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1 Drug solubilization and *in vitro* toxicity evaluation of lipoamino acid surfactants

2

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24

25 **Abstract**

26

27 To improve solubilization of a water insoluble anticancer drug, novel surfactants were
28 synthesized. All surfactants derived from lysine, with a so-called nitrilo triacetic acid (NTA)
29 polar head, and differed from the length and saturation degree of their hydrophobic moieties:
30 C19:0-NTA, C20:4-NTA, C25:0-NTA and C25:4-NTA. Self-assembling properties and
31 critical micellar concentration (CMC) values were determined using pyrene fluorescence and
32 cytotoxicity using MTT and LDH assays on endothelial cells. Surfactant haemolytic activity
33 and drug solubilization capacity were also evaluated. All surfactants self-assemble with low
34 CMC values from 0.012 to 0.430 mg/mL. Cytotoxicity assays showed that C20:4-NTA and
35 C25:0-NTA were less cytotoxic than polysorbate 80. Unsaturation and alkane chain length
36 have a marked influence on toxicity. Saturated surfactants had a similar haemolytic activity,
37 explained by their low CMC values and the linear configuration of their hydrophobic tail.
38 C20:4-NTA and C25:4-NTA were less haemolytic than polysorbate 80. Furthermore, C19:0-
39 NTA, C25:0-NTA and C25:4-NTA increased drug solubility from $< 0.15 \mu\text{g/mL}$ up to 7
40 mg/mL, with 46 % (w/w) drug loading, due to their linear and flexible hydrophobic chain
41 configuration, as evidenced by molecular modeling. Although these solubilizers are
42 promising, a compromise between drug solubilisation and toxicity remains to be found.

43

44 **Keywords:** amino acid; lipid; surfactant; micelle; self-assembly; drug solubilization

45

46 **1. Introduction**

47

48 A large number of anticancer drugs are poorly water-soluble and belong to classes II and IV
49 of the Biopharmaceutics Classification System (BCS) (Amidon et al., 1995). A large body of
50 formulation is required to get these drugs administered through the intravenous route due to
51 their poor solubility in water. Various formulations have been considered such as liposomes,
52 nanoparticles or co-solvents (Zhang et al., 2005; Cortes and Saura, 2010; Miyako et al.,
53 2010), all leading to the presence of organic solvents residues. To avoid toxic effects arising
54 from organic solvents, micellar drug delivery systems can be used (Torchilin, 2001;
55 Lukyanov and Torchilin, 2004). These nanostructures are obtained following a self-
56 assembling process of the surfactants and are able to solubilize hydrophobic drugs in their
57 core. The use of natural surfactants is of increasing interest in drug solubilization. Many
58 studies focus their attention on the synthesis of amino acid-derived surfactants. These novel
59 surfactants possess interesting properties such as a lower toxicity (Holmberg, 2001;
60 Benavides et al., 2004; Sanchez et al., 2007a; Sanchez et al., 2007b) and a higher
61 biodegradability (Infante et al., 1997; Infante et al., 2004). Lysine amino acid was previously
62 used in surfactant design (Infante et al., 1997; Sanchez et al., 2007a). In particular, Vives *et*
63 *al.* studied anionic surfactants derived from lysine varying their hydrophobic chain length
64 from C6 to C10. Shortest-chain compounds (C6) were found less haemolytic and less
65 irritating than longer ones (Vives et al., 1997).

66 In the present study, to improve water solubility of new drug candidates, innovative
67 surfactants were designed and evaluated. They were composed of the same hydrophilic
68 moiety that derives from lysine: nitrilo triacetic acid (NTA) and various fatty acids moieties.
69 Indeed, fatty acids were already used as hydrophobic chains of surfactants linked to different
70 hydrophilic moieties. Palma *et al.* have conjugated L-ascorbic acid to decanoic acid as
71 hydrophobic part. This surfactant was able to self-assemble into water. In addition, micelles
72 of ascorbyl–decanoate significantly enhanced the solubility of hydrophobic molecules
73 (phenacetin, danthron, and griseofulvin) (Palma et al., 2003). For this reason, to obtain novel
74 surfactant structures with potent solubilization properties, lipid hydrophobic moieties were
75 linked to NTA via an amide bond. The lipid moieties were composed of 19 to 25 carbon
76 atoms. In addition, their structures were saturated or contained unsaturations consisting in
77 double bonds or diacetylenic groups. Their self-assembling properties, *in vitro* toxicity and
78 ability to solubilize an insoluble drug were compared to polysorbate 80, a model solubilizer.

79 This study will allow to evaluate the influence of the hydrophobic moiety on surfactant
80 properties.

81

82 **2. Materials and methods**

83

84 *2.1 Materials*

85

86 Polysorbate 80 was obtained from Seppic. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
87 hydrochloride (EDC, HCl) was obtained from Iris Biotech GmbH. Pyrene, triton X-100, 1,3-
88 dicyclohexylcarbodiimide (DCC), palladium on carbon 5 % (w/w), phosphate buffer saline
89 (PBS) and *N*-hydroxysuccinimide (NHS) were provided by Sigma-Aldrich. Dimethyl
90 sulfoxide (DMSO), trifluoroacetic acid (TFA), EtOAc, HCl and dichloromethane (CH₂Cl₂)
91 were purchased from Carlo Erba. Water was purified using a RIOS/synergy system from
92 Millipore. Acetonitrile gradient grade for liquid chromatography, ammonia solution 25 % for
93 analysis, dimethyl formamide (DMF), methanol for HPLC and triethylamine were obtained
94 from Merck. Ammonium formate and bis(carboxymethyl)lysine (NTA) were provided by
95 Fluka analytical.

96

97 *2.2 NMR and mass spectrometry experiments*

98

99 NMR spectra were recorded in deuterated DMSO at 300 K on a Bruker AvANCE-600
100 spectrometer operating at 600.13 MHz for the ¹H and a Bruker AvANCE-400 spectrometer
101 operating at 400.24 MHz for the ¹H. The spectrometer was equipped with a 5-mm Z gradient
102 inversion probe head. The parameters used were 10200 Hz and 7200 Hz spectral width for
103 600 and 400 spectrometer respectively. 64 scans were accumulated using Bruker standard
104 sequence. Chemical shifts (CSs) were given using an external standard reference
105 (Tetramethylsilane, TMS). Mass spectrometry experiments were recorded with a Synapt
106 HDMS (Waters) using a negative electrospray mode.

107

108 2.3 Surfactant synthesis

109

110 2.3.1 Synthesis of (3S)-3-[bis(carboxymethyl)amino]-6-(nonadecanoylamino)hexanoic acid 111 (nonadecanoyl-NTA / C19:0-NTA)

112

113 Nonadecanoic acid (29.3 g, 98.2 mmol, 1 eq) and *N*-hydroxysuccinimide (16.9 g, 147.3
114 mmol, 1.5 eq) were dissolved in CH₂Cl₂ (700 mL) followed by the addition of EDC, HCl
115 (28.6 g, 147.3 mmol, 1.5 eq) (Table 1, Fig. 1). The mixture was placed under magnetic
116 stirring at room temperature for 24 h and then diluted in water (700 mL). Organic and
117 aqueous phases were then separated. The aqueous phase was extracted with CH₂Cl₂ (3 x 250
118 mL). Organic phases were collected, washed with brine (250 mL), dried with Na₂SO₄, filtered
119 and concentrated under reduced pressure. The product (nonadecanoyl-NHS) was finally
120 obtained as a white powder after recrystallisation from hot EtOAc (700 mL).

121 Nonadecanoyl-NHS (3.5 g, 9.17 mmol, 1 eq), bis(carboxymethyl)lysine (NTA) (2.4 g, 9.17
122 mmol, 1 eq) and triethylamine (9 mL, 64.2 mmol, 7 eq) were dissolved in DMF (50 mL)
123 under magnetic stirring at room temperature for 48 h. Solvent was then evaporated under
124 reduced pressure. The residue was taken in water (150 mL) and acidified with HCl 1N until
125 precipitation occurred. The obtained suspension was stirred at room temperature for 1 h and
126 filtered to yield the product as a white powder after drying under vacuum (4.17 g, 7.68 mmol,
127 84 %). The product was analysed with ¹H NMR and mass spectrometry for purity and
128 chemical structure.

129 *Nonadecanoyl-NTA (C19:0-NTA)* (C₂₉H₅₄N₂O₇): ¹H NMR (400 MHz, DMSO-d₆): δ 0.85 (3H,
130 t), 1.23-1.65 (40H, m), 2.01 (2H, t), 3.00 (2H, d), 3.30-3.52 (12H, m), 7.68 (1H, t).

131

132 2.3.2 Synthesis of (3S)-3-[bis(carboxymethyl)amino]-6-[(5Z,8Z,11Z,14Z)-icosa-5,8,11,14- 133 tetraenoylamino]hexanoic acid (arachidonoyl-NTA / C20:4-NTA)

134

135 Bis(carboxymethyl)lysine (NTA) (0.7 g, 2.51 mmol, 1 eq) was dissolved in DMF (70.3 mL)
136 and triethylamine (2.2 mL, 15.7 mmol, 6.3 eq) (Table 1, Fig. 2). Arachidonoyl-NHS (1 g, 2.49
137 mmol, 1 eq) was dissolved in DMF (34.7 mL) under magnetic stirring and nitrogen flow at
138 room temperature. Then arachidonoyl-NHS solution was added to the
139 bis(carboxymethyl)lysine (NTA) solution. The mixture was protected from light under
140 magnetic stirring and nitrogen flow at 100°C for 40 h. The reaction was monitored using
141 Ultra-Fast Liquid Chromatography (UFLC) at 210 nm. DMF was then evaporated under

142 reduced pressure. The residue was dissolved in a mixture of acetonitrile and water (50/50
143 v/v). After filtration through a membrane filter of 0.45 μm pore size (polysulfone), the
144 solution was injected onto a preparative reversed phase column chromatography. A 300 g bed
145 of grafted silica (Kromasil, AkzoNobel) was used as stationary phase. Eluting conditions
146 were as follow: 0-15 min 100 % A, 15-35 min gradient from 100 % A to 100 % B then 35-45
147 min 100 % B. A, being purified water with 0.2 % (v/v) TFA and B CH_3CN with 0.2 % (v/v)
148 TFA. The expected product was collected after 31 min elution time, concentrated under
149 pressure and freeze-dried (0.77 g, 1.4 mmol, 55 %). The product was analysed with ^1H NMR
150 and mass spectrometry for purity and chemical structure.

151 *Arachidonoyl-NTA (C20:4-NTA)* ($\text{C}_{30}\text{H}_{48}\text{N}_2\text{O}_7$): ^1H NMR (600 MHz, DMSO-d_6): δ 0.85 (3H,
152 t), 1.20-1.40 (10H, m), 1.52 (2H, qt), 1.45-1.65 (2H, m), 2.00 (4H, q), 2.03 (2H, t), 2.75-2.79
153 (4H, m), 2.80 (2H, t), 2.98 (2H, q), 3.32 (1H, t), 3.44 (2H, d), 3.50 (2H, d), 5.25-5.40 (8H, m),
154 7.71 (1H, t), 12-13 (3H, vbs).

155 **MS** (ESI-) : m/z : 547.3 $[\text{M-H}]^-$, 569.3 $[\text{M}+\text{Na}-2\text{H}]^-$, 585.3 $[\text{M}+\text{K}-2\text{H}]^-$

156

157 *2.3.3 Synthesis of (3S)-3-[bis(carboxymethyl)amino]-6-(pentacosylamino)*
158 *hexanoic acid (10,12-pentacosadiynoyl-NTA / C25:4-NTA)*

159

160 The synthesis of this surfactant was previously described by Ogier *et al.* In their study, the
161 new drug carrier system was based on self-assembly and polymerization of polydiacetylenic
162 surfactants. The authors also studied the solubilization capacity of non-polymerized analogues
163 (Ogier et al., 2010).

164

165 *2.3.4 Synthesis of (3S)-3-[bis(carboxymethyl)amino]-6-(pentacosanoylamino)hexanoic acid*
166 *(pentacosanoyl-NTA / C25:0-NTA)*

167

168 10,12-pentacosadiynoyl-NTA (C25:4-NTA, 0.5 g, 0.81 mmol, 1 eq) was dissolved in
169 methanol with 250 mg of palladium on carbon 5 % (w/w) at room temperature (Fig. 3).
170 Hydrogenation was performed in a Paar apparatus under H_2 atmosphere (3.5 atm) for 16 h.
171 The mixture was then filtered through a nylon membrane filter and concentrated under
172 reduced pressure. The residue was then solubilized in CH_2Cl_2 and filtered. After CH_2Cl_2
173 evaporation, the expected product was obtained as a white powder (0.01 g, 0.016 mmol, 2 %)
174 Taking into account the low yield after purification, it was hypothesized that a large quantity
175 of the surfactant was still chelated with paladium on carbon (Richard et al., 2003). Residual

176 Pd on carbon was added to a mixture of water (25 mL), NaOH 1N (25 mL) and toluene (25
177 mL). After sonication, the organic layer was removed and replaced by CH₂Cl₂. The solution
178 was then filtered through a membrane filter of 0.45 μm pore size. A yellowish liquid was
179 obtained after filtration and freeze-dried. The residue obtained after lyophilization was
180 solubilized in water and HCl 1N in large excess until precipitation occurred. The precipitate
181 was filtered and freeze-dried to remove the remaining water (0.3 g, 0.48 mmol, 59 %). The
182 product was analysed with ¹H NMR and mass spectrometry for purity and chemical structure.
183 *Pentacosanoyl-NTA (C25:0-NTA) (C₃₅H₆₆N₂O₇):* ¹H NMR (400 MHz, DMSO-d₆): δ 0.85
184 (3H, m), 1.23 (40H, s), 1.30-1.70 (10H, m), 2.01 (2H, t), 2.99 (2H, q), 3.42 (2H, d), 3.49 (2H,
185 d), 7.67 (1H, t), 12-13 (3H, vbs).
186 **MS (ESI-)** : m/z : 625.5 [M-H]⁻, 647.5 [M+Na-2H]⁻

187

188 2.4 Molecular modelling

189

190 Surfactants 3-D structures were estimated *in vacuo* and treated at pH 7.5. Lower energy
191 conformers and packing parameters were calculated using Volsurf+ software (1.0.6)
192 (Molecular Discovery, London). From the Israelachvili packing parameter *P*, it is possible to
193 predict the shape of the aggregate (Israelachvili and Mitchell, 1975). The molecular packing
194 parameter is defined as $P = v_0/(al_0)$ where v_0 and l_0 are the volume and the length of the
195 surfactant tail respectively and a is the area of the surfactant head group. The *P* values for
196 prediction of spherical, cylindrical and lamellar aggregates are respectively for $P \leq 0.33$, 0.33
197 $\leq P \leq 0.5$ and $0.5 \leq P \leq 1$.

198

199 2.5 Fluorescence spectroscopy: CMC determination

200

201 Pyrene was used as a fluorescent hydrophobic probe to determine the critical micellar
202 concentration (CMC) of surfactants. Pyrene would preferentially partition into hydrophobic
203 cores of micelles with a concurrent change of the photophysical properties. Experiments were
204 performed using a LS50B spectrofluorimeter (PerkinElmer). A pyrene stock solution (2×10^{-4}
205 M) was prepared in acetone. A 10 μL aliquot of this solution was introduced into empty vials
206 and the solvent was evaporated at 37°C for 1 h. After evaporation, vials were filled with 1 mL
207 of surfactant solution at a fixed concentration and gently stirred overnight at room
208 temperature to ensure pyrene incorporation into micelles. Experiments were performed in
209 triplicate. Samples were protected from light throughout the experiment. Samples were

210 excited at $\lambda = 335$ nm and pyrene emission spectra were recorded from 370 to 400 nm
211 wavelength. Both excitation and emission slit widths were set at 2.5 nm. Pyrene emission
212 spectra presented vibronic peaks at $\lambda_1 = 372$ nm (intensity I_1) and $\lambda_3 = 392$ nm (intensity I_3).
213 The fluorescence intensity ratio (I_1/I_3) was calculated and plotted as a function of surfactant
214 concentration. Experimentally, the CMC was determined from the inflection point of the plot
215 of the fluorescence intensity ratio versus surfactant concentration (semi-log plot). Using linear
216 regression, the equations describing the two linear parts of the plot were established. The
217 CMC was then obtained from the intersection of these two lines.

218

219 *2.6 In vitro toxicity assays*

220

221 Human Umbilical Vein Endothelial Cells (HUVEC) were cultured in Dulbecco's Modified
222 Eagle's Medium (DMEM) containing L-glutamine and a high glucose concentration (4,5 g/L),
223 supplemented with 10 % Fetal Bovine Serum (FBS) and antibiotics (5 IU/mL penicillin and
224 50 IU/mL streptomycin). They were maintained as an adherent culture and were grown as a
225 monolayer in a humidified incubator, with 5 % CO₂, at 37°C in Petri dishes. DMEM and FBS
226 were purchased from Lonza.

227

228 *2.6.1 Cell viability assay*

229

230 Surfactant toxicity was evaluated using the 3-[4,5-dimethylthiazol-2-yl]-3,5-diphenyl
231 tetrazolium bromide (MTT) assay. This colorimetric test measures the mitochondrial
232 dehydrogenase cell activity, an indicator of cell viability. This assay is based on the reduction,
233 by living cells, of the tetrazolium salt, MTT, which forms a blue formazan product. HUVEC
234 were seeded onto 96 well plates with 200 μ L medium per well at a density of 40000 cells/well
235 for 24 h. Then, 10 μ L of surfactant solution at the desired concentrations were added into each
236 well in triplicate and plates were incubated for 72 h in a humidified incubator, with 5 % CO₂,
237 at 37°C. After incubation, 20 μ L of MTT solution (5 mg/mL in PBS) were added into each
238 well, except for negative control. Cells were further incubated for 2.5 h. After this incubation
239 time, MTT containing media were removed and replaced with 200 μ L of dimethylsulfoxide in
240 order to dissolve the blue formazan product. MTT absorbance was measured at 570 nm
241 wavelength with a microplate reader (Metertech Σ 960, Fisher Bioblock, France). Absorbance
242 value was proportional to the number of living cells. The IC₅₀ was calculated as the sample
243 concentration which inhibits growth of 50 % of cells relative to the control cells according to

244 Unger *et al.* (Unger et al., 2007). IC₅₀ was calculated using the sigmoidal fitting function from
245 Origin[®] v 8.0 (OriginLab, Northampton, USA) when possible.

246

247 2.6.2 Membrane damage assay

248

249 Lactate dehydrogenase (LDH) is a cytosolic enzyme. During the experiment, a release of
250 LDH in the extracellular medium indicates an alteration of the cell membrane. LDH catalyzes
251 the oxidation of lactate to pyruvate with the parallel reduction of NAD⁺ into nicotinamide
252 adenine dinucleotide plus hydrogen (NADH). In turn, NADH reacts with an assay reagent
253 (tetrazolium salt) to yield a red formazan product. LDH escaping from the cytoplasm
254 compartment to the extracellular medium was measured through the formation of the red
255 formazan product in medium, measured at 492 nm using a UV/VIS spectrophotometer. As a
256 comparison, total release of LDH was measured after solubilization of HUVEC membranes
257 with triton X-100 1 % (w/v) after an incubation time of 4 h. The fraction of LDH release was
258 then used as an index of cellular membrane integrity:

259

$$\text{LDH release (\%)} = \frac{(\text{LDH in sample} - \text{LDH 0 \%})}{(\text{LDH 100 \%} - \text{LDH 0\%})} \times 100$$

260

261 where 'LDH in sample' is LDH released in external medium after treatment with surfactants,
262 'LDH 0 %' is LDH present in culture medium and 'LDH 100 %' is LDH released after
263 treatment with 1 % (w/v) triton X-100 corresponding to 100 % cell lysis. The activity of LDH
264 was determined using a LDH kit (Promega).

265

266 2.6.3 Haemolytic assay

267

268 Blood samples (4 mL) were collected in citrated tubes (0.105 M) (BD Vacutainer[®]) from
269 Wistar rats (250–350 g, Charles River Laboratories) by cardiac puncture, under anesthesia
270 with sodium pentobarbital (50 mg/kg). Blood was then centrifuged (2000 rpm, 10 min) with a
271 JOUAN MR1812 centrifuge to isolate red blood cells (RBCs). The erythrocyte suspension
272 was washed twice and dispersed in isotonic PBS buffer (123.3 mM NaCl, 22.2 mM Na₂HPO₄
273 and 5.5 mM KH₂PO₄, 300 mOsmol/L) at pH 7.4. All surfactant solutions were prepared in
274 PBS buffer in order to obtain a pH close to normal blood pH (pH 7.4). Surfactant solutions (V
275 = 100 μL) were incubated and stirred with RBCs suspension (V = 50 μL) at 37°C for 1 h.

276 Instead of surfactants, minimum (0 %) and maximum (100 %) haemolysis were determined in
277 PBS (V = 100 µL) and in triton X-100, 1 % (w/v) (V = 100 µL), respectively. Each sample
278 was prepared in triplicate. Then, 2 mL of cold PBS were added to each sample to stop
279 haemolysis, except for the 100 % haemolysis standard. For this specific standard, 2 mL of
280 purified water were added. The unlysed RBCs were removed by centrifugation (2000 rpm for
281 10 min), and the supernatant was analyzed for haemoglobin release by UV/VIS spectroscopy
282 at 450 nm wavelength (Shimadzu). Haemolysis percentage was determined from the equation
283 below and plotted as a function of surfactant concentration. HC₅₀, the concentration where 50
284 % of RBCs are lysed, was determined for each surfactant. The haemolysis percentage was
285 calculated by the equation:

$$Haemolysis (\%) = \frac{(Abs - Abs_0)}{(Abs_{100} - Abs_0)} \times 100$$

287
288 where 'Abs', 'Abs₀', and 'Abs₁₀₀' were sample absorbance, control with PBS and control in
289 the presence of haemolytic dose of triton X-100, respectively.

290

291 *2.7 Drug solubilization*

292

293 Surfactant solutions were prepared in purified water whose pH was adjusted to 12 with NaOH
294 1N. Then HCl 1N and 0.1N were added to accurately adjust the pH to 7.4 (C > CMC). An
295 insoluble drug synthesized by Technologie SERVIER (MW: 861.5 g/mol, solubility: in water
296 < 0.15 µg/mL) was used as a model insoluble drug. This anticancer drug is an antagonist of
297 Bcl-2 receptors, which is soluble in methanol and acetonitrile at 8.5 mg/mL and 1.0 mg/mL,
298 respectively. The drug was added at 20 mg/mL in the surfactant solution and magnetic stirring
299 was applied during 24 h. Filtration of the insoluble part of the drug was then performed
300 through a membrane filter of 0.45 µm pore size (polysulfone). The filtrate was diluted in
301 acetonitrile/purified water (50/50, V/V). The amount of solubilized drug was quantified by
302 HPLC system with UV detection at 250 nm. Drug loading was calculated dividing the mass of
303 the solubilized drug by the sum of the surfactant mass (10 mg/mL) and the solubilized drug
304 mass.

305

306

307

308

309

310

311 **3. Results and discussion**

312

313 *3.1 Surfactant synthesis and solubility*

314

315 All surfactants were synthesized and isolated with good purity as described above. Their
316 chemical structure was evidenced using ¹H NMR spectra and mass spectrometry analysis.
317 NTA-based surfactants water-solubility was pH-dependent due to their anionic polar head. A
318 required amount of NaOH 1N was therefore added to ensure a complete dissolution of each
319 surfactant at pH 12. Then HCl 1N and 0.1N were added to accurately adjust the pH to 7.4.
320 Surprisingly, C25:0-NTA exhibited a pronounced gelation effect increasing proportionally
321 with surfactant concentration. This gelation occurred after surfactant solubilization in basic
322 conditions and the gel was preserved at pH 7.4. Due to the monomeric structure of C25:0-
323 NTA, this phenomenon could be explained by the formation of cylindrical worm-like micelles
324 in solution (Walker, 2009; Berret, 2011). Gelification most probably occurs by entanglement
325 of wormlike micelles as their concentration increases. To strengthen this hypothesis, a
326 molecular modelling study was performed.

327

328 *3.2 Molecular modelling*

329

330 Lower energy conformers of surfactants were determined and plotted in Table 2 for easier
331 comparison. Both saturated surfactants, composed of lipid tail containing 19 or 25 carbon
332 atoms, possess extended hydrophobic moieties. Because it contains a diacetylenic group,
333 C25:4-NTA conformer presents a bend that confers a lower flexibility to the lipid chain
334 without affecting its linear conformation. By contrast, C20:4-NTA exhibits a clear different
335 behaviour with its lipid moiety totally folded on itself due to the presence of four double
336 bonds. This leads to a non-linear conformation. Regarding the packing parameter
337 (Israelachvili and Mitchell, 1975), short hydrophobic chain surfactants, C19:0-NTA and
338 C20:4-NTA, have geometric packing parameter values lower than 0.33 (Table 2). Taking into
339 account geometric parameters of the polar and apolar moieties of the surfactant, the value of
340 the packing parameter indicates the trend of both surfactants to self-assemble into spherical
341 aggregates. C25:0-NTA and C25:4-NTA geometric packing parameter values are rather close

342 to 0.5, which is characteristic of cylindrical or lamellar structures. Since alkanes chains
343 composed of 25 carbon atoms are bulkier than the shorter ones, these surfactants need to have
344 a very large headgroup to form a spherical aggregate in order to balance the effect of the
345 bulky hydrophobic tail (El Moujahid et al., 1998). For C25:0-NTA, the calculated packing
346 parameter is consistent with the hypothesis of cylindrical micelles, forming an entangled gel
347 at high concentration.

348

349 *3.3 Surfactant self-assembling capacity: pyrene fluorescence measurements*

350

351 Pyrene fluorescence measurements were performed and fluorescence intensity ratios (I_1/I_3)
352 were plotted as a function of surfactant concentration (semi-log plot, Fig. 4). The I_1/I_3 ratio is
353 initially steady with low surfactant concentrations, proving that pyrene is in a hydrophilic
354 environment. Then a significant decrease of I_1/I_3 ratio is observed indicating the formation of
355 hydrophobic domains where pyrene is solubilized, a signature of surfactant micellization.
356 CMC values were determined and reported in Table 3 for easier comparison. Polysorbate 80
357 CMC value is 14 $\mu\text{g/mL}$, consistent with data from literature (Wan, 1980). C19:0-NTA,
358 C25:0-NTA and C25:4-NTA CMC values were found of the same order of magnitude: 12, 40
359 and 24 $\mu\text{g/mL}$, respectively. The slight difference of CMC between C25:0-NTA and C25:4-
360 NTA probably arises from interactions between neighbouring diacetylenic groups of the
361 latter, leading to a lower CMC. The CMC of C20:4-NTA is an order of magnitude larger with
362 a value of 430 $\mu\text{g/mL}$, most probably due to the presence of unsaturations which induce a
363 weaker hydrophobicity of the acyl chain (Chen et al., 2007). In addition, these results are in
364 agreement with the study of Brito *et al.*, who evidenced that CMC was higher for unsaturated
365 compounds than for saturated ones. The presence of unsaturations creates larger chain
366 packing constraints in the micellar core, due to a smaller number of conformational degrees of
367 freedom. This does not favour micellization hence increasing CMC (Brito et al., 2011). All
368 surfactants tested were able to spontaneously self-assemble into water, with low CMC values
369 in the same range as polysorbate 80 for 3 of them.

370

371 *3.3 In vitro toxicity evaluation of surfactants*

372

373 *3.3.1 Cell viability assay*

374

375 HUVEC viability was then assessed (Fig. 5, Table 3). A significant decrease of HUVEC
376 viability was observed with C19:0-NTA concentrations higher than its CMC value. It is likely
377 that C19:0-NTA micelles interact with HUVEC membrane and thus caused a decrease of cell
378 viability. With an IC_{50} of 0.02 mg/mL, C19:0-NTA was more cytotoxic than polysorbate 80
379 ($IC_{50} = 0.25$ mg/mL) despite their similar CMC values. This was probably due to the higher
380 hydrophobicity of C19:0-NTA, in comparison with polysorbate 80. Surprisingly, C25:0-NTA
381 didn't decrease cell viability within the concentration range tested. The diffusion rate of
382 surfactant monomers and micelles went down in the particular case of C25:0-NTA, due to
383 gelation. Therefore, we hypothesized that gelation prevents their interaction with HUVEC, as
384 observed by the absence of toxicity of this surfactant. A decrease of cell viability was
385 observed with C25:4-NTA concentrations higher than its CMC value, leading to an IC_{50} of
386 0.04 mg/mL. As a comparison, C20:4-NTA was slightly less toxic than polysorbate 80 with
387 an IC_{50} of 0.32 mg/mL. C25:0-NTA and C25:4-NTA do not seem to interact with HUVEC in
388 the same way. Saturated surfactant, C25:0-NTA, was well tolerated whereas the unsaturated
389 one, C25:4-NTA, decreased cell viability dramatically. These results evidenced that the
390 presence of unsaturations in hydrophobic backbone influenced cytotoxicity. In the case of
391 C25:4-NTA, the diacetylenic group prevents gelation, micelles are therefore able to diffuse
392 rapidly and interact with HUVEC.

393

394 *3.3.2 Membrane damage assay*

395

396 Toxicity towards cell membrane was evaluated using LDH assay (Fig. 6, Table 3). The toxic
397 effect of C19:0-NTA towards cell membrane was slightly lower than with polysorbate 80 with
398 50 % membrane lysis for concentrations of 0.2 and 0.13 mg/mL respectively. The assay
399 evidences that C19:0-NTA is able to destabilize and disrupt HUVEC membranes probably by
400 insertion of the hydrophobic chain, into the cell membrane (Nogueira et al., 2011). As a
401 comparison, C25:0-NTA is well tolerated and does not induce cell damage: no IC_{50} could be
402 determined, probably because of its gelation properties. The network probably slows down
403 the diffusion of surfactant monomers and thus limits their interaction with HUVEC
404 membranes. Both surfactants composed of unsaturated hydrophobic chains, C20:4-NTA and
405 C25:4-NTA were less toxic than polysorbate 80, with IC_{50} values of 0.68 and 0.64 mg/mL
406 respectively (Fig. 6, Table 3). The presence of unsaturations in their hydrophobic moieties
407 decreases surfactant interactions with cell membrane. In the case of C20:4 hydrophobic part,
408 unsaturations lead to an important steric hindrance which prevents its incorporation within the

409 cell membrane. C25-based surfactants can be compared in terms of cytotoxicity: the
410 unsaturated surfactant C25:4-NTA is toxic towards cell membranes whereas in the case of the
411 saturated one, C25:0-NTA was not toxic, probably due to its gelling properties.

412 To conclude, membrane toxicity is clearly related to the length of surfactant hydrophobic
413 moiety: small and saturated hydrophobic chains (C19:0) interact strongly with cell
414 membranes whereas longer saturated chains (C25:0) do not incorporate into cell membrane.
415 Furthermore, C20:4-NTA results demonstrate that steric hindrance prevents cell membrane
416 interactions and thus cytotoxic effects.

417

418 *3.3.3 Haemolytic assay*

419

420 Haemolytic activity of surfactants was evaluated using rat RBCs. Haemolysis percentage was
421 plotted as a function of surfactant concentration (semi-log plot). For C19:0-NTA and C25:0-
422 NTA, results evidence that 50 % haemolysis occurs within the same concentration range, with
423 HC_{50} values of 0.15 and 0.13 mg/mL, respectively (Fig. 7 and Table 3). These surfactants are
424 more haemolytic than polysorbate 80, which HC_{50} value is 0.76 mg/mL. The length of the
425 hydrophobic moiety, C19:0 versus C25:0, does not seem to influence surfactant haemolytic
426 activity. C25:4-NTA is also more haemolytic than polysorbate 80 with an HC_{50} of 0.25
427 mg/mL, in the same range as C19:0-NTA and C25:0-NTA. Surprisingly, a dramatic
428 improvement was observed with C20:4-NTA which showed a 5-fold increase of the HC_{50}
429 value compared to polysorbate 80 (3.7 mg/mL). As observed for LDH release, the presence of
430 four Z-unsaturations in the hydrophobic moiety of C20:4-NTA, leads to an important steric
431 hindrance that prevents the insertion of the hydrophobic moiety within RBC membrane. The
432 other surfactants which hydrophobic moieties are rather linear, can easily insert in RBCs
433 membrane, causing haemolysis.

434

435 *3.4 Drug solubilization capacity*

436

437 Finally, drug solubilization was evaluated on a poorly soluble drug ($< 0.15 \mu\text{g/mL}$) (Fig. 8
438 and Table 4). C20:4-NTA was far less efficient than polysorbate 80 as solubilizing agent with
439 solubility of 0.27 mg/mL (x 2000), corresponding to a drug loading of 3.5 % (w/w) only. As
440 observed, on cell studies, the steric hindrance of C20:4-NTA prevents interactions with other
441 hydrophobic compounds and therefore drug solubilization. Three surfactants out of four were
442 able to increase drug solubility more efficiently than polysorbate 80 which only led to the

443 solubility of 3.16 mg/mL (x 20000), corresponding to a drug loading of 24 % (w/w) (Table 4).
444 C19:0-NTA, C25:0-NTA and C25:4-NTA increased drug solubility to concentrations higher
445 than 7 mg/mL (x 47000 to x 57000) with drug loading of 41 %, 43 % and 46 % (w/w)
446 respectively (Table 4). C25:0-NTA seems slightly more efficient than C19:0-NTA in
447 solubilizing the drug which tends to indicate that a longer chain favours solubilization.
448 Surprisingly, C25:4-NTA exhibits better solubilization efficiency than C25:0-NTA. The
449 diacetylenic group, present in C25:4-NTA chemical structure seems to be favourable to drug
450 solubilization.

451

452 **4. Conclusion**

453

454 Lysine based-surfactants synthesized in this study self-assemble into water with low CMC
455 values ranging from 0.012 to 0.430 mg/mL. C19:0-NTA and C25:4-NTA were found to be
456 more cytotoxic than polysorbate 80, whereas gelling properties of C25:0-NTA led to a low
457 cytotoxicity. In addition, C20:4-NTA also exhibits a low cytotoxicity due to the presence of
458 Z-unsaturations in its hydrophobic moiety. All surfactants exert a lower membrane toxicity
459 than polysorbate 80. Surfactants with linear and flexible hydrophobic moieties lead to
460 haemolysis at lower concentrations than polysorbate 80. On the other hand, C20:4-NTA,
461 possesses a lower haemolytic activity due to the steric hindrance of its lipidic tail that prevents
462 its insertion into RBC membranes. C19:0-NTA, C25:0-NTA and C25:4-NTA, are more
463 efficient solubilizers, than polysorbate 80, due to the high flexibility and linear conformation
464 of their hydrophobic moieties that favours interaction with the hydrophobic drug. They
465 constitute promising drug delivery systems to solubilize anticancer drugs, via their self-
466 assembling ability into spherical, cylindrical or lamellar structures. Overall, it appears that
467 selection of a hydrophobic moiety will have to be done considering the affinity for the cell
468 membrane leading to cytotoxicity and the strength of the hydrophobic interaction with the
469 drug leading to an improved solubility. The best compromise will have to be found and will
470 be tested *in vivo* in due time.

471

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473

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