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RESEARCH PAPER

Novel Surfactants with Diglutamic Acid Polar Head Group: Drug Solubilization and Toxicity Studies

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ABSTRACT

Purpose Novel surfactants made of diglutamic acid (DG) polar head linked to lithocholic, arachidonic, linoleic or stearic acids were designed for drug solubilization.

Methods Surfactants 3-D conformer and packing parameter were determined by molecular modelling and self-assembling properties by pyrene fluorescence measurements. Cytotoxicity was assessed on Human Umbilical Vein Endothelial Cells (HUVEC) and haemolyitic activity on rat red blood cells. Drug solubilization was quantified and its interaction with hydrophobic moieties was characterized using differential scanning calorimetry and X-ray diffraction. Self organisation of stearoyl-DG was observed by cryogenic transmission electron microscopy. Toxicity after repeated injections of stearoyl-DG was investigated in Wistar rats.

Results DG-based surfactants self-assemble into water and their critical micellar concentrations are comprised between 200 and 920 μg/mL. Cytotoxicity and haemolysis were lower than for polysorbate 80. At best, stearoyl-DG solubilized the drug up to 22% (w/w). Solid-state characterization evidenced drug/lipid interactions leading to the formation of a new complex. Stearoyl-DG formed spherical micelles of 20 nm, as predicted by packing parameter calculation. However, it induced a possible liver toxicity after intravenous administration in rats.

Conclusions Among the surfactants tested, stearoyl-DG is the more efficient for drug solubilization but its use is limited by its possible liver toxicity.

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INTRODUCTION

A majority of new drug candidates, intended for intravenous injection, are prematurely abandonned during their development because of their water-insolubility (drug substances classified in classes II and IV according to the Biopharmaceutics Classification System, BCS).

Marketed solubilizing agents, such as cremophor® EL and polysorbate 80 were shown to exert toxic effects such as acute hypersensitivity reactions and peripheral neuropathy (1–3) encouraging researchers to investigate the discovery of new solubilizing agents. The search of less toxic solubilizing agents is therefore of particular importance to formulate insoluble drugs.

The challenge of this study was to synthesize innovative water-soluble surfactants able to self-assemble and to solubilize insoluble drugs in their micellar cores with a low toxicity. Numerous studies reported the use of surfactants based on natural hydrophilic and hydrophobic moieties. Among hydrophilic moieties, amino acids appear to be interesting. For example, glutamic acid was previously used as a hydrophilic moiety of surfactants (4),

to study in particular the interaction with proteins (5). In a previous report, self-assembling peptides which have been derived by mimicking the structure of traditional surfactants have been described (6). In the present study, the nature of the hydrophilic moiety was peptidic since all surfactants were composed of diglutamic acid (DG) as anionic polar head.

The literature also reported the use of natural and original hydrophobic moieties. On the one hand, several publications reported the efficiency of fatty acid hydrophobic moieties such as lauric acid (7,8), palmitic acid (9), stearic acid (10,11), in solubilizing insoluble drugs. For example, Lukyanov *et al.* described the use of polymeric micelles with long-chain fatty acids as very promising drug delivery systems for poorly soluble drugs (12). On the other hand, Moroi *et al.* studied the solubilization capacity of micelles composed of natural bile acids such as sodium ursodeoxycholate, sodium glyco- and taurocholate and sodium glyco- and taurodeoxycholate as hydrophobic moieties (13,14).

To obtain amphiphilic properties, the DG polar head was covalently linked to natural hydrophobic moieties such as natural fatty acids from C18 to C20, containing 0, 2 or 4 unsaturations (stearic acid, linoleic acid and arachidonic acid) or a bile acid (lithocholic acid). The aim of the study was to evaluate the influence of hydrophobic moieties on self-assembling properties, toxicity and drug solubilization capacity.

MATERIALS AND METHODS

Materials

Polysorbate 80 was obtained from Seppic. Pyrene, triton X-100, 1,3-dicyclohexylcarbodiimide (DCC), lithocholic acid and phosphate buffer saline (PBS), $SOCl_2$, linoleic acid and *N*-hydroxysuccinimide were provided by Sigma-Aldrich. DMSO, trifuoroacetic acid (TFA) and CH_2Cl_2 were purchased from Carlo Erba. Water was purified using a RIOS/synergy system from Millipore. THF, DMF, triethylamine, acetonitrile gradient grade for liquid chromatography, ammonia solution 25% for analysis and gradient grade methanol for HPLC were obtained from Merck. Ammonium formate, nitrilotriacetic acid (NTA) and 4-dimethylamine pyridine were provided by Fluka analytical. Lithocholanoyl-DG, arachidonoyl-DG, linoleoyl-DG and stearoyl-DG were synthesized as described in Supplementary Material (Figs. S1, S2, S3, S4). All surfactant chemical formulas are reported in Table I.

Fluorescence Spectroscopy: CMC Determination

Pyrene was used as a fluorescent hydrophobic probe to determine the critical micellar concentration (CMC) of surfactants. Pyrene would preferably 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 in an incubator. After evaporation, vials were filled with 1 mL of surfactant aqueous solution at pH 7.4 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 λ=335 nm and pyrene emission spectra were recorded from 370 to 400 nm. Both excitation and emission slit widths were set at 2.5 nm. Pyrene emission spectra presented vibronic peaks at λ_1=372 nm (intensity I_1) and λ_3=392 nm (intensity I_3). The fluorescence intensity ratio (I_1/I_3) was calculated and plotted as a function of surfactant concentration (semi-log plot). The CMC was experimentally determined from the inflection point of the plot of the fluorescence intensity ratio *versus* the logarithm of surfactant concentration. 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.

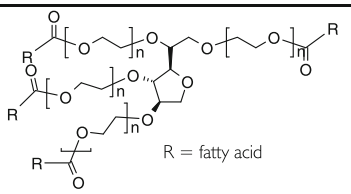
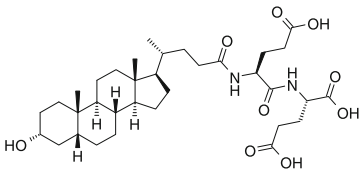
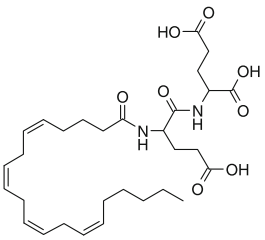
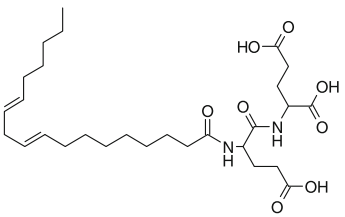
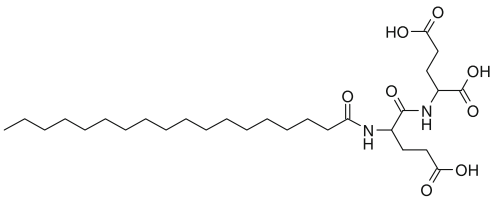
Molecular Modelling

3-D surfactant structures were estimated *in vacuo*. Structures, treated at pH 7.5, and packing parameters were calculated using Volsurf+ software (1.0.6) (Molecular Discovery, London). Lower energy conformers were determined and are presented in Table II. From the Israelachvili packing parameter P (15), it is possible to predict the aggregate shape. 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 for $P\leq0.33$, $0.33\leq P\leq0.5$ and $0.5\leq P\leq1$, respectively.

In Vitro Toxicity Assays

Human Umbilical Vein Endothelial Cells (HUVEC) were chosen to assess surfactant cytotoxicity. HUVEC were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing L-glutamine and a high glucose concentration

Table I Surfactant Composition, Hydrophobic and Hydrophilic Moieties with Molecular Weights and Their Chemical Structures

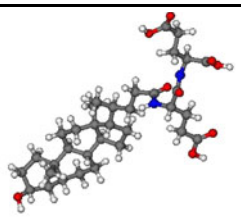
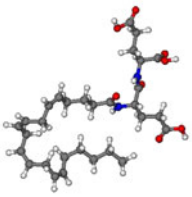
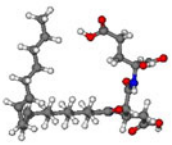
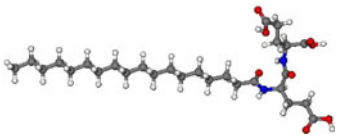
Surfactant	Mw (g/mol)	Chemical structure
Polysorbate 80	1310	
Lithocholanoyl-diglutamic acid (DG)	635	
Arachidonoyl-diglutamic acid (DG)	563	
Linoleoyl-diglutamic acid (DG)	539	
Stearoyl-diglutamic acid (DG)	543	

(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.

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, which is 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 (16,17). 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. Plates were then incubated at 37°C for 72 h in a humidified incubator with 5% CO_2. After incubation, 20 µL of MTT solution (5 mg/mL in phosphate-buffered saline, PBS) were added into each well, except for negative control. Cells were further incubated for 2.5 h. After this incubation time, 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

Table II Surfactant Molecular Modelling *In Vacuo* and Packing Parameter Values

Surfactants	Packing parameters	Lower energy conformers
Lithocholanoyl-DG	0.13	
Arachidonoyl-DG	0.16	
Linoleoyl-DG	0.20	
Stearoyl-DG	0.20	

960, Fisher Bioblock, France). Absorbance value is proportional to the number of living cells.

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 (18). LDH catalyzes the oxidation of lactate to pyruvate with the concomitant 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).

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, pH 7.4 (123.3 mM NaCl, 22.2 mM Na_2HPO_4 and 5.5 mM KH_2PO_4, 300 mOsmol/L). Minimum (0%) and maximum (100%) haemolysis were determined in PBS and in triton X-100, 1% (w/v), respectively. The haemolysis percentage was calculated by the following formula:

$$\text{Haemolysis } (\%) = \frac{(\text{Abs} - \text{Abs } 0)}{(\text{Abs } 100 - \text{Abs } 0)} \times 100$$

where 'Abs', 'Abs_0', and 'Abs_{100}' were sample absorbance, control without surfactant and control in the presence of haemolytic dose of triton X-100, respectively. 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. 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 water were added. The unlysed RBCs were removed by centrifugation (2000 rpm for 10 min), and the supernatant was analyzed for haemoglobin release by UV/VIS spectroscopy at 450 nm wavelength (Shimadzu). Haemolysis percentage was plotted as a function of surfactant concentration (semi-log plot). HC_{50}, the concentration where 50% of RBCs are lysed, was determined for each surfactant.

Drug Solubilization

Various surfactant concentrations were prepared in water (C > CMC) and pH was adjusted to 7.4 with NaOH and HCl. A water-insoluble drug synthesized by Technologie SERVIER (MW: 861.5 g/mol, solubility in water <0.15 µg/mL) was used as a model. This anticancer drug is an antagonist of Bcl-2 receptors, and 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 to surfactant solutions 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 by dividing the mass of the solubilized drug by the sum of the surfactant mass and the drug solubilized mass.

HPLC Determination

HPLC experiments were performed at 40°C on a Waters HPLC system, consisting in Waters 2487 UV detector, Waters 2695 separation module and Atlas chromatographic workstation software. The automatic injector temperature was 8°C. The volume of the injected sample was 20 µL. The Gemini NXC18 column (3 µm, 150 mm×4.6 mm) was purchased from Phenomenex®. The flow rate was fixed at 1 mL/min and the UV detector wavelength at 250 nm. The aqueous phase was composed of purified water (1 L) with ammonium formate (0.63 g). An ammonia solution, 25%, was added to adjust pH of the aqueous solution at pH 9. The mobile phase was composed of water/acetonitrile/methanol (40/50/10). Analysis were performed over a 20 min period. Under these conditions, the retention time was found to be 16 min. Quantification was allowed by comparing data with a calibration curve.

Differential Scanning Calorimetry (DSC) Measurements

Differential scanning calorimetry (DSC 2920 Modulated DSC, TA Instruments) was used to investigate drug/hydrophobic moieties interactions. The instrument was calibrated with indium (Mp=156.6°C). DSC profiles were produced with the Thermal Advantage Release software and analysed with TA Instruments Universal Analysis V3.9A (TA Instruments). Aluminium pans (TA Instruments) were used to contain the samples. Measurements were carried out in an inert gas stream (nitrogen) within a temperature range from −20 to +200°C with a heating rate of 10°C/min. Samples contained 0, 10, 30, 50, 70 or 100% (w/w) drug/hydrophobic moiety ratios. The drug was first dissolved in ethanol. Then, the surfactant hydrophobic moiety was solubilized in ethanol. Specific volumes of each were mixed together under magnetic stirring to obtain the desired ratio. Ethanol was then removed by evaporation under reduced pressure. These measurements were performed with stearic acid, linoleic acid and arachidonic acid hydrophobic moieties. Samples were directly weighted in the DSC pans which were then hermetically closed. A quantity of 4–7 mg was used for the DSC measurements.

X-Ray Diffraction (XRD)

To study the polymorphic and crystalline properties of stearic acid and its mixture with insoluble drug, XRD was carried out in an X-ray diffractometer (X'Pert Pro diffractometer PANalytical, Netherlands), working at 45 kV tube voltage and 40 mA intensity and using a copper anode emitting a 1.54 Å wavelength. XRD measurements were taken from 3° to 35° in 0.017° steps (1 s per step). Samples were sandwiched between 3 µm thick films, analyzed in transmission geometry, and rotated to optimize orientation statistics.

Cryogenic Transmission Electron Microscopy (Cryo-TEM)

Samples for cryo-TEM observations were prepared in a chamber at ambient temperature. Surfactant solutions (5 µL) prepared in purified water, containing or not the drug, were deposited on a lacey carbon film supported by a copper grid, which was gently blotted with filter paper to obtain a thin liquid film. The grid was then rapidly cryo-fixed by immersion in liquid ethane maintained at −180°C, before being transferred into liquid nitrogen and stored until observation. Specimens were examined using a transmission

electron microscope (JEOL JEM2100) equipped with a camera (GIF Tridiem GATAN camera 2 k).

Formulation for the Evaluation of the Stearoyl-DG Surfactant Safety Profile

For the evaluation of the safety profile of stearoyl-DG surfactant, solutions were prepared extemporeanously in 0.9% (w/v) sodium chloride injection. For surfactant solubilization, basic conditions were required. NaOH 1 N was thus added to the solution followed by sonication. Osmolarity was then measured using a micro-osmometer (model 210 from Fiske® Associates) and adjusted with 0.9% (w/v) sodium chloride solution. pH was measured using a pH-meter from Mettler Toledo® Seven Multi and adjusted to 7.4 using a hydrochloric acid solution from 0.1 N to 1 N. The solution was then filtered through a non-pyrogenic single use filter unit of 0.2 µm pore size (cellulose acetate) to ensure its sterility for intravenous injection.

In Vivo Toxicity of Stearoyl-DG Surfactant

The safety profile of stearoyl-DG was evaluated after daily repeated intravenous administration for a minimum of 5 days to Wistar rats at dose levels of 0, 4.5, 9 and 18 mg/kg (Fig. 6). Rats were obtained from Charles River Laboratory France and acclimated for a minimum period of 6 days. Three rats of the same gender were housed per cage. The study was conducted on 9 weeks old rats at the start of the dosing period. Each group was composed of 3 males and 3 females. On the day before dosing, bodyweights were comprised between 243 and 254 g and between 148 and 167 g for males and females respectively. The formulation containing stearoyl-DG micelles was injected intravenously in a caudal vein during 2 min as a slow bolus without rinsing. In a preliminar study, a single intravenous injection of stearoyl-DG in the rat at dose level of 248.5 mg/kg, caused immediate death. Moreover, a single administration of the dose 35 mg/kg in a male and two females led to clinical signs as partial blepharoptosis, decreased motor activity and piloerection associated with changes in hepatic parameters (ALAT, GLDH). Taking into account these results, three dose levels of stearoyl-DG were daily injected intravenously (IV) for a minimum of 5 days: 4.5, 9 and 18 mg/kg (Fig. 6). Animals were compared to a control group receiving a sodium chloride solution 0.9% (w/v). Throughout the study, clinical observations, including check for mortality and signs of morbidity were daily performed. Bodyweight and food intake were checked. At 24 h after intravenous administration, haematological and clinical chemistry analyses were performed on blood samples collected from jugular vein of all isoflurane-anaesthetised animals after an overnight food deprivation of approximately 16 h. EDTA was used as an anticoagulant for blood cell counts (0.5 mL minimum). Blood collected into tubes containing a serum separator were rapidly centrifuged after clotting. The following haematological parameters were evaluated with the haematology analyser Advia 120 (Bayer Diagnostics): RBC count, packed cell volume, mean corpuscular volume, haemoglobin concentration, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, total and differential leucocytes counts and platelet count. The following clinical chemistry parameters were evaluated with the Cobas c501 (Roche) automaton: electrolytes (Na^+, K^+, Cl^-), calcium, inorganic phosphorus, glucose, urea, creatinine, albumin, total protein, total cholesterol, triglycerides, total bilirubin, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase and glutamate dehydrogenase. All surviving animals were weighed and sacrificed by exsanguination under isoflurane anaesthesia up to 2 days after the last dosing. A detailed necropsy was performed for all animals. Macroscopic anomalies of their organs were detected. The bodyweight at necropsy was used to calculate relative organ weights. For surviving animals, the following organs were weighed: adrenal glands, kidneys, spleen, heart, liver and thymus. All tissues selected for microscopic examination were included in paraffin wax, cut at approximately 4 µm of thickness and stained with hemalun eosin saffron. Microscopic examination was performed on bone marrow (sternum), duodenum, forestomach, glandular stomach, heart, injection site (tail), kidneys, liver, lungs, spleen, thymus.

RESULTS

Surfactant Water-Solubility and Critical Micellar Concentration

DG-surfactants were successfully synthesized (Supplementary Material, Figs. S1, S2, S3 and S4). Their water-solubility was pH-dependent due to the presence of three carboxylic acid functions on the hydrophilic moieties. NaOH 1 N was therefore added in order to solubilize the surfactant in basic conditions. The pH was then adjusted to 7.4 with the addition of HCl 1 N and 0.1 N. CMC values of designed surfactants were determined through pyrene fluorescence measurements by plotting I_1/I_3 fluorescence ratios as a function of surfactant concentration (semi-log plot) (Supplementary Material, Fig. S5), and compared with polysorbate 80. All surfactant were able to self-assemble, as evidenced by the decrease of I_1/I_3 fluorescence ratio. CMC values are ranked in the following order: stearoyl-DG < arachidonoyl-DG < lithocholanoyl-DG < linoleoyl-DG. CMC values were comprised

between 200 and 920 µg/mL (Table III), which are much higher than for polysorbate 80 (CMC=14 µg/mL).

Molecular Modelling

Surfactant packing parameters were determined by molecular modelling. All of them possess geometric packing parameter values lower than 0.33 (Table II). Lower energy conformers of surfactants were determined and presented in Table II. Unsaturated fatty acid based-surfactant conformers present folded hydrophobic moieties. Stearoyl-DG presents an extended and linear hydrophobic moiety whereas lithocholanoyl-DG conformer exhibits a clear different behaviour with a rigid and bulky hydrophobic moiety.

In Vitro Toxicity Assays: Cell Viability Assay

In this study, HUVEC cell model was chosen to investigate the cytotoxicity of new designed surfactants because it allows the determination of surfactant interactions with endothelial cells, constitutive of blood vessel walls. HUVEC are thus an interesting model to predict surfactant toxicity after intravenous injection. To assess surfactant cytotoxicity, MTT assay was performed (Table III). Cell viability percentage was plotted as a function of surfactant concentration (semi-log plot) (Supplementary Material, Fig. S6) and compared to polysorbate 80 (IC_{50}=250 µg/mL). Lithocholanoyl-DG exhibits a similar IC_{50} as polysorbate 80 (230 µg/mL). Arachidonoyl-DG and linoleoyl-DG possess slightly higher IC_{50} values of 450 µg/mL and 400 µg/mL, respectively. On the other hand, stearoyl-DG exhibits a higher cytotoxicity than polysorbate 80 with an IC_{50} value of 100 µg/mL. Surfactant cytotoxicity can therefore be ordered as follows: arachidonoyl-DG < linoleoyl-DG < polysorbate 80 < lithocholanoyl-DG < stearoyl-DG.

Membrane Damage Assay

LDH assay was performed to investigate the membrane specific toxicity of all surfactants. Interestingly, three surfactants out of four did not interact with HUVEC membranes, as attested by the absence of LDH release: lithocholanoyl-DG, arachidonoyl-DG and linoleoyl-DG. Those are therefore not toxic to the membrane in the concentration range tested (Table III and Supplementary Material, Fig. S7). On the contrary, stearoyl-DG interacted with HUVEC membranes and led to an increase of LDH release with an IC_{50} of 190 µg/mL. Membrane toxicity of stearoyl-DG is slightly lower than polysorbate 80, for which the IC_{50} was 130 µg/mL. In conclusion, all DG based-surfactants possess a lower membrane toxicity than polysorbate 80.

Haemolytic Assay

To further investigate surfactant toxicity, their haemolytic activity was evaluated on rat RBCs. HC_{50} values have been determined and allow the comparison of surfactants with each others (Table III). All surfactants are less haemolytic than polysorbate 80, whose HC_{50} is 760 µg/mL. Lithocholanoyl-DG, stearoyl-DG, linoleoyl-DG and arachidonoyl-DG have the following HC_{50} values: 4.24, 2.15, 2.92 and 4.60 mg/mL. The order of surfactant haemolytic activity is as follows: arachidonoyl-DG < lithocholanoyl-DG < linoleoyl-DG < stearoyl-DG < polysorbate 80. In the case of arachidonoyl-DG and linoleoyl-DG, at higher concentrations where 100% haemolysis was observed, a decrease of haemolysis percentage was noted (Supplementary Material, Fig. S8).

Drug Solubilization Capacity

Surfactant solubilizing capacity was evaluated with a model insoluble drug (S < 0.15 µg/mL), according to the specifications for solubility of the European Pharmacopoeia 7.2 (Insoluble drug, solubility < 0.1 mg/mL). Surfactants solubilization capacity was tested as a function of the concentration and compared to polysorbate 80 (Fig. 1). Lithocholanoyl-DG and arachidonoyl-DG do not allow a significant increase of drug solubility (× 4 and × 100 respectively), which remains lower than 17 µg/mL (Table IV). As a comparison, linoleoyl-DG, with only two unsaturations in the fatty acid chain, is able to solubilize the drug tested up to a concentration of 1,27 mg/mL (× 8000) with a drug loading of 3.1% (w/w). Stearoyl-DG

Table III Comparison of Innovative Surfactants with Polysorbate 80 in Term of CMC, IC_{50} (µg/mL) of HUVEC Treated by Surfactants Determined with MTT and LDH Assays. HC_{50} (µg/mL) of Rat RBCs Treated by Surfactants. NA: Not Applicable

Surfactants tested	CMC (µg/mL)	CMC (mol/L)	MTT assay IC_{50} (µg/mL)	LDH assay IC_{50} (µg/mL)	Haemolytic assay HC_{50} (µg/mL)
Polysorbate 80	14	1.1×10^{-5}	250	130	760
Lithocholanoyl-DG	780	1.23×10^{-3}	230	NA	4240
Arachidonoyl-DG	430	7.64×10^{-4}	450	NA	4600
Linoleoyl-DG	920	1.71×10^{-3}	400	NA	2920
Stearoyl-DG	200	3.69×10^{-4}	100	190	2150

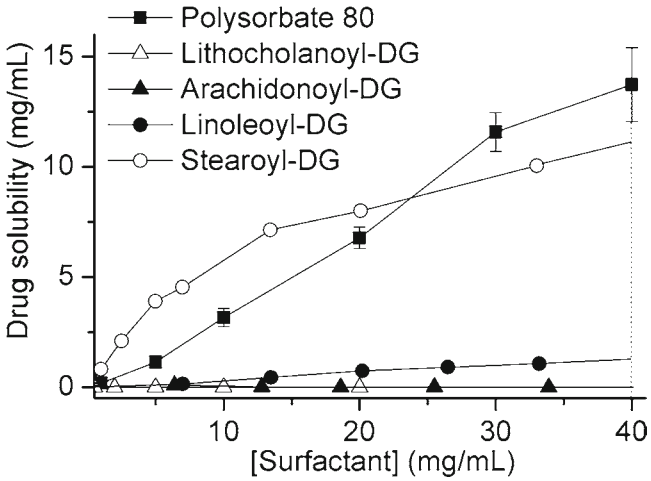


Fig. 1 Drug solubilization with polysorbate 80 (■), lithocholanoyl-DG (Δ), arachidonoyl-DG (▲), linoleoyl-DG (●) and stearoyl-DG (○) at room temperature (20°C ± 2), after 24 h magnetic stirring.

exhibits a high solubilization capacity up to 11.1 mg/mL (× 70000) with a drug loading of 21.8% (w/w). This value is close from what is obtained with polysorbate 80 (13.7 mg/mL with a drug loading of 25.6% w/w). Surfactant solubilization capacity is in the following order: lithocholanoyl-DG < arachidonoyl-DG < linoleoyl-DG < stearoyl-DG < polysorbate 80.

Solid State Characterization

DSC Measurements

DSC experiments were conducted to assess drug interactions with hydrophobic moieties and explain high drug solubilization capacity of particular surfactants. DSC profiles are presented in Fig. 2. Pure lithocholic acid was characterized by an endothermic peak at 191°C corresponding to its melting temperature. As the drug was progressively added to lithocholic acid, a decrease of the enthalpy, from 98 J/g to 26 J/g, was observed accompanied by a decrease of the melting temperature from 191°C to 181°C. As reported in Fig. 3, the enthalpy variation decreased before

Table IV Comparison of Surfactants with Polysorbate 80 in Term of Drug Solubilization and Drug Loading with an Extrapolated Surfactant Concentration of 40 mg/mL. Drug Solubility in Water is Lower than 0.15 μg/mL

Surfactants tested	Drug solubility in micelles (mg/mL)	Drug loading (mass balance,%)
Polysorbate 80	13.73	25.6
Lithocholanoyl-DG	0.00065	0.0016
Arachidonoyl-DG	0.017	0.042
Linoleoyl-DG	1.27	3.1
Stearoyl-DG	11.13	21.8

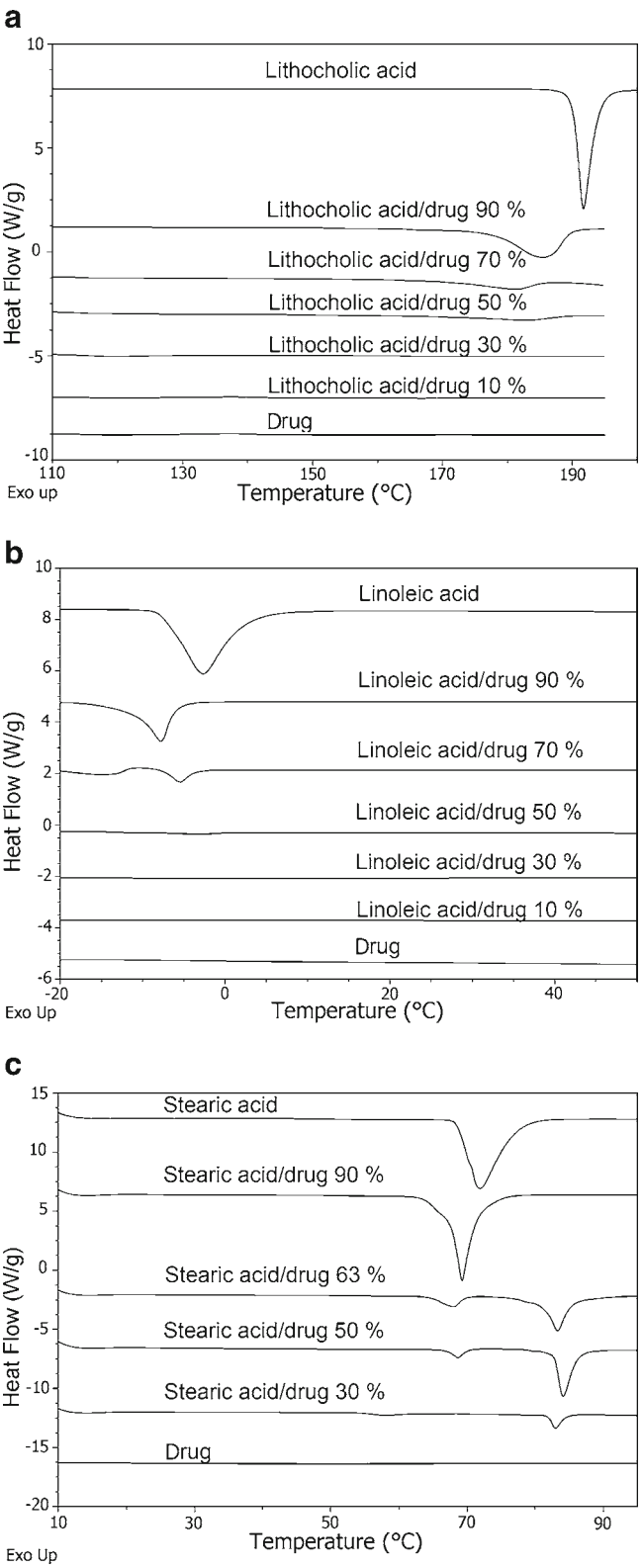


Fig. 2 DSC profiles of heating cycle of hydrophobic moieties/drug mixtures after their solubilization in ethanol followed by solvent evaporation. DSC profiles were recorded from -20°C to 200°C with a heating rate of 10°C/min. Hydrophobic moieties studied were lithocholic acid (**a**), linoleic acid (**b**) and stearic acid (**c**).

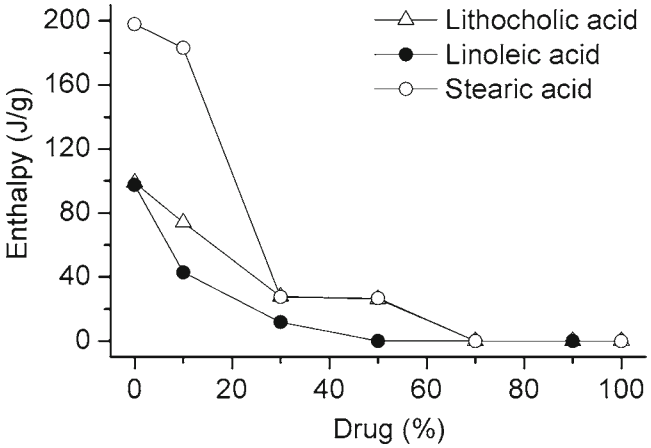


Fig. 3 Enthalpy values of lithocholic acid (Δ), linoleic acid (●) and stearic acid (○) as a function of drug content (%).

reaching a plateau corresponding to a level around 30% of drug (w/w). Pure linoleic acid was characterized by an endothermic peak at −3°C corresponding to its melting temperature. As the drug was progressively added to linoleic acid, the enthalpy decreased from 97 J/g to 12 J/g when the proportion of drug increased (drug/linoleic acid ratios from 0 to 30% w/w) (Fig. 3). For easier comparison, phase transition temperatures (T_m) of the pure stearic acid and stearic acid/drug mixtures were reported in Table V. Pure stearic acid was characterized by an endothermic peak corresponding to its melting point at 72°C, in accordance with literature (19). As the drug was progressively mixed with stearic acid, a decrease of enthalpy was observed (Fig. 3). As reported in Fig. 3, the enthalpy variation decreased from 198 J/g to 27 J/g when the proportion of drug increased (drug/stearic acid ratios from 0 to 50% w/w) and reached a plateau around 37%. At the ratio 37% drug/stearic acid (w/w), a second endothermic peak appeared at 83°C (Fig. 2). This endothermic peak remained present in DSC profiles from 37% to 70% (w/w) drug/stearic acid with a constant onset temperature (Fig. 2).

XRD Characterization

In order to explain the appearance of the new endothermic peak during DSC experiments with stearic acid/drug mixtures, XRD measurements were performed. Figure 4 shows XRD results for mixtures containing different stearic acid/drug ratios. Pure drug and pure stearic acid diffractograms were compared to drug/stearic acid 1:1 physical mixture and mixtures after evaporation from ethanol. Stearic acid diffraction peaks are sharp. In the case of the drug, a few diffraction peaks are observed with low intensity. The stearic acid/drug 1:1 physical mixture shows the characteristic diffraction peaks of both the drug and stearic acid. Interestingly, spectrum of the mixture after ethanol evaporation presents new diffraction peaks located at two-theta angles of about 5.2, 5.6, 14.9, and 25.8°, which did not exist in the spectrum of stearic acid and the drug respectively. Diffraction peaks located at two-theta angles of about 6.4, 15.7, 17 and 17.3°, corresponding to drug, were absent in this sample. In addition, this spectrum also indicates the absence of peaks located at two-theta angles of about 15.7, 17.9, 18.4, 26.2, 26.3, 27.4 and 30.2° corresponding to those of pure stearic acid. Stearic acid diffraction peak shifts were observed from 19° to 19.2° and from 19.3° to 19.35° in this mixture.

Cryo-TEM Observations

Self-assembling capacity of stearoyl-DG surfactant was evaluated with cryo-TEM experiments. Observations were performed with 5 and 35 mg/mL surfactant concentrations, before and after drug encapsulation (Fig. 5). Stearoyl-DG self-assembled into nano-sized spherical micelles at both concentrations with a monodispersed size repartition. Before drug encapsulation, micelle diameters were around 20 nm, for both concentrations and were not modified after drug encapsulation.

Table V Synthesis of Melting Temperature and Enthalpy of Different Drug/Hydrophobic Moieties Mixtures Determined from DSC Profiles

Drug/hydrophobic moiety% (w/w)	Stearic acid		Lithocholic acid		Linoleic acid	
	Melting point (°C)	Enthalpy (J/g)	Melting point (°C)	Enthalpy (J/g)	Melting point (°C)	Enthalpy (J/g)
0%	72	198	192	99	−3	97
10%	69	183	185	74	−8	39
30%	ND	ND	181	28	−5	8
37%	68	28	ND	ND	ND	ND
50%	69	27	183	26	−3	2
70%	NA	0	NA	0	NA	0
90%	ND	ND	NA	0	NA	0
100%	NA	0	NA	0	NA	0

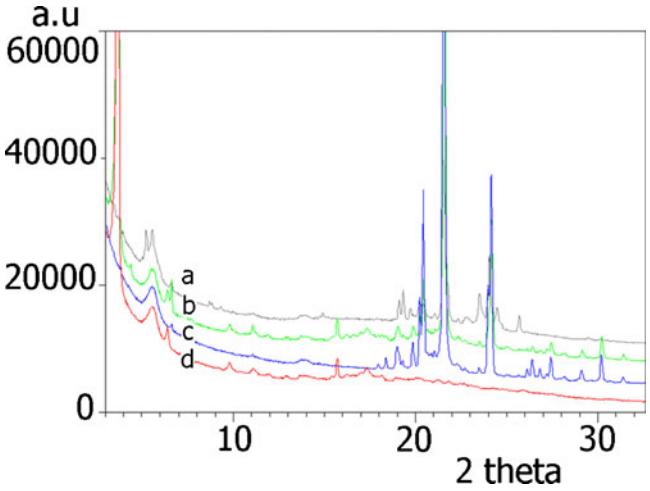


Fig. 4 XRD characterization of stearic acid/drug 1:1 mixture after their solubilization in ethanol followed by solvent evaporation (**a**), stearic acid/drug 1:1 physical mixture (**b**), pure stearic acid (**c**), pure drug (**d**) to assess crystallinity and amorphous state.

In Vivo Toxicity of Stearoyl-DG Surfactant

One female, dosed with stearoyl-DG at 9 mg/kg, was found dead on morning of Day 8. No relevant microscopic changes were observed and the cause of death was not determined. No other mortality occurred in this study. At clinical examination, doses of 4.5, 9 and 18 mg/kg induced reddish and blackish cutaneous areas at the injection site. At gross examination, relevant changes were observed at the injection site (tail) only: haematoma, dark red discolouration and/or oedema among the males from 4.5 mg/kg and for the females at 18 mg/kg (Table VI). In addition, a crust (1–2 cm) at the tail base and a wound at the tip of tail were seen for 2 male rats at 18 mg/kg. Moreover the dose of 9 mg/kg induced a superficial swelling and a decreased motor activity. At the highest dose of 18 mg/kg, more marked clinical signs were observed such as aggressive behaviours and vocalizations after injection in males on Days 4 and 5. Only the highest dose of 18 mg/kg induced a bodyweight decrease up to –6% and a decrease in food intake up to –22%. For ethical reasons, animals of intermediate dose group and all males of high dose group were only dosed over a 5 or 6 days period instead of the 7 days originally planned (Fig. 6). Haematologic and clinical chemistry parameters were measured (Table VII). Neutrophils and GLDH were increased in few animals dosed at 18 mg/kg. Moreover thymus weight decreased for males dosed at 18 mg/kg (–27% for relative thymus weights *vs* control group). Several microscopic changes were observed at the intravenous injection site, mainly vascular thrombi, dilated vein with congestion, perivenous exudate (Fig. 7), with a higher incidence and/or intensity in dosed groups than in control group, as indicated in Table VIII. For a male at 18 mg/kg, a marked cutaneous ulceration was also observed at the base of the tail (correlating with a crust at necropsy). In addition, a minimal to slight increase in extramedullary haematopoiesis was seen in the spleen of all rats dosed at 18 mg/kg. A female dosed at 18 mg/kg had several foci of enlarged and acidophilic hepatocytes in one lobe (out of 2 examined), seen without a precise distribution within the hepatic lobules or acini. No relevant microscopic changes were seen in the liver of rats given 4.5 or 9 mg/kg. No other relevant microscopic changes were observed in the other examined tissues (including the thymus) of rats dosed at 18 mg/kg.

DISCUSSION

Influence of Hydrophobic Moiety Conformation on Surfactant Self-Assembly

All DG based-surfactants were water-soluble and able to self-assemble with low CMC values. Surfactants with unsaturated hydrophobic moieties tend to self-assemble with slightly higher CMC values than surfactants with saturated hydrophobic tails. These results are in agreement with data from

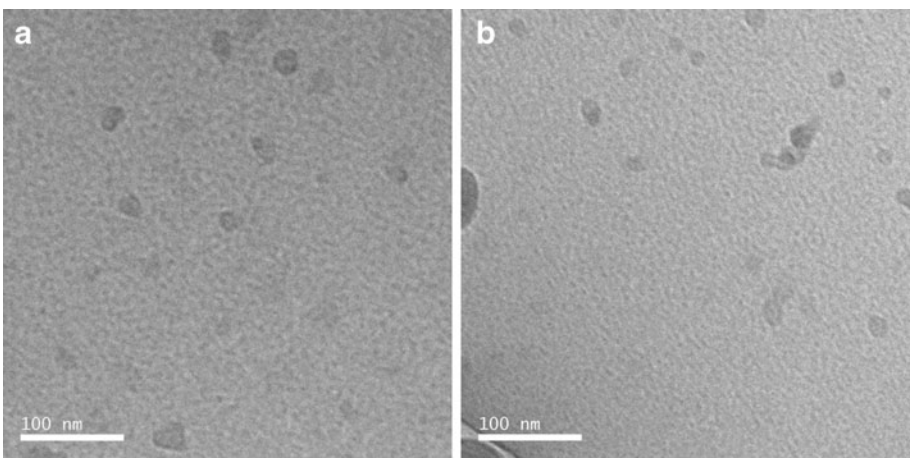


Fig. 5 Cryo-TEM observations of stearoyl-DG micelles in presence of 35 mg/mL surfactant without drug (**a**) and with drug (**b**). Scale bar represents 100 nm.

Table VI Macroscopic Changes at the Injection Site (tail) After Repeated Intravenous Administration of stearoyl-DG. -: No Animal Affected, Relevant Changes are Indicated in Bold

Injection site (Tail)	Male				Female			
Dose (mg/kg)	0	4.5	9	18	0	4.5	9	18
Number of animals	3	3	3	3	3	3	3	3
Haematoma	–	2	1	2	–	–	–	1
Dark red discolouration	–	–	–	1	–	–	–	2
Oedema	–		**1**	**3**	–	–	–	–

litterature. It was previously shown by Folmer *et al.* and by Brito *et al.* that surfactants based on unsaturated lipid moieties self-assemble with higher CMC values than surfactants based on saturated lipid moities. The presence of double bonds decreases surfactant hydrophobicity due to an increase of chain polarization. In addition, double bonds increase steric hindrance and decrease surfactant flexibility. Indeed, they lead to the formation of bends in lipid chains causing an increase of constraint packing in the micellar core due to a lower freedom degree (20,21). All surfactants possess geometric packing parameter values lower than 0.33. This would fit with a self-assembly into spherical micelles. Unsaturated fatty acid based-surfactants present folded hydrophobic moieties. The linearity and extended conformation of stearic acid moiety induces an important flexibility. In comparison, lithocholanoyl-DG presents a high rigidity and compacity.

Influence of Hydrophobic Moiety Conformation on Surfactant Cytotoxicity

Surfactant 3-D conformations were helpful in understanding their respective toxicities. Surfactants composed of polyunsaturated fatty-acids, as hydrophobic moiety, were less cytotoxic than polysorbate 80. An increase of unsaturations in the hydrophobic moiety increased surfactant compatibility with HUVEC. On the other hand, two surfactants out of four, lithocholanoyl-DG and stearoyl-DG, were more cytotoxic than polysorbate 80. Their interactions with HUVEC were probably due to the nature of their hydrophobic moieties. All surfactants, except arachidonoyl-DG, were toxic at concentrations below their CMC value, indicating that their action does not necessarily involve cell membrane disruption. LDH assay evidenced that the linear and flexible hydrophobic moiety of stearoyl-DG is able to interact with HUVEC membranes and destabilize them until disruption as described by Vives *et al.* (22). The absence of membrane cytotoxicity observed with lithocholanoyl-DG, arachidonoyl-DG and linoleoyl-DG arises from the configuration of their hydrophobic moieties. Unsaturations in fatty acid compositions and the steroidal ring system of lithocholic acid lead to an important steric hindrance, therefore preventing insertion of these surfactants into HUVEC membrane. Some studies report the ability of bile salts to associate to membrane phospholipids inducing the breaking of membrane integrity (23). From their nature, bile salts possess high partition coefficients which are correlated with a high affinity for cell membranes and thus possible cytotoxic effects (24). In our study, surprisingly, the bile salt lithocholic acid does not exert a high cytotoxicity, probably due to its coupling with diglutamic acid which decreases its partition coefficient and thus its interaction with cell membranes. Concerning haemolysis activity of fatty acid based-surfactants, the presence of unsaturations leads to an increase of HC_{50} values and therefore, increased surfactant haemocompatibility. This finding is in agreement with the LDH release results. In the case of arachidonoyl-DG and linoleoyl-DG, surprisingly a decrease of haemolysis was observed for the highest concentrations tested (Supplementary Material, Fig. S4). This could be explained by the rapid aggregation of RBCs that prevents total release of haemoglobin from RBCs present inside aggregates, as confirmed by microscopic observations (data not shown).

Influence of Hydrophobic Moiety Conformation on Surfactant Solubilization Capacity

After *in vitro* toxicity assessment, solubilization capacity of surfactants was evaluated and relationships existing between hydrophobic moiety configurations and drug solubilization capacity were evidenced. Lithocholanoyl-DG hydrophobic moiety is composed of a steroidal ring system that is rather rigid and does not enhance significantly drug solubilization. For arachidonoyl-DG, the presence of four unsaturations in the fatty acid chain confers a high steric hindrance to the surfactant, as evidenced by conformer determination, that does not favor drug incorporation in the micellar core. The drug solubility increase, obtained with linoleoyl-DG, can be explained by the higher flexibility of the hydrophobic moiety compared to

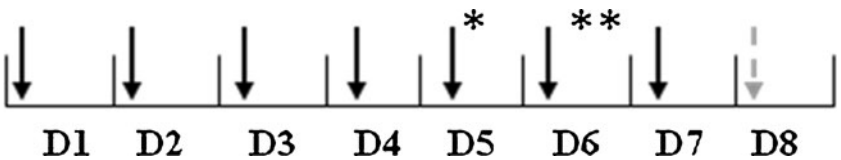


Fig. 6 Repeated toxicity study protocols with rats. *Black arrows* indicate intravenous injections of stearoyl-DG in caudal vein. All animals were dosed for 7 days except one male given 9 mg/kg and all males given 18 mg/kg, dosed for 5 days instead of 7 days (*). One male given 9 mg/kg was dosed for 6 days instead of 7 days (**). *Dotted grey* arrow indicates blood collection.

Table VII Haematologic and Clinical Chemistry Parameters After Repeated Intravenous Administration of stearoyl-DG in Rats for a Minimum Period of 5 days. Daily Doses (DD) and Cumulative Doses (CD) are Presented. Indicated Values Represent the Minimum and Maximum Values of Each Group. Values in Bold are Out of the Historical Data and Considered as Significant Changes, - : No Noteworthy Findings

	Historical data		DD=0 mg/kg		DD=4.5 mg/kg		DD=9 mg/kg		DD=18 mg/kg	
			CD=0 mg/kg		CD=31.5 mg/kg		CD=45–63 mg/kg		CD=90–126 mg/kg	
	M	F	M	F	M	F	M	F	M	F
RBCs (T/L)	6.59–8.68	6.66–8.80	–	**6.42**–8.62	–	**6.14**–8.06	–	6.93–8.07	–	**6.39**–7.04
Haemoglobin concentration (mmol/L)	8.2–10.6	8.5–10.8	–	**7.1**–9.6	–	**7.1**–9.0	–	8.6–9.4	–	7.3–8.4
Packed cell volume (%)	39.1–49.1	39.1–49.0	–	**33.6**–45.6	–	**33.5**–42.6	–	40.5–45.2	–	**34.7**–39.9
Leukocytes (G/L)	1.76–12.97	1.92–9.68	6.99–11.54	–	–	–	–		4.49–**14.93**	–
Neutrophils (G/L)	0.23–2.27	0.17–2.40	0.89–1.56	–	–	–	–		1.52–**3.42**	–
Albumine (g/L)	36.4–48.4	39.6–50.4	45.1–50.2	–	–	–	–		36.5–40.3	–
GLDH (U/L)	3–10	4–9	7–10	4–6	–	–	–		6–**20**	6–**10**

Fig. 7 Microscopic observations of injection site (tail) of rats treated with stearoyl-DG, 18 mg/kg, for 7 days. (**a**) Intravenous thrombus (*black arrow*; objective x4), (**b**) venous dilation and congestion (*black arrow*, objective x4), (**c**) perivenous exudate and oedema (*black arrows*, objective x10).

lithocholic acid and arachidonic acid ones. The effect of hydrophobic moiety flexibility is even more marked for stearoyl-DG, which is a very efficient solubilizer. These results are in agreement with conformer determination. This evidences that

Table VIII Microscopic Observations at the Injection Site (tail) After Repeated Intravenous Administration of stearoyl-DG. -: No Animal Affected, Relevant Changes are Indicated in Bold, §: Parietal (one), Those Seen in Dosed Rats Often Seen in Several Veins, Mainly Occlusive

Intravenous injection site (Tail)	Male				Female			
Dose (mg/kg)	0	4.5	9	18	0	4.5	9	18
Number of animals	3	3	3	3	3	3	2	3
Vascular thrombus	–	**3**	**3**	**2**	1§	**3**	**1**	**2**
Dilated veins with congestion	–	**1**	–	**1**	–	–	–	**1**
Exudate, perivenous	3	3	3	3	3	3	2	3
Minimal	3	1	–	–	3	2	2	1
Slight	–	**2**	**2**	**1**	–	**1**	–	**2**
Moderate	–	–	**1**	**2**	–	–	–	–
Haemorrhage, perivenous	3	3	2	2	1	3	2	3
Minimal	3	3	2	1	1	3	1	2
Slight	–			**1**	–	–	**1**	**1**
Inflammatory cell infiltrate, perivenous	3	3	3	3	3	3	2	3
Minimal	3	1	2	2	3	1	–	2
Slight	–	**2**	**1**	**1**	–	**2**	**2**	**1**
Ulceration (with erosion or vacuolar degeneration), overlying epidermis	–	–	–	**1**	–	–	–	**2**

the more flexible and linear the hydrophobic moiety is, the better the solubilization will be. Some studies report an increase of surfactant solubilizing properties as the lipid chain length increases, due to a more voluminous hydrophobic core (25). In our study, surprisingly we noticed a better solubilization ability with shorter lipidic chains based surfactants, C18:0 and C18:2, in comparison with longer ones C20:4. This is probably due to the presence of unsaturations in lipidic chains which increase steric hindrance, as demonstrated by the molecular modelling study. This particular conformation reduces potential interactions with the insoluble drug and thus decreases drug loadings. To evidence drug/hydrophobic moieties interactions and thus explain solubilization results, DSC and XRD characterization were conducted. First, DSC evidenced the presence of an interaction between lithocholic acid or linoleic acid and drug, due to the stabilization of enthalpy values when increasing drug concentration in the mixture. With stearoyl-DG, DSC experiments evidenced the appearance of a new endothermic peak, probably due to stearic acid-drug interactions, leading to the formation of a specific complex. Interactions were observed at the specific ratio for which drug solubilization was notably more efficient. Results also indicated the absence of the first endotherm corresponding to the melting of stearic acid at high drug/stearic acid ratios. The second endotherm at 83°C could correspond to another polymorph or to a co-crystal formed between the drug and the stearic acid. To conclude, all hydrophobic moieties appear to interact with the insoluble drug. However, in the case of the stearic acid study, an additional interaction was evidenced by the presence of a second endothermic peak. In order to confirm and further investigate the evidenced interaction between stearic acid and the drug, XRD experiments were conducted with pure stearic acid, pure drug, physical dry mixture (stearic acid/drug 1:1) and intimate mixture after ethanol evaporation of stearic acid/drug 1:1 solution. Diffraction peaks of stearic acid indicated negligible amorphous content and high crystallinity of stearic acid, whereas in the case of the drug, a low crystallinity was evidenced. The stearic acid/drug 1:1 physical mixture showed characteristic diffraction peaks of both drug and stearic acid, indicating that both components were present. New diffraction peaks were observed in the spectrum of the mixture after ethanol evaporation. This indicates that drug and stearic acid formed an original crystalline entity. The existence of specific interactions between the drug and the stearic acid could explain the high drug loadings obtained due to the *in situ* formation of the complex within hydrophobic micellar core of stearoyl-DG micelles.

The high drug solubilization capacity of stearoyl-DG and the evidence of its interaction with the insoluble drug led us to investigate the size and morphology of self-assemblies and the *in vivo* toxicity.

Stearoyl-DG Self Assembly and *In Vivo* Toxicity Profile

Cryo-TEM showed the formation of spherical micelles in solution with a mean diameter around 20 nm with 5 mg/mL of stearoyl-DG. In addition, some micelles aggregates were also detected. At 35 mg/mL, a higher number of micelles are formed but their diameters remained around 20 nm. Micelle size did not increase after drug incorporation and the spherical form of micelles was retained at both concentrations. Size of micelles encapsulating the hydrophobic drug is compatible with an intravenous injection (size < 1 μm). Repeated toxicity study with stearoyl-DG evidenced a poor tolerance of the surfactant at the injection site after intravenous administration inducing an increasing number of clinical signs as a function of surfactant dose.

Considering the different observations and measurements including clinical signs, bodyweight and food intake, it was shown that the administration was better tolerated by females than males. This is probably due to the flow rate which was much higher for males, as a result of the administration of higher doses within the same time of administration (2 min). The administration of stearoyl-DG resulted in an inflammation process as demonstrated by neutrophils increase as well as a potential primary vascular endothelium injury, characterized by vascular thrombi and perivenous exudates. This inflammation process resulted in an extramedullary haematopoiesis in the spleen of all rats dosed at 18 mg/kg. Liver was also affected since GLDH was increased. The presence of foci of enlarged and acidophilic hepatocytes in the liver of a female also indicates a probable toxic effect. It cannot be clearly determined whether it corresponded either to a simple hepatocellular hypertrophy or to an early degenerative change. Finally, the ulcerative cutaneous tail changes seen in rats at 18 mg/kg were considered as a degenerative change secondary to the vascular thrombi and perivenous exudate or the result of a potential scratching.

CONCLUSION

All DG based-surfactants were water-soluble. In addition, they were able to self-assemble into micelles with CMC values comprised between 0.2 and 0.92 mg/mL.

Cell viability with poly-unsaturated fatty-acid based surfactants is higher than with polysorbate 80. All surfactants are less cytotoxic against HUVEC membranes and less haemolytic than polysorbate 80. Cytotoxicity of surfactants is related to the nature of their hydrophobic moiety and to their spatial configuration, as evidenced by conformer calculations. A folded configuration of the hydrophobic moiety reduces its interaction with HUVEC and RBC membranes, therefore increasing surfactant haemocompatibility.

In particular, two surfactants out of four are efficient solubilizers: linoleoyl-DG and stearoyl-DG. The high flexibility of their hydrophobic moieties allows drug-surfactant interactions and thus leads to an easier drug incorporation into micellar cores. In addition, DSC and XRD measurements evidenced a specific interaction between stearic acid and the drug.

Stearoyl-DG self-assembles into spherical micelles, as predicted from packing parameter determination. After its intravenous administration in rat, the maximum tolerated dose (MTD) is 4.5 mg/kg. Moreover, stearoyl-DG exhibits a poor tolerance at the injection site when administered intravenously and a potential liver toxicity. Despite its solubilizing ability the *in vivo* toxicity of stearoyl-DG limits its use for pharmaceutical applications.

This study was very useful in order to understand the relationships existing between surfactant structure, cytotoxicity and solubilization capacity. Surfactant membrane toxicity seems to increase with drug solubilization capacity. It is of particular importance to balance these two main properties to define the safe and efficient concentration of the surfactant that can be used in formulations.

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