact, the increase of PEC chain length could enhance the
 448 coacervation process, as previously observed for complex coacervation between PEC and
 449 mixed micelles.⁴³

450 pH ϕ is classically defined as the point of abrupt turbidity within a very small change in pH
 451 (Figure 2) and it is determined as the intercept of *Region 1* and *Region 2* in the turbidity curve
 452 (Figure S 6) and it has currently been interpreted as the pH of the appearance of turbidity, or
 453 visual phase separation.⁷⁷ For a better understanding of the influence of PEC concentration,
 454 the pH ϕ was plotted against the reduced ratio (r'), introduced previously by Kaibara et al.,⁷⁷
 455 and which is the result of the multiplication of the SL-PEC weight ratio by the factor [PEC
 456 monomer unit mass/SL molecular weight] (Figure 4). Since r' is related to the reciprocal
 457 number of charged sites of PEC per SL molecule, only the half of the CHL monomer unit
 458 mass was considered, since it possesses two functional amino-groups (Figure 1c).



459
 460 **Figure 4 – Evolution of the pH ϕ as function of the reduced ratio (r') of SL-PEC. pH ϕ is defined as the**
 461 **point of abrupt turbidity as indicated in Figure 2, while r' is the multiplication of the SL-PEC weight ratio**
 462 **by the factor [PEC monomer unit mass/SL molecular weight]. [SL] = 5mg/mL**

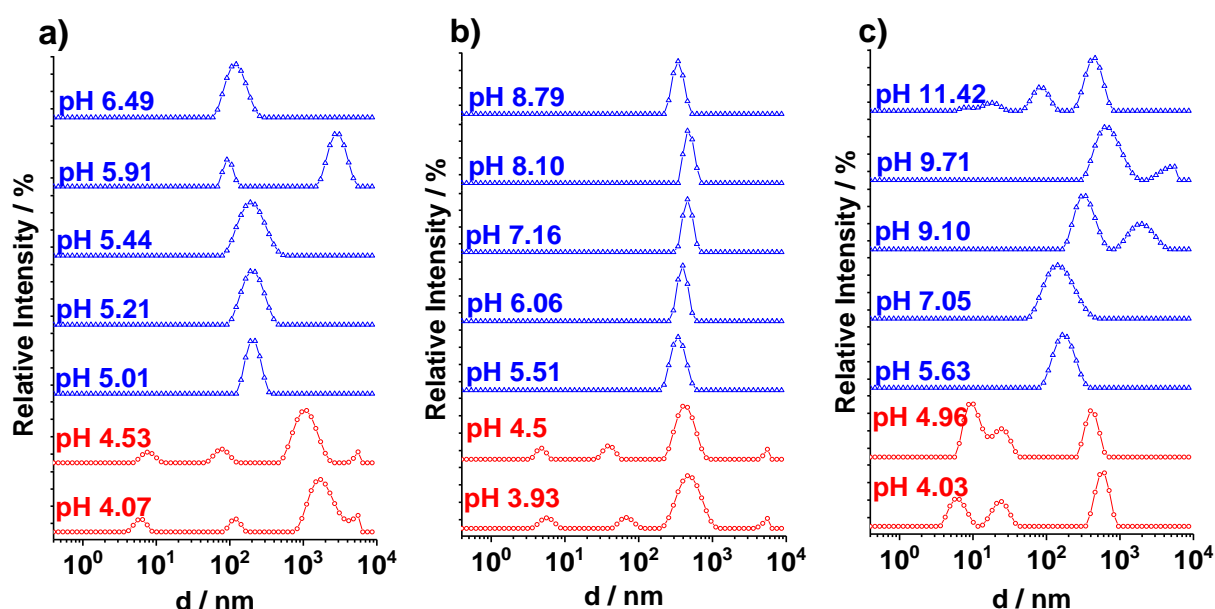
463

464 CHL, PLL and PAA systems exhibit practically comparable plots, where the pH_{ϕ} varies
 465 slightly for $r' \geq 0.6$ due to the saturation of PEC chains with the biosurfactant micelles but it
 466 increases sharply for $r' < 0.6$, due to the possible existence of free PEC chains in the solution.
 467 This tendency could be generalized by expanding the studied SL-PEC ratios as previously
 468 performed for polymer/protein systems.⁸⁶ But from Figure 4 it can be established that the
 469 evolution of pH_{ϕ} as a function of the PEC type is minimized. This result suggests that the
 470 number of polymeric charged sites per SL micelles controls the coacervation process.
 471 Consequently, the structural differences of the PEC charged site had only a small effect on the
 472 phase separation, which is mainly driven by electrostatic interaction and it demonstrates the
 473 crucial role of pH by modulating the charge density and the strength of complexation. One
 474 should note that investigation of SL-CHL complex coacervation at lower reduced ratio ($r' <$
 475 0.4), and where pH_{ϕ} is expected to be higher than $pH 7$, is not possible due to CHL
 476 insolubility in this pH range.

477

478 Evolution of SL-PEC complexes with pH

479 The complexation process of the SL biosurfactant with the different PEC was probed by
 480 monitoring the size evolution of SL-PEC complexes as function of pH by DLS measurements.
 481 The data are presented in Figure 5 in terms of hydrodynamic diameter as a function of the
 482 relative intensity.



483

484 **Figure 5 - Distribution of the hydrodynamic diameters of (a) SL-CHL, (b) SL-PLL and (c) SL-PAA**
 485 **complex coacervates as a function of pH at 25 °C. [SL]= 5 mg/mL, [CHL]= 1.4 mg/mL, [PLL]= 2 mg/mL,**
 486 **and [PAA]= 0.75 mg/mL.**

487 At $\text{pH} < \text{pH}_\phi$ (*Region 1*, here $\text{pH} < 5$), all samples practically display three distinctive
488 populations. The smallest diameter value is below 10 nm, a size which is in agreement with
489 the presence of free SL micelles.⁶³ The size of the second and third populations vary with the
490 SL-PEC system: SL-CHL (Figure 5a) shows two distributions at about 150 nm and $>1 \mu\text{m}$
491 (beyond the limitation of the DLS instrument); SL-PLL (Figure 5b) at around 80 nm 500 nm
492 while SL-PAA (Figure 5c) at about 25 and 600 nm. Similar complexes were previously
493 observed for mixed micelles and PEC mixtures during temperature-induced coacervation.
494 Authors suggested that the coacervation process can be viewed as a clustering mechanism of
495 soft colloidal particles which precedes coacervation.^{81,87} Indeed, soluble complexes from intra
496 PEC-micelles and inter PEC-micelles are considered as precursors of coacervation.⁵⁹
497 Nonetheless, it should be mentioned that relative number or volume size distributions only
498 show free SL micelles at $\text{pH} < \text{pH}_\phi$ in *Region 1*, as shown in Figure S 7, thus indicating the
499 majority of the sample is practically composed of free micelles.

500 The solution becomes cloudy above pH_ϕ (*Region 2* and *Region 3*), and the DLS
501 measurements reveal the appearance of one single peak (hydrodynamic diameter > 100 nm),
502 the size of which is quite stable with pH. These results reflect the formation of insoluble SL-
503 PEC complexes. By increasing the pH, the size distribution of the complex coacervates shifts
504 to larger aggregates for all SL-PEC complexes. Visually, the size of droplets increases and the
505 solution becomes more opalescent due to coacervate coalescence and to the evolution from
506 submicronic to microscopic droplets. When the pH is increased further in the vicinity or even
507 beyond the PEC pK_a , the hydrodynamic diameter becomes smaller again, as this can be
508 observed for SL-CHL (Figure 5a) and SL-PAA (Figure 5c) in both the intensity and
509 number/volume distributions (Figure S 7a,b). For instance, at pH 10.42 for the SL-PAA
510 system, one can observe again that the majority of the population has a hydrodynamic
511 diameter below 10 nm (Figure S 7a), suggesting the massive presence of micelles and the
512 dissolution of the SL-PAA complex.

513 From DLS, one could suppose that at the starting pH ($\text{pH} \sim 4$) in *Region 1*, the surface
514 charge density of the SL micelles is strong enough to lead to soluble complexes formation
515 with the different cationic PEC by Coulombic interaction. This seems to occur despite the
516 very small amount of protonated C=O groups, and in the absence of obvious screened
517 Coulomb interaction among the micelles, otherwise observed at higher pH values by SAXS
518 and SANS experiments.^{63,67,68} In this situation, the pH-induced complex coacervation
519 mechanism takes place in four steps as follow: (i) formation of soluble complex from the free
520 SL micelles and PEC chains at $\text{pH} \sim 4$, (ii) the SL-PEC complexes become insoluble at pH_ϕ ,

521 when the interaction between both species becomes sufficiently high, (iii) precipitation when
522 the interaction strength become too high and finally (iv) the dissolution of the precipitates.
523 Combined electrophoretic mobility and turbidity data presented in Figure S 2a for SL-CHL
524 and in Figure S 8 for SL-PAA show the strong correlation between the decrease of the
525 electrophoretic mobility from $+2.4 \mu\text{m cm/Vs}$ to zero, expected in a pH region rich in NH_3^+
526 groups, and the increase in turbidity, that is, complex formation. The coacervation is
527 maximum for SL-CHL ($P_{\text{CHL}} = 0.25 \text{ mg/mL}$: low concentration) for an electrophoretic
528 mobility of almost one; while all CHL chains are expected to be involved in the coacervates
529 and are not expected to be free in solution (Figure S 2). Likewise for SL-PAA ($C_{\text{PAA}}=0.75$
530 mg/mL), with a theoretical stoichiometry equals to one, the maximum coacervation region
531 started significantly far from the point of electroneutrality, as also commented above. These
532 observations are in line with the results reported in Figure S 4b, where the maximum
533 coacervation region was reported for charge ratios less than one. Theoretically, the absence of
534 charge neutralization could be explained by the model proposed by Zhang and Shklovskii,⁸⁸
535 who predicted that the oppositely charged macroions could form a neutral macroscopic drop
536 by intracomplex, or intercomplex, disproportionation when the macroion charge
537 stoichiometry deviates from unity.

538

539 **Quantification of the coacervation process**

540 The efficiency of coacervation and composition of coacervates are estimated by solution ^1H
541 NMR, as detailed in the experimental section and presented in Figure S 9. In particular, the
542 integral ratio between the resonances $\alpha\text{-CH}_2$ ($\text{R-CH}_2\text{-C=O}$) of SL and $\alpha\text{-CH}_z$ ($\text{R-CH}_z\text{-NH}_x$, $z=$
543 1 for CHL, $z= 2$ for PAA, PLL) of the polyelectrolyte is used to quantify the C=O/NH_x molar
544 ratio in the coacervate (C=O and NH_x refer to the carboxylic/carboxylate and
545 amine/ammonium groups). On the contrary, since solution NMR is not sensitive to the sample
546 involved in the coacervates, the loss in the spectral intensity after coacervation between 3 ppm
547 and 4 ppm is used to quantify the extent of coacervation (residual method in Table 1b). The
548 extent of coacervation is also verified by measuring the intensity loss (between 3 ppm and 4
549 ppm) after recovery of the coacervate through centrifugation and disassembling it below the
550 coacervation pH (centrifugation method in Table 1b).

551 Table 1a shows that the C=O/NH_x molar ratio before and after coacervation stays practically
552 unchanged, thus indicating that the fraction of SL and polyelectrolyte is stoichiometric in
553 terms of charge pairing, as expected. In terms of the extent of coacervation (Table 1b), both

554 methods used in this work nicely agree on the fact that about 25% of the initial
 555 polyelectrolyte-SL concentration is involved in the complex coacervation process. If a
 556 discrepancy seems to exist between the two methods for the SL-PAA system, we believe that
 557 incomplete recovery of coacervate during and after centrifugation is at its origin.

558

559 **Table 1 – Quantification of the a) C=O/NH_x molar ratio before (pH < 5) and after (pH > 5) coacervation**
 560 **(error is estimated to 10%) and b) extent of coacervation, measured using two complementary methods.**
 561 **Centrifugation: the solution above pH 5 is centrifuged to separate the coacervate, which is eventually**
 562 **redispersed in quantified. Residual: the amount of coacervate is estimated from the signal loss before and**
 563 **after coacervation pH. All data are collected through solution ¹H NMR spectroscopy, which implied the**
 564 **use of a fully deuterated solvent (D₂O). One can refer to Figure S 9 for the typical NMR data.**

<i>Polyelectrolyte/SL</i>	C=O/NH _x molar ratio		Extent of coacervation (%)	
	Before	After	Centrifugation	Residual
CHL	0.8	1.0	24 ± 1	24 ± 1
PLL	1.1	1.3	22 ± 1	23 ± 2
PAA	0.7	0.5	27 ± 2	37 ± 2

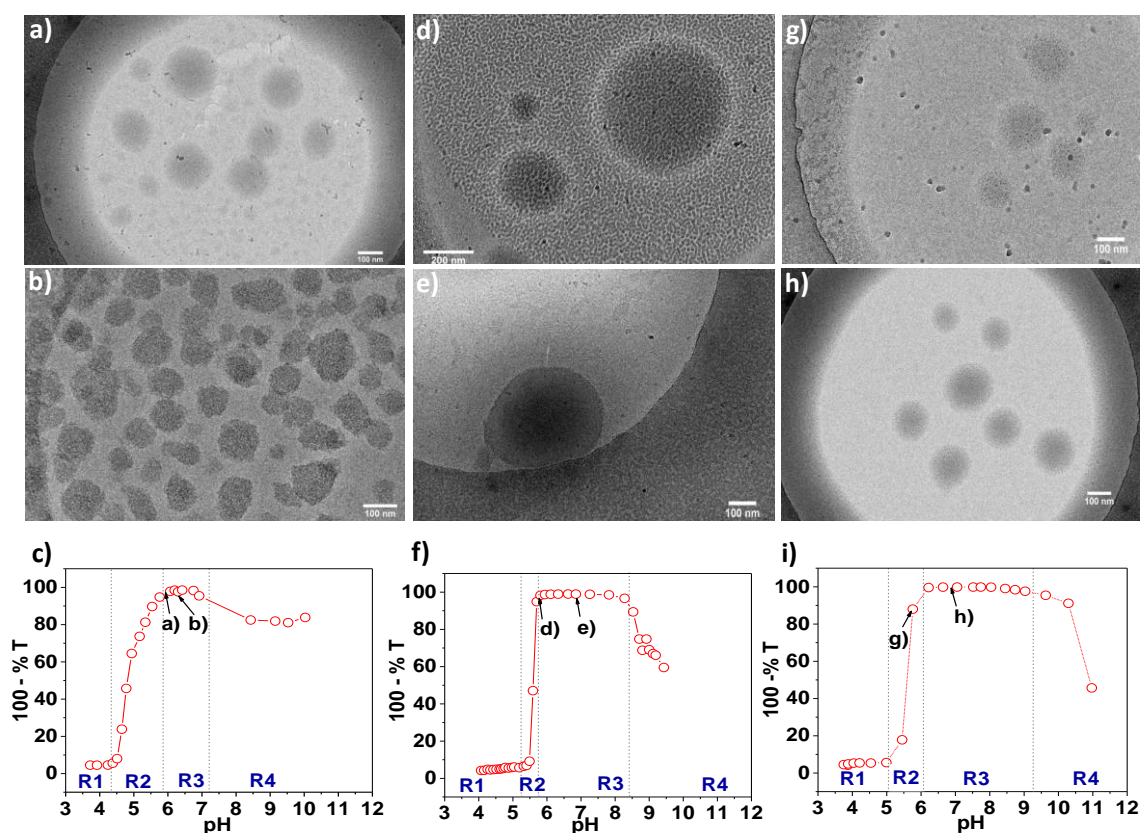
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567 **Structure of the coacervates phase**

568 In order to investigate the structures of the complex coacervates during their formation, cryo-
 569 TEM was carried out at different pH-values for each SL-PEC system (Figure 6). The SL-CHL
 570 system at pH 5.94 (*Region 3*, Figure 6a,c) shows spherical particles of variable size (mainly of
 571 50 and 200 nm). These particles are homogeneous in texture and do not exhibit internal
 572 ordering or evidence of a particular organization. This type of structure is in a good agreement
 573 with coacervates observed in previous studies.²² Upon pH increasing to 6.33 (*Region 3*,
 574 Figure 6b), the structures keep the same size but they become denser, strongly contrasted, and
 575 lose their spherical shape. These aggregates could be generated by a dehydration
 576 phenomenon, which is associated to counterion expulsion and entropy loss, when SL-PEC
 577 interactions are promoted, as previously observed for polysaccharide-protein coacervation,⁸⁹
 578 and by analogy to complex coacervation of PEC-mixed micelles with temperature.⁹⁰ For SL-
 579 PLL at pH 5.77 (Figure 6d), at the *Region 2/Region 3* frontier (Figure 6f), the coacervates
 580 exhibit also a spherical shape with a relative larger size (several hundred nm); when the pH is
 581 increased to 7.38 (*Region 3*, Figure 6e,f), the droplets show, again, a higher apparent electron
 582 density. Finally, SL-PAA at pH 5.77 (*Region 2*, Figure 6g,i) displays discrete spherical
 583 particles in the 100 nm size and, in agreement with the other samples, denser particles at pH
 584 6.83 (*Region 3*, Figure 6h,i). Interestingly, in practically all samples, a poorly-contrasted

585 phase, most likely composed of free micelles and polymer, is observed at the frontier between
 586 *Region 2* and *Region 3*. Coacervates in their initial stage of formation generally coexist with
 587 polymer and micelles, which seems the reason for the poor contrast, as suggested by the SL-
 588 PLL system at pH 5.77 in Figure 6d, where the three spherical particles are superimposed to a
 589 broad continuum of matter. Once the pH defines *Region 3*, all samples show a much more
 590 contrasted, denser, phase composed of spheroidal particles; In this case, the background is
 591 much clearer, that is rich mainly composed of icy water and less rich in residual matter
 592 (polymer, micelles).
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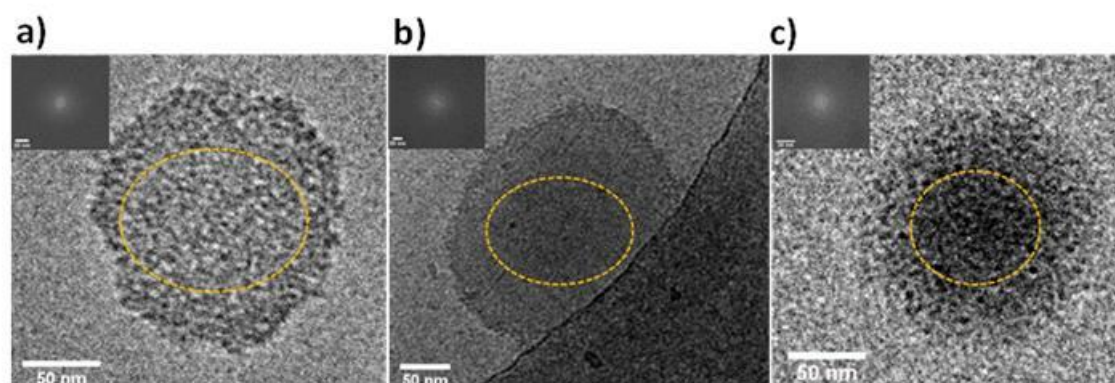


594
 595 **Figure 6 - Cryo-TEM images of SL-CHL coacervates at (a) pH 5.94 and (b) pH 6.33, SL-PLL coacervates**
 596 **at (d) pH 5.77 and (e) pH 7.38 and SL-PAA coacervates at (g) pH 5.77 and (h) pH 6.83. The corresponding**
 597 **turbidity of the observed SL-CHL, SL-PLL and SL-PAA systems is shown respectively in (c), (f) and (i).**
 598 **[SL]= 5 mg/mL, [CHL]= 1.4 mg/mL, [PLL]= 2 mg/mL, and [PAA]= 0.75 mg/mL. R stands for Region.**
 599

600 Cryo-TEM shows that the coacervate structures critically depend on the pH and their
 601 evolution could be described as follow: at the early stage of coacervation, i.e. for pH just
 602 above the pH_{ϕ} (*Region 2*) and until pH_{max} (boundary between *Region 2* and *Region 3*),
 603 spherical discrete droplets with a relatively low electron density are formed and are
 604 surrounded by a rich micellar phase. When $pH > pH_{max}$, micelles gradually disappear due to

605 their interactions to free PEC chains, as observed for SL-CHL at pH 5.94 and SL-PAA at pH
606 6.83. At later stages of coacervation, the droplets exhibit a more electron dense structure (e.g.,
607 SL-PLL at pH 7.38) due to the higher concentration of matter due to dehydration resulting
608 from the release of counterions and water molecules from the molecular complex. The
609 difference between the SL-PLL and SL-CHL structures is possibly related to the pH of the
610 latter compared to the pH limit of coacervation (limitation between *Region 3* and *Region 4*).
611 In fact, SL-CHL and SL-PLL coacervates were respectively imaged at pH 6.33 and pH 7.38
612 while the pH limits are 6.92 and 8.53, respectively. Therefore SL-CHL coacervates were
613 imaged at a later coacervation stage. Other parameters like the intrinsic molecular properties
614 of CHL and PLL could also affect the fine coacervates structure, the description of which is
615 out of the scope of this manuscript.

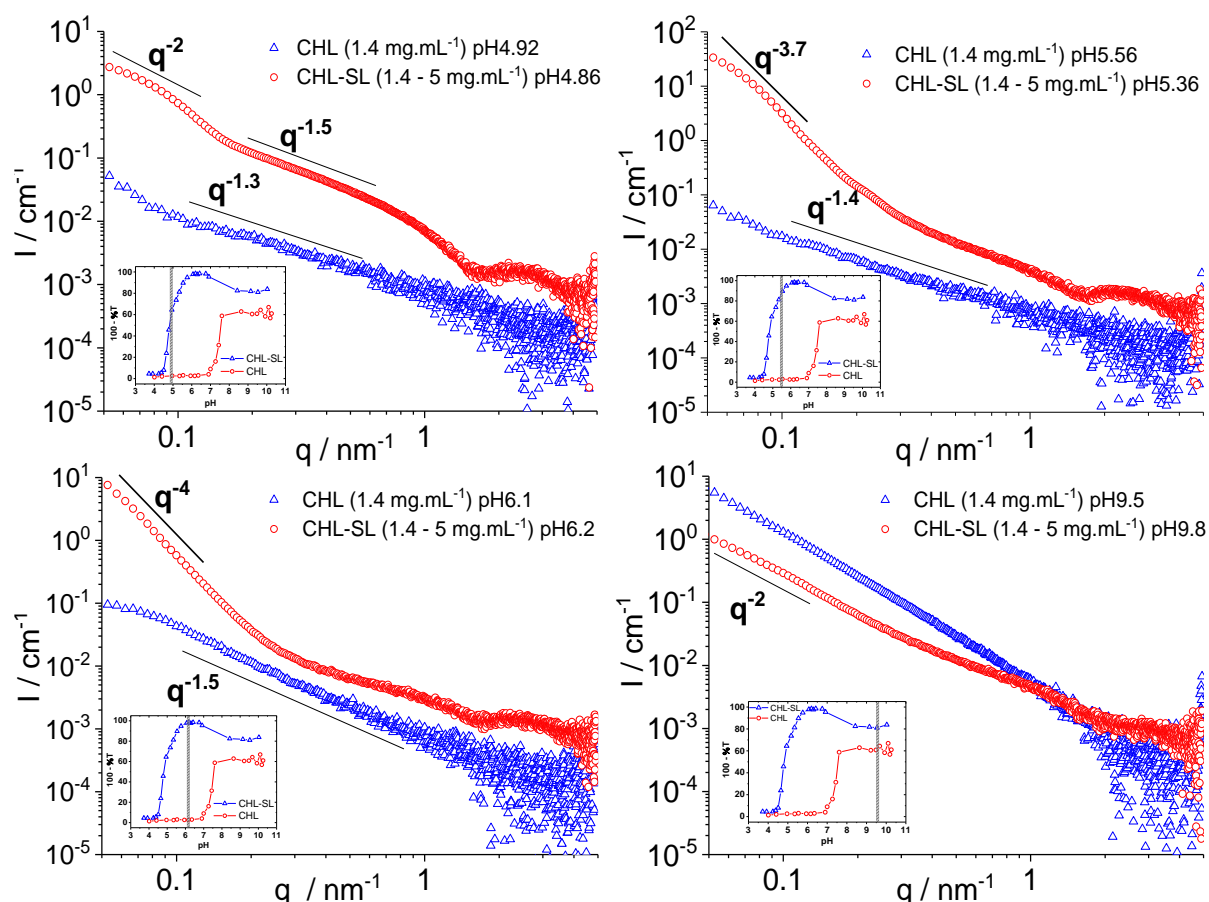
616 One should note that for SL-CHL system, the coexistence of both spherical coacervates and
617 aggregates structures at pH 5.94 and pH 6.33 were detected (Figure S 9). The coacervate
618 structures of SL-CHL, SL-PLL and SL-PAA at respectively pH 6.33, pH 7.38 and pH 6.83
619 are shown in Figure 7 at higher magnification with the corresponding Fast Fourier Transform
620 (FFT). One can notice that the internal organization depends strongly on the PEC. Moreover,
621 the coacervates from SL-CHL and SL-PLL exhibit a well-defined interface compared to the
622 SL-PAA. This observation is related to the coacervation stage. In fact SL-CHL and SL-PLL
623 coacervates were imaged at pH-values corresponding to 63% and 65% of the Region 3, while
624 SL-PAA coacervates were imaged at only 24% of the optimal coacervation region. Further
625 cryo-TEM images of SL-PEC coacervates at different pH are given in the electronic
626 supplementary information (Figure S 10
627 **Figure S 11**, **Figure S 12**, **Figure S 13**).



628
629 **Figure 7 – Zoomed cryo-TEM images of complex coacervate particles of SL-CHL at (a) pH 6.33, (b) SL-**
630 **PLL at pH 7.38 and (c) SL-PAA at pH 6.83. [SL]= 5 mg/mL, [CHL]= 1.4 mg/mL, [PLL]= 2 mg/mL, and**
631 **[PAA]= 0.75 mg/mL.**

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To further investigate the structure of SL-PEC coacervates and their pH evolution, SAXS measurements were conducted on SL-CHL and free CHL at different pH values (Figure 8), whereas SAXS/SANS data on SL micelles under similar conditions are reported elsewhere for comparison.^{63,67,68} In this study, the experimental q -range 0.05 nm^{-1} to 5 nm^{-1} is equivalent to sizes from 1.25 nm to 125 nm ($d \approx 2\pi/q$), which is suitable for studying the organization of the coacervates phase but not their entire structure, since some coacervates are larger than 200 nm , as previously shown by DLS, light microscopy and Cryo-TEM.



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Figure 8 - SAXS profiles of CHL solution and SL-CHL mixtures at different pH-values. [SL]= 5 mg/mL; [CHL]= 1.4 mg/mL

645 In the beginning of *Region 2*, below pH 5 (Figure 8a), SL-CHL is characterized by a
 646 scattering pattern typical of micelles in water for $q > \sim 0.8 \text{ nm}^{-1}$ and an increasing signal in the
 647 $\sim 0.1 < q/\text{nm}^{-1} < \sim 0.8 \text{ nm}^{-1}$ region (slope: -1.5), which most likely corresponds to the CHL
 648 contribution; its characteristic scattering profile recorded under similar conditions is given by
 649 the blue curve and it shows a slope of about -1.3 . A further increase in the scattering signal for

650 the SL-CHL system below $q \sim 0.8 \text{ nm}^{-1}$ indicates the presence of larger objects, thus
651 confirming the intensity-weighted DLS data in Figure 5a, discussed previously. Considering
652 the fact that free SL micelles in solution at acidic pH do not generally provide a pronounced
653 low- q signal,⁹¹ one could attribute such a signal to either the free CHL (the SAXS profile of
654 which in water also displays a small increase in the scattering signal at $q < 5 \text{ nm}^{-1}$, Figure 8a)
655 or to what was hypothesized before, that is pre-formed SL-CHL complexes. However, such a
656 contribution is small and, all in all, the SL-CHL pattern confirms that the system is mainly
657 constituted by micelles and free polymer in solution, in agreement with the previous DLS data
658 (Figure 5a, Figure S 7).

659 When the pH is set to be in the middle of *Region 2* (pH 5.56, Figure 8b), the SL-CHL
660 signal is still visible, although the low- q scattering contribution becomes more and more
661 important. The low- q slope is close to -4, which is expected in a scattering profile by a smooth
662 interface, attributed to the coacervate droplet surface. As a comparison, the signal of free CHL
663 has a slope of about -1.3, which has practically not evolved in *Region 3*, when the formation
664 of the complex coacervate is optimized, the SL-CHL SAXS pattern at $q < 0.2 \text{ nm}^{-1}$ is again
665 characterized by a frank -4 slope, while the high- q portion of the curve reflects the core of the
666 coacervate assembly (Figure 8c). Although the interpretation is not straightforward, one can
667 still observe the presence of an oscillation characteristics of a micellar form factor at $q > 1 \text{ nm}^{-1}$,
668 showing that the micellar structure is kept intact within the coacervate. The stability of SL
669 micelles during their binding to PEC comes from the absence of change of the solubilizing
670 capacity of micelles.⁹² Again, the scattering pattern of free CHL in the same pH range is not
671 comparable with the coacervate signal and it has not evolved since acidic pH: the Porod
672 exponent of CHL ranges between -1.3 and -1.5. Generally, a Porod exponent of ≈ -1 refers to
673 a rod-like structure, although deviations from a -1 power law may occur and they can be
674 explained by deviations of polymer chain linearity due to, e.g., intra-chain electrostatic
675 repulsion affecting the expected rigid rod-like chain conformation for a semi-flexible polymer
676 like CHL. One should note that the Porod exponent found here is close to -5/3, which is
677 generally attributed to the scattering profile of a swollen chain, corresponding to a polymer in
678 a good solvent,^{93,94} which is the case of CHL in water below pH 7. Finally, well above the
679 pKa of CHL (pH > 9.5, *Region 4*), the signal of both CHL and SL-CHL are now comparable at
680 $q < \sim 0.8 \text{ nm}^{-1}$, both characterized by a Porod exponent of -2, while one can still observe a mild
681 contribution of the micelles to the SAXS signal above $q \sim 0.8 \text{ nm}^{-1}$. The different oscillating
682 profile of SAXS signal for SL micelles observed at acidic and basic pH ($q > \sim 0.8 \text{ nm}^{-1}$) should

683 not be surprising because it has been observed before and described as a difference in terms of
684 neutral and charged SL arrangement within the micelle itself.⁹¹ In the end, the SAXS analysis
685 shows that in *Region 4* the system is composed of the free CHL polymer and in a Gaussian
686 chain conformation,^{93,94} and free charged SL micelles, as hypothesized above.

687 **Conclusions**

688 In this work, we demonstrate the ability of biobased sophorolipid bolaform
689 biosurfactant micelles to form complex coacervates with different cationic polyelectrolytes,
690 i.e. a naturally derived oligosaccharide and two synthetic polymers. The coacervation process
691 is mainly driven by pH and turbidimetric titration revealed that the coacervates can be formed
692 in a large pH range as function of the cationic polyelectrolyte type and concentration. The
693 charge-pairing mechanism is confirmed by quantitative NMR analysis, which also shows that
694 25% of the initial SL-polyelectrolyte concentration is involved in the coacervates.

695 The coacervation structure investigated by cryo-TEM shows the coexistence of polymer
696 and micelles upon coacervate formation and the presence of well-defined coacervates in their
697 stability region. Cryo-TEM suggests that micelles compose the coacervate and this piece of
698 evidence is confirmed by SAXS experiments, which show that micelles and free polymer
699 coexist and probably interact out of the coacervate-formation window. SAXS also shows that
700 coacervates are themselves composed of micelles. This description of the complex coacervate
701 formation between a chargeable bolaform surfactant and chargeable polyelectrolytes is
702 consistent with what has been described for more classical ionic surfactants-polyelectrolyte
703 systems.

704 Finally, this study offers new prospects for the use of bolaform sophorolipid micelles to
705 prepare complex coacervates which could be useful for pollutants and dye removal^{54,55} or like
706 an encapsulation matrix for drug delivery applications. In a general view, the valorization of
707 such bolaamphiphile molecules through the investigation of their binding behavior to further
708 macromolecules seems to be a promising approach to prepare future functional soft materials.

710 **Acknowledgments**

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1 ***ELECTRONIC SUPPLEMENTARY INFORMATION***

2

3 **Complex coacervation of natural sophorolipid bolaamphiphile**
4 **micelles with cationic polyelectrolytes**

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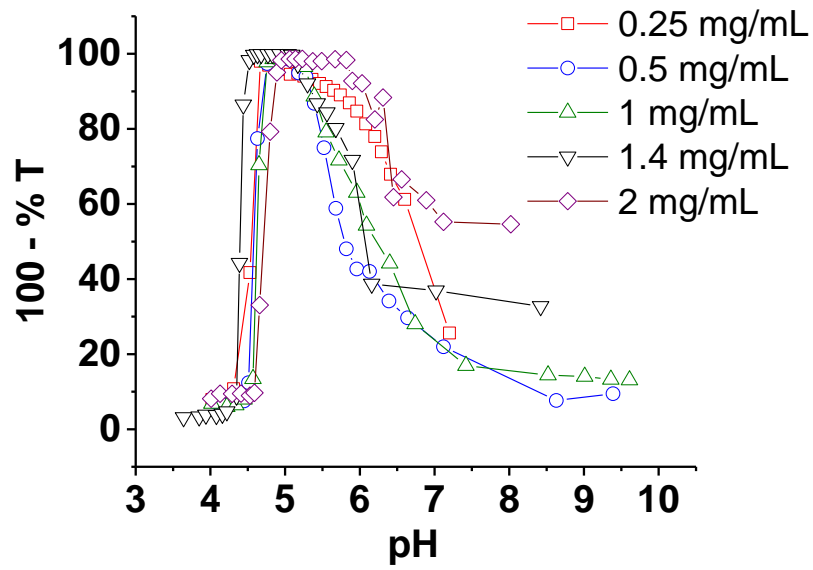
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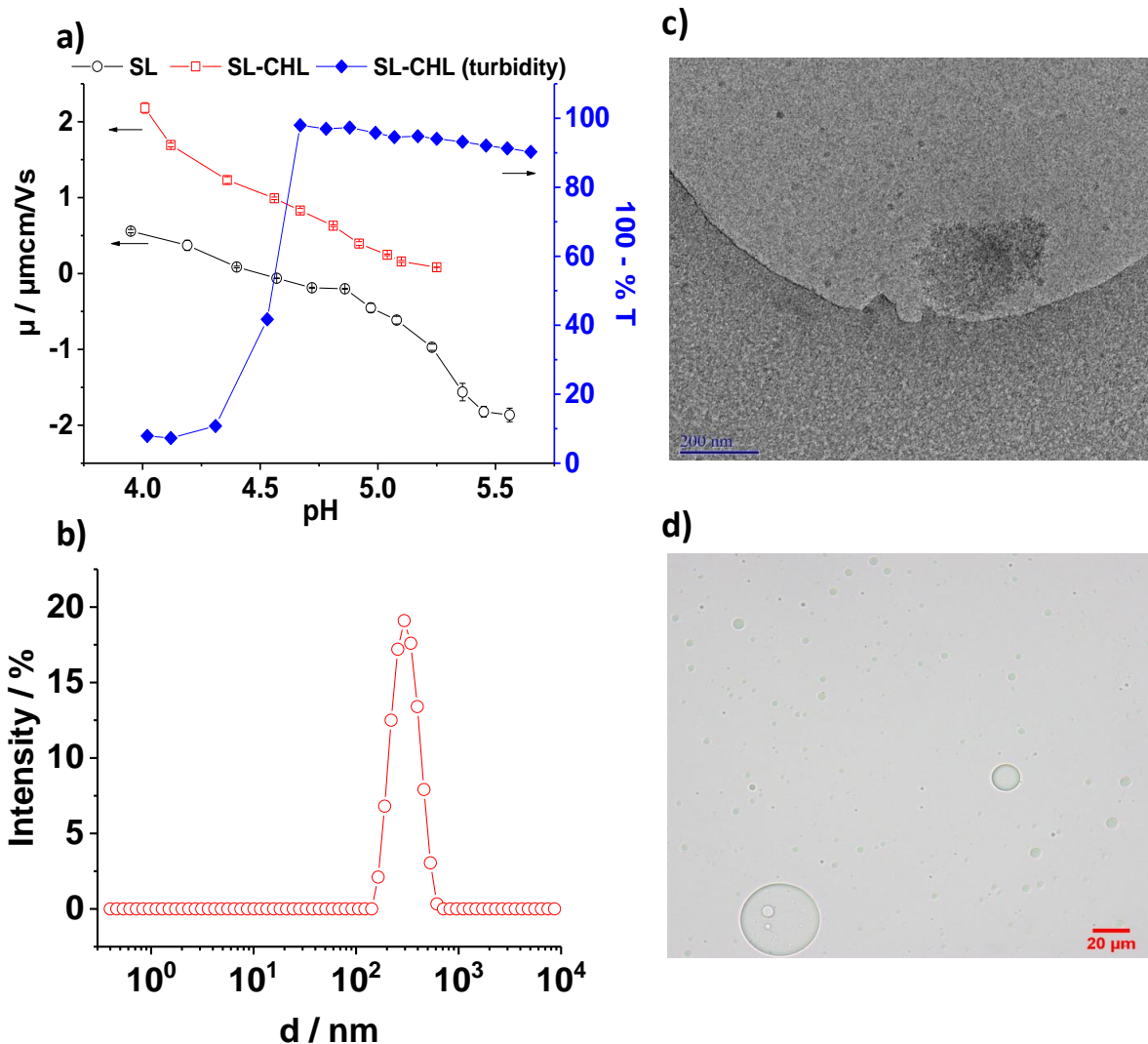
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26 **Figure S 1 - Evolution of turbidity during coacervation of SL micelles ([SL]= 10 mg/mL) with CHL**
 27 **([CHL]= 0.25 - 2 mg/mL) as function of pH.**

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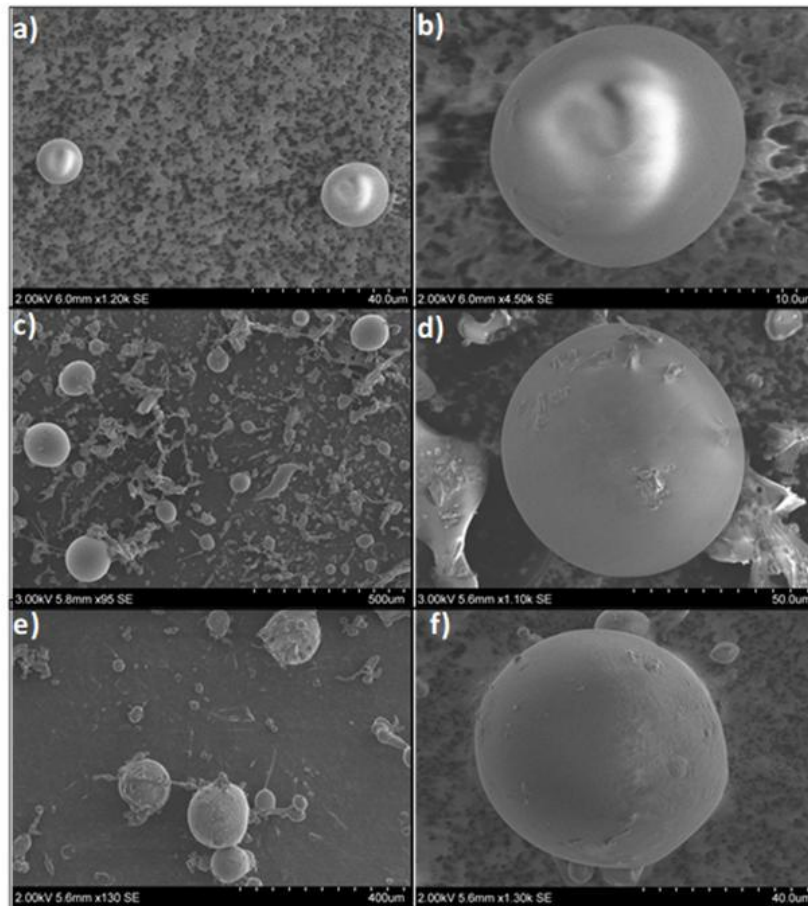


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 30 **Figure S 2 – SL-CHL ([SL]= 0.25; [CHL]= 10 mg/mL) sample: (a) evolution of the electrophoretic**
 31 **mobility and turbidity (100-%T) as function of pH; (b) size distribution at pH 4.56; (c) cryo-TEM image**
 32 **at pH 4.56 and (d) light microscopy large droplets at pH 5.2.**

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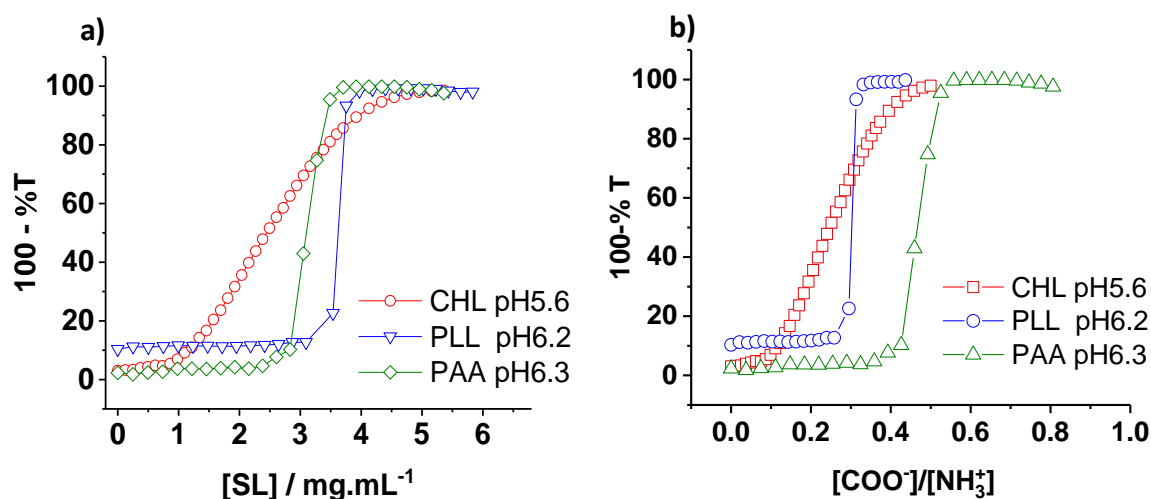
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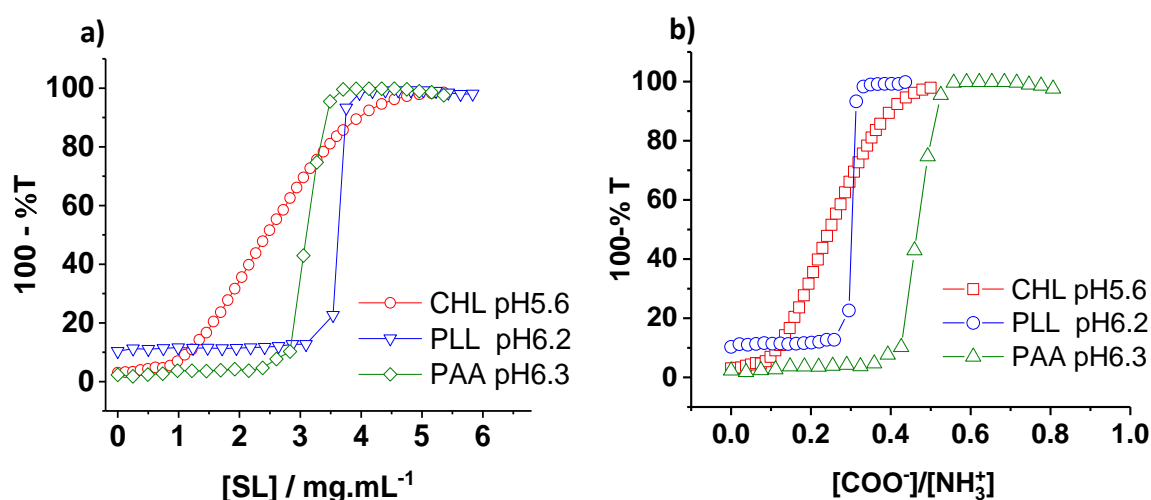
39 **Figure S 3 - SEM images of (a,b) SL-CHL ([CHL]= 1.4 mg/mL, pH 6.2), (c,d) SL-PLL ([PLL]= 2 mg/mL,**
40 **pH 7) and (e,f) SL-PAA ([PAA]= 0.75 mg/mL, pH 8) complex coacervates prepared with [SL]=5 mg/mL.**

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 43 **Figure S 4 - Evolution of the turbidity of PEC solutions during stepwise addition of SL solution as**
 44 **function of (a) SL concentration and (b) charge ratio. [CHL]= 1.4 mg/mL, [PLL]= 2 mg/mL, and [PAA]=**
 45 **0.75 mg/mL**
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47 The difference of the critical aggregation concentration (cac) among the three systems (



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 49 Figure S 4b) could be related to the molecular structure of each PEC. In theory, cac is usually
 50 lower than the cmc, however we are not able to explain the high cac compared to the cmc (0.1
 51 mg/mL) due to the complex behavior of SL. Indeed, during gradual addition of SL, free
 52 molecules can exist in solution or can even preferentially adsorb to the air-water interface to
 53 expose the intermediate aliphatic chain to the hydrophobic air phase. Furthermore, for
 54 bolaform surfactants, the relationship between the cmc and the free energy of micellization is
 55 complex and it requires taking into account the contribution of counterions.¹ The analysis

¹ R. Zana, Critical Micellization Concentration of Surfactants in Aqueous Solution and Free Energy of Micellization. *Langmuir*, 1996, 12, 1208-1211.

56 becomes even more complex due to the pH-sensitive nature of SL. In fact, the degree of
 57 ionization, also called the apparent degree of counterions dissociation,² will vary strongly with
 58 pH. In addition, the counterions distribution (condensation and release during electrostatic
 59 interaction) will also be affected by the binding process of SL micelles to each PEC.

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65 Determination of the ionization degrees

66 The evolution as function of the pH of the theoretical degrees of ionization values α for (SL,
 67 pKa 5.8) and β for (CHL, pKa 6.5), (PLL, pKa 10) and (PAA, pKa 9.5) were calculated as
 68 function of pH from the modified Henderson–Hasselbalch equation:

69 For SL:

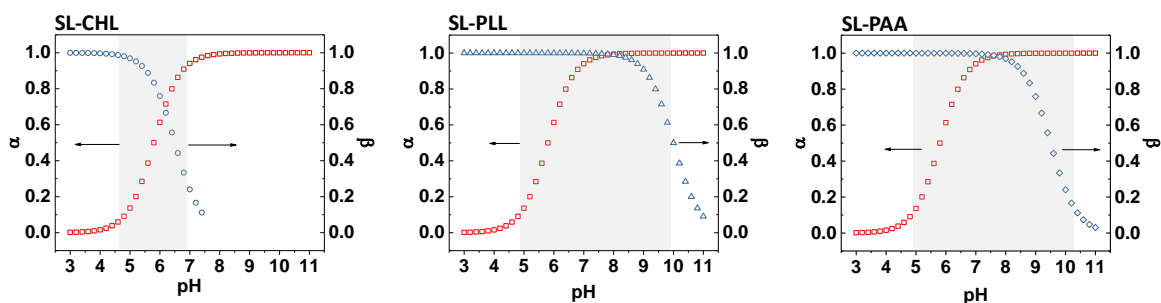
$$70 \quad pKa = pH + \log \frac{(1 - \alpha)}{\alpha}$$

$$71 \quad \text{Where} \quad \alpha = \frac{[COO^-]}{[COOH] + [COO^-]}$$

72 For PEC:

$$73 \quad pKa = pH + \log \frac{\beta}{(1 - \beta)}$$

$$74 \quad \text{Where} \quad \beta = \frac{[NH_3^+]}{[NH_2] + [NH_3^+]}$$



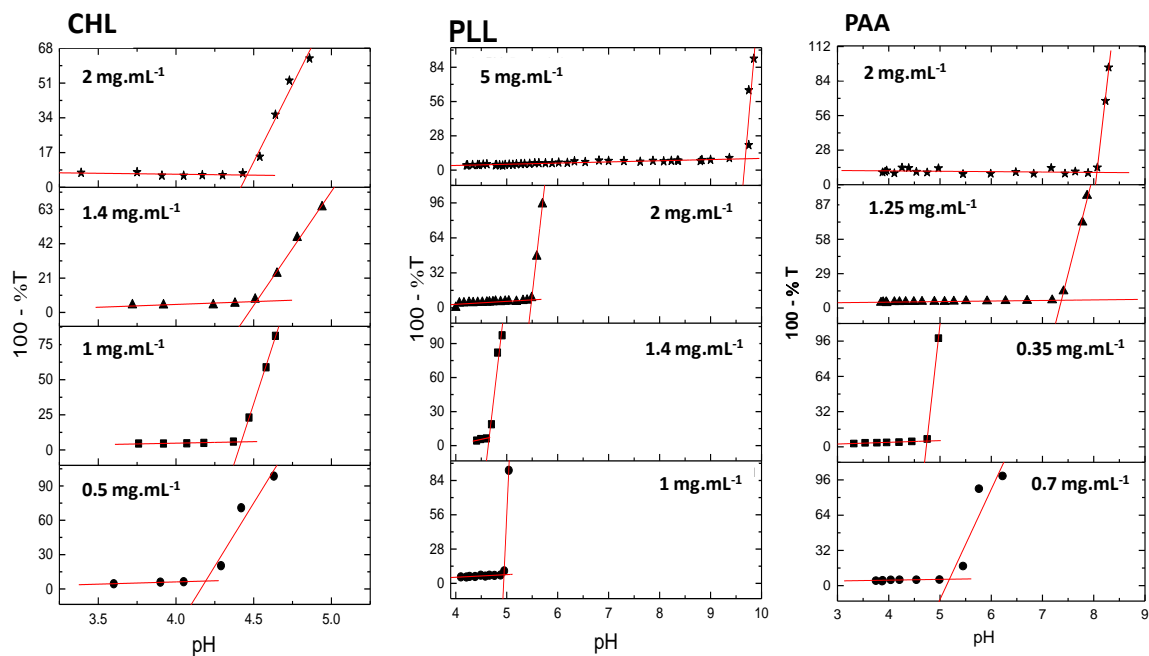
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Figure S 5 - Evolution of the theoretical degrees of ionization α and β as function of pH for SL and polymers

Determination of the charge ratio: $[COO^-]:[NH_3^+]$

² B. L. Bales, A Definition of the Degree of Ionization of a Micelle Based on Its Aggregation Number. *J. Phys. Chem. B*, 2001, 105, 6798–6804

79 The stoichiometric ratio for chargeable groups (-/+) and defined = $[S] / (n \times [P])^{3,4,5}$ where [S]
 80 and [P] are the molar concentrations for the SL and for the PEC, respectively and n is the
 81 number of PEC monomers or binding sites. If we consider that the average molecular weight
 82 of SL, CHL, PLL and PAA are respectively 633, 4000, 5000 and 17,500 g/mol. The CHL,
 83 PLL and PAA are therefore made from average monomers of 15, 23 and 128, respectively.
 84 However, for CHL the number of binding sites is assumed to be (15 x 2) because each
 85 monomer contains two amino groups. By including the theoretical ionization degrees (α and
 86 β) is therefore possible to estimate the charge ratio Z (-/+) or $[\text{COO}^-]:[\text{NH}_3^+]$ which will be
 87 equal to: $Z = (\alpha \times [S]) / (n \times \beta \times [P])$.
 88



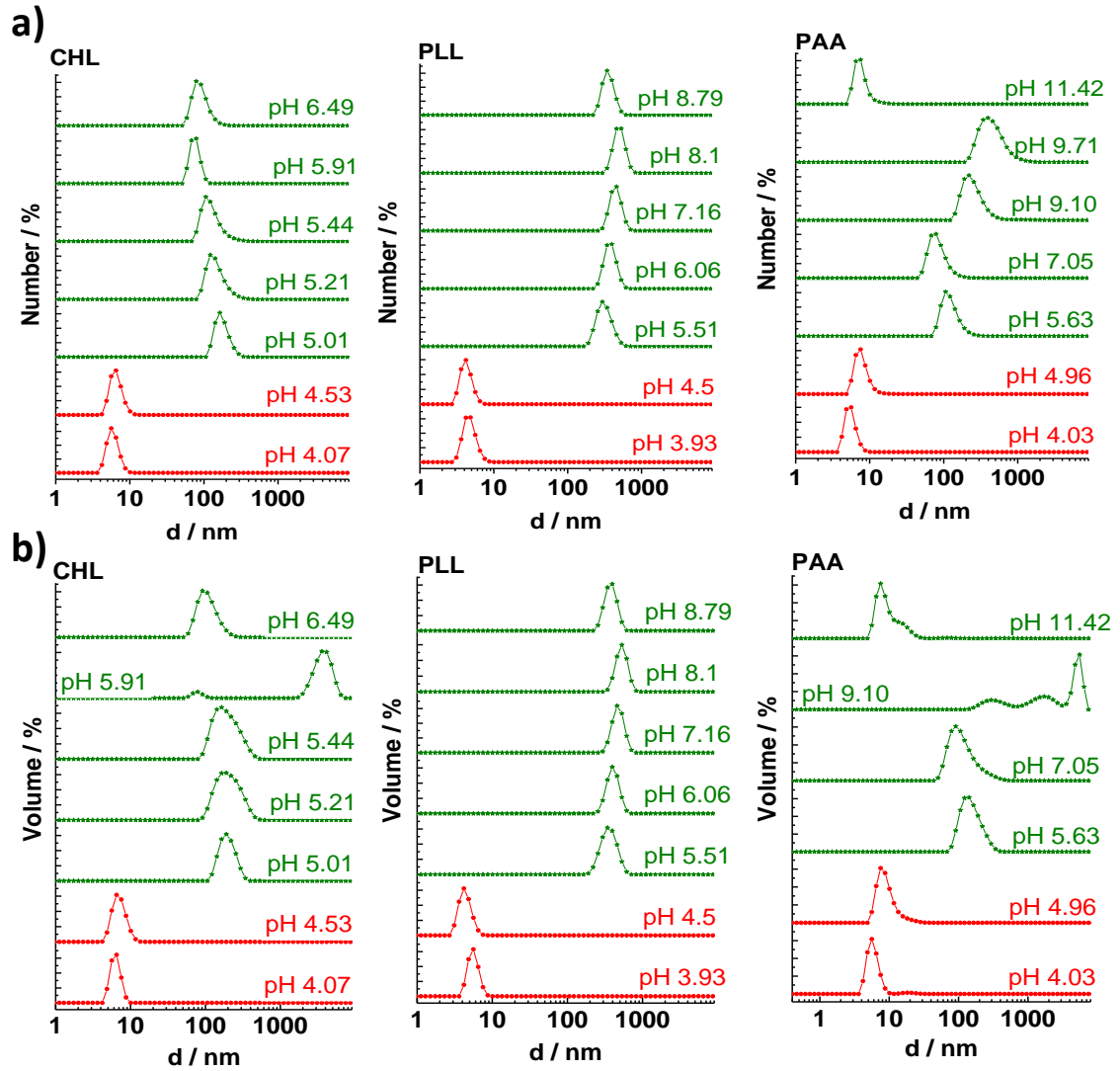
89
 90 **Figure S 6 - Determination of $\text{pH}\phi$ as points as the intercept of the initial linear portion of the curve with**
 91 **the tangent to the rapidly increasing portion of the curve. [SL]= 5 mg/mL.**

92
 93
 94

³ Hervé, P., et al. "Novel core-shell structure for colloids made of neutral/polyelectrolyte diblock copolymers and oppositely charged surfactants." *EPL (Europhysics Letters)* 58.6 (2002): 912

⁴ Berret, Jean-Francois, et al. "Colloidal complexes obtained from charged block copolymers and surfactants: A comparison between small-angle neutron scattering, Cryo-TEM, and simulations." *The Journal of Physical Chemistry B* 107.32 (2003): 8111-8118

⁵ Berret, Jean-François, et al. "Electrostatic self-assembly of oppositely charged copolymers and surfactants: A light, neutron, and X-ray scattering study." *Macromolecules* 37.13 (2004): 4922-4930



95

96 Figure S 7 - Evolution of the hydrodynamic diameter of SL- PEC complexes with pH, as function of the

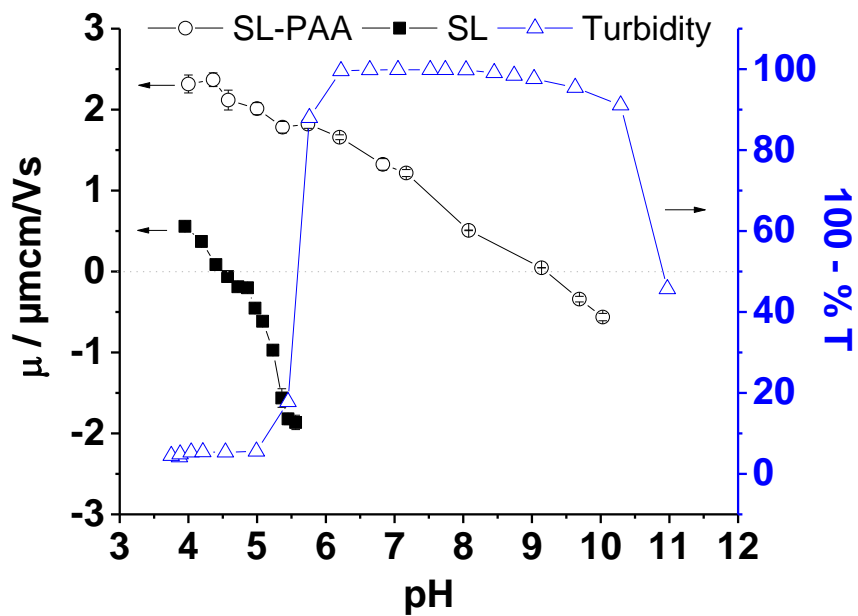
97 (a) relative number and (b) relative volume at 25 °C. The pH values are indicated above the curves. [SL]=

98 5 mg/mL, [CHL]= 1.4 mg/mL, [PLL]= 2 mg/mL, and [PAA]= 0.75 mg/mL

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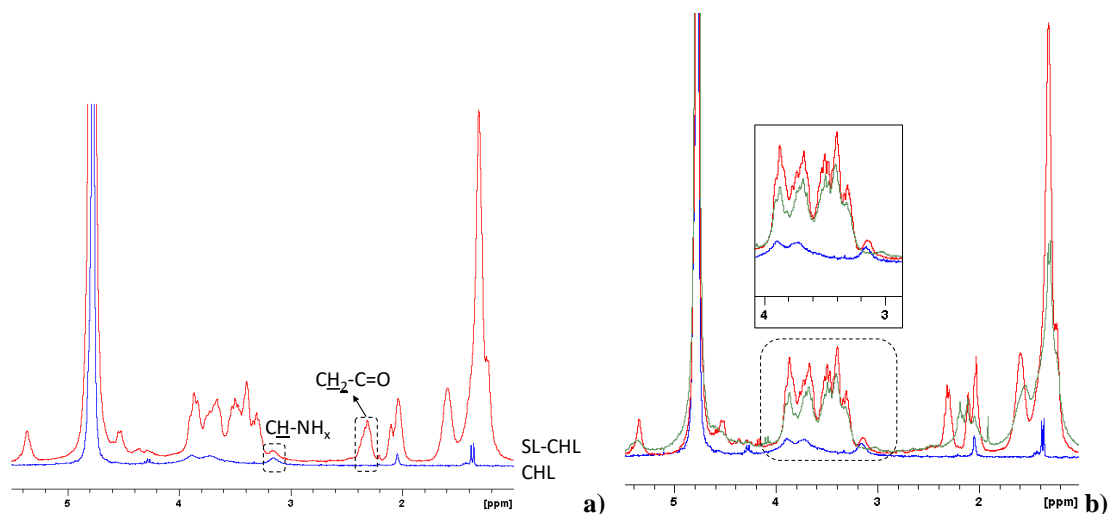
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 103 **Figure S 8 - Evolution of turbidity and electrophoretic mobility as function of pH during complex**
 104 **coacervation of SL-PAA ([SL]= 5 mg/mL, [PAA]= 0.75 mg/mL).**

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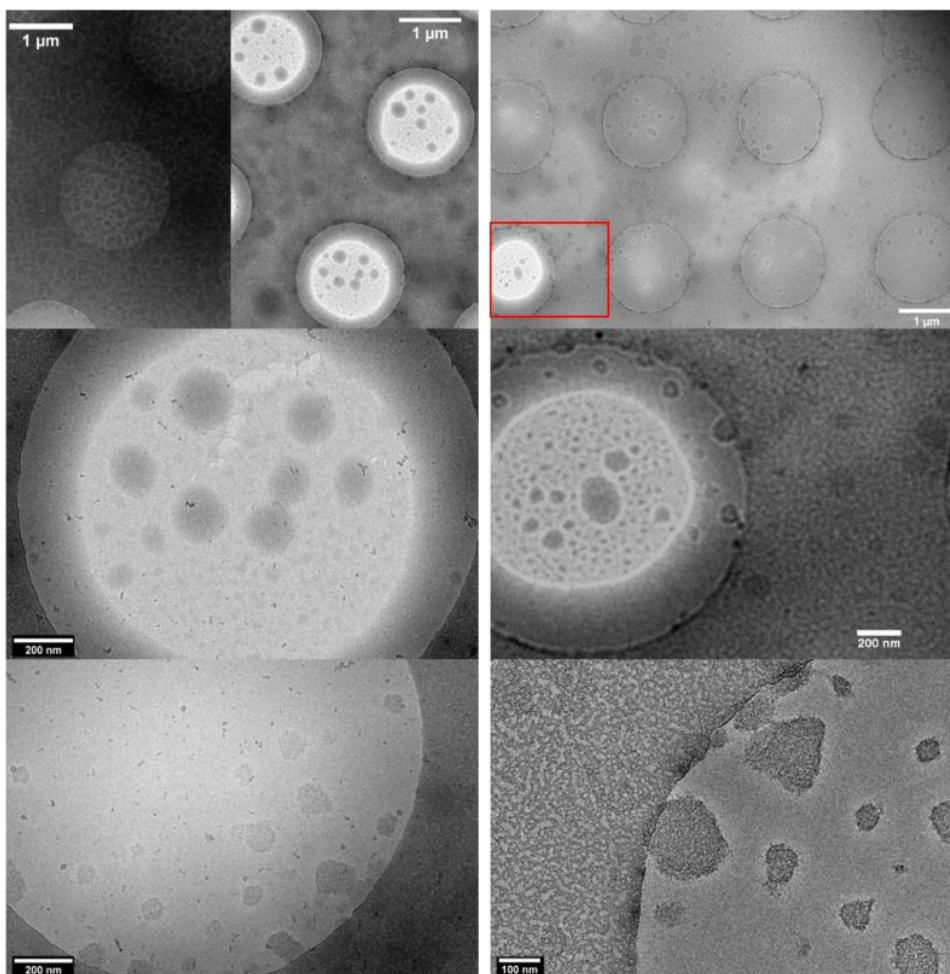


109 **Figure S 9 – Quantification of the a) C=O/NH_x molar ratio in the SL-polyelectrolyte mixture in the**
 110 **coacervation region and b) coacervation extent. Experiments are provided for the SL-CHL system. All**
 111 **experiments are carried out in D₂O. pH is adjusted with DCl and NaOD. The numerical data and relative**
 112 **discussion are provided in the main text.**

113 **a) ¹H NMR spectra of CHL solution (blue line, C = 1.4 mg/mL, pH 6.09). The red signal corresponds to a**
 114 **SL-CHL solution initially prepared in Region 3, on the coacervation plateau, (C_{SL} = 5 mg/mL, C_{CHL} = 1.4**
 115 **mg/mL, pH 6.12) and eventually centrifuged (3000 rpm, 1h) to recover the coacervate phase only; the**
 116 **coacervate is finally dispersed in 500 μL D₂O, intentionally set at pD < 5, out of the coacervation plateau.**
 117 **The CH-NH_x and CH₂-C=O integrals are used to quantify the C=O/NH_x molar ratio. b) ¹H NMR spectra**
 118 **of SL-CHL solution (C_{SL} = 5 mg/mL, C_{CHL} = 1.4 mg/mL) before (red line, pH 4.46) and after (green line,**
 119 **pH 6.12) coacervation. In blue, the signal of CHL (C = 1.4 mg/mL, pH 6.09). The spectra are superimposed**
 120 **as such; no adjustment of the relative intensity is operated. The highlighted region between 4 ppm and 3**
 121 **ppm is used to measure the intensity loss before and after coacervation.**

a) SL-CHL pH 5.94

b) SL-CHL pH 6.33



123

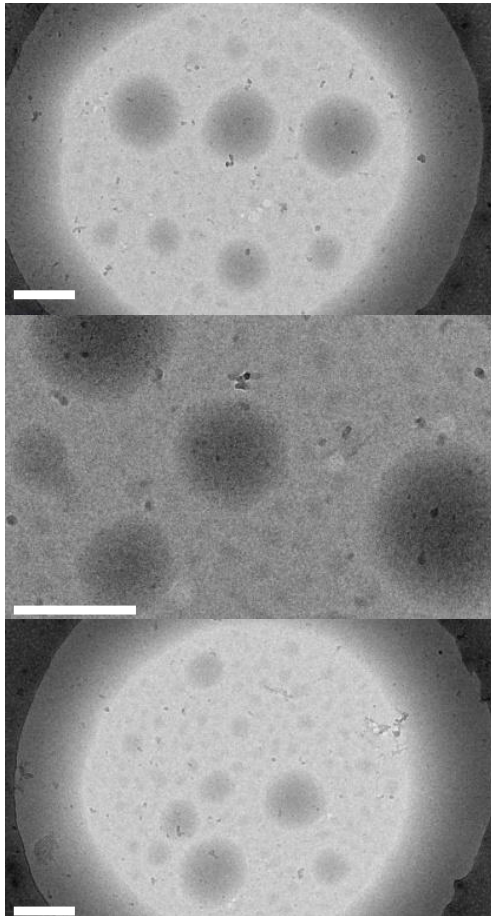
124 Figure S 10 - Cryo-TEM images of SL-CHL coacervates at pH 5.94 and pH 6.33 ([SL]= 5 mg/mL, [CHL]=
125 1.4 mg/mL).

126

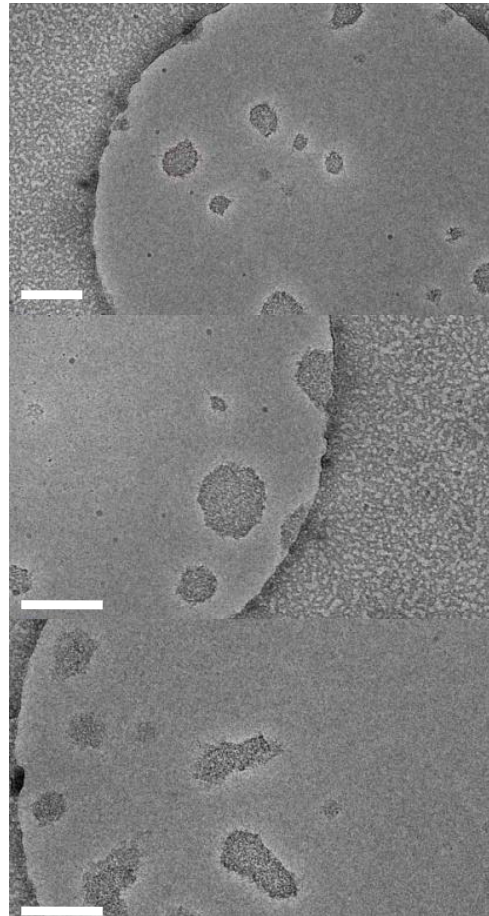
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SL- CHL pH 5.94



SL- CHL pH 6.33



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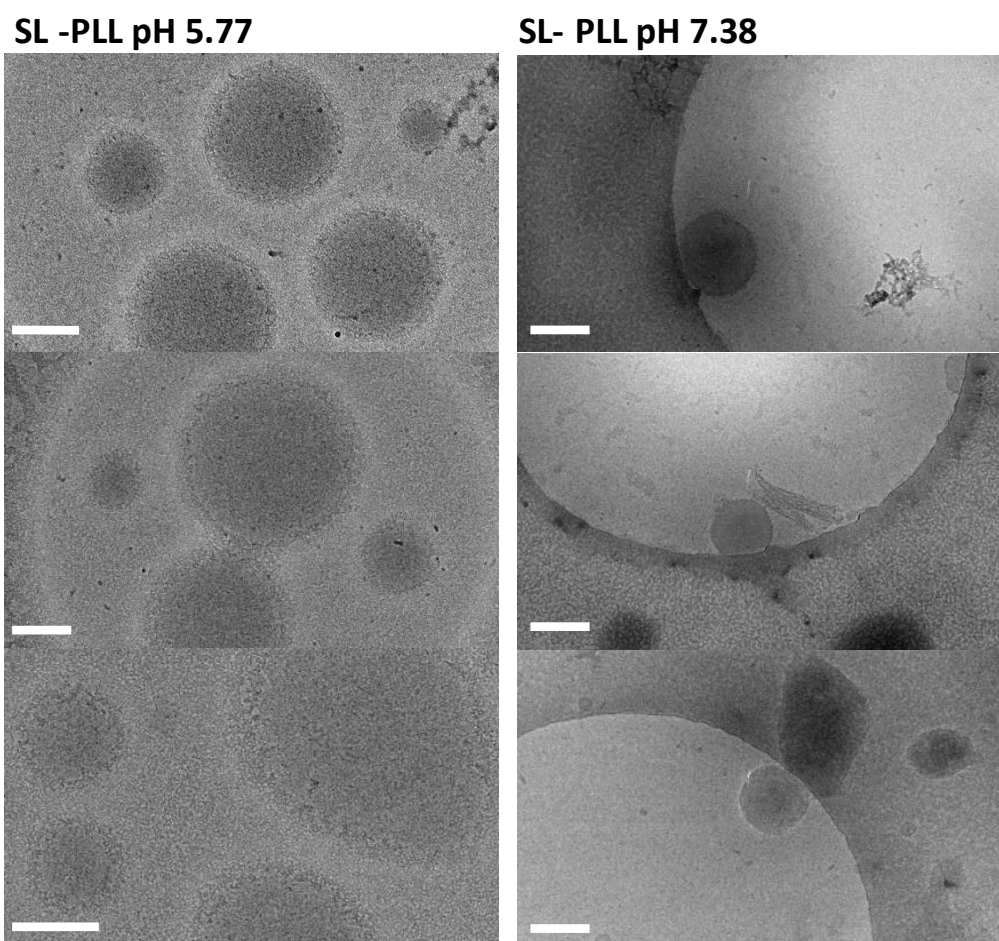
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Figure S 11 - Cryo-TEM images of SL-CHL coacervates ([SL]= 5 mg/mL, [CHL]= 1.4 mg/mL). Scale bar is 200 nm

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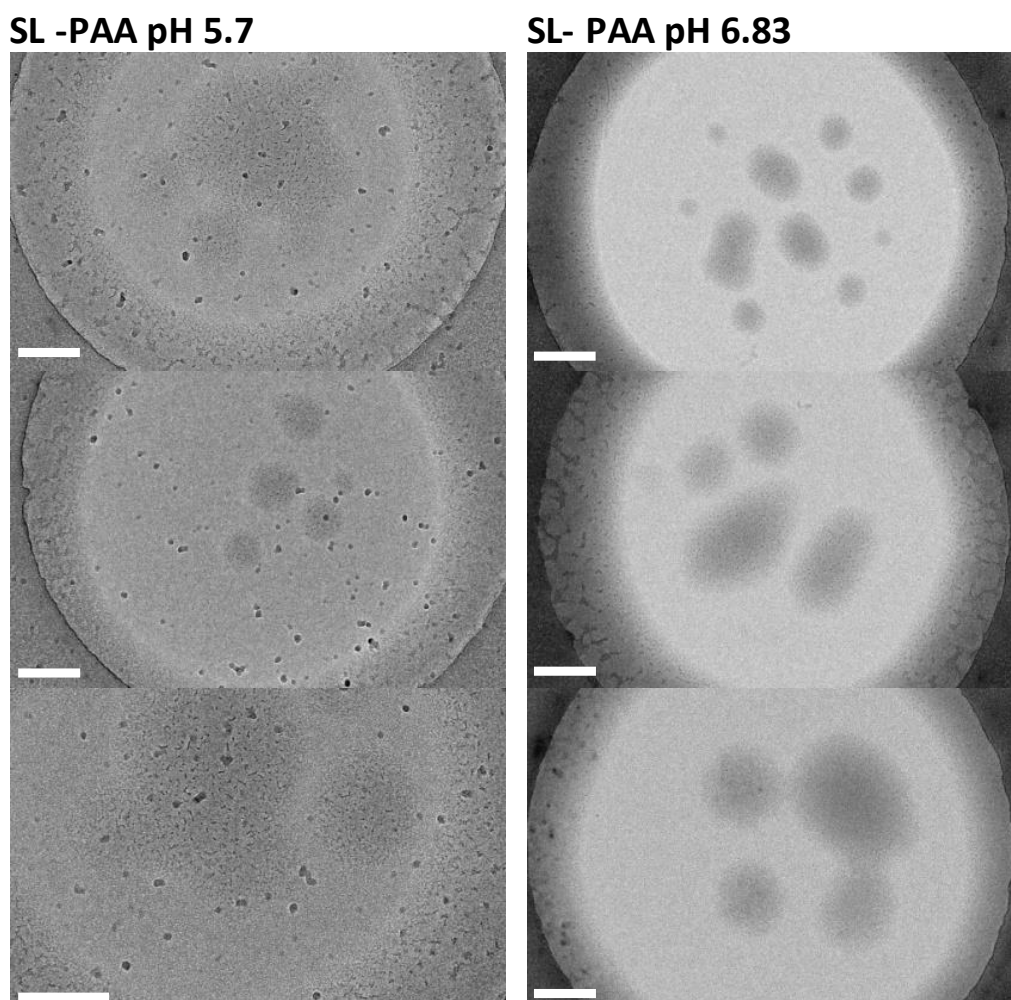
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137 **Figure S 12 - Cryo-TEM images of SL-PLL coacervates ([SL]= 5 mg/mL, [PLL]= 2 mg/mL). Scale bar is**

138

200 nm

139



141

142 **Figure S 13 - Cryo-TEM images of SL-PAA coacervates ([SL]= 5 mg/mL, [PAA]= 0.75 mg/mL). Scale bar**

143

is 200 nm

144