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Drug solubilization and in vitro toxicity evaluation of lipoamino acid surfactants

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ABSTRACT

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

1. Introduction

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

In the present study, to improve water solubility of new drug candidates, innovative surfactants were designed and evaluated. They were composed of the same hydrophilic moiety that derives from lysine: nitrilo triacetic acid (NTA) and various fatty acids moieties. Indeed, fatty acids were already used as hydrophobic chains of surfactants linked to different hydrophilic moieties. Palma et al. (2003) have conjugated L-ascorbic acid to decanoic acid as hydrophobic part. This surfactant was able to self-assemble into water. In addition, micelles of ascorbyl-decanoate significantly enhanced the solubility of hydrophobic molecules (phenacetin, danthron, and griseofulvin). For this reason, to obtain novel surfactant structures with potent solubilization properties, lipid hydrophobic moieties were linked to NTA via an amide bond. The lipid moieties were composed of 19–25 carbon atoms. In addition,

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their structures were saturated or contained unsaturations consisting in double bonds or diacetylenic groups. Their self-assembling properties, in vitro toxicity and ability to solubilize an insoluble drug were compared to polysorbate 80, a model solubilizer. This study will allow to evaluate the influence of the hydrophobic moiety on surfactant properties.

2. Materials and methods

2.1. Materials

Polysorbate 80 was obtained from Seppic. 1-Ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride (EDC, HCl) was obtained from Iris Biotech GmbH. Pyrene, triton X-100, 1,3-dicyclohexylcarbodiimide (DCC), palladium on carbon 5% (w/w), phosphate buffer saline (PBS) and *N*-hydroxysuccinimide (NHS) were provided by Sigma–Aldrich. Dimethyl sulfoxide (DMSO), trifuoroacetic acid (TFA), EtOAc, HCl and dichloromethane (CH_2Cl_2) were purchased from Carlo Erba. Water was purified using a RIOS/synergy system from Millipore. Acetonitrile gradient grade for liquid chromatography, ammonia solution 25% for analysis, dimethyl formamide (DMF), methanol for HPLC and triethylamine were obtained from Merck. Ammonium formate and bis(carboxymethyl)lysine (NTA) were provided by Fluka analytical.

2.2. NMR and mass spectrometry experiments

NMR spectra were recorded in deuterated DMSO at 300 K on a Bruker AvANCE-600 spectrometer operating at 600.13 MHz for the ^1H and a Bruker AvANCE-400 spectrometer operating at 400.24 MHz for the ^1H. The spectrometer was equipped with a 5-mm Z gradient inversion probe head. The parameters used were 10,200 Hz and 7200 Hz spectral width for 600 and 400 spectrometer respectively. 64 scans were accumulated using Bruker standard sequence. Chemical shifts (CSs) were given using an external standard reference (Tetramethylsilane, TMS). Mass spectrometry experiments were recorded with a Synapt HDMS (Waters) using a negative electrospray mode.

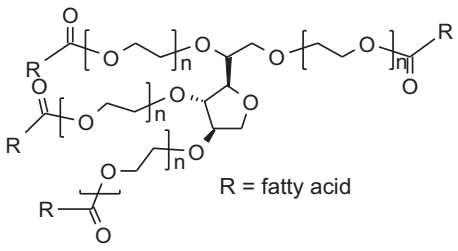
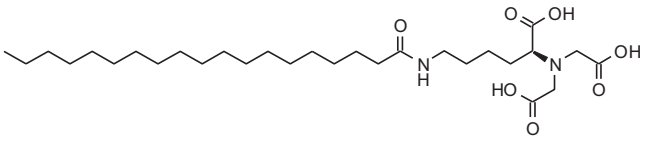
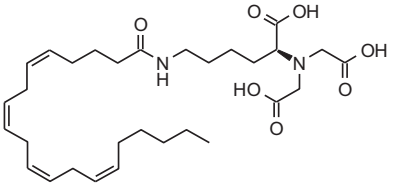
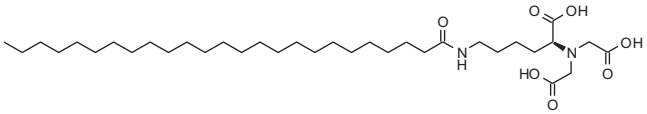
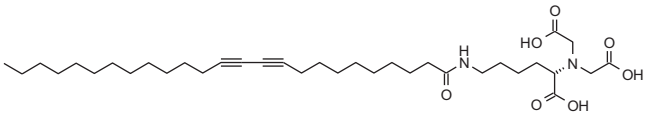
2.3. Surfactant synthesis

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

Nonadecanoic acid (29.3 g, 98.2 mmol, 1 equiv.) and *N*-hydroxysuccinimide (16.9 g, 147.3 mmol, 1.5 equiv.) were dissolved in CH_2Cl_2 (700 mL) followed by the addition of EDC, HCl (28.6 g, 147.3 mmol, 1.5 equiv.) (Table 1 and Fig. 1). The mixture was placed under magnetic stirring at room temperature for 24 h and then diluted in water (700 mL). Organic and aqueous phases were then separated. The aqueous phase was extracted with CH_2Cl_2 (3×250 mL). Organic phases were collected, washed with brine (250 mL), dried with Na_2SO_4, filtered and concentrated under reduced pressure. The product (nonadecanoyl-NHS) was finally obtained as a white powder after recrystallisation from hot EtOAc (700 mL).

Nonadecanoyl-NHS (3.5 g, 9.17 mmol, 1 equiv.), bis(carboxymethyl)lysine (NTA) (2.4 g, 9.17 mmol, 1 equiv.)

Table 1
Nitrilo triacetic acid (NTA) based-surfactants composition, hydrophobic and hydrophilic moieties, molecular weights and chemical structures.

Surfactant	Hydrophobic moiety	Hydrophilic moiety	*M* (g/mol)	Chemical structure
Polysorbate 80	Sorbitan fatty acid esters	Polyoxy ethylene	1310	
C19:0-NTA	Nonadecanoyl	NTA	543	
C20:4-NTA	Arachidonoyl	NTA	549	
C25:0-NTA	Pentacosanoyl	NTA	627	
C25:4-NTA	Pentacosadiynoyl	NTA	619	

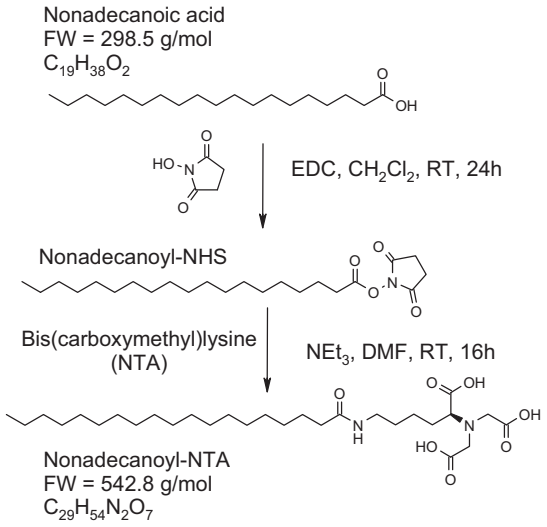


Fig. 1. Synthesis of nonadecanoyl-NTA (C19:0-NTA).

and triethylamine (9 mL, 64.2 mmol, 7 equiv.) were dissolved in DMF (50 mL) under magnetic stirring at room temperature for 48 h. Solvent was then evaporated under reduced pressure. The residue was taken in water (150 mL) and acidified with HCl 1 N until precipitation occured. The obtained suspension was stirred at room temperature for 1 h and filtered to yield the product as a white powder after drying under vacuum (4.17 g, 7.68 mmol, 84%). The product was analysed with ^{1}H NMR and mass spectrometry for purity and chemical structure.

Nonadecanoyl-NTA (C19:0-NTA) ($C_{29}H_{54}N_2O_7$): ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.85 (3H, 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).

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

Bis(carboxymethyl)lysine (NTA) (0.7 g, 2.51 mmol, 1 equiv.) was dissolved in DMF (70.3 mL) and triethylamine (2.2 mL, 15.7 mmol, 6.3 equiv.) (Table 1 and Fig. 2). Arachidonoyl-NHS (1 g, 2.49 mmol, 1 equiv.) was dissolved in DMF (34.7 mL) under magnetic stirring and nitrogen flow at room temperature. Then arachidonoyl-NHS solution was added to the bis(carboxymethyl)lysine (NTA) solution. The mixture was protected from light under magnetic stirring and nitrogen flow at 100 °C for 40 h. The reaction was monitored using Ultra-Fast Liquid Chromatography (UFLC) at 210 nm. DMF was then evaporated under reduced pressure. The residue was dissolved in a mixture of acetonitrile and water (50/50, v/v). After filtration through a membrane filter of 0.45 μm pore size (polysulfone), the solution was injected onto a preparative reversed phase column chromatography. A 300 g bed of grafted silica (Kromasil, AkzoNobel) was used as stationnary phase. Eluting conditions were as follows: 0–15 min 100% A, 15–35 min gradient from 100% A to 100% B then 35–45 min 100% B. A, being purified water with 0.2% (v/v) TFA and B CH_3CN with 0.2% (v/v) TFA. The expected product was collected after 31 min elution time, concentrated under pressure and freeze-dried (0.77 g, 1.4 mmol, 55%). The product was analysed with ^{1}H NMR and mass spectrometry for purity and chemical structure.

Arachidonoyl-NTA (C20:4-NTA) ($C_{30}H_{48}N_2O_7$): ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.85 (3H, 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 (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), 7.71 (1H, t), 12–13 (3H, vbs).

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

2.3.3. Synthesis of (3S)-3-[bis(carboxymethyl)amino]-6-(pentacosa-10,12-diynoylamino) hexanoic acid (10,12-pentacosadiynoyl-NTA/C25:4-NTA)

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

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

10,12-pentacosadiynoyl-NTA (C25:4-NTA, 0.5 g, 0.81 mmol, 1 equiv.) was dissolved in methanol with 250 mg of palladium on carbon 5% (w/w) at room temperature (Fig. 3). Hydrogenation was performed in a Paar apparatus under H_2 atmosphere (3.5 atm) for 16 h. The mixture was then filtered through a nylon membrane filter and concentrated under reduced pressure. The residue was then solubilized in CH_2Cl_2 and filtered. After CH_2Cl_2 evaporation, the expected product was obtained as a white powder (0.01 g, 0.016 mmol, 2%) Taking into account the low yield after

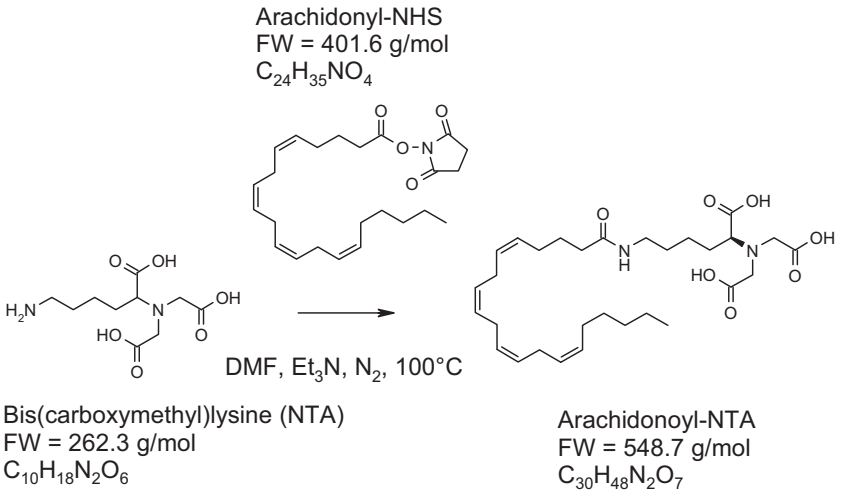


Fig. 2. Synthesis of arachidonoyl-NTA (C20:4-NTA).

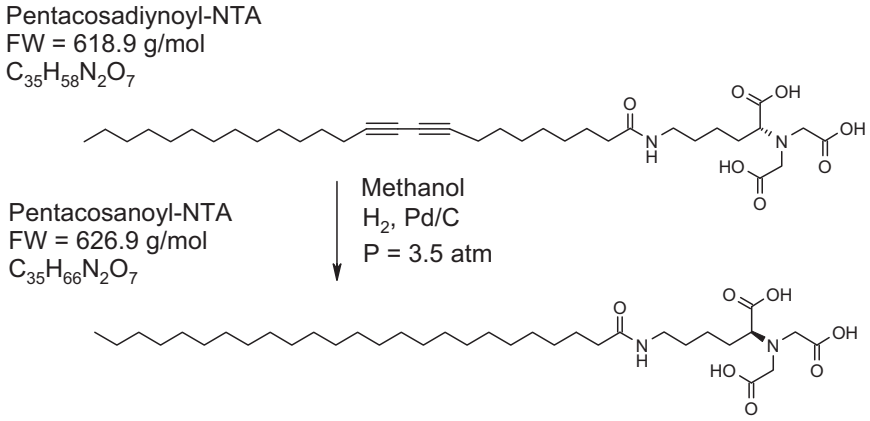


Fig. 3. Synthesis of pentacosanoyl-NTA (C25:0-NTA).

purification, it was hypothesized that a large quantity of the surfactant was still chelated with paladium on carbon (Richard et al., 2003). Residual Pd on carbon was added to a mixture of water (25 mL), NaOH 1 N (25 mL) and toluene (25 mL). After sonication, the organic layer was removed and replaced by CH_2Cl_2. The solution was then filtered through a membrane filter of 0.45 μm pore size. A yellowish liquid was obtained after filtration and freeze-dried. The residue obtained after lyophilization was solubilized in water and HCl 1 N in large excess until precipitation occured. The precipitate was filtered and freeze-dried to remove the remaining water (0.3 g, 0.48 mmol, 59%). The product was analysed with ^{1}H NMR and mass spectrometry for purity and chemical structure.

Pentacosanoyl-NTA (C25:0-NTA) ($C_{35}H_{66}N_2O_7$): ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.85 (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, d), 7.67 (1H, t), 12–13 (3H, vbs).

MS (ESI−): m/z: 625.5 $[M-H]^-$, 647.5 $[M+Na-2H]^-$

2.4. Molecular modelling

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

2.5. Fluorescence spectroscopy: CMC determination

Pyrene was used as a fluorescent hydrophobic probe to determine the critical micellar concentration (CMC) of surfactants. Pyrene would preferentially partition into hydrophobic cores of micelles with a concurrent change of the photophysical properties. Experiments were performed using a LS50B spectrofluorimeter (PerkinElmer). A pyrene stock solution (2×10^{-4} M) was prepared in acetone. A 10 μL aliquot of this solution was introduced into empty vials and the solvent was evaporated at 37 °C for 1 h. After evaporation, vials were filled with 1 mL of surfactant solution at a fixed concentration and gently stirred overnight at room temperature to ensure pyrene incorporation into micelles. Experiments were performed in triplicate. Samples were protected from light throughout the experiment. Samples were excited at $\lambda = 335$ nm and pyrene emission spectra were recorded from 370 to 400 nm wavelength. Both excitation and emission slit widths were set at 2.5 nm. Pyrene emission spectra presented vibronic peaks at $\lambda_1 = 372$ nm (intensity I_1) and $\lambda_3 = 392$ nm (intensity I_3). The fluorescence intensity ratio (I_1/I_3) was calculated and plotted as a function of surfactant concentration. Experimentally, the CMC was determined from the inflection point of the plot of the fluorescence intensity ratio versus surfactant concentration (semi-log plot). Using linear regression, the equations describing the two linear parts of the plot were established. The CMC was then obtained from the intersection of these two lines.

2.6. In vitro toxicity assays

Human Umbilical Vein Endothelial Cells (HUVEC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing L-glutamine and a high glucose concentration (4.5 g/L), supplemented with 10% Fetal Bovine Serum (FBS) and antibiotics (5 IU/mL penicillin and 50 IU/mL streptomycin). They were maintained as an adherent culture and were grown as a monolayer in a humidified incubator, with 5% CO_2, at 37 °C in Petri dishes. DMEM and FBS were purchased from Lonza.

2.6.1. Cell viability assay

Surfactant toxicity was evaluated using the 3-[4,5-dimethylthiazol-2-yl]-3,5-diphenyl tetrazolium bromide (MTT) assay. This colorimetric test measures the mitochondrial dehydrogenase cell activity, an indicator of cell viability. This assay is based on the reduction, by living cells, of the tetrazolium salt, MTT, which forms a blue formazan product. HUVEC were seeded onto 96 well plates with 200 μL medium per well at a density of 40,000 cells/well for 24 h. Then, 10 μL of surfactant solution at the desired concentrations were added into each well in triplicate and plates were incubated for 72 h in a humidified incubator, with 5% CO_2, at 37 °C. After incubation, 20 μL of MTT solution (5 mg/mL in PBS) were added into each well, except for negative control. Cells were further incubated for 2.5 h. After this incubation time, MTT containing media were removed and replaced with 200 μL of dimethylsulfoxide in order to dissolve the blue formazan product. MTT absorbance was measured at 570 nm wavelength with a microplate reader (Metertech Σ 960, Fisher Bioblock, France). Absorbance value was proportional to the number of living cells. The IC_{50} was calculated as the sample concentration which inhibits growth of 50% of cells relative to the control cells according to Unger et al. (2007). IC_{50} was calculated using the sigmoidal fitting function from Origin® v 8.0 (OriginLab, Northampton, USA) when possible.

2.6.2. Membrane damage assay

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

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

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

2.6.3. Haemolytic assay

Blood samples (4 mL) were collected in citrated tubes (0.105 M) (BD Vacutainer®) from Wistar rats (250–350 g, Charles River Laboratories) by cardiac puncture, under anesthesia with sodium pentobarbital (50 mg/kg). Blood was then centrifuged (2000 rpm, 10 min) with a JOUAN MR1812 centrifuge to isolate red blood cells (RBCs). The erythrocyte suspension was washed twice and dispersed in isotonic PBS buffer (123.3 mM NaCl, 22.2 mM Na_2HPO_4 and 5.5 mM KH_2PO_4, 300 mOsmol/L) at pH 7.4. All surfactant solutions were prepared in PBS buffer in order to obtain a pH close to normal blood pH (pH 7.4). Surfactant solutions (V = 100 μL) were incubated and stirred with RBCs suspension (V = 50 μL) at 37 °C for 1 h. Instead of surfactants, minimum (0%) and maximum (100%) haemolysis were determined in PBS (V = 100 μL) and in triton X-100, 1% (w/v) (V = 100 μL), respectively. Each sample was prepared in triplicate. Then, 2 mL of cold PBS were added to each sample to stop haemolysis, except for the 100% haemolysis standard. For this specific standard, 2 mL of purified water were added. The unlysed RBCs were removed by centrifugation (2000 rpm for 10 min), and the supernatant was analysed for haemoglobin release by UV/VIS spectroscopy at 450 nm wavelength (Shimadzu). Haemolysis percentage was determined from the equation below and plotted as a function of surfactant concentration. HC_{50}, the concentration where 50% of RBCs are lysed, was determined for each surfactant. The haemolysis percentage was calculated by the equation:

$$\text{Haemolysis}\ \ (\%) = \frac{(\text{Abs} - \text{Abs0})}{(\text{Abs100} - \text{Abs0})} \times 100$$

where 'Abs', 'Abs_0', and 'Abs_{100}' were sample absorbance, control with PBS and control in the presence of haemolytic dose of triton X-100, respectively.

2.7. Drug solubilization

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

3. Results and discussion

3.1. Surfactant synthesis and solubility

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

3.2. Molecular modelling

Lower energy conformers of surfactants were determined and plotted in Table 2 for easier comparison. Both saturated surfactants, composed of lipid tail containing 19 or 25 carbon atoms, possess extended hydrophobic moieties. Because it contains a diacetylenic group, C25:4-NTA conformer presents a bend that confers a lower flexibility to the lipid chain without affecting its linear conformation. By contrast, C20:4-NTA exhibits a clear different behaviour with its lipid moiety totally folded on itself due to the presence of four double bonds. This leads to a non-linear conformation. Regarding the packing parameter (Israelachvili and Mitchell, 1975), short hydrophobic chain surfactants, C19:0-NTA and C20:4-NTA, have geometric packing parameter values lower than 0.33 (Table 2). Taking into account geometric parameters of the polar and apolar moieties of the surfactant, the value of the packing parameter indicates the trend of both surfactants to self-assemble into spherical aggregates. C25:0-NTA and C25:4-NTA geometric packing parameter values are rather close to 0.5, which is characteristic of cylindrical or lamellar structures. Since alkanes chains composed of 25 carbon atoms are bulkier than the shorter ones, these surfactants need to have a very large headgroup to form a spherical aggregate in order to balance the effect of the bulky hydrophobic tail (El Moujahid et al., 1998). For C25:0-NTA, the calculated packing parameter is consistent with the hypothesis of cylindrical micelles, forming an entangled gel at high concentration.

3.3. Surfactant self-assembling capacity: pyrene fluorescence measurements

Pyrene fluorescence measurements were performed and fluorescence intensitiy ratios (I_1/I_3) were plotted as a function of surfactant concentration (semi-log plot, Fig. 4). The I_1/I_3 ratio is initially steady with low surfactant concentrations, proving that pyrene is in a hydrophilic environment. Then a significant decrease

Table 2
Surfactant molecular modelling *in vacuo*: packing parameters and lower energy conformers.

Surfactants	Packing parameter	Lower energy conformers
C19:0-NTA	0.24	
C20:4-NTA	0.24	
C25:0-NTA	0.37	
C25:4-NTA	0.50	

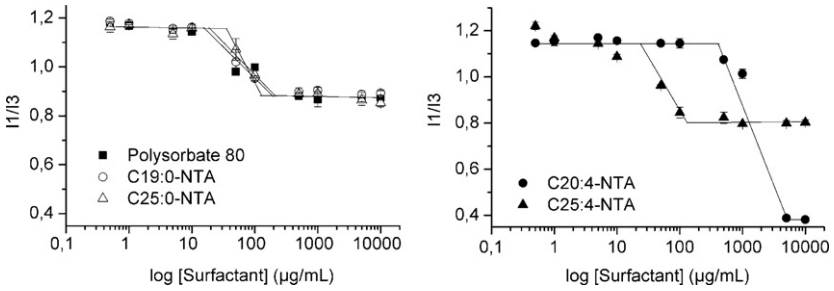


Fig. 4. Pyrene fluorescence I_1/I_3 ratio versus surfactant concentration for polysorbate 80 (■), C19:0-NTA (○), C25:0-NTA (△), C20:4-NTA (●) and C25:4-NTA (▲). All data are mean ± S. D., $n = 3$.

of I_1/I_3 ratio is observed indicating the formation of hydrophobic domains where pyrene is solubilized, a signature of surfactant micellization. CMC values were determined and reported in Table 3 for easier comparison. Polysorbate 80 CMC value is 14 µg/mL, consistent with data from literature (Wan, 1980). C19:0-NTA, C25:0-NTA and C25:4-NTA CMC values were found in the same order of magnitude: 12, 40 and 24 µg/mL, respectively. The slight difference of CMC between C25:0-NTA and C25:4-NTA probably arises from interactions between neighbouring diacetylenic groups of the latter, leading to a lower CMC. The CMC of C20:4-NTA is an order of magnitude larger with a value of 430 µg/mL, most probably due to the presence of unsaturations which induce a weaker hydrophobicity of the acyl chain (Chen et al., 2007). In addition, these results are in agreement with the study of Brito et al. (2011), who evidenced that CMC was higher for unsaturated compounds than for saturated ones. The presence of unsaturations

Table 3
Comparison of novel surfactants with polysorbate 80 in terms of CMC, IC_{50} of determined by MTT and LDH assays and HC_{50} of rat red blood cells. NA, not applicable.

Surfactants	CMC (mg/mL)	CMC (mol/L)	MTT assay IC_{50} (mg/mL)	LDH assay IC_{50} (mg/mL)	Haemolytic assay HC_{50} (mg/mL)
Polysorbate 80	0.014	1.1×10^{-5}	0.25	0.13	0.76
C19:0-NTA	0.012	2.2×10^{-5}	0.02	0.2	0.15
C20:4-NTA	0.430	7.8×10^{-4}	0.32	0.68	3.715
C25:0-NTA	0.040	6.4×10^{-5}	NA	NA	0.13
C25:4-NTA	0.024	3.8×10^{-5}	0.04	0.64	0.25

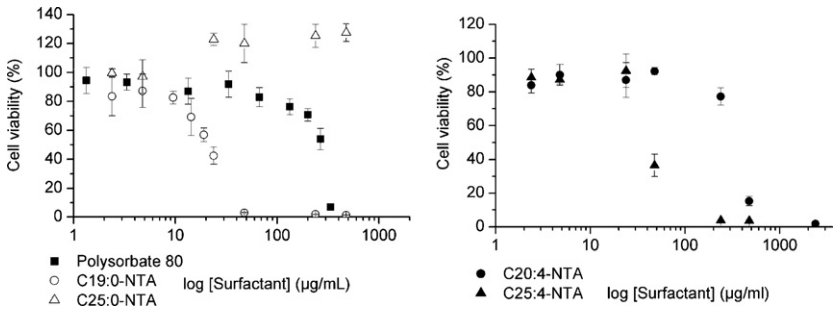


Fig. 5. Viability of HUVEC treated by polysorbate 80 (■), C19:0-NTA (○), C25:0-NTA (Δ), C20:4-NTA (●) and C25:4-NTA (▲). All data are mean ± S. D., $n = 8$.

creates larger chain packing constraints in the micellar core, due to a smaller number of conformational degrees of freedom. This does not favour micellization hence increasing CMC. All surfactants tested were able to spontaneously self-assemble into water, with low CMC values in the same range as polysorbate 80 for 3 of them.

3.4. In vitro toxicity evaluation of surfactants

3.4.1. Cell viability assay

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

3.4.2. Membrane damage assay

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

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

3.4.3. Haemolytic assay

Haemolytic activity of surfactants was evaluated using rat RBCs. Haemolysis percentage was plotted as a function of surfactant concentration (semi-log plot). For C19:0-NTA and C25:0-NTA, results evidence that 50% haemolysis occurs within the same concentration range, with HC_{50} values of 0.15 and 0.13 mg/mL, respectively (Fig. 7 and Table 3). These surfactants are more haemolytic than polysorbate 80, which HC_{50} value is 0.76 mg/mL. The length of the hydrophobic moiety, C19:0 versus C25:0, does not seem to

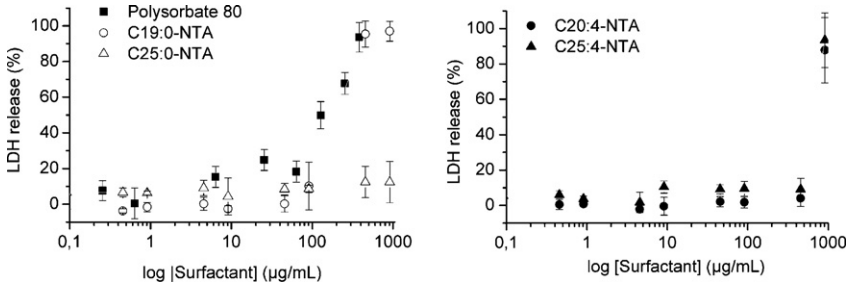


Fig. 6. Membrane toxicity of polysorbate 80 (■), C19:0-NTA (○), C25:0-NTA (Δ), C20:4-NTA (●) and C25:4-NTA (▲), using LDH release in HUVEC after an incubation time of 4 h. All data are mean ± S. D., $n = 8$.

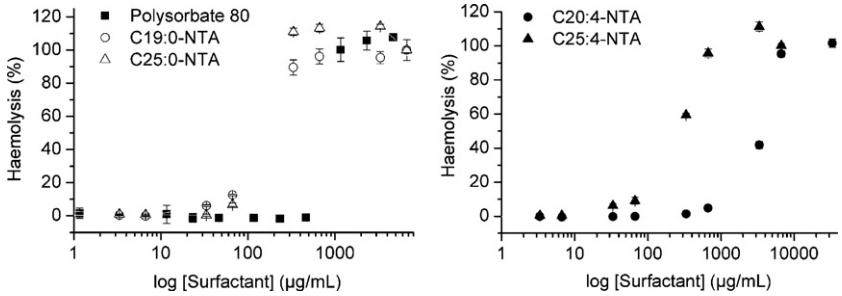


Fig. 7. Haemolytic activity of polysorbate 80 (■), C19:0-NTA (○), C25:0-NTA (△), C20:4-NTA (●) and C25:4-NTA (▲) surfactant aqueous solutions with rat red blood cells after an incubation time of 1 h at 37 °C. All data are mean ± S. D., $n = 3$.

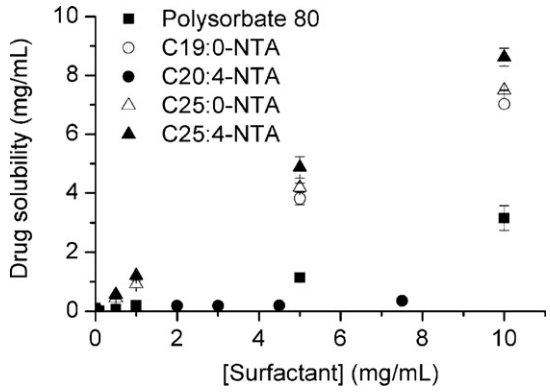


Fig. 8. Drug solubilization with polysorbate 80 (■), C19:0-NTA (○), C20:4-NTA (●), C25:0-NTA (△) and C25:4-NTA (▲) at room temperature, after 24 h magnetic stirring. All data are mean ± S. D., $n = 3$.

influence surfactant haemolytic activity. C25:4-NTA is also more haemolytic than polysorbate 80 with an HC_{50} of 0.25 mg/mL, in the same range as C19:0-NTA and C25:0-NTA. Surprisingly, a dramatic improvement was observed with C20:4-NTA which showed a 5-fold increase of the HC_{50} value compared to polysorbate 80 (3.7 mg/mL). As observed for LDH release, the presence of four Z-unsaturations in the hydrophobic moiety of C20:4-NTA, leads to an important steric hindrance that prevents the insertion of the hydrophobic moiety within RBC membrane. The other surfactants which hydrophobic moieties are rather linear, can easily insert in RBCs membrane, causing haemolysis.

3.5. Drug solubilization capacity

Finally, drug solubilization was evaluated on a poorly soluble drug (<0.15 µg/mL) (Fig. 8 and Table 4). C20:4-NTA was far less efficient than polysorbate 80 as solubilizing agent with solubility of 0.27 mg/mL (×2000), corresponding to a drug loading of 3.5% (w/w) only. As observed, on cell studies, the steric hindrance of C20:4-NTA prevents interactions with other hydrophobic compounds and therefore drug solubilization. Three surfactants out of four were able to increase drug solubility more efficiently than polysorbate 80 which only led to the solubility of 3.16 mg/mL (x 20,000), corresponding to a drug loading of 24% (w/w) (Table 4). C19:0-NTA, C25:0-NTA and C25:4-NTA increased drug solubility to concentrations higher than 7 mg/mL (×47,000–×57,000) with drug loading of 41%, 43% and 46% (w/w) respectively (Table 4). C25:0-NTA seems slightly more efficient than C19:0-NTA in solubilizing the drug which tends to indicate that a longer chain favours solubilization. Surprisingly, C25:4-NTA exhibits better solubilization efficiency than C25:0-NTA. The diacetylenic group, present in C25:4-NTA chemical structure seems to be favourable to drug solubilization.

Table 4
Comparison of novel surfactants with polysorbate 80 in terms of drug solubilization and drug loading. Drug solubility in water is lower than 0.15 µg/mL. All data are mean ± S. D., $n = 3$.

Surfactants	Drug solubility (mg/mL)	Drug loading (mass balance, %)
Polysorbate 80	3.16	24
C19:0-NTA	7.02	41
C20:4-NTA	0.27	3.5
C25:0-NTA	7.50	43
C25:4-NTA	8.62	46

4. Conclusion

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

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