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3

4 **Probing the organization of fulvic acid using a cationic surfactant**

5

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40

41 **Abstract**

42

43 The organization of Suwannee River Fulvic Acid (SRFA) is investigated through the
44 interaction of SRFA with a cationic surfactant molecule (Dodecyl-trimethylammonium
45 chloride (DTAC)). Turbidity measurements, Dynamic light scattering, electrophoretic
46 mobility, surface tension, and cryo-transmission electron microscopy are combined to
47 describe the SRFA/DTAC molecular structures thus obtained. Increasing DTAC
48 concentration, fulvic acid-rich unilamellar vesicles, globules, aggregates of globules,
49 disks, DTAC-rich unilamellar vesicles, and loosely aggregated spheroidal structures, are
50 successively observed. Such sequence of molecular structures is typically found in
51 phase diagrams of catanionic systems (mixture of oppositely charged surfactants). The
52 strong surface activity of fulvic acid/DTAC complexes and the geometrical constraints
53 associated with the formation of vesicles imply that a major component of SRFA is an
54 individual amphiphile negatively charged of molecular size similar to that of DTAC.
55 This soft matter approach supports a supramolecular organization for SRFA.

56

57 **Keywords:** Suwannee River Fulvic Acid, cationic surfactant, supramolecular
58 organization, catanionic systems, cryo-transmission electron microscopy.

59

60 **1. Introduction**

61

62 Humic substances (HS), operationally classified according to their solubility into fulvic
63 acid, humic acid, and humin, are ubiquitous organic compounds in soils and aquatic
64 systems [1]. They are formed from the by-products of organic material degradation (*e.g.*

65 plant debris, microorganisms) but also in part from the condensation of small organic
66 molecules [2][3][4][5]. Besides their obvious role in carbon geocycling, HS have been
67 shown to be key components in the transport/sequestration of xenobiotics and metal
68 contaminants in the environment [6][7].

69

70 Extensive characterization of HS revealed that those compounds not only contain a
71 large proportion of carboxylic and phenolic groups that make them hydrophilic, but also
72 aliphatic moieties that give them surface active and hydrophobic-binding properties
73 [8][9]. A characteristic size scale of HS determined by various techniques is about 1-2.5
74 nm [10][11][12][13][14][15]. Changes in solution pH, adsorption experiments and
75 complexation experiments with polycations, have revealed the flexibility of HS
76 structure [16][17][18][19][20]. Furthermore, modelling of their charge characteristics
77 suggests that HS can be regarded as soft and porous structures [21].

78

79 Still, the detailed organization of HS remains a matter of debate: molecular weight
80 measurements performed at different stages of extensive fractionation schemes suggest
81 that HS can be regarded as macromolecules or slightly branched polymers that can coil
82 or adopt an extended conformation according to solution properties [1][22][23][24],
83 whereas some Size Exclusion Chromatography experiments and NMR results suggest
84 that HS correspond to supramolecular associations of small heterogeneous molecules
85 that can be disrupted in the presence of organic acids [6][10][25][71][72][73]. To
86 account for the surface-active properties of HS and the enhanced solubility of
87 hydrophobic organic compounds in their presence [26], such molecular aggregates have
88 also been described as micelles with a hydrophilic exterior and a hydrophobic core [9].

89 A-middle-of-the-road view has also been taken, the fulvic fraction being identified as a
90 supramolecular assembly, and the humic fraction corresponding to the coexistence of
91 both macromolecular and supramolecular patterns [27].

92

93 The main goal of the present paper is to assess which of the two structural concepts,
94 polymeric or supramolecular, is the more appropriate for Suwanee river fulvic acid
95 (SRFA), a reference organic material provided by IHSS. For that purpose, we
96 investigate the association between SRFA and various concentrations of Dodecyl-
97 trimethylammonium chloride (DTAC), a cationic surfactant molecule. Adding an
98 oppositely charged amphiphilic molecule to SRFA is expected to induce drastic
99 restructurations of the fulvic colloid driven by both electrostatic and hydrophobic
100 interactions. Previous works followed a similar strategy by exploring the structure of
101 humate/alkylammonium chloride precipitates using X-ray diffraction [28] and Small-
102 Angle X-ray Scattering [29], whereas most other studies dealing with the association of
103 a surfactant with humic substances were carried out in the context of contaminant
104 transport in the environment, and focused on the binding of the detergent to humic
105 colloids [30][31][32][33].

106

107 The various molecular structures formed upon addition of DTAC to a SRFA suspension
108 are investigated using turbidity and Dynamic Light Scattering (DLS) measurements that
109 provide a rapid assessment of complex formation, electrophoretic mobility and surface
110 tension measurements that yield information about the extent of neutralization and
111 surface active properties of SRFA/DTAC complexes, and Cryogenic Transmission
112 Electron Microscopy (Cryo-TEM) that directly images those complexes. We show that

113 such soft matter approach provides additional tools to assess the organization of humic
114 substances.

115

116 **2. Experimental Section**

117

118 *2.1 Materials*

119

120 Suwannee River Fulvic acid (SRFA) was obtained from the International Humic
121 Substances Society (IHSS) (Fulvic Acid Standard 2S101F). SRFA is extracted from the
122 Suwannee river, a blackwater river that flows from the Okefenokee Swamp (Georgia),
123 characterized by a pH of 4 and a Dissolved Organic Carbon (DOC) content ranging
124 from 25 to 75 mg/L [34]. Dodecyltrimethyl ammonium chloride ($C_{15}H_{34}ClN$ - DTAC)
125 was purchased from Sigma-Aldrich (purity 99%). With a Krafft temperature below 0
126 °C, DTAC is readily soluble in water at ambient temperature [35][36] and its critical
127 micelle concentration (CMC) at 30 °C is 21.7 mmol/L [37]. Both fulvic acid and
128 surfactant were used without further purification.

129

130 *2.2 Sample preparation*

131

132 The DTAC stock solution was prepared at 10 g/L in deionised water (Millipore-MilliQ
133 18.2 Ω .cm). The concentration of the SRFA stock solution, and hence the range of
134 DTAC concentrations, were adapted to the technique of investigation. For Turbidity,
135 Dynamic Light Scattering, Surface Tension and Electrophoretic Mobility
136 measurements, a SRFA suspension of 20 mg/L, *i.e.* 12 mg C/L, was prepared by adding

137 the appropriate mass of fulvic acid into 500 mL of deionised water. For Cryo-TEM,
138 stock suspensions of 20 mg/L and 1g/L were used. The SRFA stock solutions were
139 prepared daily and mechanically mixed overnight in the dark to obtain a well-dispersed
140 suspension. The dissolved organic carbon was checked for each prepared stock solution
141 (Shimadzu TOC-Vcsn).

142

143 SRFA/DTAC complexes were prepared by adding aliquots of the surfactant stock
144 solution to 10 mL of SRFA suspension and by submitting the mixture to gentle
145 overhead agitation (3 times). Both DTAC and fulvic acid stock solutions as well as
146 SRFA/DTAC samples were maintained at 30°C in a heating bath during the
147 experiments, *i.e.* a temperature at which the alkyl chain of the surfactant is flexible.

148

149 *2.3 Characterization of SRFA/DTAC complexes*

150

151 The turbidity of SRFA/DTAC suspensions was measured as a function of time using a
152 HACH LANGE 2100Q turbidimeter. Conductivity and pH (WTW Multiline F/SET),
153 surface tension (KRUSS K12 Processor Tensiometer using a Wilhelmy plate), the
154 electrophoretic mobility and the size of the fulvic acid-surfactant aggregates (Malvern
155 Zetasizer Nano ZS) were measured at the end of the experiment.

156

157 Cryo-TEM observations were carried out at the Multiscale Electron Imaging (METI)
158 platform at the University of Toulouse using a *JEOL 2100 Lab6* transmission electron
159 microscope equipped with a *Gatan 626* cryoTEM grids holder. Images were recorded
160 with a *Gatan ultrascan1000* camera under low dose conditions (about 10 electrons per

161 Å²). 4 µL of SRFA/DTAC suspension was deposited onto a LACEY/CARBON FILM
162 grid previously ionized using PELCO-easi Glow 91000 glow discharging-cleaning
163 system. After blotting for 0.2 second to remove the excess added volume (*Leica* EM GP
164 controlled vitrification system at 30 °C and 85% relative humidity), the grid was
165 immediately plunged into liquid ethane at its freezing point -185 °C and then stored in
166 liquid nitrogen. The size distribution of SRFA/DTAC complexes was obtained by
167 counting the aggregates in logarithmically spaced size bins such that $d_{i+1} = 2^{1/3} d_i$, which
168 yields equally spaced size intervals on a x-axis log scale [38]. The mean aggregate size
169 D was calculated from:

$$D = \frac{\sum_{i=1}^{i=k} n_i D_i}{\sum_{i=1}^{i=k} n_i}$$

170 where n_i is the number of aggregates measured in range i , D_i is the middle of class
171 range, and k is the total number of bins.

172

173 3. RESULTS

174

175 3.1 Turbidity Measurements

176

177 Figure 1 shows the temporal evolution of the turbidity of SRFA/DTAC mixtures
178 obtained after various DTAC additions to a 20 mg/L fulvic acid suspension at natural
179 pH (4.32). At low and high DTAC concentration, the turbidity increases to reach a
180 plateau after about 25 minutes, whereas in the 2.8-4.7 mmol/L range, the turbidity
181 evolution is characterized by a rapid early overshoot followed by a slow stabilizing

182 trend. For all surfactant additions, the pH of the SRFA/DTAC mixtures remains close to
183 that of the initial fulvic acid solution (figure S1 in Supporting Information).

184

185 At steady-state, *i.e.* after about two hours, the variation of turbidity with DTAC
186 concentration is characterized by two well-defined peaks (fig. 2a). Such variation may
187 be the result of SRFA/DTAC interactions but, as DTAC is a salt, could also be related
188 to a change in solution ionic strength. As illustrated in figure 2, substituting either NaCl
189 or the analogous surfactant of shorter alkyl chain Octyltrimethylammonium chloride
190 (OTAC) to DTAC, led to an equivalent linear increase in solution conductivity without
191 any change in the turbidity of SRFA suspensions. Therefore, the main features of the
192 SRFA/DTAC turbidity curve should be predominantly explained by the association of
193 SRFA with both oppositely-charged polar head groups and hydrophobic alkyl chains of
194 DTAC.

195

196 *3.2 Electrophoretic mobility, DLS and Surface Tension measurements*

197

198 Figure 3 shows the electrophoretic mobility and the particle size of SRFA/DTAC
199 complexes as a function of surfactant concentration. In each graph, the turbidity curve is
200 superimposed for comparison. Unexpectedly, at low DTAC concentration, the
201 electrophoretic mobility becomes more negative upon the addition of cationic surfactant
202 to reach a minimum that coincides with the first turbidity peak. The electrophoretic
203 mobility then increases to reach positive values above 3.5 mmol/L, and finally stabilizes
204 in the decreasing part of the second turbidity peak. The opposite patterns followed by
205 turbidity and electrophoretic mobility curves imply a significant reorganization of the

206 fulvic colloid since new negatively charged sites have become electrokinetically-active
207 during the charge neutralization of the anionic functional groups of fulvic acid with
208 DTAC. Such result is consistent with Duval et al. [21] view of humic substances as
209 permeable and flexible structures, the neutralization of SRFA "external" sites by the
210 surfactant turning the fulvic structure inside out and exposing "internal" ionogenic sites.
211 On the other hand, the lack of correlation between those same curves in the
212 concentration range of the second aggregation peak suggests an association between
213 DTAC and SRFA essentially driven by hydrophobic interactions in the presence of
214 excess surfactant. Those hydrophobic interactions may originate either from previously
215 hidden hydrophobic moieties displayed by the reorganization of SRFA, or from the
216 hydrophobicity of SRFA/DTAC complexes that facilitates the sorption of
217 supplementary surfactant molecules.

218

219 The particle size measured by DLS at 2 h remains in the 80-130 nm range at the start of
220 the first turbidity peak, reaches a maximum in the valley between the two turbidity
221 peaks when neutral SRFA/DTAC complexes are formed, and then decreases
222 substantially to stabilize around 150 nm in the DTAC concentration range of the second
223 turbidity peak (fig. 3b). Such behaviour implies that the two increases in turbidity
224 correspond to the formation of an increased number of individual entities rather than the
225 formation of aggregated structures, which actually occurs around a DTAC concentration
226 of 3.5 mmol/L in the valley between the turbidity peaks. All DLS plots exhibited a
227 monomodal size distribution except at a DTAC concentration of 0.19 mmol/L where
228 two populations centered on 16 nm and 80 nm were detected (inset of fig.3b).

229

230 According to Ritchie and Perdue [39], the potentiometric proton titration of SRFA (0.36
231 g in NaCl 0.1 M) yields an acidity of 6.44 meq/g Carbon at pH 4.2. This should
232 correspond to an initial charge of our fulvic acid solution of less than 0.077 meq
233 (Dissolved organic carbon of 12 mgC/L), which then implies that most of the added
234 surfactant remains in solution when neutral SRFA/DTAC complexes are obtained. Such
235 result is consistent with the adsorption isotherms reported in the literature for similar
236 cationic C12-surfactant/fulvic acid systems [33].

237

238 Figure 4 shows that the surface tension is drastically reduced at very low DTAC
239 concentration, and then further diminishes gradually in successive small steps
240 corresponding to the concentration range of turbidity peaks and the valley in between.
241 This reveals that fulvic acid/DTAC complexes are highly surface active, since values
242 around 40 mJ/m^2 are only attained around CMC with pure DTAC [40]. Such effect,
243 already noted by Gamboa and Olea [32], is reminiscent of the strong cooperative effect
244 observed between oppositely charged surfactants at the air-water interface [41].

245

246 *3.3 Cryo-TEM observations*

247

248 CryoTEM observations performed with 20 mg/L SRFA suspensions at DTAC
249 concentrations of 1.8 mmol/L and 7.58 mmol/L, allow the identification of
250 SRFA/DTAC molecular structures that make up the turbidity peaks. As illustrated in
251 figure 5, poorly formed unilamellar vesicles and spongy networks are formed at those
252 DTAC concentrations. The term vesicle is used here to describe the general vesicular-
253 like architecture and in no way implies that the wall of the vesicle is made from a lipid

254 bilayer. It is worth noting that the size of vesicles is comparable with that of the minor
255 population at 16 nm detected by DLS. From the electrophoretic mobility measurements,
256 it appears that the fulvic-rich vesicles formed at low DTAC concentration are negatively
257 charged whereas the DTAC-rich vesicles obtained at higher surfactant concentration are
258 positively charged.

259

260 Further cryo-TEM experiments conducted with a SRFA concentration of 1g/L provide a
261 more complete picture of the interaction of fulvic acid with DTAC. At 1g/L, the pH of
262 the SRFA suspension is 2.95, hence the charge of fulvic acid is smaller, and the DTAC
263 concentration range that destabilizes SRFA is much lower. Figure 6 presents the various
264 molecular structures obtained upon mixing SRFA with various DTAC concentrations.
265 Unlike DTAC solutions that yield featureless micrographs (see figure S2 of the
266 supporting information), the reference fulvic acid suspension (fig. 6a), *i.e.* 0 mmol/L
267 DTAC, exhibits globular structures about 25-32 nm in diameter, as well as small
268 aggregates of those globules (inset of fig. 6a). The size range obtained for the individual
269 globules is in good agreement with previous SANS measurements performed with
270 fulvic and humic acids in similar conditions (pH 5 and about 3 g/L concentration) [42].
271 Smaller values of radii-of-gyration have been reported for SRFA at higher pHs, which
272 suggests that the globular structures correspond to aggregates of SRFA [43][44][45].
273 Addition of $7.6 \cdot 10^{-3}$ mmol/L of DTAC leads to the formation of unilamellar vesicles,
274 both complete and open, mostly in the 12-20 nm range (fig. 6b1 and 6b2), as well as
275 sparse spongy networks with budding vesicles attached on the branches (fig. 6b3 and
276 6b4). Further addition of cationic surfactant, *i.e.* $3.03 \cdot 10^{-2}$ mmol/L, increases the average
277 size of the vesicles (30.3 nm) that now coexist with globular structures of similar size

278 and aggregates of those globules (inset of fig. 6c). At a DTAC concentration of $5.67 \cdot 10^{-2}$
279 mmol/L, the unilamellar vesicles can no longer be identified and only merging
280 aggregates of globules are found (fig. 6d).

281

282 Another kind of morphology, *i.e.* aggregates of disk-like structures, is observed after 45
283 minutes at a surfactant concentration of $7.56 \cdot 10^{-2}$ mmol/L (fig. 6e1). At longer times
284 (101 min), cryo-TEM examination reveals that the platelets become larger and that they
285 tend to adopt a hexagonal shape implying a well-defined local structure (fig. 6e2).
286 When the surfactant concentration is further increased ($18.8 \cdot 10^{-2}$ mmol/L), unilamellar
287 vesicles (mean size around 19 nm) are again spontaneously formed (fig. 6f1 and 6f2).
288 As for the 20 mg/L SRFA concentration, those vesicles are generally closed and more
289 abundant than at lower DTAC concentration. Less developed spongy-like networks with
290 nascent vesicles are also frequently observed (fig. 6f3 and 6f4). Finally, at the highest
291 surfactant concentration investigated ($74.3 \cdot 10^{-2}$ mmol/L), loosely aggregated spheroidal
292 structures (~ 22.8 nm) are found (fig. 6g).

293

294 The size histograms of SRFA/DTAC complexes observed by cryo-TEM and the
295 evolution of their mean diameter as a function of surfactant concentration are shown in
296 figure S3 (Supporting Information). All distributions are monomodal with a slight
297 skewness towards larger sizes when an aggregation phenomenon has been detected on
298 the micrographs. In those cases, the size distributions are certainly biased because the
299 large aggregates can be drained away during the blotting step of sample preparation
300 [46].

301

302 **4. DISCUSSION**

303

304 *4.1 Self-assemblies of fulvic acid and DTAC*

305

306 Over the years, many studies have reported a variety of molecular structures adopted by
307 humic and fulvic acids under various conditions of pH, background electrolyte, and
308 coagulant concentration. Using TEM, Baalousha et al. [15] and Wilkinson et al.
309 [47][48] evidenced networks of fibrils and globules ranging in size from 50 to 300 nm.
310 Above a 1gC/L in humic concentration, Myneni et al. [49], using X-ray microscopy,
311 found globules, ring-like, sheet-like, and thread-like structures in the 200-1200 nm size
312 range, by varying the pH, ionic strength of the solution, and nature of the counterion.
313 Using the same technique, Sieliechi et al. [20] also observed the formation of 80 nm
314 globules during the coagulation of a 10 mgC/L aquagenic humic acid in presence of
315 hydrolyzed-Fe species. Maurice and Namjesnik-Dejanovic [50], Plaschke et al. [51],
316 used Atomic Force Microscopy to image fulvic and humic acid adsorbed on the basal
317 plane of a freshly cleaved muscovite. Spongy-like structures consisting of 15 nm rings
318 along with 10-50 nm spheres were found at low humic concentration, whereas
319 aggregates of spheres, chain-like assemblies, and torus-like structures, were obtained at
320 higher concentration. Thieme and Niemeyer [52] investigated by X-ray microscopy an
321 experimental system very similar to ours, *i.e.* a 10g/L soil humic acid suspension
322 coagulated with various amounts of either Dodecyltrimethylammonium bromide
323 (DTAB) or Hexadecyltrimethylammonium bromide (CTAB). In presence of low DTAB
324 amounts, nearly monodispersed spheres (224 nm) were observed; those spheres doubled
325 in size with increasing surfactant concentration. At high DTAB concentration, spongy

326 networks were obtained. In the case of CTAB additions, micron size hollow spheres
327 with about 240 nm thick walls were detected at low surfactant concentration.

328

329 Many of the molecular structures described in the literature are reminiscent of those
330 found in this study. However, the formation of small unilamellar vesicles was, to our
331 knowledge, never reported for either fulvic or humic colloids. In systems involving a
332 cationic surfactant and an oppositely charged component, vesicles have been found to
333 form either with an anionic surfactant, *i.e.* a catanionic system [53], or an anionic
334 polymer [54]. Therefore, the observation of vesicles in the mixture SRFA/DTAC is not
335 in itself an argument for either view - supramolecular or polymeric - of SRFA.
336 However, the sequence of molecular structures in which our vesicles are successively
337 found, *i.e.* SRFA-rich vesicles, globules, disks, DTAC-rich vesicles, is fully consistent
338 with phase diagrams typically encountered in catanionic systems [55][56]. The presence
339 of two DTAC concentration domains with disperse vesicles on both sides of neutral
340 aggregated globules at both 20 mg/L and 1g/L, is also reminiscent of such phase
341 diagrams. Moreover, the morphology of spongy networks observed in association with
342 vesicles bears a close resemblance to that of the branched wormlike micelles found in
343 aqueous surfactants solutions [57].

344

345 The amphiphilic character of humic substances has long been recognized [8][9].
346 According to Wershaw [3], such property is even intrinsic to the formation of HS by
347 enzymatic degradation of plant biopolymers. Recent NMR spectroscopy studies have
348 revealed the existence of more or less flexible alkyl segments in humic substances
349 [58][59][60]. DOSY experiments performed on soil HS even indicated that the diffusion

350 coefficients of aliphatic components are consistent with the presence of monomers to
351 tetramers of C₁₆ – C₁₈ fatty ester units [10]. Electrospray ionization also suggested that
352 fulvic acid consists of more or less saturated carbon skeleton with carboxylate
353 functional groups or even lipids [61][62][63]. Therefore, it does not seem unreasonable
354 to consider SRFA as a supramolecular assembly of various anionic amphiphile
355 molecules and the SRFA/DTAC complexes as resulting from a catanionic surfactant
356 system.

357

358 *4.2 Towards average geometric characteristics of SRFA constituents*

359

360 The nature of a self-assembly is essentially controlled by both electrostatic and
361 geometric packing effects. According to the framework introduced by Israelachvili et al.
362 [64], the self-organization of amphiphiles can be reasonably predicted from the critical
363 packing parameter P defined as the ratio between the volume v_c of the hydrophobic tail
364 and the product of the polar head surface area a_o with the critical chain length l_c of the
365 surfactant:

$$P = \frac{v_c}{a_o l_c}$$

366 Thus, for cone shaped surfactants, *i.e.* $p \leq 1/3$, spherical micelles are formed, whereas
367 for cuplike amphiphile molecules, *i.e.* $1/2 < p < 1$, vesicles are formed. Bilayers are
368 obtained when the packing parameter is equal to 1. For DTAC, according to the
369 experimental values of geometrical parameters reported in the literature, $v_{DTAC} = 0.482$
370 nm^3 , $l_{DTAC} = 1.643 \text{ nm}$, $a_{DTAC} = 0.88 \text{ nm}^2$, $p_{DTAC} \sim 0.33$ and DTAC therefore forms
371 spherical micelles [36].

372

373 To carry out that kind of calculation for SRFA amphiphiles is more questionable. Even
 374 though a micelle-like organization for HS has previously been proposed in the literature
 375 [8][9][74], a pure hydrophobic interior is highly unlikely in view of the electrokinetic
 376 behaviour of SRFA/DTAC complexes at low surfactant concentration (fig. 3a).
 377 Furthermore, the polydisperse distribution of molecular weights and a likely variety in
 378 the nature of polar head groups, can only lead to a broad estimate of the geometrical
 379 characteristics of SRFA amphiphiles. Still, an average polar head area of SRFA can be
 380 obtained from surface tension measurements (fig. S4) using Gibbs adsorption equation
 381 at the maximal value of $d\gamma/d\ln C$ [65]:

$$382 \quad a_{SRFA} = \frac{1}{\Gamma_{\max} N} \text{ and } \Gamma_{\max} = -\frac{1}{RT} \frac{d\gamma}{d\ln C}$$

383 where Γ_{\max} is the maximal surface adsorption of SRFA, N Avogadro number, R the
 384 ideal gas constant, T the absolute temperature, γ the surface tension, and C the SRFA
 385 concentration. In our case, $\Gamma_{\max} = 2.35 \cdot 10^{-10} \text{ mol/cm}^2$ and hence $a_{SRFA} = 0.71 \text{ nm}^2$, which
 386 is consistent with previous results in the literature [66]. An average chain volume V_{SRFA}
 387 of SRFA constituents can also be assessed by assuming a spherical micelle
 388 organization: knowing that the volume V of the micelle is $V = n V_{SRFA} = 4/3\pi r_{SRFA}^3$ and
 389 the surface S of the micelle is $S = n a_{SRFA} = 4\pi r_{SRFA}^2$, with n the number of SRFA
 390 constituents in a micelle, $V_{SRFA} = \frac{a_{SRFA} r_{SRFA}}{3}$. Using the average radius-of-gyration r_{SRFA}
 391 of 7.5 \AA reported in the literature for SRFA [44][67], we obtained $V_{SRFA} = 0.177 \text{ nm}^3$. An
 392 estimate of the number n_c of alkyl groups in the hydrophobic portion of SRFA
 393 constituents can also be inferred using the following expression: $V_{SRFA} = (27.4 + 26.9 n_c)$
 394 $\times 10^{-3} \text{ nm}^3$ [68]. Interestingly, the n_c value of about 5 thus obtained, is consistent with

395 the picture of short-chained or highly branched aliphatic structures provided by NMR
396 studies of SRFA [69].

397

398 The complex formed from DTAC and SRFA constituents possesses its own packing
399 parameter p_c . Following Manohar and Narayanan approach [70], it is convenient to
400 consider two cases: (i) the anionic surfactants (N_1 moles), *i.e.* the SRFA constituents,
401 are in excess and all the cationic surfactants (N_2 moles) are complexed; the average

402 packing parameter P is then:
$$P = \frac{2N_2}{N_1 + N_2} p_c + \frac{N_1 - N_2}{N_1 + N_2} p_{SRFA}$$
 or

403 $P = 2x p_c + (1 - 2x) p_{SRFA}$ where $x = \frac{N_2}{N_1 + N_2}$ is the mole fraction of cationic surfactant.

404 (ii) The cationic surfactants are in excess and all the SRFA constituents are complexed,

405
$$P = \frac{2N_1}{N_1 + N_2} p_c + \frac{N_2 - N_1}{N_2 + N_1} p_{DTAC}$$
 or $P = 2(1 - x) p_c + (2x - 1) p_{DTAC}$.

406

407 This simple analysis readily accounts for the variety of aggregate structures (micelles,
408 vesicles, disks) that were found in the SRFA/DTAC system, P changing with the molar
409 fraction of cationic surfactant. Actually, both anionic and cationic surfactants should be
410 distributed between the bulk and the self-assemblies, which complicate the exact
411 calculation of the packing parameter. At the moment, not knowing the amount of DTAC
412 associated in any self-assembly, it is not possible to infer the architectural
413 characteristics of the SRFA/DTAC complex from the geometrical constraints associated
414 with the formation of a given molecular structure. The polar head of the complex may
415 however be determined using the Gibbs adsorption equation, *i.e.* $a_c \sim 0.17 \text{ nm}^2$ (fig. 4).
416 In addition, the thin thickness of vesicles, less than 1.15 nm according to the width of

417 the dark rim on the micrographs (fig. 6b2 and 6f2), and hence much less than $l_{DTAC} =$
418 1.643 nm, suggests (i) a mismatch in size between the cationic surfactant and the SRFA
419 constituent, and (ii) a deformation of the DTAC hydrocarbon chain around the SRFA
420 constituent.

421

422 **5. Conclusions**

423 The surface active behaviour of complexes formed during the interaction of SRFA with
424 DTAC, the sequence of molecular structures observed by cryoTEM, and the size of
425 constituents involved in those molecular structures, concur to describe SRFA as a
426 supramolecular assembly of amphiphiles molecules. Taking into account the variety of
427 functional groups evidenced in humic material [6], and the various compounds detected
428 in SRFA by coupling solvent extraction with ESI FTICR MS [63], it seems a bit
429 simplistic to assimilate SRFA to a single type of amphiphile molecule, and hence to
430 consider the SRFA/DTAC mixture as a well-defined binary system. In addition, it is
431 highly plausible that only part of SRFA constituents participate to the molecular
432 structures observed here. Nevertheless, such comments do not fundamentally challenge
433 the picture of SRFA as a supramolecular structure. To assess the dynamics of such
434 molecular aggregate, *i.e.* its formation and evolution in the natural environment, or its
435 relationships to the other DOM components, in particular humic acid, appear to be key
436 elements for a better understanding of global carbon cycle.

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439

440

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673

674 **Figure captions**

675

676 **Figure 1.** (a) Typical turbidity curves of SRFA/DTAC suspensions as a function of time
677 for various DTAC additions; SRFA concentration = 20 mg/L.

678

679 **Figure 2.** Interaction of SRFA with DTAC (•), OTAC (•), and NaCl (•) as a function of
680 salt concentration: (a) Turbidity curves; (b) Conductivity curves.

681

682 **Figure 3.** (a) Electrophoretic mobility of SRFA/DTAC complexes versus DTAC
683 concentration (•); (b) Number-average particle size of SRFA/DTAC complexes (DLS)
684 versus DTAC concentration (•) for a SRFA concentration of 20 mg/L. The turbidity
685 curve (•) is superimposed for comparison. The inset shows the bimodal size distribution
686 obtained for a DTAC addition of 0.19 mmol/L.

687

688 **Figure 4.** Surface tension of SRFA/DTAC suspensions versus DTAC concentration (•)
689 for a SRFA concentration of 20 mg/L. The turbidity curve (•) for a SRFA concentration
690 of 20 mg/L is shown in order to position the main variations of surface tension.

691

692 **Figure 5.** CryoTEM micrographs of SRFA in the presence of DTAC concentrations of
693 1.8 mmol/L and 7.58 mmol/L (red dots) showing spongy networks and vesicles. The
694 arrows indicate the walls of the vesicles. SRFA concentration = 20 mg/L.

695 **Figure 6.** CryoTEM micrographs of SRFA/DTAC molecular structures; all scale bars
696 equal 100 nm unless otherwise indicated. (a) [DTAC] = 0 mmol/L. (b1-4) [DTAC] =
697 $7.6 \cdot 10^{-3}$ mmol/L; the arrow indicates an open vesicle. (c) [DTAC] = $30.2 \cdot 10^{-3}$ mmol/L.
698 (d) [DTAC] = $56.7 \cdot 10^{-3}$ mmol/L. (e1-2) [DTAC] = $75.6 \cdot 10^{-3}$ mmol/L. (f1-4) [DTAC] =
699 $18.8 \cdot 10^{-2}$ mmol/L. (g) [DTAC] = $74.3 \cdot 10^{-2}$ mmol/L.

700

701 **Figure S1.** pH variation of SRFA/DTAC suspensions as a function of DTAC
702 concentration.

703

704 **Figure S2.** CryoTEM micrographs of (a) DTAC stock solution (10g/L or 37.9 mmol/L);
705 (b) DTAC solution at a concentration of $7.6 \cdot 10^{-3}$ mmol/L, i.e. the concentration at which
706 fulvic-rich vesicles are formed.

707

708 **Figure S3.** Size histograms of molecular structures viewed by cryoTEM and evolution
709 of mean particle size with DTAC concentration. The DTAC concentration is indicated
710 in the histograms in mmol/L. The histograms in red are associated with the presence of
711 vesicles.

712

713 **Figure S4.** Surface tension of SRFA suspensions as a function of SRFA concentration.