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Separation of unsaturated C18 fatty acids using perfluorinated-micellar electrokinetic chromatography:

II) Role of nano-structuration

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Highlights

- Free fatty acids are separated by capillary electrophoresis using fluorosurfactants
- Scattering experiments evidence aggregation from small micelles to large structures
- Micelle structuration in injection buffer and background electrolytes is affected by fatty acids
- Selective interactions between fatty acids and fluorosurfactant allow CE separation

Abstract

Three fatty acids were separated by micellar electrokinetic chromatography (MEKC) using ammonium perfluorooctanoate as volatile surfactant. Characterizations of both injection buffer and background electrolyte have been performed by complementary techniques including scattering experiments (light, x-rays) and NMR. Different structures have been observed such as small micelles at low methanol content and larger aggregates at high methanol content. In both cases fatty acids interact specifically with the perfluorinated self-assemblies due to different geometrical constraints and different interactions between polar head groups, ensuring separation by MEKC.

30 **Abbreviations:** Alpha linolenic acid ALA, Ammonium perfluorooctanoate APFOA, Background
31 electrolyte BGE, Elaidic acid EA, Fatty acids FAs, Linoleic acid LA, Micellar electrokinetic
32 chromatography MEKC, Oleic acid OA, Perfluorooctanoic acid PFOA.

33 **Keywords**

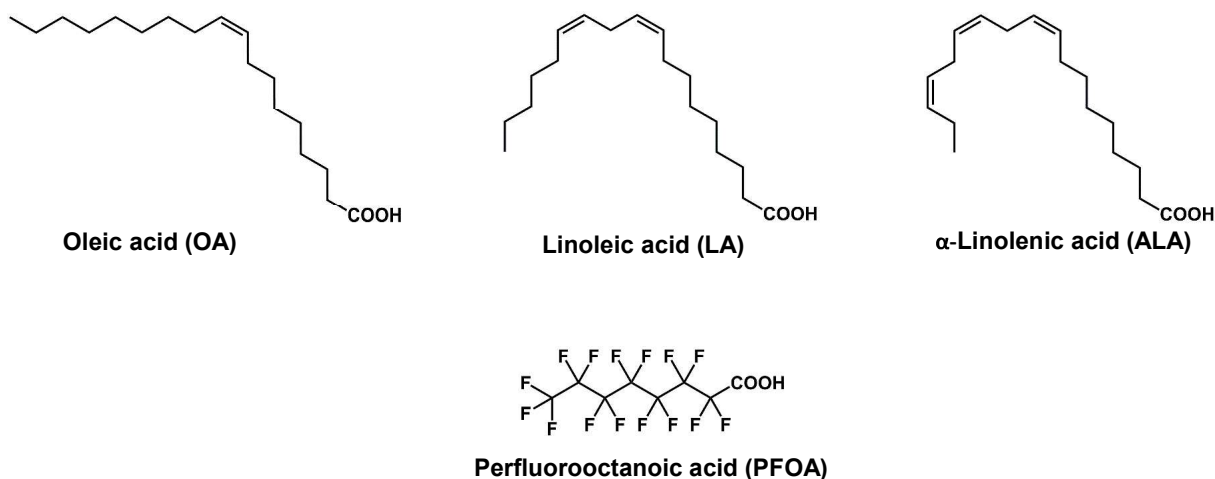
34 Self-assembly, surfactant, MEKC, light scattering, SAXS, NMR, fatty acids

35

36 **1. Introduction**

37 Capillary electrophoresis (CE) is an analytical technique which allows separation of charged analytes
38 based on their electrophoretic mobilities in an applied electric field. In the case of neutral compounds,
39 additional structured charged fluids are required to achieve separation. The use of charged micelles
40 has been largely developed in Micellar Electro Kinetic Chromatography (MEKC) [1]. Separation is there
41 determined by competitive hydrophobic interactions and equilibrium between aqueous bulk and
42 hydrophobic micellar cores acting as pseudo-stationary phase. Sodium dodecyl sulfate is the most
43 widely used surfactant in MEKC, most of the time associated to cyclodextrins. Although efficient, this
44 surfactant is not volatile and does not allow mass spectrometry (MS) detection in the case of trace
45 amounts. One of the less developed application of CE is the separation of lipids [2]. However, this
46 technique was recently shown to be particularly powerful to separate unsaturated fatty acids using a
47 very simple buffer composed of a buffering salt, Brij 35 as surfactant, and large amount of organic
48 solvent (50%) [3]. Adding sodium dodecylbenzenesulfonate to supplement the latter buffer, the
49 separation of saturated and unsaturated fatty acids was realized using indirect UV detection [4]. Why
50 the use of CE for free fatty acids analysis would be better than the one of the very classical and robust
51 gas chromatography (GC)? Because fatty acids have to be derivatized into fatty acid methyl esters
52 (FAMES) prior to GC analysis. The preparation of FAMES is long, because of the extraction/methylation
53 steps and might cause autooxidation of fatty acids when polyunsaturated. Moreover, the CE can be
54 easily applied in routine applications, such as analysis of food supplements in the form of oil, mainly
55 containing triglycerides of fatty acids. In this case, a simple saponification followed by a dilution is
56 enough to prepare the sample before injection. More generally, compared to other separation
57 techniques (*e.g.* HPLC or gel electrophoresis), CE is a very attractive analytical method from the view
58 of environmental and economical aspects. Indeed, this simple and miniaturized techniques requires
59 only very small volumes of analytes and solvents (few μLs per analysis) [5]. In a previous paper, we
60 have shown that SDS/cyclodextrin can be efficiently replaced by ammonium perfluorooctanoate
61 (APFOA), a volatile perfluorinated surfactant, for the separation of a series of fatty acids [6]. It could
62 be mentioned here that perfluorocarboxylates are persistent compounds which have been detected

63 in various natural environments. Methods have been reported for the removal and recycling of APFOA
64 from waste water, especially membrane nanofiltration [7], which can be envisaged here for the
65 reusability of APFOA after CE analysis and sample collection. We showed that the most unsaturated
66 species were migrating first while the less unsaturated ones were eluted with the highest migration
67 times. We wanted to understand how separation of free fatty acids could be possible in such
68 perfluorinated medium while perhydrogenated/perfluorinated interactions are known to be not
69 favored. Only few studies report the mesostructures of such micellar electrolytes although this is
70 important for understanding how separation proceeds [8,9]. The aim of the present study is thus to
71 characterize the structuration of the complex fluids involved during the separation of fatty acids,
72 namely oleic acid (OA), linoleic acid (LA), and alpha-linolenic acid (ALA) in the presence of APFOA as
73 surfactant (the structures of these compounds are reported in Figure 1). We have used complementary
74 techniques such as scattering experiments (x-rays, light), microscopy, CE, and NMR. We propose a
75 mechanism to rationalize the observed separation.



76

77 **Figure 1.** Structures of the studied compounds: fatty acids and perfluorosurfactant

78 2. Materials and methods

79

80 2.1. Materials

81 Perfluorooctanoic acid (PFOA, 96 %), oleic acid (OA, $\geq 99\%$), linoleic acid (LA, $\geq 99\%$), alpha-linolenic
82 acid (ALA, $\geq 99\%$), 2,2,2-trifluoroethanol (TFE), sodium hydroxide, ammonium hydroxide (25 M in
83 water), methanol (HPLC grade), and water (HPLC grade) were purchased from Sigma-Aldrich (Saint-
84 Quentin-Fallavier, France). Deuterated solvents were supplied from Eurisotop (Saint-Aubin, France).

85 2.2. Sample preparation

86 Stock solutions of ammonium perfluorooctanoate (APFOA) were prepared by dissolving the
87 appropriate amount of PFOA in aqueous NH_4OH at a given ratio $R = [\text{NH}_4\text{OH}]/[\text{PFOA}]$. The pHs of these
88 stock solutions were measured using a Seven Compact S220 pH-meter (Mettler Toledo). Stock
89 solutions of FAs (30 mM) were prepared in MeOH and kept at 5 °C before use. The background
90 electrolytes (BGEs) and the injection buffer (INJ) were obtained from the stock solutions of APFOA and
91 FAs, adjusting the target concentrations by addition of water and MeOH.

92 **2.3. Multi-angle light scattering (MALS)**

93 MALS measurements were performed on a 3D LS Spectrometer from LS Instruments (Fribourg,
94 Switzerland) equipped with a 100 mW high performance DPSS Laser (Cobolt, $\lambda = 660$ nm), an
95 automated laser attenuation system combined with on-line laser intensity measurement, a two-
96 channel multiple tau correlator (auto and cross correlation, 1088 real time channels), a 3D cross-
97 correlation module, and a variable-angle detection system equipped with two high sensitivity APD
98 detectors. Samples were introduced into cylindrical glass cells (10 mm diameter) that were placed in a
99 sample chamber filled with index-matching fluid (mixed cis-trans decaline); temperature control was
100 ensured by an external circulator (Julabo CF31). Measurements were performed at 25°C for scattering
101 angles θ ranging from 12° to 150°, corresponding to scattering wave vectors $q = [4\pi \cdot n \cdot \sin(\theta/2)]/\lambda$
102 ranging from $3 \times 10^{-4} \text{ \AA}^{-1}$ to $2 \times 10^{-3} \text{ \AA}^{-1}$ ($n=1.330$ is the refractive index of water). When preliminary
103 measurements indicated multiple scattering, the 3D cross-correlation configuration[10] was required
104 to discard the multiple scattering contribution from the total scattered light. At each angle, 3 to 10
105 measurements were carried out and averaged. Analysis of dynamic light scattering measurements
106 were performed using the home-made MULTI-STORMS software developed by Dr. C. Mingotaud (see
107 *Supporting Information*), while static light scattering measurements were analyzed using the SasView
108 software (<http://www.sasview.org/>), version 4.2.2. (M. Doucet et al. SasView Version 4.2.2, Zenodo,
109 <http://doi.org/10.5281/zenodo.2652478>). Full equations are reported in the *Supporting Information*
110 S2-S3.

111

112 **2.4. Small-angle x-ray scattering (SAXS)**

113 SAXS measurements were performed on a Xeus 2.0 SAXS beamline for laboratory from Xenocs
114 (FERMAT Federation, Toulouse), equipped with an internal copper source producing 8 keV x-rays ($\lambda =$
115 1.54 \AA) and a Pilatus 1 M pixel detector from Dectris. The sample-to-detector distance was 1216.5 mm
116 corresponding to a covered q range from $6 \times 10^{-3} \text{ \AA}^{-1}$ to $5 \times 10^{-1} \text{ \AA}^{-1}$. For each sample, 6 scans of 10 min
117 were recorded and averaged. The observed intensities $I(q)$ were corrected for transmission, solvent,
118 and detector response. Absolute intensities $I(q) (\text{cm}^{-1})$ were obtained after standard normalization. As

119 for static light scattering, analyses of the $I(q)$ profiles were realized using SasView. Full equations are
120 reported in the *Supporting Information*.

121 **2.5. Nuclear magnetic resonance (NMR)**

122 Solutions were prepared using deuterated solvents. NMR experiments were performed on a Bruker
123 Avance 400 spectrometer (Bruker Biospin AG, Fallanden, Switzerland) equipped with a 5 mm triple
124 resonance probe (TXO) at 298 K. All ^1H NMR and DOSY experiments were performed on mixtures
125 containing FAs and trifluoroethanol (TFE) as a reference.

126 Quantitative ^1H NMR spectra with pre-saturation pulse for HOD signal suppression were acquired with
127 the following parameters: acquisition time of 2.93 s, 30° pulse, relaxation delay of 12s, for a spectral
128 width of 14 ppm and a number of scans of 64. Under these recording conditions, the ^1H resonances
129 were fully relaxed since a repetition time upper than 15 s did not modify the signal intensities.

130 The DOSY spectra were acquired with the *stebpgp1s* program from Bruker library pulse
131 sequences. All spectra were recorded with 32K data points, 12 ppm for spectral width, 3 s for relaxation
132 delay and 16 scans. The pulse field gradient length and the diffusion time were optimized for each
133 samples and were 3.6-4.0 s and 160-250 ms respectively. The gradient strength was linearly
134 incremented in 16 steps from 5 % up to 95 % of the maximum gradient strength.

135 All NMR data were processed using the TOPSPIN 4.0.8 software with one level of zero-filling
136 and an exponential line-broadening function of 0.3 Hz. Spectra were calibrated at 3.88 ppm on the CH_2
137 NMR signal of TFE. The data sets of diffusion NMR experiments were processed with TOPSPIN software
138 and transferred to the Origin 9.1 software. The exponential fit tool was then used to determine the
139 values of the self-diffusion coefficients (D).

140

141 **2.6. Capillary electrophoresis (CE)**

142 CE analysis were performed on an Agilent Technologies CE7100 instrument (Waldbronn Germany).
143 Separations of FAs were carried out on a $50\ \mu\text{m}$ id x $365\ \mu\text{m}$ od x 51 cm effective length (59 cm total
144 length) bare-fused silica capillary (Picometrics - Adelis Technologies, Labège, France).

145 New capillaries were rinsed with 1 M NaOH for 60 min, water (HPLC grade) for 30 min, and background
146 electrolyte (BGE) for 15 min. Between analysis, capillaries were rinsed with methanol for 3 min, 1 M
147 NaOH for 3 min, water for 2 min, and BGE for 3 min. At the end of each day, capillary was rinsed with
148 methanol for 3 min, 1 M NaOH for 15 min, and water for 5 min. Samples were hydrodynamically
149 injected at 50 mbar for 5 s. All analyses were carried out at $25\ ^\circ\text{C}$ and the wavelength of UV direct
150 detection was set at 195 nm. The samples were prepared in 53 % (v/v) MeOH APFOA 23 mM, to ensure
151 solubilization of FAs.

2.7. Cryo-Transmission electron microscopy (cryo-TEM)

For cryo-TEM analysis, samples were prepared and analyzed at METi, Toulouse. Samples were deposited onto carbon grids and loaded into the thermostatic chamber of a Leica EMGP automatic plunge Freezer, set at 20 °C and 95 % humidity. Excess solution was blotted with a Whatman filter paper, and the grid was immediately flash frozen in liquid ethane, cooled at -183 °C. Sample observations were performed on a Jeol 2100-EX electron microscope operating at 200 kV. Cryo-TEM image acquisitions were performed with a CCD camera US4000 4k×4k (Gatan) equipped with a post-column energy-filter (Gatan GIF Quantum). Images were recorded with a low electron dose ($< 10e^-/\text{Å}^2$) at a nominal magnification corresponding to a calibrated pixel size of 1.71 Å. Nominal defocus values ranged from 800 nm to 1200 nm.

3. Results and discussion

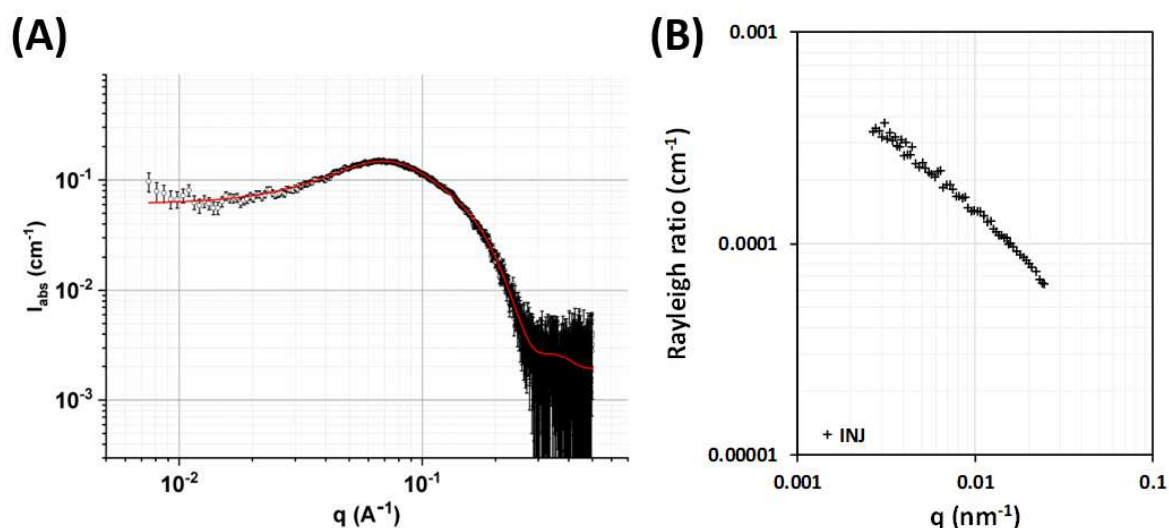
3.1. Self-organization of APFOA as a function of methanol content leads to various nanostructuring in buffers

In water (0% MeOH), APFOA has been shown to self-assemble into small micelles as already reported [11]. In the present case, CE separations were achieved using an excess of ammonia with respect to PFOA (ratio $R = [\text{NH}_4^+]/[\text{PFOA}^-] = 5.1$, pH = 9.9). As ammonium counter-ions can affect APFOA self-assembly [11–13], we first checked this nano-structuration in water keeping $R = 5.1$. NMR experiments allowed cmc determination by measuring the variation of the chemical shift as a function of the reciprocal of the concentration (see *Supporting Information S5*). The value found in the absence of methanol was $\text{cmc} = 28.3 \pm 0.2$ mM, which is in good agreement with the literature [14]. Light scattering experiments were then conducted but gave no relevant signal due to the very low optical contrast of APFOA (refractive index increment dn/dc close to 0) [15], even at $[\text{APFOA}] = 100$ mM. In contrast, SAXS experiments easily afforded APFOA scattering profiles thanks to the electronic contrast provided by fluorine atoms. They confirmed the formation of small ellipsoidal micelles, in good agreement with the reported data (see *Supporting Information S11*) [16]. Therefore, we can assume that high ammonia concentration has no significant impact on APFOA micellization in water.

In order to study to role of MeOH, the next measurements have been conducted on the solutions used for the CE separation, namely the background electrolyte (BGE, 10% MeOH, 50 mM APFOA, $R=5.1$) and the injection medium (INJ, 53% MeOH, 23 mM APFOA, $R=5.1$).

At 10 % MeOH (BGE), the cmc decreased to 19 ± 0.9 mM (as already determined by CE and NMR) [6], evidencing the promoting effect of MeOH on micelle formation. This could be related to the preferential solvation of APFOA by MeOH than by water [17], in a similar way as 10 % ethanol promotes

185 APFOA micellization, as reported recently [18]. Indeed, the localization of alcohol at the micelles
186 reduces the repulsive interactions between charged head groups by decreasing the surface charge
187 density, which leads to a decrease of the cmc. As observed in pure water, the BGE did not produce
188 relevant light scattering. This was not surprising as the refractive index of the solution (1.335) remained
189 very close to the one of water (1.330) and therefore the optical contrast was not enhanced.
190 Nevertheless, SAXS experiments were successfully performed on this sample, evidencing that small
191 ellipsoidal micelles were still there even in the presence of 10% MeOH in the solution (Figure 2A). The
192 structural parameters have been determined and only show a marginal change compared to the
193 micelles in pure water. This was confirmed by CE analysis, which could also be used to determine the
194 micellar volume, the aggregation number, and the degree of counterion dissociation from the global
195 charge of the micelles (see *Supporting Information S6-S7*). This shows that the APFOA initial micellar
196 structure is maintained in the BGE.



197
198 **Figure 2.** (A, left) SAXS profile of BGE indicating structuration into small ellipsoidal micelles; (B, right)
199 SLS profile of INJ indicating structuration of APFOA into large aggregates

200 The case of the injection buffer (53% MeOH) was radically different. In SAXS, the signals
201 corresponding to the small micelles completely vanished thus indicating their dissolution. Only an
202 increase of the scattered intensity at very low q values could be the sign of the presence of much larger
203 aggregates. Indeed, light scattering was this time much stronger and both DLS and SLS measurements
204 could be accurately performed. As the refractive index of the solution did not increase much ($n =$
205 1.341), the increase of light scattering indicated that larger objects were present in the solution.
206 Indeed, the $I(q)$ profile radically changed as illustrated by SLS in Figure 2B. Moreover, both the
207 hydrodynamic radius and the radius of gyration ($R_h, R_g = 30\text{-}55$ nm) observed by CE analysis could not
208 correspond to small micelles (*Supporting Information S7*).

209 We can conclude that increasing the MeOH content induced a phase transition from small micelles (in
210 BGE) to much larger aggregates (in INJ).

211 In INJ, the local concentration of MeOH in the solvation sphere of APFOA is close to 75% [17]. We
212 observe a transition from small micelles to larger objects when the amount of alcohol increases. This
213 could be explained first by the local reduction of the dielectric constant which induces a lowering of
214 electrostatic repulsions and therefore a decrease of the interface curvature, like observed in the
215 presence of large counterions or when the ionic strength is high. Moreover, MeOH could also interact
216 with polar heads by forming H-bonds resulting again in a flatter interface by bridging effect [19].

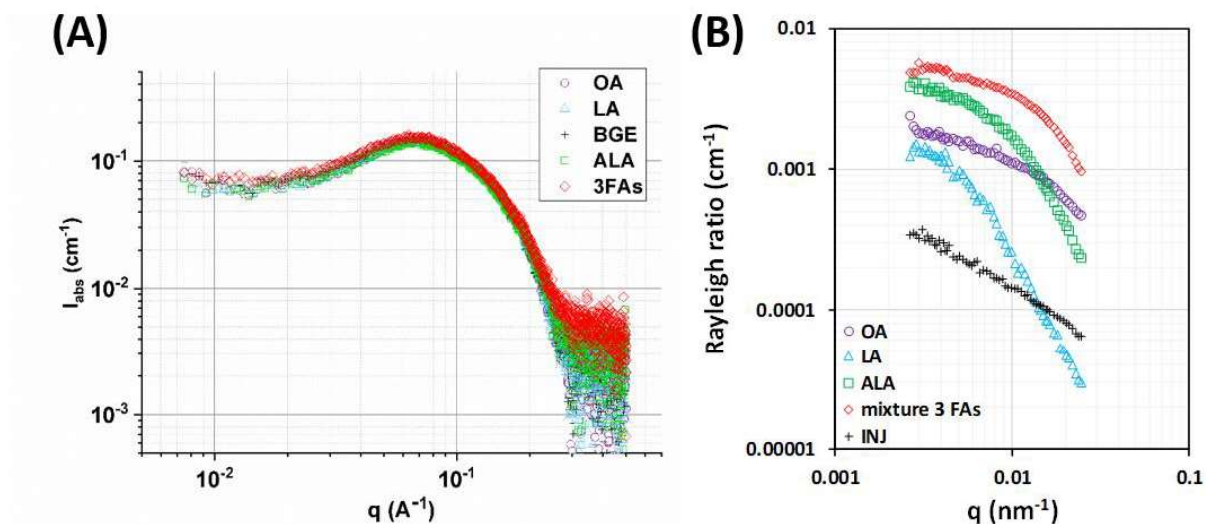
217 This first set of scattering experiments show that APFOA can self-assemble into different sizes of
218 aggregates, depending on the amount of MeOH. From the CE point of view, the injection buffer initially
219 contains large objects that converted into small ellipsoid micelles upon dilution in the background
220 electrolyte.

221

222 **3.2. Structuration of the complex fluids (BGE, INJ) in the presence of fatty acids**

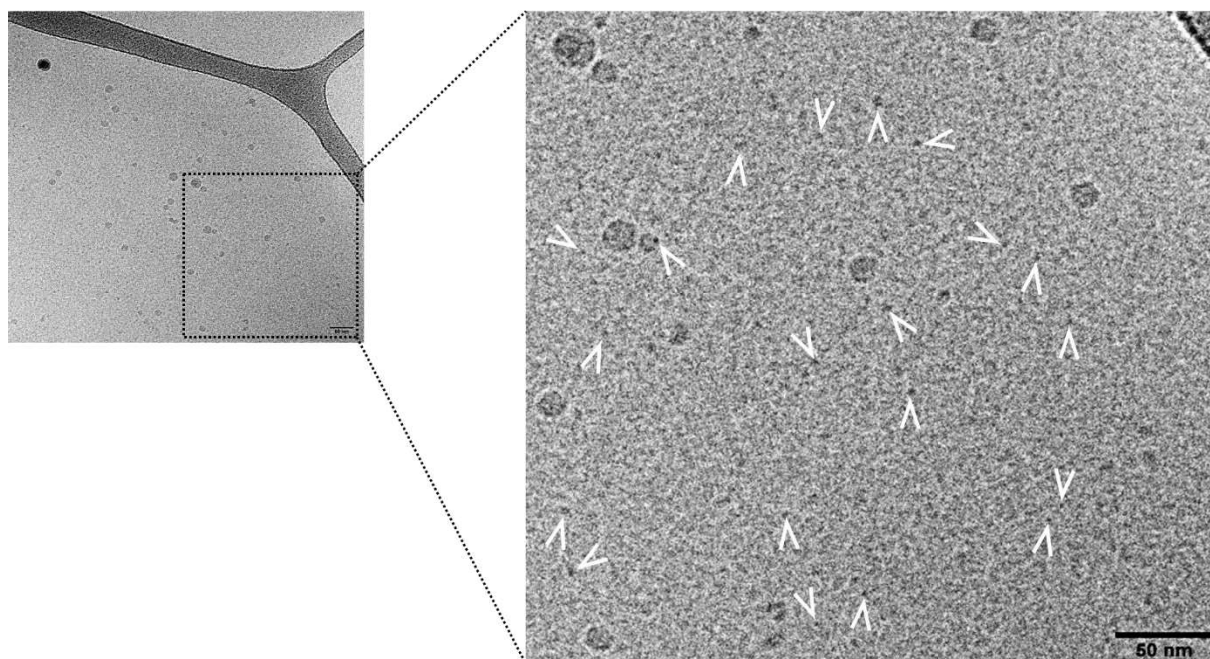
223 Here we investigated the impact of dissolved FAs on the microstructures of both BGE and INJ.

224 Introducing 1mM FA in the BGE did not produced more relevant DLS signal than what observed in pure
225 BGE, while SAXS shows the presence of small micelles nearly identical to APFOA alone (Figure 3A).
226 Although CE separation is the sign that FAs interact with APFOA micelles, the insignificant structural
227 changes observed here could be due to the large ratio between the surfactant and the analytes:
228 $[APFOA]/[FA]=50$, corresponding to about one FA molecule per APFOA micelle. This could also be due
229 to the dynamic character of micelles with fast exchange between molecules. Nevertheless, this
230 observation indicates that aggregation into small dynamic micelles still occurs in the presence of FA,
231 allowing CE separation.



232
 233 **Figure 3.** (A, left) SAXS profile of BGE containing FAs indicating structuration into small ellipsoidal
 234 micelles; (B, right) SLS profile of INJ indicating structuration of APFOA into large aggregates whose
 235 nature depends on FAs.

236 The structuration into small micelles could be detected by cryo-TEM observations as illustrated in
 237 Figure 4 in the case of 3mM OA in BGE.



238
 239 **Figure 4.** Cryo-TEM micrographs of BGE containing OA (3 mM) and illustrating the structuration into
 240 small micelles mainly.

241

242 At 23 mM APFOA, 53% MeOH (INJ), DLS and SLS measurements evidenced the presence of large
243 aggregates (as observed without FAs), as illustrated for SLS in Figure 3B (DLS are reported in the
244 *Supporting Information*).

245 SLS profiles show that the presence of FAs impacts the structure of these large aggregates even if the
246 relative amount of FA compared to APFOA remains very low. This is the sign that FAs are present in
247 these large aggregates confirming that hydrogenated FAs can be incorporated in fluorinated APFOA
248 structures. Moreover, the influence of the FA depends on its nature. Large aggregates are only weakly
249 modified by OA; this minor perturbation due to OA incorporation in APFOA aggregates could be related
250 to a matrix effect [20]. Major structural modifications are observed with other FAs probably due to
251 geometric constraints (repulsive interactions): this induced size/shape transitions to structures where
252 FAs (LA, ALA) could be less embedded than OA. The number of unsaturations is responsible for FA
253 different structural geometries: the molecules are bended, in the order OA > LA > ALA (Figure 1), and
254 could explain the easier incorporation of OA in APFOA structures as compared to LA and ALA.

255 This set of experiments indicates a selective interaction between FAs and APFOA.

256

257 **3.3. Structuration in the absence of APFOA micelles**

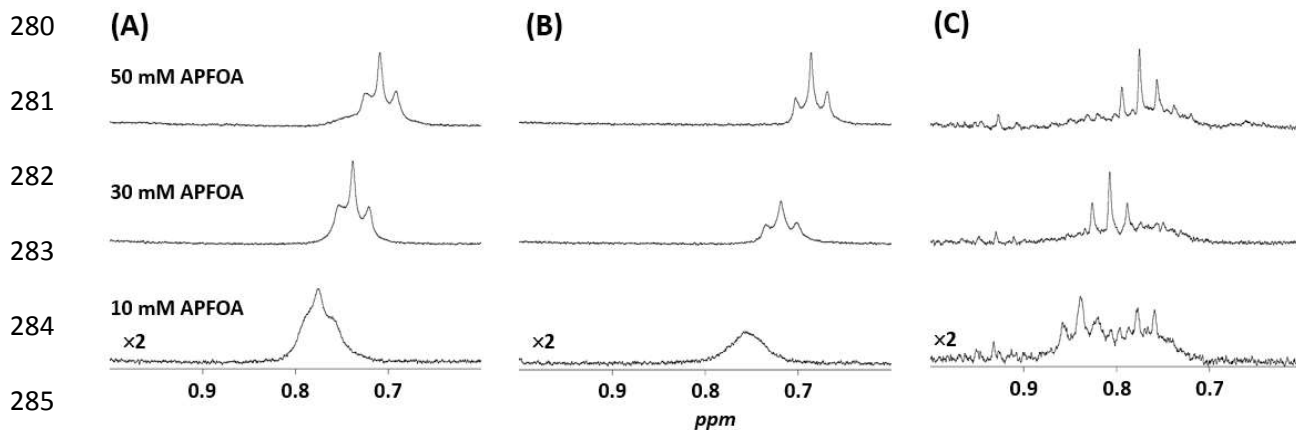
258 We have seen that small APFOA micelles are required to allow CE separation. Here we investigated the
259 behavior of FAs in the absence of APFOA micelles.

260 First, we replaced APFOA by 50 mM TFA (which cannot form micelles) in the BGE.
261 Unprotonated FAs (soaps) are known to self-assemble in water in various structures depending on pH.
262 At high pH (pH > pKa) small micelles are formed with low cmcs (0.1-0.5 mM) [21–23]. At pH close to
263 pKa, rodlike micelles are formed and they transit to vesicle if pH is further decreased [24]. In the
264 present case, the pH is just above the pKas of FAs which are supposed to potentially self-assemble into
265 micelles. Indeed, DLS showed that FAs formed small spherical micelles (ALA and LA), or elongated ones
266 (OA, pH close to pKa). When mixing the three FAs an average micellization was observed (see
267 *Supporting Information S11*). This evidences that FAs still form dynamic micelles at 10% MeOH, but
268 that the rapid exchange between FA micelles could not allow CE separation in the absence of APFOA
269 micelles. To ensure CE separation, the presence of micelles is required but not sufficient: a selective
270 interaction between FAs and APFOA is needed.

271 In order to further characterize this selective interaction, we conducted complementary
272 experiments in an intermediate situation where APFOA is present but not in a micellar form (c=10 mM
273 < cmc). In these conditions, CE separation could not be achieved. DLS and SLS measurements showed
274 the formation of large structures (Rh=43 nm, Rg=75 nm) which could not correspond to small micelles

275 (see *Supporting Information S12*). Additional NMR experiment showed that FAs in the mixed FAs-
 276 APFOA aggregates have not the same line width for the NMR signals above or below the cmc of APFOA.
 277 A difference in dynamics for these aggregates can explain this observation. Figure 5 presents the
 278 comparative ^1H NMR signals of terminal CH_3 for each FA at various APFOA concentration.

279



286 **Figure 5.** NMR signal of CH_3 (0.6-1.0 ppm) for (A) OA, (B) LA, and (C) ALA at 10, 30, and 50 mM APFOA,
 287 respectively.

288

289 Below APFOA cmc, the NMR signals for the 3 FAs present large line broadenings: 15.3 Hz, 17.8 Hz, and
 290 4.8 Hz for the CH_3 of OA, LA, and ALA respectively; this indicates that molecules do not diffuse freely
 291 and are embedded in weakly dynamic aggregates that do not ensure CE separation. On the contrary,
 292 higher dynamics are observed for the CH_3 above the cmc of APFOA (50 mM) with smaller line
 293 broadenings at 3.4 Hz, 2.4 Hz, and 1.5 Hz respectively, closer to what observed for free FAs in 100 %
 294 MeOD (1.2-1.5 Hz). Moreover, considering the right situation for CE separation (APFOA at 50mM >
 295 cmc), we see that the integration of the NMR signals of FA, recorded under the same conditions, are
 296 not the same. They increase in the order $\text{ALA} < \text{LA} < \text{OA}$ and the integration ratios OA/ALA and LA/ALA
 297 are 2.2 and 1.25 respectively. This observation shows a favored solubilization of OA (followed by LA
 298 and ALA), indicating a selective incorporation of FAs in APFOA micelles. This was confirmed by DOSY
 299 experiments, giving access to the diffusion coefficients (D) of FAs as a function of APFOA concentration.
 300 First in 100% MeOD, the diffusion coefficients of FAs are in the range $D = 10.4\text{-}11.1 \cdot 10^{-10} \text{ m}^2/\text{s}$.
 301 Considering the viscosities of methanol/water mixtures at 25°C reported in the literature ($\eta=1.16 \text{ cP}$
 302 for $\text{MeOH}:\text{H}_2\text{O} = 10/90$ and $\eta=0.54 \text{ cP}$ for $\text{MeOH}:\text{H}_2\text{O} = 100/0$) [25], the diffusion coefficients of free
 303 FAs in situation of CE separation were estimated to be about $D = 4.6 \pm 0.2 \cdot 10^{-10} \text{ m}^2/\text{s}$. However, in
 304 mixed FA-APFOA aggregates at 50 mM of APFOA, experimental D are measured at 0.8 ± 0.1 , 1.5 ± 0.1
 305 and $2.6 \pm 0.1 \cdot 10^{-10} \text{ m}^2/\text{s}$ for OA, LA, and ALA, respectively. NMR has evidenced slow diffusion

306 coefficients for FAs in the order $OA < LA < ALA$. This again evidences a selective interaction between FAs
307 and APFOA aggregates. This controls the ease of FA incorporation in APFOA micelles as well as the
308 residence time (longer for OA than for LA than for ALA).

309

310 **3.4. Selective interactions between FAs and APFOA**

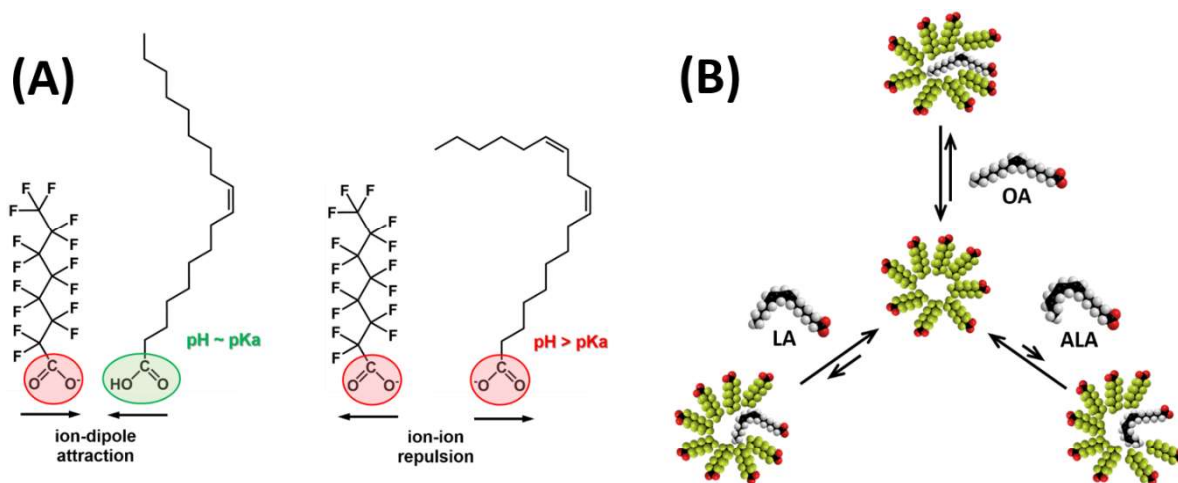
311

312 The selective association between FAs and APFOA result from the contribution of different
313 interactions.

314 First, at the level of polar head groups, interactions depend on the charge and therefore on the pKa
315 value. It has been reported for FAs that when the pH is close to the pKa (this is here the case for OA,
316 $pK_{a_{OA}}=9.85$ [26]) then the molecules are only partially charged and interact at the interface in a close
317 way via ion-dipole interactions (Figure 6A) [26–28]. On the other hand, when pH is higher than pKa
318 (case of LA and ALA with $pK_{a_{LA}}=9.24$ and $pK_{a_{ALA}}=8.28$ [26]), ionic repulsions between charged polar
319 head groups tend to increase the distance between the molecules at the interface. This might support
320 the fact that OA interacts with APFOA micelles in a more favored way than LA and ALA.

321 Then at the level of fatty chains (in the micelle core) the hydrophobic interactions depend also on the
322 distance between the tails. From geometric considerations it is clear that it will be impacted by the
323 geometries of FAs, which depend on the number of saturations in the chain. Therefore, the steric
324 hindrance will increase in the order $OA < LA < ALA$ and, together with the differences in polar
325 interactions, it will control the differential association of FAs with APFOA micelles (Figure 6B). This
326 general analysis could be extended to other structurally related analytes such as saturated FAs. For
327 such compounds, both higher pKa values and lower steric hindrance (compared to unsaturated FAs)
328 would result in more favorable interactions with APFOA micelles: in that case, we expect a longer
329 elution time. The situation could be more complex for other analytes for which interactions with
330 APFOA might compete in opposite ways. It could be the case for shorter FAs with lower pKas
331 (unfavorable interactions) and reduced steric hindrance (favorable interactions): in that case, the
332 resulting balance of interactions could be difficult to predict.

333



334

335 **Figure 6.** Differential interactions between polar heads and fatty chains that control the overall
 336 preferential incorporation of FAs in APFOA micelles. (A) left: when pH is close to pKa, half of FAs
 337 molecules are in neutral form and can interact with APFOA by attractive ion-dipole interactions; right:
 338 when pH is higher than pKa only ion-ion repulsions occur between charged anionic species. (B)
 339 representative scheme illustrating the increasing difficulty of incorporating FAs in APFOA micelles as
 340 the molecules become bender and bender in the series OA < LA < ALA (based on geometrical
 341 considerations).

342

343 Conclusion

344 Meso-scale characterizations of electrolytes used in CE separation of FAs by APFOA have evidenced
 345 the presence of structured complex fluids both in the injection medium (INJ) and in the background
 346 electrolyte (BGE). The structuration has been characterized by complementary techniques such as
 347 scattering experiments (light, x-rays) and NMR. It has been shown to depend on the amount of MeOH
 348 and on the concentration of the surfactant APFOA. First, in INJ (53% MeOH), large aggregates were
 349 formed with structures depending on the nature of the incorporated FA, revealing selective
 350 interactions between the perfluorosurfactant and the perhydrogenated analytes. Then in BGE (10%
 351 MeOH, [APFOA]>cmc), small APFOA micelles were observed which ensured CE separation of FAs. In
 352 both cases selective interactions have been related to a subtle interplay between pH, MeOH amount,
 353 and APFOA concentration. Considering both chemical and structural parameters of the analytes (pKa,
 354 shape), we could propose a global depiction of these systems allowing to rationalize the observed
 355 separation. This shows that using micellar systems in MEKC could lead to more complex structures
 356 than expected and that structural characterization of the separating fluids could be a prerequisite to
 357 understand and potentially anticipate the analytic separation. This work could now be extended not
 358 only to the analysis of other important FAs or analytes, but also especially to the use of mass

359 spectrometry (MS) as detection mode thanks to the volatility of APFOA allowing downscaling of
360 analysis to trace levels.

361

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