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1 Physicochemical, foaming and biological properties of lowly irritant anionic sugar-based

2 surfactants

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5

6 Abstract

Surface-active compounds derived from biomass, especially sugar-based amphiphiles, have received wide attention regarding their biodegradability, low toxicity and ecological acceptability. Compared to nonionic sugar-based surfactants, the anionic ones show significantly better solubility, higher surface activity and foaming performance. Thus they are largely used in personal care formulations and many technological applications. However, anionic surfactants are well known to induce skin and eye irritation.

In this study, three sugar-based anionic surfactants, bearing a lipidic chain grafted to the 13 anomeric position of a monosaccharide (glucose or xylose) and a sulfate group on the primary 14 hydroxyl, were synthesized: 6-O-sulfo-N-(β-D-glucopyranosyl) dodecanamide (GlcNC12S), 15 N-dodecyl-6-O-sulfo-D-gluconamide (GlcCC12S) and N-dodecyl-6-O-sulfo-D-xylonamide 16 (XylCC12S). These molecules were investigated in details for their self-assembling behavior, 17 foaming properties and biological effects. All their properties were compared to those of two 18 commercially available anionic surfactants, sodium laureth sulfate (SLES) and sodium 19 20 dodecylsulfate (SDS).

Results revealed that the three anionic glycolipids show surface properties and foaming
behavior comparable to those of SDS. Furthermore, their cytotoxic and irritation potentials

are significantly lower compared to commercial molecules, which make these renewable
 molecules potential candidates for replacement of petroleum-based compounds.

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Keywords: anionic surfactant, sugar-based surfactant, glycolipid, foaming properties,
cytotoxic activity, irritation potential

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29 Introduction

Surface-active compounds performing detergency, wetting, emulsifying, solubilizing, 30 31 dispersing and foaming effects are among the most representative chemical products consumed on a world scale [1, 2]. Depending on the charge of the hydrophilic group, 32 33 surfactants can be nonionic, anionic, cationic or amphoteric. Anionic surfactants are used in greater volume compared to other surfactants (about 60% of worldwide surfactant production) 34 35 because of their excellent performance in cleaning processes and low cost manufacture [3]. During the transition period from 1940 to 1970 due to the shift from the use of powder 36 37 detergents to liquid ones, anionic amphiphiles became widely used in formulation of laundry detergents, domestic and industrial cleaning products, agricultural and pharmaceutical 38 39 formulations, cosmetic and personal care products. Anionic surfactants are mainly constituted of a hydrophobic part (linear aliphatic hydrocarbon chain with length between C_8 and C_{18} , 40 alkylphenyl ethers, alkylbenzenes, etc) linked to a hydrophilic part containing a sulphate, 41 sulfonate, carboxyl or phosphate group, associated to a counter ion (for example, Na⁺, K⁺, 42 43 NH_4^+ or an alkanolamine cation) [4-6].

However, the use of these surfactants is somewhat limited by their irritation profiles. Anionic surfactants show an important skin and eye irritation potential [6]. Moreover, cytotoxicity studies showed that anionic surfactants were more toxic than nonionic ones [4]. The development of new anionic amphiphiles with tailored functionalities and showing at the same time skin tolerance, has become a priority in the field of surface and colloid science.

Over the past few decades, the pressure from legislation and growing consumer trend toward environmentally benign chemicals had a strong impact on surfactants development. There is a growing need for the substitution of anionic surfactants of particular concern in different applications. The biodegradability and biocompatibility of surfactants have become very important not only to the consumer but also to its functional performance. To increase the environmental sustainability of amphiphilic molecules, new surface-active compounds, commonly referred as "green surfactants", have been intensively developed [7]. Different biomass substrates such as peptides and amino acids [8, 9][8, 10], sugars [10], fatty acids and
alcohols [11] [12, 13]have been extensively studied for the production of these surfactants.

Sugar-based surfactants have increasingly become a viable bio-based alternative to 58 petrochemical-based amphiphiles. Particularly, nonionic ones showed high performance, 59 environmental compatibility, low toxicity and low irritancy towards living organisms [10, 12-60 15][11, 14-16, 18]. The raw material sources used are mainly sucrose from sugar beet or 61 sugarcane, carbohydrate-based residues from hemicellulose and starch derivatives (glucose, 62 63 galactose, mannose, xylose, sorbitol, lactose). Nowadays, most important sugar-based 64 surfactants used for different applications are nonionic alkylpolyglucosides, sorbitan esters and sucrose esters. Numerous syntheses of such amphiphilics molecules have been described 65 66 [10, 16-18]. Nevertheless, to the best of our knowledge, only few studies on anionic sugarbased surfactants have been reported. Among them, several articles dealt with the synthesis 67 68 and properties characterization of anionic D-glucopyranoside derivatives; these molecules showed good adsorption properties similar to other ionic surfactants [19-21] and can also been 69 70 applied for enantiomeric separation of racemic compounds [22, 23]. Otherwise, the analysis of literature data demonstrates a significant decrease in Krafft temperature (T_K) for anionic 71 72 sugar derived surfactants [20] compared to their nonionic homologous with the same chain 73 length [24]. The micelle formation is also impacted by addition of anionic group, higher value of critical micelle concentration (CMC) is observed for sulfated alkyl glucoside [25] 74 compared to nonionic one [26]. Besides, anionic sulfosucrose derivatives showed very 75 76 promising surface-active properties with CMC values from two to three orders of magnitude lower than those of commercial anionic molecules [27]. Other studies demonstrated that 77 anionic galactoside [28] and glucoside [17] derivatives had interesting wetting properties for, 78 respectively, textile treatment applications and enhanced oil recovery. 79

Nevertheless, there is no data in the literature dealing with neither foaming behavior, which is an important property for various surfactants applications, nor dermal irritation potential of anionic sugar-based amphiphiles. Based on the facts mentioned above, studying the potential of eco-friendly anionic glycolipids for the substitution of petroleum-derived anionic surfactants is worthy of experimental study.

The present contribution reports the complete characterization of three anionic surfactants based on sugar-derived hydrophilic units. These molecules were chosen from among a number of glycolipids synthesized in our laboratory after pre-sreening of physicochemical properties. The main objective of this work is to identify new surfactants which can potentially replace analogues petroleum-derived amphiphiles for specific applications. Thus, 90 all the properties analyzed were compared to commercial standard anionic surfactants with 91 the same alkyl chain length. Surface tension was measured to study micellar and interfacial 92 phenomena. Foaming behavior was studied using gas-sparging method. To evaluate the 93 biological effects of surfactants, cellular and tissue models (mouse dermal fibroblasts and 94 reconstituted human epidermis (RHE)) were treated with the molecules and the cell metabolic 95 activity and IL-1 α release (skin irritation) were measured.

96

97 2. Materials and methods

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99 2.1. Synthesis of anionic glycolipids

100 Three sugar-based surfactants with anionic sulfated polar head and alkyl chain, constituted of 12 carbon atoms, were synthetized in this study. 6-O-Sulfo-N-(β -D-glucopyranosyl) 101 dodecanamide (GlcNC12S, M_w=463.5 g.mol⁻¹) was prepared from commercial glucose 102 following the procedure described in literature [29]. N-dodecyl-6-O-sulfo-D-gluconamide 103 (GlcCC12S, M_w=465.5 g.mol⁻¹) and *N*-dodecyl-6-*O*-sulfo-D-xylonamide (XylCC12S, 104 M_w =435.5 g.mol⁻¹) were produced in three steps, by 1) oxidation of commercial glucose and 105 xylose [30], 2) ring-opening aminolysis of the as-obtained glyconolactone and 3) sulfation 106 107 using Pyr-SO₃ complex [25]. The monosulfated sugars were separated from disulfated compounds using reverse phase chromatography. Produced anionic surfactants were stored at 108 109 -20°C.

In addition, two commercially available anionic surfactants were used: Sodium Laureth Sulfate (SLES, M_w =370 g.mol⁻¹) provided from THOR Personal Care SAS (La Croix Saint-Ouen, France) as 27 wt% aqueous solution (Texapon® NSO UP) and Sodium Dodecylsulfate (SDS, M_w =288 g.mol⁻¹, purity>99%) was purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). The chemical structures of studied anionic surfactants are presented in Fig. 1.

116

117 2.2. Preparation of surfactant solutions

Water from Milli-Q Millipore system with a resistivity of 18.2 MΩcm produced by a PureLab Classic purification chain (Elga/Veolia, Wissous, France) was used to prepare aqueous solutions of surfactants for surface tension and foaming properties evaluation. Cell-culture medium was prepared as described previously [31] and used for preparation of solutions for biological responses evaluation. Surfactant solutions with different concentrations were prepared by dissolving a known quantity of pure surfactant in water or cell-culture aqueous
medium. All surfactants were soluble in water at 25°C.

125

126 2.3. Surface tension measurement

127 2.3.1. Equilibrium surface tension

Equilibrium surface tensions (γ_{eq}) of aqueous surfactant solutions prepared in pure water and 128 cell-culture medium were measured using K100 KRÜSS tensiometer (Hamburg, Germany) by 129 the Wilhelmy plate method. The Wilhelmy plate has a length, width and thickness of 130 131 19.9 mm, 10 mm and 0.2 mm, respectively and is made of platinum. The tensiometer operates by holding a plate in a fixed vertical position attached to a microbalance (resolution 10 µg) 132 measuring the force acting on the plate. The surface tension is directly determined from the 133 force-balance measurement. The surfactant solution was put in a glass vessel surrounded by a 134 circular thermostated system maintained at 25±1°C throughout the duration of the test. Prior 135 to each determination, the plate was thoroughly cleaned and burnt to red hot conditions in a 136 blue flame to ensure perfect wetting (zero contact angle). The measurement of surface tension 137 at the air/liquid interface was conducted over 12 000s. The surface tension values are reported 138 as the mean of three repeat measurements. The Critical Micelle Concentration (CMC) of each 139 140 surfactant was determined from the minimum point of the plot of the surface tension with respect to the logarithm of the concentration. The efficiency of surfactant adsorption (pC_{20}) 141 was associated to the surfactant concentration required to reduce the surface tension of pure 142 water by 20 mN/m. The surface tension of pure water measured at 25°C was equal to 72.8 143 144 $mN.m^{-1}$.

145 The maximum surface excess of surfactant at the air/water interface, Γ_{max} , was calculated 146 using the Gibbs adsorption isotherm equation [32]:

147
$$\Gamma_{\max} = \frac{-1}{nRT} \frac{d\gamma}{d\ln(C)}$$
(1)

where n=2 for a conventional ionic surfactant, assuming electrical neutrality of the interface [33], R is the universal gas constant (8.314 J.mol⁻¹.K⁻¹), T is the absolute Kelvin temperature and $\left(\frac{d\gamma}{d\ln(C)}\right)$ is the slope of γ versus ln(C) determined at the CMC. The minimum area occupied by a surfactant molecule, A_{min}, at the interface:

152
$$A_{\min} = \frac{1}{\mathcal{N}\Gamma_{\max}}$$
(2)

153 where \mathcal{N} is Avogadro's number.

154

155 2.3.2. Dynamic surface tension

Measurement of dynamic surface activity of the surfactant solutions were performed using the 156 maximal bubble pressure method (BP2 Krüss tensiometer, Hamburg, Germany). This method 157 is the most appropriate one for the determination the kinetics of surface tension decrease at 158 very short times. Briefly, an inert gas (nitrogen) is slowly bubbled through a fine capillary, 159 which is immersed in the test aqueous solution. Hydrophobically modified glass capillary 160 with an inner diameter of 0.24 mm was used for the measurements. When the bubble area 161 growths at the tip of the capillary, the pressure within the bubble increases and passes through 162 163 a maximum. The surface tension can be calculated from the measured maximum capillary pressure using the Laplace equation [34]. The time associated with the interface formation 164 165 (surface age) was varied from 30 ms to 50 s in every single experiment. The constant temperature of 25±1°C during measurements was assured by the external thermostat. 166 167 Analyses were performed in triplicate.

168

169 2.4. Foaming properties

The determination of foaming properties of surfactant solutions was performed using an 170 171 automated foaming device (FoamScan, Teclis, France). This instrument produces foam by 172 blowing gas through a porous frit at a controlled gas flow rate. The conductivity measurement and image analysis are used to monitor different foam properties such as foam ability, foam 173 stability, liquid fractions, drainage rate, and bubble size distribution. The experimental setup 174 consisted of a glass column (diameter 3.5 cm, height 30 cm) equipped with a porous glass frit 175 at its bottom. The foam generated was observed with two CDD cameras: one camera allowing 176 the measurement of total foam and liquid amounts and a second camera equipped with a 177 macro objective used to record the variation of the air bubbles size. The experimental 178 conditions for determination of foam-related properties using gas-sparging method were 179 established in our previous work [35]. Low frit porosity, low concentration and high gas flow 180 rate turned out to the best choice for the screening of foaming performances of surfactants. In 181 182 the present study, 40 mL of surfactant solution at a concentration of C=0.1CMC was introduced into the column equipped with frit porosity of grade P3 (ϕ =16-40 µm), at ambient 183 temperature $(24 \pm 2^{\circ}C)$. Foam was generated by injection of pressurized air (0.5 bar) at a flow 184 rate of 200 mL.min⁻¹ during 60 s. The total duration of one experiment was set to 30 minutes 185 186 for measurement of foam stability. All experiments were carried out in triplicate. Several foaming properties were examined, namely foaming capacity, foam stability, half-life time of 187 188 foam and maximum foam density.

189 The foamability at the end of bubbling is defined by the foam capacity, FC:

190
$$FC = \frac{V_{foam,0}}{V_{gas}}$$
(3)

where $V_{foam,0}$ is the volume of foam at the end of gas injection and V_{gas} is the volume of the sparged gas.

Foam stability is a parameter taking into account the whole behaviour of the foam collapse process as a function of time. For evaluation of foam stability (S) the Experimental area under the plot V_{foam} =f(time) starting at the end of gas-injection was calculated and compared to the Maximal area (ideal behaviour in which the foam would remain stable all along the experiment).

198
$$S = \frac{\text{Experimental area}}{\text{Maximal area}}$$
 (4)

Foam half-life time $(t_{1/2})$ corresponds to the time (seconds) needed for the reduction of the foam volume to half of the initial volume value (V_{foam,0}).

Maximum foam density (MD), related to the amount of liquid in the foam, was measured at the end of gas bubbling. The density was determined by conductivity measurements within the bottom solution.

204 MD (%) =
$$\frac{V_{\text{solution},0} - V_{\text{solution},f}}{V_{\text{foam},0}} \times 100$$
 (5)

 $V_{solution,0}$ is the initial volume of liquid, $V_{solution,f}$ is the volume of liquid at the end of gasinjection.

207

208 2.5. Biological responses on cellular and tissue models

209 2.5.1. Cell and tissue models

The mouse fibroblast L929 cell line was obtained from ATCC (ATCC-CCL-1, LGC Standards, France). Cells (passage 15-40) were cultured in 75 cm² Falcon® cell culture flasks with DMEM (Gibco, Thermofisher scientific, France) supplemented with fetal bovine serum

213 (FBS, Gibco), 2 mM L-glutamine (Gibco), 100 U/mL penicillin (Gibco) and 100 µg/mL

- streptomycin (Gibco). Cells were maintained in a humid atmosphere at 37°C with 5% CO₂.
- 215 Reconstituted Human Epidermis (RHE) model (EpiDermTM EPI-200-SIT kit) was purchased

216 from MATTEK (Bratislava, Slovakia). This model consists of normal human epithelial

217 keratinocytes, which have been cultured at air/liquid interface to form a multilayered, highly

- skin-like structure on specially prepared permeable cell culture inserts. Upon reception, RHE
- 219 were pre-conditioned by standard protocol following manufacturer's instructions.

220

221 2.5.2. Cell metabolic activity assay

The cytotoxic potential of surfactants was evaluated by measuring the cell metabolic activity of both fibroblasts L929 and RHE. Cells and RHE were cultured and treated by surfactants at different concentrations following the procedure described previously [31, 36].

Shortly, L929 cells (2600 cells/cm²) were seeded in Costar® culture plate with culture medium (2 mL). After 24h cell adhesion, surfactant dissolved in culture medium was added to the cells at different concentrations for 48h. The tetrazolium salt (200 μ L), commonly referred as MTS (Cell titer 96® Aqueous One solution cell proliferation assay, Promega, France), was added to each well. After 4 h, 3 aliquots (100 μ L) from each well were transferred into a 96 well cell culture plate.

The effects of surfactants on RHE were evaluated according to the OECD N° 439 guideline 231 232 for *in vitro* skin irritation (OECD 439, 2019). Surfactants dissolved in phosphate buffer saline (PBS) (30 µL, 1 mg/mL) or negative control (30 µL PBS) were applied for 1h (25 min at 233 234 room temperature and 35 min at 37°C) on the top of each tissue with nylon meshes used to cover the tissue surface and to guarantee the spreading of liquids. Tissues in inserts were 235 236 extensively washed to discard surfactants and RHE were incubated for 24h. Then the culture 237 media were harvested and stored for a second end point, release of the cytokine IL1- α . Fresh culture medium was added on tissues, RHE were cultured for 18h and then were transferred 238 into wells of Costar 24-well plate containing 270 µL of Hank's buffered salt solution (HBSS, 239 240 Life Technologies) and 30 µL of MTS. After incubation period (3h at 37°C), 3 aliquots (100 µL) from each well were transferred into a 96 well cell culture plate. 241

The amount of viable cells was evaluated by the measuring their dehydrogenase activity. The 242 MTS was reduced by living cells into a formazan compound with characteristic adsorption 243 band at 490 nm. The absorbance (A) of all samples was read at 490 nm using microplate 244 reader (Bio-Rad Model 680, Bio-Rad, USA). Cell-less controls were used in each test to avoid 245 any background absorbance due to possible interactions between MTS and tested molecules. 246 247 Metabolic activity of L929 cells and RHE model related to the amount of living cells in culture was determined as: Cell metabolic activity = (A of sample treated with surfactant -A248 of culture medium with surfactants without sample)/(A untreated sample - A of culture 249 medium). All experiments were performed six times. 250

251

252 2.5.2. Irritation test on RHE

The irritant effect of commercial and synthetized anionic surfactants on the RHE model was evaluated by measuring the secretion rate of human interleukin-1 α (IL-1 α) in culture medium. IL-1 α cytokine concentrations were quantified after 1h surfactant exposure (followed by 24h post-incubation according to the RHE manufacturer's instructions) by ELISA test according to the kit manufacturer's instructions (Human IL-1 α Platinum ELISA kit, ThermoFisher Scientific, France). Six repetitions were performed for each surfactant solution.

260

261 2.6. Statistical analysis

Statistical analyses of foaming properties and biological responses were performed using GraphPad InStat software. Different variables are expressed as means \pm standard deviation. For foaming properties, experimental data was studied using one-way analysis of variance (ANOVA). Tukey's test was used for the multiple comparison procedure and the significance was accepted at the P<0.05 level.

For biological responses, non-parametric Dunn and Kruskal-Wallis tests were used for comparisons as most of the continuous values measured had a non-Gaussian distribution. A value of P<0.05 was taken as significant.

270

271 **3. Results**

272

273 *3.1. Surface tension reduction and CMC*

For comparison purposes, different surface properties of the three selected glycolipids were studied and the two classical anionic surfactants (SDS and SLES). The CMC values of anionic glycolipids were determined using equilibrium surface tension measurement at different concentrations and are taken in consideration at the minimum point of the decreasing surface tension curve. Plots of surface tension versus glycolipids and model anionic surfactants concentration are shown in Fig. 2A. Physicochemical properties of surfactants in water determined from these experimental curves are reported in Table 1.

When compared to commercial surfactants, sugar-based molecules have the same order of magnitude of CMC values as that of SDS. However, the CMC of SLES is significantly lower. For instance, the CMC of GlcNC12S is 20 times higher than that of SLES. The minimum observed for all curves indicated the presence of small amount of residual molecules with amphiphilic properties participating in the surface tension reduction. This particularity is

generally observed for SDS solutions due to the presence of dodecanol impurity [37, 38]. The 286 surface tension values at CMC (γ_{CMC}) indicate the effectiveness in surface tension reducing. 287 By comparing the γ_{CMC} of five anionic surfactants, results indicate that highest value of 34.9 288 mN.m⁻¹ was obtained for SDS and the lowest of 27.3 mN.m⁻¹ for SLES. The γ_{CMC} of sugar-289 based molecules were comprised between those of commercial surfactants. Similar tendencies 290 can be observed for the results of calculated adsorption efficiency (pC_{20}) , maximum surface 291 excess (Γ_{max}) and corresponding minimal area per molecule (A_{min}). For these physicochemical 292 parameters, the values obtained for sugar-based molecules were comprised between those of 293 294 SLES and SDS. Results reveal that GlcCC12S was characterized by highest pC₂₀ value of 3.92, followed by XylCC12S with pC_{20} of 3.43 and GlcNC12S with pC_{20} of 2.89. In regards 295 296 to Γ_{max} and A_{min} parameters, estimated by applying Gibbs adsorption isotherm to the surface tension curves, the values obtained for anionic glycolipids were in the range of 1.68×10^{-10} -297 2.33×10^{-10} mol.cm⁻² and 71.3–99.1 Å²/molecule respectively. 298

The dynamic surface adsorption of surfactant was characterized using maximum bubble 299 300 pressure method. This technique allows an accurate regulation of air/solution surface adsorption with time, ranges from 0.03 to 50 s. Fig. 2B presents the plots of dynamic surface 301 302 tension of studied anionic surfactant as a function of bubble age at concentration equal to 0.1CMC. For a new liquid/gas interface formation, the initial surface tension (γ_0) corresponds 303 to 72.8 mN.m⁻¹, which is a surface tension for pure water/nitrogen interface at room 304 305 temperature and at atmospheric pressure. It can be observed that at short times, before surface 306 age of 1 s, the dynamic surface tension of SLES and sugar-based surfactants changes very little and remains close to that of pure water, whereas SDS is characterized by lower surface 307 tension of about 68–70 mN.m⁻¹, suggesting faster adsorption at the air/water interface. For 308 higher surface ages, a little decrease in dynamic surface tension can be noticed for SLES after 309 1 s and for XylCC12S after 20 s. In addition, results show that dynamic surface tension is not 310 significantly affected by the increase in air bubble age for GlcCC12S and GlcNC12S. 311

From the results presented in Figure 2 and Table 1, it can be concluded that for the same alkyl chain length, the structure of the hydrophilic head group significantly affects adsorption at the air/water interface of anionic surfactants.

315

316 *3.2. Foaming properties*

Foaming behavior of the three synthesized anionic sugar-based surfactants and the two anionic reference surfactants was studied using gas-sparging method. Fig. 3 displays the foam

volume and liquid volume as a function of time for surfactant solutions and Table 2 presents 319 the summary of experimental data on foaming properties. In order to compare foaming 320 properties of different surfactant systems, it is more appropriate to consider these systems in 321 the same thermodynamic state. Below CMC, surfactants are under a monomer form. Thus the 322 flux of surfactant molecules to the interface and the foaming behavior of solution are not 323 influenced by kinetic of micellization and break-time of micelles [39]. Moreover, the results 324 of our previous study showed that low concentration conditions are favorable for the 325 discrimination of foaming behavior of different surfactants [35]. Thus, solution concentration 326 327 applied for all surfactants was 0.1CMC.

The results obtained demonstrated that the whole foam process consists of two stages: the generation of the foam (from 0s to 60s) and the destabilization of the foam (from 60s to 1800s). In the first stage, i.e. gas sparging into the liquid, the liquid volume decreases and the foam volume increases gradually. In the decay stage, the foam volume decreases because of different destabilization processes, and the liquid volume increases due to the liquid drainage through foam.

As can be seen on Fig.3A, the volume of formed foam is close to 200 mL (maximal volume 334 of produced foam at gas flow rate of 200 mL.min⁻¹ during 60 s) for all surfactants except 335 GlcCC12S and GlcNC12S. Another information which can be observed from this figure is the 336 collapsing rate or foam stability expressed by area under the curve (S, Table 2). This property 337 is closely related to the half-life time of foam $(t_{1/2})$. SLES solution showed significantly 338 higher foam stability S of 0.79 compared to other surfactants. The stability of the foams 339 produced by XylCC12S (0.36) and SDS (0.27) was intermediate, whilst in the case of 340 GlcCC12S and GlcNC12S a significant reduction in the foam stability was observed. 341

The variation of liquid volume with time (Fig. 3B) showed that the liquid volume of 342 GlcCC12S slightly decreased during bubbling and remained higher than that of other 343 surfactants throughout the experience. The liquid drainage rate for foams produced with SDS, 344 XylCC12S and GlcNC12S was similar. These observations are in agreement with maximum 345 foam density values (MD, Table 2) representing the amount of liquid in the foam and 346 measured at the end of bubbling. It can be seen that GlcCC12S formed very dry foam 347 (MD=2.0%) whereas liquid content in foams produced by other anionic surfactants varied 348 from 16.9 to 20.6%. 349

350

351 *3.3. Cell metabolic activity and irritation response of RHE after surfactant treatment*

The interaction of surfactants with L929 model cells was assessed to study surfactant-352 membrane interaction at low surfactant concentration (below CMC). The results reported on 353 Fig.4 illustrates the metabolic activity of cells treated with anionic glycolipids at different 354 concentrations. As can be seen, in the studied concentration range, the results show that cell 355 viability decreased with increase in surfactant concentration, but for the concentrations below 356 0.006 mM, the three molecules did not modify significantly the cell metabolic activity 357 compared to untreated cells. A significant cytotoxic effect is observed for 0.06 mM of 358 surfactants, with no difference observed between the three anionic glycolipids. The biological 359 360 effects of anionic sugar-based surfactants were compared to responses of commercial anionic surfactants at the same molar concentration, 0.2 mM. This maximal concentration was fixed 361 362 based on our previous studies, as at this concentration a significant cytotoxic effect of commercial surfactants on the cell metabolic activity was observed [36, 40]. The anionic 363 364 glycolipids show the same cytotoxicity than SDS and SLES. However, at this concentration, the metabolic activity of cell treated with SLES seems to be lower compared to those of other 365 366 molecules.

Table 3 summarizes the results obtained after treatment of RHE tissues with surfactant 367 368 solutions, according to the OECD 439 guideline for the surfactant concentration and exposure 369 conditions. These results show that the treatment with the anionic sugar-based surfactants did 370 not modify significantly the cell metabolic activity of keratinocytes in RHE tissue compared to untreated RHE. Cells exposed to anionic glycolipids still maintain high metabolic activity 371 (75.3-89%) even at high surfactant concentrations (1 mg.mL⁻¹). Metabolic activity of cells 372 seems to be affected by SLES, but considerable variability of obtained results $(51.1\pm34.6\%)$ 373 leads to no significant statistical difference between SLES and glycolipids. In contrast, the 374 commercial anionic surfactant SDS shows clear cytotoxic effect on cells. 375

Simultaneously, the irritation effect of surfactants on RHE tissue was evaluated by measuring the release of IL-1 α in culture medium after 24h of surfactant treatment. A significant increase of IL-1 α concentration in RHE models treated by SDS and SLES was observed, compared to non-treated control (PBS), indicating a strong irritant effect of both commercial surfactants on the *in vitro* skin models. Synthetized anionic glycolipids showed a lower release of IL-1 α , and were not considered different from negative control PBS.

382

383 4. Discussion

384

385 *4.1. Surface active properties in water*

To act as an efficient surfactant, an amphiphilic molecule is usually characterized by adsorption properties leading to decrease in surface tension and by formation of aggregates from a certain concentration. Since these properties are mainly affected by the structure, it is fundamental to characterize the potential on new surfactants from an amphiphilic point of view.

The particular low CMC value of SLES compared to anionic glycolipids can be attributed to 391 intermolecular and intramolecular attractive ion-dipole interactions between the sulfate group 392 393 (SO_4) and the O \rightarrow CH₂ dipole of oxyethylene group [41]. Such attractive interaction is very specific and arising only with oxyethylene group adjacent to sulfate ion. It is not occurring for 394 395 SDS and anionic sugar-based surfactants micelles, where strong self-repulsion between sulfate groups is dominant. Consequently, the self-assembling for these molecules was 396 397 observed at higher concentrations. A higher value of pC_{20} usually indicates a higher ability for the reduction of surface tension, assuming that lower energy is needed to bring the surfactant 398 399 molecule from the bulk to the air/water interface [42]. pC₂₀ values of anionic glycolipids presented in Table 1 suggest a high surfactant efficiency in lowering the surface tension of 400 401 water comparable to commercial anionic surfactants.

402 The size of hydrophilic head group is dominant factor to determine the adsorption quantity of surfactant molecules at the air/water interface, Γ_{max} , and corresponding area occupied by a 403 single surfactant molecule, A_{min} [32]. Γ_{max} and A_{min} values calculated for anionic surfactants 404 405 can be correlated with their molecular structure. SDS with lowest size of polar moiety has high packing density at the interface air/water. The cross-sectional area of the hydrophilic 406 group increases for SLES and glycolipids resulting in lower Γ_{max} value and thereby, larger 407 Amin value. The same tendency, where an adsorption quantity of surfactant molecules at the 408 air/water interface decreases with the increasing of the polar part size, was reported for 409 410 anionic polyethylene oxide derivatives [43, 44]. Moreover, for sugar-based surfactants the intermolecular interactions of the -OH groups and their steric hindrance can be responsible for 411 higher distance between the molecules in the adsorption layer and increased Amin value. For 412 the same length of hydrophobic chain, the surface adsorption properties of sugar-based 413 surfactants depend not only on headgroup size, but also on the linker type and structure [45]. 414 Consequently, the differences in physiochemical properties of studied anionic glycolipids can 415 be related to the nature of linker group. Similar results on surface adsorption properties for 416 other families anionic surfactants have been reported in the recent literature [17, 42, 43, 46]. 417

Dynamic surface tension measurements have been performed in order to get information on 418 419 the instantaneous decrease of surface tension at very short times. After creation of the fresh surface, the dynamic tension decreases with time as amphiphilic molecules diffuse and adsorb 420 421 at the interface. The results demonstrate that SLES exhibits faster adsorption kinetics compared to other surfactants. Surprisingly, the dynamic surface tension of three sugar-based 422 surfactants was not changing significantly over time period considered, indicating the absence 423 of surfactant adsorption at short times. In this case, it can be suggested that the time required 424 to attain the equilibrium surface tension is higher for glycolipids compared to commercial 425 426 molecules, and the adsorption of sugar-based surfactants is controlled not only by the diffusion of molecules. The repulsive electrostatic interactions of anionic groups might 427 428 explain the higher potential barrier of glycolipids adsorption and slower adsorption rate at the gas/liquid interface for these surfactants [33, 47]. Additionally, this low surfactant diffusivity 429 430 and slow reduction in dynamic surface tension can be linked to the low concentration of analyzed solutions (C = 0.1CMC). 431

432

433 *4.2. Evaluation of foaming behavior*

434 Aqueous foams are thermodynamically unstable disperse systems. They consisted of gas bubbles separated by liquid films where gas/liquid interface is generally stabilized by 435 surfactants. Different self-destructive processes take place simultaneously in foam during 436 ageing. The main processes limiting the foam stability are drainage of the liquid under the 437 action of gravity, and foam ageing phenomena like coalescence (film rupture) and ripening 438 (exchange of gas between bubbles due to differences in Laplace pressure) [48, 49]. These 439 factors cause the transformation of foams to their thermodynamically most stable state -440 441 complete destabilization to initial liquid.

Foaming capacity describes the ability of a surfactant solution to produce foam. It depends on 442 both the rate of transport of the surfactant to the gas/liquid interface and the total adsorbed 443 amount of surfactant [50]. High foam capacity of SLES and SDS could be related to the 444 445 decrease of dynamic surface tension for these solutions. This result indicates that the diffusion of the surfactant to the surface is fast enough for the formation of interfacial layer in the foam 446 generation process. However, despite low adsorption rate and dynamic surface tension 447 reduction, anionic glycolipids XylCC12S and GlcNC12S showed high foaming capacity close 448 to those of commercial molecules. Significant differences in foam properties, such as foam 449 stability (S), maximum foam density (MD) and foam half-life time $(t_{1/2})$ can be attributed to 450 451 the variation of head group structure, since all surfactants studied have the same length of

hydrophobic chain. The organization of the hydration water around an ethylene oxide and 452 sugar group under similar conditions, were found to be completely different [51, 52]. 453 Moreover, flexible hydrophilic part of negatively charged surfactants can cause the adoption 454 of various molecular configurations on the air/water interface. The compactness of adsorbed 455 molecules on the two air/water interfaces of the foam lamellae affects both foamability and 456 foam stability [42]. While SDS and SLES have short and flexible hydrophilic units, 457 XylCC12S and GlcCC12S have more voluminous open chain sugar part, and the cyclic sugar 458 GlcNC12S head group behaves like a hard disk with reduced conformational flexibility. 459 460 These mentioned differences influence the behavior of surfactants in solution as well as their adsorption and foaming properties. It is important to indicate that the viscosity of aqueous 461 surfactant solution could significantly affect its foaming properties. Typically, the foam 462 stability increased for bulk solutions with higher viscosity [53]. In this study, very diluted 463 464 solutions were analyzed in foaming experiments (C = 0.1 CMC), the viscosity remained constant and very close to that of pure water. 465

To sum up, foaming properties of two of the three synthetized anionic glycolipids (XylCC12S and GlcNC12S) are close to those of commercial anionic surfactants. They form the similar amount of foam that SDS and SLES, and exhibit foaming stability comparable to that of SDS. Since foaming performances of anionic surfactants are of great importance in formulation of personal care products, an interesting approach is to study the biological activity of new glycolipids and to evaluate their potential use in such applications.

472

473 *4.3. Biological responses*

The potential use of surfactants in several cosmetic and pharmaceutical applications depends 474 on their cytotoxicity, biocompatibility and toxic effects. Our previous studies revealed a 475 significant difference in biological responses, such as metabolic activity, cytotoxicity and 476 477 irritancy, between commercial model surfactant and bio-based nonionic glycolipids [31, 36]. Biological effects of anionic glycolipids have not been investigated before. In the present 478 479 study, the metabolic activity related to the amount of viable cells was studied on L929 model cells and on RHE tissue. As expected, cell metabolic activity decreases with the rise of 480 surfactant concentration. While at fixed concentration, cells treated with sugar-based 481 surfactants showed similar metabolic activity. We used an in vitro RHE EpiDerm system and 482 the OECD guidelines for the testing of chemicals and cosmetic formulations (irritant effect) 483 were strictly applied to characterize the surfactant's effect on a human skin model [54]. The 484 485 significant differences in keratinocyte metabolic activity observed in RHE models treated

with commercial or synthesized anionic glycolipids revealed that glycolipids are mild and 486 tolerant towards epidermal cells. Notably no significant difference between the three 487 glycolipids was found, suggesting that the structure of polar part (cyclic or acyclic) of 488 surfactant cannot be directly correlated to biological responses. In contrast, significant loss in 489 metabolic activity was found after the exposure of human keratinocytes to SDS and SLES 490 solutions suggesting an important amount of damaged cells. The irritancy potential 491 492 measurements showed clear difference in IL-1 α release between the three anionic glycolipids and model anionic surfactants, highlighting that SDS and SLES had significantly higher 493 494 irritation potential. Previous study reported that skin irritation profile of SDS was significantly higher compared with 15 other nonionic, amphoteric and anionic surfactants [55]. 495

Overall, all obtained results confirmed that in the concentration range studied, GlcCC12S, GlcNC12S and XylCC12S are significantly less toxic than SLES or SDS. *In vitro* test systems used here were particularly interesting for pre-screening of new surfactants for novel formulation development in cosmetics, avoiding the use of animals for testing. Obviously, surfactant combinations are used in formulation, and it is necessary to evaluate biological responses of fully formulated products.

502

503 5. Conclusion

A multidisciplinary approach taking into account surface properties, foaming behavior and 504 505 biological responses of three anionic surfactants (GlcNC12S, GlcCC12S, XylCC12S), obtained from renewable raw materials (carbohydrates) comparatively to model commercial 506 507 anionic surfactants (SDS and SLES) has been chosen. Sugar-based surfactants were found to exhibit good surface activities including low surface tension and CMC values close to those 508 509 of commercial surfactant (SDS). Foam capacity and foam stability of XylCC12S and 510 GlcNC12S were found to be similar to those of SDS at the same C/CMC concentration. The analysis of biological responses on model cells and RHE tissues indicated significantly lower 511 toxic effects of the three anionic glycolipids compared to those of commercial anionic 512 surfactants. The lowest irritant effect, evaluated on a human skin model, was also observed 513 for the new synthesized surfactants, GlcNC12S and XylCC12S. The analysis of obtained data 514 515 demonstrates that the three sugar-derived anionic surfactants can be categorized as bio-based compounds and considered as excellent potential candidates for the substitution of petroleum-516 517 based surfactants for cosmetic and detergence applications. These promising results contribute to the knowledge about sugar-based surfactants and encourage us to explore other anionic 518 sugar-based structures for future work. 519

520

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Fig. 1. Anionic surfactants studied for their surface-active properties, foaming behavior and biological effects.

Fig. 2. (A) Plots of surface tension versus concentration of anionic surfactants. Dotted lines are displayed as a visual guide. (B) Dynamic surface tension behavior of anionic surfactants at C=0.1CMC; standard deviation bars are smaller than used symbols (n=3).

Fig. 3. Variation of the foam volume (A) and liquid volume (B) with time for different surfactants at C=0.1CMC. The bubbling rate is 200 mL.min-1.

Fig. 4. Metabolic activity of L929 cells after surfactant treatment. n=6 per group, data are presented as mean value with standard deviation. * P<0.05 and *** P<0.001 indicate significant difference *versus* untreated cells.









Table 1.	CMC (mM)	$\gamma_{CMC}(mN.m^{-1})$	pC ₂₀	10^{10} . Γ_{max}	A_{min} (Å ²)
Summary				(mol.cm ⁻²)	
of surface					
properties					
and					
foaming					
properties					
of					
surfactants					
: the					
critical					
micelle					
concentrati					
on (CMC),					
the					
equilibriu					
m surface					
tensions at					
CMC					
$(\gamma_{\rm CMC})$, the					
adsorption					
efficiency					
(pC_{20}) , the					
maximum					
surface					
excess					
(Γ_{max}) and					
correspond					
ing					
minimal					
area per					
molecule					
(A _{min})					

determined					
from plots					
in					
Fig.2A.Surf					
actant					
SDS	8.0	34.9	2.52	3.86	42.9
SLES	0.5	27.3	4.40	1.31	127.0
GlcCC12S	3.0	31.5	3.92	1.68	99.1
XylCC12S	2.0	31.0	3.43	2.33	71.3
GlcNC12S	10.0	28.7	2.89	1.76	94.2

Table 2. Summary of foaming properties of surfactants: the foaming capacity (FC), the foaming stability (S), the maximum foam density (MD) and the half-life time of foam $(t_{1/2})$ determined from curves in Fig.3.

	Foaming properties*					
Surfactant	FC	S	MD (%)	$t_{1/2}(s)$		
SDS	1.05±0.01 ^a	0.28±0.01°	18.8±0.2 ^b	271±46 ^{bc}		
SLES	1.05±0.01 ^a	0.79±0.01ª	17.8±0.2°	1502±85 ^a		
GlcCC12S	0.57±0.05 ^c	0.23±0.01 ^{cd}	2.0±0.4 ^d	133±2°		
XylCC12S	1.04±0.01ª	0.36±0.06 ^b	16.9±0.5°	356±58 ^b		
GlcNC12S	0.98 ± 0.02^{b}	0.20±0.02 ^d	20.6±0.5ª	324±50 ^b		

^{a-e} Mean values in the same column followed by different letters indicate a statistical difference (P<0.05, n=3) *Measurement of foaming properties was realized at C=0.1CMC

Table 3.	Metabolic	activity of	f cells an	d IL-1α	concentrations	detected	in culture	medium	of
RHE afte	er surfactan	t treatment	- -						

Surfactant	PBS (negative	GlcCC12S	GlcNC12S	XylCC12S	SDS	SLES
	control)					
Metabolic	100±4.9	89.0±12.3	85.4±10.0	75.3±19.6	$2.9\pm2.7^{***}$	51.1±34.6
activity (%) ^a						
IL-1α	5.3±2.7	68.7±9.0	35.1±9.0	54.8±8.4	157.4±23.0***	155.5±26.7***
concentration						
$(pg.mL^{-1})^b$						

^a Metabolic activity was measured by absorbance of cells in RHE models at 490 nm

^b IL-1 α concentration of RHE model was assessed by ELISA test after 1h surfactant exposure *** P< 0.001 indicate significant difference versus untreated cells

