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Lactose oleate as new biocompatible surfactant for pharmaceutical applications

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Abstract

Sugar fatty acid esters are an interesting class of non-ionic, biocompatible and biodegradable sugar-based surfactants, recently emerged as a valid alternative to the traditional commonly employed (e.g. polysorbates and polyethylene glycol derivatives). By varying the polar head (carbohydrate moiety) and the hydrophobic tail (fatty acid), surfactants with different physicochemical characteristics can be easily prepared. While many research papers have focused on sucrose derivatives, relatively few studies have been carried out on lactose-based surfactants. In this work, we present the synthesis and the physico-chemical characterization of lactose oleate. The new derivative was obtained by enzymatic mono-esterification of lactose with oleic acid. Thermal, surface, and aggregation properties of the surfactant were studied in detail and the cytotoxicity profile was investigated by MTS and LDH assays on intestinal Caco-2 monolayers. Transepithelial electrical resistance (TEER) measurements on Caco-2 cells showed a transient and reversible effect on the tight junctions opening, which correlates with the increased permeability of 4kDa fluorescein-labelled dextran (as model for macromolecular drugs) in a concentration dependent manner. Moreover, lactose oleate displayed a satisfactory antimicrobial activity over a range of Gram-positive and Gram-negative bacteria. Overall, the obtained results are promising for a further development of lactose oleate as an intestinal absorption enhancer and/or an alternative biodegradable preservative for pharmaceutical and food applications.

Keywords: permeability enhancer; antimicrobial agent; enzymatic synthesis; non-ionic surfactants; lactose monoester; unsaturated fatty acid.

1. Introduction

Sugar-based surfactants are an emerging broad class of non-ionic amphiphilic molecules characterized by a carbohydrate polar moiety, linked to one or more hydrophobic alkyl or acyl chains of different lengths [1].

These surfactants have attracted great attention in different fields thanks to their promising physico-chemical properties, linked to a desirable biodegradability and a safe biocompatibility profile [2, 3] Among all sugar-based surfactants, sucrose esters and alkyl glucosides are the most studied and applied derivatives, particularly in the pharmaceutical, cosmetic and food formulations [4-7].

As regards the pharmaceutical perspective, this class of amphiphilic molecules can represent a suitable alternative to the commonly employed non-ionic surfactants (e.g. polysorbates).

For instance, alkyl glycosides have been proposed as replacers of polysorbates in biologics commercial formulations, because of their ability not to induce progressive protein degradation or increased immunogenicity, during manufacturing or storage time prior to administration [8]. Moreover, they may prevent biologics aggregation through the formation of a hydrophilic shield around the exposed hydrophobic sites of partially folded proteins or peptides [9].

Sugar-based surfactants have been also demonstrated to modify the bioavailability of drugs in different dosage forms by influencing the absorption, penetration and dissolution of the payload [10].

As such, new amphiphilic molecules (e.g. alkyl maltosides, lactose esters, rhamnolipids) have been recently explored as permeability enhancers for biologics, to improve the absorption of macromolecular drug across the epithelia [11][12, 13].

Several molecules such as medium chain fatty acids (MCFAs), sodium *N*-[8-(2-hydroxybenzoyl)amino] caprylate (SNAC), carnitines, bile salts or polysaccharides like chitosan have been recognised to act as permeation enhancers either by facilitating transcellular transport or modulating the paracellular route through a reversible effect on tight junctions opening [10, 14, 15].

Among the biological properties, sugar-based surfactants also exhibit an interesting antimicrobial activity, mainly due to the interactions of the surfactants with cell membranes of bacteria [16]. An antiproliferative action has been also observed, which is also attributable to the interaction/inhibition of these amphiphiles with enzymes involved in the mono/oligosaccharides microbial metabolism. [17].

Despite the conspicuous evidences available in the literature about the potential use of sugar-based surfactants for pharmaceutical applications, a limited number of studies has been performed regarding lactose-based surfactants in this field [18].

Our group previously demonstrated the permeability enhancing effect and the antibacterial activity of novel unsaturated fatty acid monoesters based on lactose as polar moiety. Specifically, the

synthesized lactose palmitoleate and lactose nervonate showed a marked effect on the permeability of fluorescein isothiocyanate-labeled ovalbumin across Caco-2 cell monolayer at not-toxic concentrations and resulted to be more effective than parabens as preservatives [19].

This paper reports, for the first time, the synthesis and characterization of a new lactose derivative obtained from the enzymatic mono-esterification of lactose with the oleic acid. The chemo- and regioselective mild esterification of lactose was ensured by Lipozyme®, an immobilized lipase obtained from Mucor miehei. The use of biocatalysts to promote ester formation is an example of achievable sustainable chemistry in the field of surfactants synthesis.

The oleic acid was selected as unsaturated fatty acid due to its well-characterized absorption enhancing properties [20]. Moreover, it has an intermediate hydrophobic chain length (C18) that lies between the previously investigate palmitoleic (C16) and nervonic (C24) acids [19].

Lactose oleate (URB1383) was successfully synthetized and widely characterized in terms of physicochemical properties (nuclear magnetic resonance, NMR; mass spectrometry, MS; infrared spectroscopy, IR; differential scanning calorimetry, DSC; dynamic light scattering, DLS).

Further experiments were performed to assess the cytotoxicity profile (MTS and LDH assays) of this lactose-based surfactant and to evaluate its possible use in pharmaceutical formulations as macromolecular absorption enhancer (Trans-Epithelial Electrical Resistance measurements, TEER; and fluorescent-labelled dextran 4kDa permeability studies on Caco-2 cell monolayers) and preservative agent (Minimum Inhibitory Concentration, MIC).

2. Material and methods

2.1 Materials

Oleic acid was purchased from TCI, lactose monohydrate from Carlo Erba, while Lipozyme® (immobilized from *Mucor miehei*), *p*-toluenesulfonic acid, 2,2-dimethoxypropane, tetrafluoroboric acid diethyl ether complex and all organic solvents used in the study were purchased from Sigma. Prior to use, acetonitrile was dried with molecular sieves with an effective pore diameter of 4 Å and toluene was saturated with water. Caco-2 cells were obtained from the European Collection of Cell Cultures. Dulbecco's Modified Eagles Medium (DMEM), Hank's Balanced Salt Solution (HBSS, with sodium bicarbonate and without phenol red), non-essential amino acids (100%), L-glutamine (200 mM), fetal bovine serum (FBS), antibiotic/antimycotic solution (10–12,000 U/mL penicillin, 10–12 mg/mL streptomycin, 25–30 μg/mL amphotericin B), trypsin–EDTA solution (2.5 mg/mL trypsin, 0.2 mg/mL EDTA) and fluorescein isothiocyanate-labelled dextran (FD-4) were supplied by Sigma (Poole, UK). MTS reagent, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (commercially known as CellTiter96® AQ_{ueous} One Solution Cell

Proliferation Assay) was purchased from Promega (USA). Tissue culture flasks (75 cm^3 with ventilated caps), black 96-well plates and Transwell® inserts (12 mm diameter, 0.4 μm pore size, were purchased from Corning (USA).

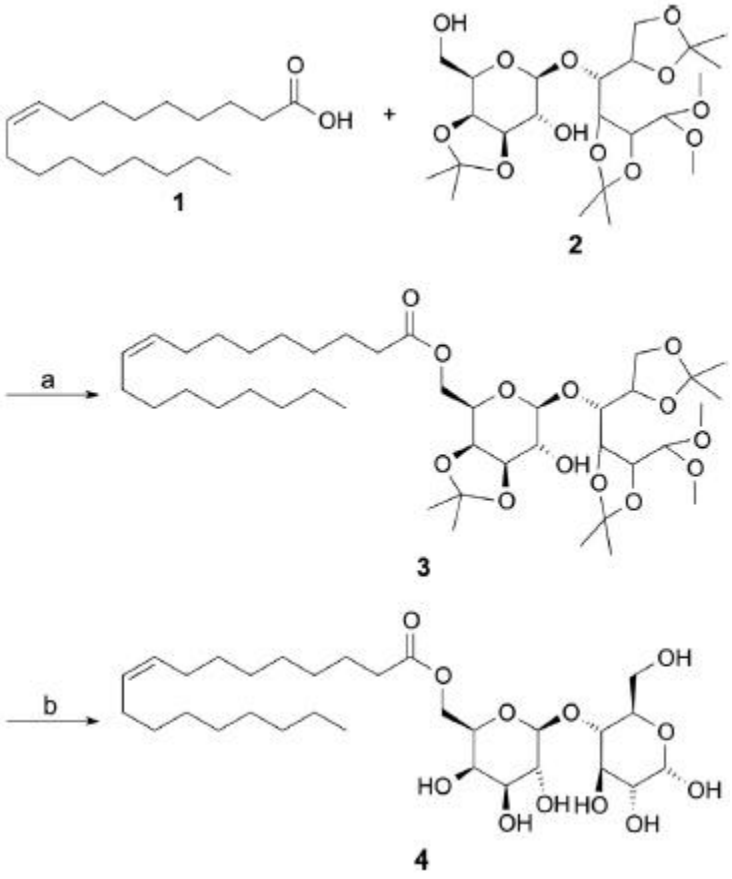
2.2 Synthesis of (*Z*)-6'-*O*-octadec-9-enoyl-4-*O*-(3',4'-*O*-isopropylidene-β-D-galactopyranosyl)-2,3:5,6-di-*O*-isopropylidene-1,1-di-*O*-methyl-D-glucopyranose (3).

Lipozyme® (0.078 g) was added to a solution of oleic acid (**1**) (0.223 g, 0.79 mmol, 0.249 mL) and 4-*O*-(3',4'-*O*-isopropylidene-β-D-galactopyranosyl)-2,3:5,6-di-*O*-isopropylidene-1,1-di-*O*-methyl-D-glucopyranose (lactose tetra acetate, LTA, previously synthesized according to [21]) (**2**) (0.401 g, 0.79 mmol) in water-saturated toluene (50% v/w) (0.5 mL) at 25 °C [19]. The mixture was stirred at 75 °C for 12 h, cooled, diluted with acetone, then filtered, and the filtrate was concentrated. The purification of the residue by column chromatography (cyclohexane/ethyl acetate 8:2) gave **3** as a pale yellow oil. Yield: 62% (0.379 g). ^{1}H NMR (400 MHz, MeOD) δ: 0.92 (t, 3H, J = 7.0 Hz, CH_3), 1.29-1.38 (m, 26H), 1.39 (s, 3H, CH_3), 1.41 (s, 3H, CH_3), 1.44 (s, 3H, CH_3), 1.49 (s, 3H, CH_3),1.59-1.70 (m, 2H, CH_2CH_2COOR), 2.01-2.09 (m, 4H, $CH_2CH{=}CHCH_2$), 2.40 (t, 2H, J = 7.0 Hz, CH_2COOR), 3.30-3.47 (m, 6H, 2 -OCH_3), 3.47 (dd, 1H, $J_{8\text{-}9}$ = 7.0 Hz, $J_{8\text{-}7}$ = 8.0 Hz, H^8), 3.91 (dd, 1H, $J_{4\text{-}3}$ = 1.5 Hz, $J_{4\text{-}5}$ = 6.0 Hz, H^4), 4.04 (ddd, 1H, $J_{11\text{-}12a}$ = 1.5 Hz, $J_{11\text{-}10}$ = 2.5 Hz, $J_{11\text{-}12b}$ = 6.8 Hz, H^{11}), 4.05 (dd, 1H, $J_{6b\text{-}5}$ = 6.0 Hz, $J_{6b\text{-}6a}$ = 8.5 Hz, H^{6b}),4.08 (dd, 1H, $J_{9\text{-}10}$ = 5.5 Hz, $J_{9\text{-}8}$ = 7.0 Hz, H^9), 4.14 (dd, 1H, $J_{3\text{-}4}$ = 1.5 Hz, $J_{3\text{-}2}$ = 7.6 Hz, H^3), 4.17 (dd, 1H, $J_{6a\text{-}5}$ = 6.0 Hz, $J_{6a\text{-}6b}$ = 8.5 Hz, H^{6a}), 4.21 (dd, 1H, $J_{10\text{-}11}$ = 2.5 Hz, $J_{10\text{-}9}$ = 5.5 Hz, H^{10}), 4.27 (dd, 1H, $J_{12b\text{-}11}$ = 6.8 Hz, $J_{12b\text{-}12a}$ = 11.5 Hz, H^{12b}), 4.30 (dd, 1H, $J_{12a\text{-}11}$ = 1.5 Hz, $J_{12a\text{-}12b}$ = 11.5 Hz, H^{12a}), 4.31 (ddd, $J_{5\text{-}4} \cong J_{5\text{-}6a} \cong J_{5\text{-}6b}$ = 6.0 Hz, H^5), 4.41 (d, 1H, $J_{1\text{-}2}$ = 6.4 Hz, H^1), 4.49 (d, 1H, $J_{7\text{-}8}$ = 8.0 Hz, H^7), 4.51 (dd, 1H, $J_{2\text{-}1}$ = 6.4 Hz, $J_{2\text{-}3}$ = 7.5 Hz, H^2), 5.35 (ddd, 1H, $J_{22\text{-}23a} \cong J_{22\text{-}23b}$ = 6.0 Hz, $J_{22\text{-}21}$ = 11.0 Hz, CH=CH), 5.39 (ddd, 1H, $J_{21\text{-}20a} \cong J_{21\text{-}20b}$ = 6.0 Hz, $J_{21\text{-}22}$ = 11.0 Hz, CH=CH) ppm. ^{13}C NMR (400 MHz, MeOD) δ: 13.0, 22.3, 24.2, 24.6, 25.1, 25.5, 25.6, 26.2, 26.7, 26.7, 27.0, 28.8, 28.8, 28.9, 28.9, 29.0,29.2, 29.4, 29.4, 31.7, 33.5, 53.0, 55.1, 63.0, 65.5, 70.8, 73.3, 73.5, 75.4, 76.4, 76.8, 77.6, 79.4, 103.1, 105.7, 108.4, 109.7, 109.8, 129.4, 129.5, 173.8 ppm. ESI-MS: *m/z* 772 $(M\text{-}H)^-$. IR (Nujol): 2955, 1730, 1711 cm^{-1}.

2.3 Synthesis of (*Z*)-6'-*O*-octadec-9-enoyl-4-*O*-(β-D-galactopyranosyl)-D-glucopyranose (4, URB1383, lactose oleate).

3 (0.332 g, 0.43 mmol) was dissolved in tetrafluoroboric acid diethyl ether complex/water/acetonitrile (3.5 mL, 1:5:500) and the mixture was stirred at 30 °C for 4 h. The product precipitated during the reaction as white solid were subsequently filtered, washed with acetonitrile, and then dried. The

purification by recrystallization from methanol gave **4** (Scheme 1) as white solid. Yield: 30% (0.078 g). ^{1}H NMR (400 MHz, DMSO) δ: 0.86 (t, 3H, J = 6.5 Hz, CH_3), 1.16-1.36 (m, 20H, $(-CH_2-)_n$), 1.47-1.57 (m, 2H, CH_2CH_2COOR), 194-2.04 (m, 4H, $CH_2CH{=}CHCH_2$), 2.30 (t, 2H, J = 7.5 Hz, CH_2COOR), 3.17 (ddd, 1H, $J_{2\text{-}1}$ = 4.0 Hz, $J_{2\text{-}OH2}$ = 7.0 Hz, $J_{2\text{-}3}$ = 9.5 Hz, H^2), 3.27 (dd, 1H, $J_{4\text{-}3} \cong J_{4\text{-}5}$ = 9.5 Hz, H^4), 3.32-3.38 (m, 2H, H^8, H^9), 3.57 (dd, 1H, $J_{3\text{-}2} \cong J_{3\text{-}4}$ = 9.5 Hz, H^3), 3.60-3.67 (m, 3 H, H^{6a}, H^{6b}, H^{10}), 3.67-3.76 (m, 2H, H^5, H^{11}), 4.08 (dd, 1H, $J_{12b\text{-}11}$ = 4.5 Hz, $J_{12b\text{-}12a}$ =11.5 Hz, H^{12b}), 4.16 (dd, 1H, $J_{12a\text{-}11}$ = 8.5 Hz, $J_{12a\text{-}12b}$ = 11.5 Hz, H^{12a}), 4.20-4.27 (m, 2H, H^7, OH^3), 4.42 (dd, 1H, $J_{OH6\text{-}6a} \cong J_{OH6\text{-}6b}$ = 6.0 Hz, OH^6), 4.55 (d, 1H, $J_{OH2\text{-}2}$ = 7.0 Hz, OH^2), 4.78 (d, 1H, $J_{OH10\text{-}10}$ = 5.0 Hz, OH^{10}), 4.86 (brs, 1H, OH), 4.90 (dd, 1H, $J_{1\text{-}OH1} \cong J_{1\text{-}2}$ = 4.0 Hz, H^1), 5.15 (brs, 1H, OH), 5.30 (ddd, 1H, $J_{22\text{-}23a} \cong J_{22\text{-}23b}$ = 6.0 Hz, $J_{22\text{-}21}$ = 11.0 Hz, CH=CH), 5.35 (ddd, 1H, $J_{21\text{-}20a} \cong J_{21\text{-}20b}$ = 6.0 Hz, $J_{21\text{-}22}$ = 11.0 Hz, CH=CH), 6.33 (d, 1H, $J_{OH1\text{-}1}$ = 4.0 Hz, OH^1) ppm. ^{13}C NMR (400 MHz, DMSO) δ: 14.4, 22.5, 24.8, 27.0, 27.1, 28.9, 29.0, 29.05, 29.06, 29.1, 29.3, 29.6, 31.7, 33.7, 60.9, 63.7, 68.7, 70.2, 70.8, 71.7, 72.7, 72.9, 73.3, 81.6, 92.5, 104.0, 130.1, 130.1, 173.4 ppm. ESI-MS: *m/z* 605 $(M\text{-}H)^-$. IR (Nujol): 3400, 2954, 1734, 1709 cm^{-1}.



Scheme 1. Reagents and conditions: (a) toluene, 75 °C, 12 h; (b) $HBF_4{\cdot}Et_2O$, CH_3CN, 30 °C, 4 h.

2.4 Electrospray ionization mass spectrometry (EI-MS), nuclear magnetic resonance (NMR) and infrared spectroscopy (IR)

The structures of compounds were unambiguously characterized by EI-MS, ^{1}H NMR, ^{13}C NMR, and IR. ESI-MS spectra were recorded with a Waters Micromass ZQ spectrometer in a negative or positive mode using a nebulizing nitrogen gas at 400 L/min and a temperature of 250 °C, cone flow 40 mL/min, capillary 3.5 Kvolts and cone voltage 60 V. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AC 400 or 101, respectively, spectrometer and analyzed using the TopSpin software package. Chemical shifts were measured by using the central peak of the solvent. IR spectra were obtained on a Nicolet Atavar 360 FT spectrometer. Column chromatography purifications were performed under "flash" conditions using Merck 230–400 mesh silica gel. TLC was carried out on Merck silica gel 60 F254 plates, which were visualized by exposure to ultraviolet light and by exposure to an aqueous solution of ceric ammonium molibdate.

2.5 Differential scanning calorimetry (DSC)

Thermal analysis was carried out in a DSC 8500 (PerkinElmer, USA), equipped with an intracooler (Intracooler 2, PerkinElmer, USA) under an inert nitrogen atmosphere (flow rate of 20 mL/min). A small amount (2–4 mg) of lactose oleate was placed in a sealed aluminium pan and analysed with respect to the reference.

For the analysis, the following thermal programme was applied: heating from 20 °C to 150 °C, cooling down to 20 °C, isotherm at 20 °C for 3 minutes and heating again to 150 °C. The heating/cooling rate was 10 °C/min. The instrument was calibrated following the manufacturer's procedure using indium and zinc as standards. Thermal parameters were calculated from the thermograms collected from the second heating scan. All runs were performed at least in triplicate.

2.6 Dynamic light scattering (DLS) measurement of the critical micelle concentration (CMC).

CMC and the hydrodynamic diameter of surfactant micelles and/or aggregates were determined by DLS technique using a Malvern Zetasizer Nano S (Malvern, Worcestershire, UK). Counts (Kcps), which are a measure of the scattering intensity to the detector, were recorded at different concentrations of lactose oleate solutions and the CMC value was determined by the straight-line interception method as previously reported [22]. Particle size and distribution of surfactants micelles and/or aggregates are expressed as hydrodynamic diameter (nm; from volume distribution %) and width (nm; width of the distribution at half height), respectively. All measurements were performed in triplicate.

2.7 Caco-2 cell culture

Caco-2 were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% v/v FBS, 1% v/v antibiotic-antimycotic solution and L-glutamine. Cells were grown to confluence in T75 flasks at 5% CO_2 and 37 °C, detached from the flasks (using trypsin) and seeded on 96-well plates (for cytotoxicity assays) at 10,000 cells per well or on filter inserts (Transwell®, 0.4 µm pore size, 1.1 cm diameter) at 100,000 cells/cm^2. For transepithelial electrical resistance (TEER) and permeability studies, cells were cultured on Transwell® inserts for 21 days (typical requirement for Caco-2 differentiation) at the conditions detailed above prior to use. The culture medium was changed regularly (every other day). Cell growth, tight junction formation and cell monolayer integrity was ensured through measurement of TEER.

2.8 MTS cell proliferation colorimetric assay

The effect of lactose oleate on cell viability was measured using the MTS colorimetric assay. Caco-2 cells were cultured on 96-well plates in DMEM for 24 h. Culture medium was replaced with lactose oleate at different concentrations in Hank's Balanced Salt Solution (HBSS). Triton X-100 (0.1% v/v, in HBSS) and HBSS were used as a positive and negative control assuming 0% and 100% cell death, respectively. Cells were incubated (at 37 °C, 5% CO_2) with lactose oleate for 3 h. Thereafter, the assay was performed according to the manufacturer's instructions, with four repeats for each sample. Relative cell viability (%) was calculated using the following equation:

$$\text{Relative cell viability (\%)} = \frac{S-T}{H-T}\, x100 \qquad \textbf{(Equation 1)}$$

where S is the absorbance of the cells treated with the sample, T is the absorbance of cells treated with Triton X-100, and H is the absorbance of cells incubated with HBSS.

EC_{50} values were calculated by the non-linear regression analysis of the experimental data using a dose–response model (utilizing Prism version 6.0b, GraphPad Software).

2.9 LDH cytotoxicity assay

LDH assay was used in the study to assess any potential membrane disruption effect exerted by lactose oleate. Caco-2 cells were cultured on 96-well plates. Lactose oleate solution in HBSS were added to the cells at the same concentrations as in MTS assay. Triton X-100 at 1% v/v and HBSS were used as controls. Cells were treated with lactose oleate at 37 °C for 3h. Thereafter, LDH release assay was carried out according to the manufacturer's instructions. LDH release was calculated

relative to the controls following the assumption that cells treated with Triton X-100 resulted in complete LHD release and HBSS-treated cells did not release LDH. The concentration of lactose oleate that induced 50% of LDH release (EC_{50}) was calculated by fitting the experimental data with dose–response model (Prism version 6.0b, GraphPad Software).

2.10 Trans-Epithelial Electrical Resistance (TEER) measurement

Differentiated Caco-2 cell monolayers with a TEER above 800 Ωcm^2 (after 21 days from seeding cells on Transwell® inserts) were used in these experiments. Prior to the application of lactose oleate to the cells, culture medium was replaced with HBSS and cells equilibrated in this (at 37 °C, 5% CO_2) for 45 min. An initial TEER recording was then conducted; this was treated as the baseline TEER. Lactose oleate solution at 0.015–0.25 mg/mL concentration range was applied to the apical side of the Caco-2 monolayers for 3 h and TEER measured every 30 min, taking care to ensure consistency in TEER measurement conditions (e.g. measurement time following removal from the incubator) at each interval. Lactose oleate solutions were removed from the cells after 3 h and cells washed with PBS. Culture medium was then added to both sides of the Caco-2 monolayers for overnight incubation. A further TEER recording was conducted 24 h following cell treatment with lactose oleate in order to establish TEER reversibility (if applicable). An EVOM Voltohmmeter (World Precision Instruments, UK), equipped with a pair of chopstick electrodes, was utilized for this study. Background TEER due to the filter (~100–110 Ω cm^2) was considered in all cases. All experiments were performed in triplicates.

2.11 Permeability experiments across Caco-2 cell monolayer

FITC-Dextran 4 kDa (FD4) was used as model macromolecular drug in conjunction with differentiated Caco-2 cell monolayers with TEER above 800 Ω cm^2. Prior to the application of FD4 and lactose oleate, culture medium was removed and cells washed with PBS. Caco-2 monolayers were then equilibrated in HBSS for 45 min. Lactose oleate at 0.25, 0.12, 0.06 or 0.03 mg/mL and FD4 at 100 μg/mL (in HBSS) were then applied in combination to the apical side of the cell monolayers. Basolateral solution was thereafter sampled (100 μL volumes) at 30, 60, 90, 120, 150 and 180 min after sample application, with replenishment of sampled solution with fresh HBSS. FD4 was quantified by fluorescence, using a Tecan M200 Pro plate reader. After the final sampling, Caco-2 monolayers were washed with PBS and TEER measured to ensure that cell monolayer integrity remained intact during the permeability experiments. The experiment was conducted in four

replicates. FD4 permeability is expressed as apparent permeability coefficient (P_{app}), calculated using the following equation:

$$P_{app} = \left(\frac{\Delta Q}{\Delta t}\right) \times \left(\frac{1}{A \times C_o}\right) \qquad \textbf{(Equation 2)}$$

P_{app}, apparent permeability (cm/s); $\Delta Q/\Delta t$, permeability rate (amount of FD traversing cell monolayers over time); A, diffusion area of the cell monolayer (cm^2); C_0, apically added FD4 concentration.

2.12 Bacterial strains and culture conditions

Eight reference human pathogens were used in this study, namely *Escherichia coli* O157:H7 ATCC 35150, *Listeria monocytogenes* ATCC 7644, *Salmonella enteritidis* ATCC 13076, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 43387, *Yersinia enterocolitica* ATCC 27729 and *Candida albicans* ATCC 14053. All the strains were routinely maintained in Tryptic Soy Agar (TSA, Oxoid, Milan, Italy) at 37 °C, while stock cultures were kept at -80 °C in Nutrient broth (Oxoid) with 15% of glycerol.

2.13 Determination of minimum inhibitory concentration (MIC)

MIC determination of lactose oleate was performed by microdilution method. Briefly, 1.28 mg of the test compound was dissolved in DMSO (1 mL); several colonies of each bacterial strain were inoculated in 10 ml of Mueller-Hinton Broth (MHB) (Oxoid) and incubated at 37 °C for 18–24 h. Bacterial suspensions were adjusted to a turbidity (spectrophotometrically determined) corresponding to 10^6 cfu/mL (OD_{610nm} 0.13–0.15). Then, 100 µL of each bacterial suspension were added to a 96-well plate, together with the test solution to obtain final lactose oleate concentrations of 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5 µg/mL. Positive (bacteria alone) and negative (MHB alone) controls as well as gentamicin (128–0.125 µg/mL) and a standard preservative mixture (methylparaben and propylparaben, ratio 9:1) (1024–0.5 µg/mL) were added as controls. A preliminary assay with DMSO was performed to exclude its possible bacteriostatic and/or bactericidal activity. For this reason, the volume of DMSO never exceeded 5% (v/v) of the final total volume. MIC was defined as the lowest concentration of compound inhibiting the visible bacterial growth after 24 h incubation. All the experiments were performed in duplicate.

3. Results and discussion

3.1 Synthetic procedure to obtain lactose oleate

Lactose oleate (URB1383) was synthesized by a reaction coupling from oleic acid (**1**) and LTA (**2**) catalyzed by Lipozyme®. The deprotection of the acetalic adducts **3** was performed by using tetrafluoroboric acid diethyl ether complex to obtain the desired product **4** (Scheme 1).

3.2 Thermal analyses characterization by DSC

The thermal properties of sugar-based surfactants are very interesting to be investigated since sucrose esters have been demonstrated to act as promising hydrophilic carriers for the preparation of solid dispersions by hot-melt technology in order to increase the dissolution rate of hydrophobic drugs [23]. As such, lactose-based surfactants should display the same applicability in this field of technology. Moreover, several sugars surfactants, including lactose-based surfactants, have shown a thermotropic behaviour, thereby forming liquid crystalline phases by heating, thanks to the hydrogen bonding ability of the polyhydroxy polar heads [24].

According to the literature, sucrose esters with low HLB values (generally polyesters) are crystalline and have a melting points around 40-80 °C, while surfactants with high or moderate HLB value (generally monoesters) are amorphous and display a glass transition temperature (*T*g) [25, 26].

Figure 1 shows the thermogram obtained from lactose oleate from the second DSC heating ramp. The thermogram, clearly indicated a variation in the heat capacity (Cp) of the surfactant at the solid state over temperatures, denoting the amorphous state of lactose oleate. The calculated *T*g value from DSC was 62.49±1.45 °C with a ΔCp of 0.265±0.02 J/g °C.

The inset of the figure 1 reports the thermograms obtained from the starting materials as oleic acid and lactose monohydrate for comparison.

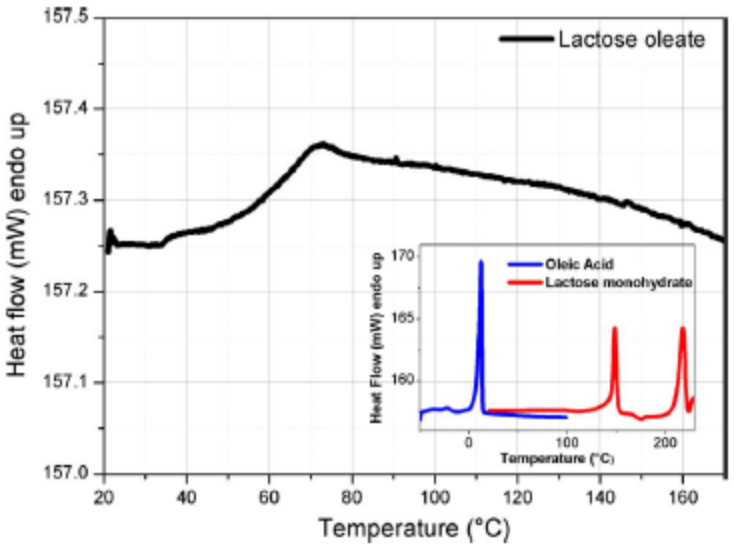


Figure 1. DSC thermogram of lactose oleate recorded from the second heating ramp (10°C/min). The inset shows the thermograms of oleic acid and lactose monohydrate for comparison.

3.3 Determination of critical micelle concentration (CMC) by DLS

CMC is one of the main parameters describing the aggregation behaviour of surfactants, and dynamic light scattering (DLS) is one of the methods by which CMC can be determined. Figure 2 shows the variation of the scattering intensities to the detector (counts) as a function of surfactant concentration. This pattern has a typical profile. For surfactant concentration below CMC counts are low and comparable to those of HBSS. This is because the contribution to scattering intensity of surfactants as unimers is negligible. On the other hand, micelles formation is associated with a sudden increase in scattering intensity as a consequence of the modified optical properties of the solution. Thus, above CMC, counts are proportional to the number of the micelles in the samples. CMC can be determined by the sharp increase in counts (i.e. breakpoint of the plot in Figure 2). For lactose oleate, the calculated CMC was 0.148±0.006 mg/mL (0.244±0.01 mM; MW 607.74), which is comparable with the value reported in the literature for sucrose oleate [27]. This denotes the major effect exerted by the nature of the hydrophobic tails (length, presence of unsaturation) with the respect to the hydrophilic heads in affecting the CMC values, as reported for other classes of surfactants [28, 29]. The size of micelles and/or aggregates above CMC was also calculated by DLS (figure 2 inset). A mean hydrodynamic diameter of 10.3±1.2 nm with a width of the size distribution of 3.09±0.04 nm was measured at a concentration of lactose oleate of 1.5 mg/mL (approximately 10-times CMC).

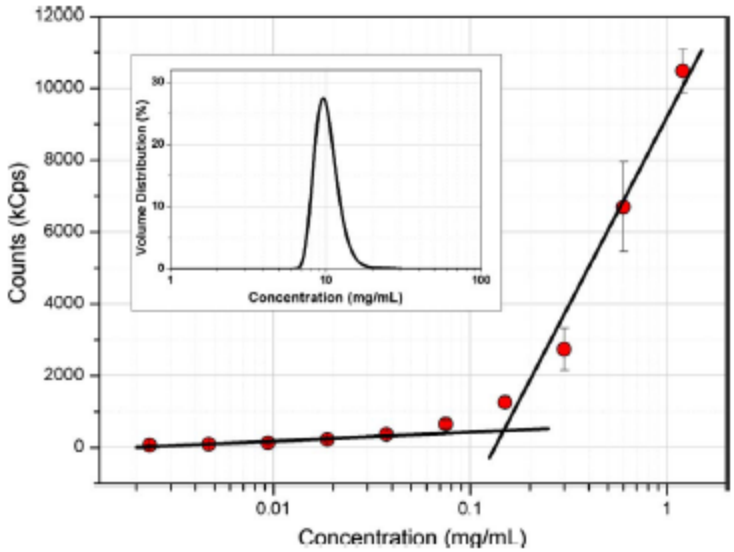


Figure 2. Counts *vs* surfactant concentration (mg/mL) plot obtained from DLS measurements. The interception point of the two fitting lines represents the CMC value. Particle size distribution (volume %) of lactose oleate is reported in the inset.

3.4 Biocompatibility evaluation on Caco-2 cell monolayer

Cytotoxicity of lactose oleate was assessed by MTS and LDH assays on the intestinal human cell line, Caco-2. The calculated EC_{50} values represent the concentration of surfactants causing 50% cell death for MTS and 50% release of the cytoplamatic enzyme lactate dehydrogenase for the LDH assay. The calculated EC_{50} values, based on the data from Figure 3, were 0.138±0.03 mg/mL (0.227± 0.05 mM; MW 607.74) for MTS and 0.255±0.05 mg/mL (0.420±0.05 mM; MW 607.74) for the LDH assay. These values were found to be comparable (MTS assay) or slightly higher (LDH assay) than CMC, configuring lactose oleate as a relatively safe surfactant. In fact, surfactants, which generally display a high-level of cytotoxicity, showed EC_{50} values lower than CMC [22]. A limited number of studies have been performed to evaluate the cytotoxicity profiles of sugar ester surfactants (generally sucrose esters) on different cell lines [19, 23, 30, 31], despite several data on animal model about their acute toxicity after oral administration or about their mild irritancy capacity on skin or mucosa are available [1]. Some sucrose esters, are in fact, have been approved by European Food Safety Authority (EFSA) as food additives [32] and by Food and Drug Administration (FDA) as excipients for pharmaceutical products [33].

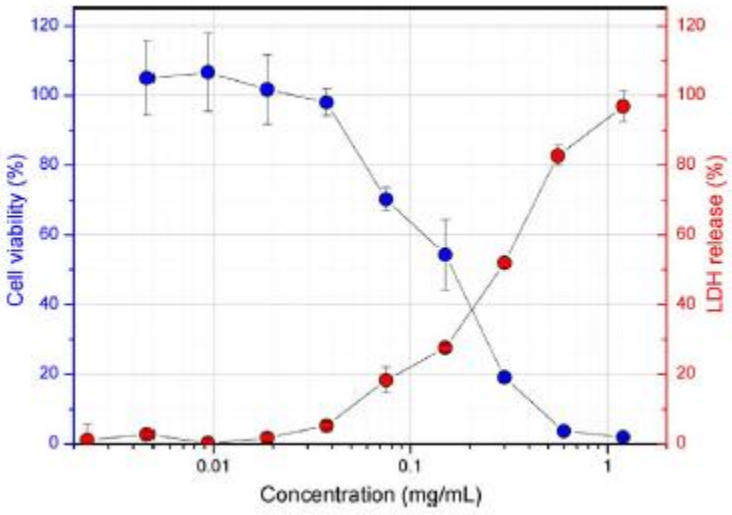


Figure 3. Cytotoxicity assays results. The plot shows the cell viability (%; from MTS assay) and the LDH release (%; from LDH assay) values for different concentrations of lactose oleate using Caco-2 cells.

3.5 Determination of Trans-Epithelial Electrical Resistance (TEER)

TEER was measured to assess the ability of lactose oleate to open intercellular epithelial tight junctions in Caco-2 cell monolayer. Concentrations of lactose oleate selected from the cytotoxicity assays (0.015 mg/mL, 0.025 mM; 0.03 mg/mL, 0.05 mM; 0.6 mg/mL, 0.1 mM; 0.125 mg/mL, 0.21 mM; 0.25 mg/mL; 0.41 mM) up to the EC_{50} values, were tested. TEER was found to decrease as a function of lactose oleate concentration, particularly above 0.06 mg/mL (Figure 4). The maximum

decrease in TEER was observed after 120 min, with TEER remaining stable from that point and up to 180 min following the application of the surfactant. TEER values at 120 min were approximately 70%, 55% and 40% of the baseline value for lactose oleate concentrations of 0.06 mg/mL, 0.125 mg/mL and 0.25 mg/mL, respectively. TEER recovered after 24 h with all the tested concentrations, indicating the reversible effect of lactose oleate on tight junction opening and no appreciable toxic effect on the cell monolayer.

A comparable effect in decreasing TEER at around 50% of the baseline values is reported for sucrose oleate (O-1570; composed of 70% monoester, 30% di-, tri-, poly-ester) on Caco-2 cell monolayers [12]. The results obtained from TEER measurements confirmed the ability of the unsaturated fatty acid mono ester of lactose to affect the electrical resistance of Caco-2 monolayer, at relatively not-toxic concentrations, by interacting with tight junctions [19].

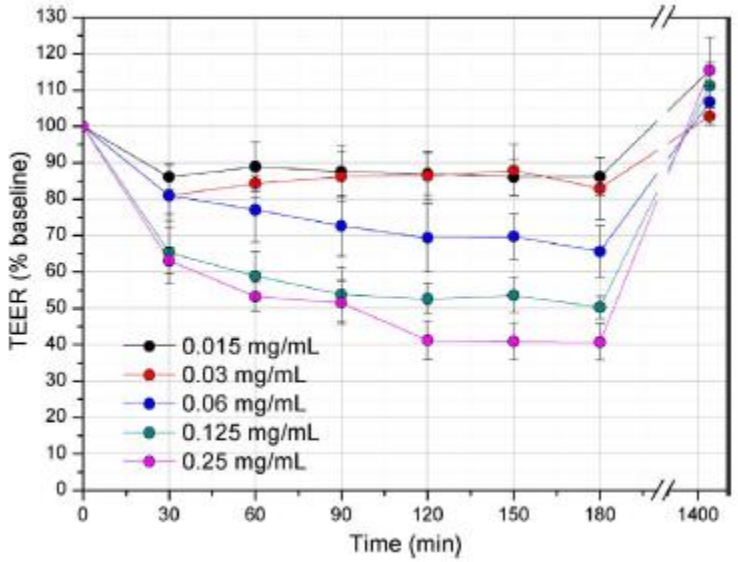


Figure 4. Effect of different concentrations of lactose oleate (from 0.015 mg/mL; 0.025 mM to 0.25 mg/mL; 0.41 mM) on transepithelial electrical resistance (TEER) of Caco-2 monolayers. Data are presented as the mean ± SEM (n = 3).

3.6 Evaluation of FITC-dextran (FD-4) permeability across Caco-2 cell monolayer

The effect of sugar esters (generally sucrose esters) as mucosal enhancers has been only partially explored, since most of the available data are related to the increase in permeability of small molecular drugs. In this regards, the enhancement effect has been demonstrated *in vitro* using different cell lines (e.g. Caco-2, RPMI 2650) [31, 34] but also *ex-vivo* and *in-vivo* [12, 35].

On the other side, the non-invasive administration of biologic therapeutics (as pharmaceutically active proteins and peptides) have attracted a great interest, following the release in the market of several biotechnological products. In this context, sugar esters could represent a promising option, despite a

limited number of studies, mainly focused on the permeability of insulin, has been conducted [36, 37].

Lactose oleate, at the same concentrations used for TEER experiments, was subsequently tested for its ability to improve the *in vitro* permeability of FD-4, as model macromolecular drug. Results are expressed in terms of apparent permeability across Caco-2 monolayers (Figure 5). The observed effect was found to be dependent on the applied surfactant concentration. In fact, FD-4 permeability in presence of the lowest tested concentration of lactose oleate (0.03 mg/mL, 0.05 mM) was comparable to the control (apparent permeabilities of 1.86×10^{-8} and 1.74×10^{-8} cm/s, respectively). On the other hand, the application of larger concentrations (from 0.06 mg/mL to 0.25 mg/mL) led to an increase in FD-4 permeability statistically different from the control (ANOVA test followed by Dunnett test for multiple comparisons). Specifically, the calculated permeability enhancement was of 2.7-fold, 3.5-fold and 7.7-fold for surfactant concentrations of 0.06 mg/mL (0.1 mM), 0.125 mg/mL (0.21 mM) and 0.25 mg/mL (0.41 mM), respectively.

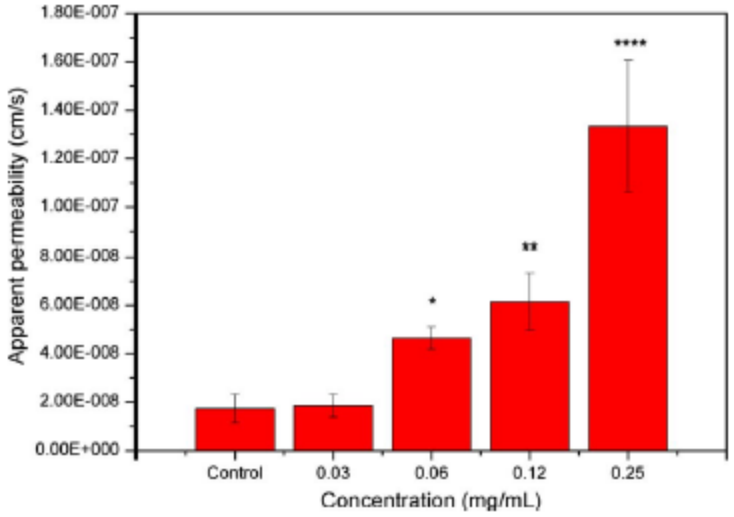


Figure 5. Effect of different concentrations of lactose oleate (from 0.03 mg/mL, 0.05 mM to 0.25 mg/mL, 0.41 mM) on the apparent permeability of FITC-labelled dextran of 4kDa across Caco-2 cell monolayers. Data are presented as the mean ± SEM (n = 4). ****P<0.0001; ***0.0001<P <0.005; ** 0.005<P<0.01; *0.01<P<0.05.

Taken together, both TEER decrease and FD-4 permeability enhancement showed a trend, dependent on lactose oleate concentration. In fact, a statistically significant increase in FD-4 permeability was only evident at concentration of lactose oleate inducing at least a 30% drop in TEER. Moreover, the more pronounced enhancement in permeability was achieved with the higher tested concentration of lactose oleate (0.25 mg/mL, which also exerted the major observed effect on TEER (60% drop in TEER). These considerations point to a tight junction-related mechanism for the FD-4 absorption enhancing the effect of lactose oleate across Caco-2 cell (i.e. paracellular route), despite a possible

involvement of the transcellular route cannot be excluded, as already proposed for other sugar-based surfactants [31].

3.7 Antimicrobial activity

Sugar esters have been shown to inhibit microbial growth, but there are conflicting data on the susceptibility of the tested microorganisms. In some studies, the inhibition of Gram-negative or Gram-positive bacteria, when tested alone, was reported [38, 39], while in others the comparison regarding the inhibitory effect was performed only on a limited number of bacterial species [40, 41]. As regards lactose derivatives, they are increasingly of interest to the pharmaceutical and food industry since many compounds, such as lactose monolaurate, lactose monodecanoate and others lactose fatty acid esters, have shown a wide antimicrobial activity [41, 42]. In the present work, the MICs of lactose oleate were determined against *E. coli* O157:H7 ATCC 35150, *L. monocytogenes* ATCC 7644, *S. enteritidis* AC 13076, *E. fecalis* ATCC 29212, *P. aeruginosa* ATCC 9027, *S. aureus* ATCC 43387, *Y. enterocolitica* ATCC 27729 and *C. albicans* ATCC 14053 according to the National Committee for Clinical Laboratory Standards (NCCLS) document M100-S12 method. The data are summarized in Table 1. As shown, lactose oleate demonstrated a similar antimicrobial activity against the tested food-borne pathogens with MIC values of 0.128 mg/mL, except for *L. monocytogenes* ATCC 7644 (MIC 0.256 mg/mL). This different antimicrobial activity is not clearly understood, but it is supposed to be related to the sugar group attached to the ester, the number and type of fatty acids esterified, and the degree of esterification, as referred by other authors [40, 43].

As regards the internal controls, gentamicin showed the lowest MIC value for *S. enteritidis* ATCC 13076 (0.004 mg/mL) and the highest MIC value for *E. coli* O157:H7 ATCC 35150 (0.128 mg/mL), while the used parabens mixture determined MIC values >1.024 mg/mL for all the examined bacterial species.

It is worth to underline that these amphiphiles showed an antibacterial effect (according to the calculated MIC values) over a panel of Gram-negative and Gram-positive bacteria at concentrations comparable to those they are able to exert an *in vitro* permeability enhancer effect on Caco-2 cells. This consideration encourages further studies aimed to clarify the molecular mechanism upon the interesting properties of lactose oleate surfactant.

Table 1. MIC values (mg/mL) of the lactose oleate against selected bacterial strains. Gentamicin and parabens mixture were used as internal controls.

Specie target	MICs (mg/mL)		
	Lactose oleate	Gentamicin	Parabens mix
E. coli O157:H7 ATCC 35150	0.128	0.128	> 1.024
E. faecalis ATCC 29212	0.128	0.064	> 1.024
L. monocytogenes ATCC 7644	0.256	0.008	> 1.024
P. aeruginosa ATCC 9027	0.128	0.016	> 1.024
S. aureus ATCC 43387	0.128	0.016	> 1.024
S. enteritidis ATCC 13076	0.128	0.004	> 1.024
Y. enterocolitica ATCC 27729	0.128	0.008	> 1.024
C. albicans ATCC 10231	0.128	nd	> 1.024

nd: not determined.

4. Conclusions

This work reports the synthesis and a comprehensive physico-chemical characterization (NMR, ESI-MS, DSC and DLS) of a new sugar-based surfactant, namely lactose oleate, obtained from the enzymatic mono-esterification of lactose and oleic acid. Lactose oleate displayed an acceptable cytotoxicity profile (EC_{50} comparable or higher than CMC) and a concentration-dependent absorption enhancing effect on intestinal Caco-2 monolayers using FD-4 as model for macromolecular drugs. The permeability effect was most pronounced at the highest tested surfactant concentration (0.25 mg/mL, 0.41 mM), which also determined the most remarkable decrease in TEER value. The obtained results suggest the involvement of the intercellular tight junction opening (paracellular route) in the increased permeability of FD-4 in presence of lactose oleate, although a combined effect, correlated to other mechanisms (e.g. transcytosis) cannot be excluded. Moreover, this work also introduces lactose oleate as antimicrobial agent (MIC values) intermediate between those of some antibiotics and common preservatives (i.e. parabens). Overall, this study highlights the potential use of lactose oleate in pharmaceutical formulations as an absorption enhancer and/or an alternative preservative.

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