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Membrane-lytic Actions of Sulfonated Methyl Ester Surfactants and Implications to Bactericidal Effect and Cytotoxicity

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ZL and FP made equal contribution

Abstract

Surfactants are multifunctional molecules widely used in personal care and healthcare formulations to cleanse, help disperse active ingredients (e.g., forming emulsions) and stabilise products. With increasing demands on improving biosafety, there is now mounting pressure to understand how different surfactants elicit toxicities at molecular and cellular levels. This work reports the membrane-lytic behaviour of a group of sulphonated methyl ester (SME) surfactants together with representative conventional surfactants. All surfactants displayed the clear rise of lysis of the model lipid bilayer membranes around their CMCs, but the two ionic surfactants SDS and C₁₂TAB even caused measurable lysis below their CMCs, with membrane-lytic actions increasing with monomer concentration. Furthermore, whilst ionic and nonionic surfactants could achieve full membrane lysis once above their CMCs, this ability was weak from the SME surfactants and decreased with increasing the acyl chain length. In contrast to the conventional anionic surfactants such as SDS and SLES, the protein solubilizing capability of the SME surfactants was also low. On the other hand, MTT assays against 3T3 fibroblast cells and human chondrocyte cells revealed high toxicity from SDS and C₁₂TAB against the other surfactants studied, but the difference between SME and the rest of conventional surfactants was small. Similar behaviour was also observed in their bactericidal effect against *E. coli* and *S. aureus*. The trend is broadly consistent with their membrane-lytic behaviour, indicating little selectivity in their cytotoxicity and bactericidal action. These results thus reveal different toxicities implicated from different surfactant head groups. Increase in acyl chain length as observed from SME surfactants could help improve surfactant biocompatibility.

1. Introduction

Surfactants are widely used in personal care, healthcare and hygiene products [1]. They are also widely used in skin based drug delivery systems and formulation [2]. Their main physical role is to help disperse components that may have poor water solubility and improve product stability, irrespective of their appearances, e.g., dispersions, foams, emulsions or gels. In addition to product stabilization, surfactants can also work as emollients and moisturisers to soften skin by reducing moisture evaporation. For the products that are dedicated to cleansing, their contacts with skin are short. In many personal care and healthcare applications, however, where the products are left on skin (so-called leave-ons), their mildness or biocompatibility must be more carefully assessed as prolonged skin contact might cause skin irritancy or toxicity.

A typical formulated personal care or infection control cream often contains more than a dozen of ingredients that have different levels of toxicity, but surfactants are usually the most abundant. Surfactants and other ingredients can be synthetic or naturally occurring. Because of increasing demands on biosafety and environmental concern, there is now growing requirement for understanding which type of surfactants is better suited for a particular use. The European Union (EU) has the most restrictive regulations to control chemicals used in personal care, healthcare and hygiene; products sold in the EU must comply with these regulations [3]. Despite these restrictions, new surfactant based products may still be developed by using existing and newly developed chemicals with biosafety information available, demonstrating that benefits outweigh hazards [4].

Human skin acts as a barrier to resist the penetration of many molecules, particularly those with molecular weights (MWs) below 500 Dalton [5, 6]. Because most surfactants currently used in personal care and healthcare have MWs below 500 Da they have been examined by various test models investigating their effects in mediating permeation across the skin barrier. Extensive research has provided evidence to support the view that most known contact allergens are under 500 Da and that larger ones usually can't act as contact sensitizers. In addition, common pharmacological agents for topical skin treatment are usually under 500 Da [5]. In contrast, immune suppressants aimed at topical applications such as cyclosporine, tacrolimus and ascomycins have MWs above 500 Da, thus augmenting this point from the opposite side. [6]. However, it should be noted that marking the MW of 500 Da as the limit is largely empirical as there are some known allergens that have MWs above 500 Da. On the

other hand, the exact chemical allergens can be person specific. Various dermatological tests have been developed including the routine patch test series advised by the International Contact Dermatitis Research Group (ICDRG) to diagnose contact allergy from potential sensitizing agents [6].

Extensive research over the past 2 decades using cell models and clinical studies have revealed controversial results suggesting that certain cosmetic ingredients such as parabens, aluminium salts, phthalates, or bisphenol A could be carcinogenic and mutagenic to humans [7]. They could act as xenoestrogens to disrupt the normal metabolism of the natural estrogen and result in DNA damage in animal and human mammary epithelial cells. In contrast, surfactants such as nonyl phenol ethoxylates (the Triton series) have also been reported to be toxic to mammalian cells and aquatic species by lysing cell membranes [8]. In vitro and in vivo tests on different skin models have suggested that cationic surfactants are more toxic than anionic ones whilst nonionic surfactants were not-toxic for the skin [9-12].

In addition to membrane disruption, the irritancy of ionic surfactants could be enhanced by their ability to bind to keratin and lead to membrane swelling because ionic surfactant molecules can initiate their binding to proteins through electrostatic attraction and the process is then promoted by hydrophobic interaction [13]. The nature of the polar head group appears as a significant factor governing the irritancy. Whilst both anionic and cationic surfactants can bind to protein molecules due to the presence of cationic and anionic amino acids in their structure the exact strength of binding and structural disruption is also dependent on the proteins concerned and their physical properties such as the isoelectric points, the net numbers of positive and negative amino acids and their structural stability (tertiary structure). On the other hand, ionic surfactants with different sizes and CMCs may impose different extent of interaction, resulting in different skin irritancy and cytotoxicity [4].

In spite of extensive studies of biosafety of surfactants used in personal care and healthcare, there is still a lack of understanding of how surfactant structures affect their cytotoxicities. Furthermore, as surfactants can attack bacterial membranes and kill them as well, it would be highly desirable to understand how to optimize their actions against bacteria whilst minimizing their side effects on host cells [14,15]. The sulfonated methyl esters (SME) have recently been reported to show attractive surface adsorption behavior [16,17,18]. Methyl esters are shown to be easier to degrade than other conventional surfactants [19]. They could

thus be considered as alternatives to replace some conventional surfactants but a key criterion that must also be considered is their cytotoxicity. In this study we investigated the mildness or biocompatibility of SME-C_n (where n stands for the number of carbon atoms in the fatty acid chain, n = 12, 14, 16) against other conventional surfactants. Through measurements of their lysis of model vesicles, capacity in solubilizing zein and their ability to kill representative bacteria and mammalian cell models, the working mechanism underlying membrane-lytic actions and potential benefits from these surfactants are discussed.

2. Materials and methods

2.1 Materials

Chemicals and surfactants The acyl sulfonated methyl esters (SME) were prepared by sulphonation of methyl esters with different fatty acid chains (dodecanoic, myristic and palmitic acids) and denoted as SME-C12, SME-C14 and SME-C16, respectively. They were provided by KLK Oleo, with their molecular structures shown in Scheme 1. At ambient conditions, SME-C12 appeared in the form of thick pastes, SME-C14 in the form of dry powders and SME-C16 in the form of flakes. They were used as received without any further purification. These SME samples were of the same batches as used by Danov et al [16,17] who showed the purity above 98% and 96.0% for SME-C14 and SME-C16, respectively by liquid chromatography–mass spectrometry (LC/MS) analysis. They suggested that the samples might contain a small amount of unsulfonated methyl esters and other compounds as impurities. However, the LC-MS characterisations revealed a small amount of homologues with neighbouring chain lengths in each sample but with only traces of unsulfonated methyl esters present. These observations were further confirmed by their combined measurements of surface tension and electric conductivity, as will be explained later.

Other surfactants including sodium dodecylsulphate (SDS), dodecyltrimethylammonium bromide (C₁₂TAB), hexaethyleneglycol monododecyl ether (C₁₂E₆) and Triton X-100 (octylphenol ethoxylates, used as reference in membrane lysis) were all analytical reagents from Sigma-Aldrich. Sodium lauryl ethoxylate sulphate (SLES), linear benzyl-alkyl sulphonate (LAS) and zwitterionic surfactant cocamidopropyl betaine (CAPB) were also provided by KLK Oleo. The molecular structures of these surfactants are also shown in Scheme 1. SLES, LAS and CAPB were commercial samples. SLES contained mixed mono- and di-ethoxylate units and LAS contained different alkyl chain branching. SDS was

recrystallized 3 times from ethanol-water mixture by heating and cooling and C₁₂TAB was recrystallized also 3 times from acetone-absolute ethanol mixture by heating and cooling, following the established procedures as described previously [20,21]. The other surfactants were used without any further purification. All solutions were prepared in phosphate buffered saline solution at pH 7.4 (10 mM PBS) containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄ and 1.9 mM KH₂PO₄ to mimic physiological conditions.

Lipids Both 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (sodium salt, DOPG) were purchased from Avanti Lipids (Alabaster, AL) and used without further purification. Their molecular structures are depicted in Scheme S1(i).

Other chemicals Phosphate buffered saline tablet, NaCl, Sephadex® G-50 gels (Sigma G5080) and QuantiPro™ BCA Assay Kit were also analytical reagents from Sigma-Aldrich. 5(6)-Carboxyfluorescein (CF, Sigma-21877) was of analytical grade from Sigma-Aldrich and used as supplied. Its molecular structure is shown in Scheme S1(ii).

Scheme 1. Chemical structures of all surfactants selected to study in this work.

Bacteria and mammalian cells The representative bacteria used in this work were the Gram-negative (G-) strain *Escherichia coli* (*E. coli* 25922) and the Gram-positive (G+) strain *Staphylococcus aureus* (*S. aureus* 6538). *E. coli* was grown in the LB medium (tryptone at 10 g L⁻¹, yeast extract at 5 g L⁻¹, NaCl at 10 g L⁻¹, pH 7.0) and *S. aureus* was incubated in beef extract peptone medium (glucose at 60 g L⁻¹, beef extract at 10 g L⁻¹, peptone at 10 g L⁻¹, yeast extract at 10 g L⁻¹, NaCl at 5 g L⁻¹, pH 7.0). The reagents used for bacterial culture were

bought from Sigma and ThermoFisher Scientific. NIH 3T3 fibroblasts (NIH/3T3 ATCC[®] CRL-1658[™]) were cultured at 37 °C in a 5% CO₂ atmosphere in the DMEM medium (ATCC[®] 30-2002[™]) containing 10% heat-inactivated fetal bovine serum (FBS). HCa cells (human chondrocytes from articular, #4650) and the corresponding medium (#4651) were purchased from ScienCell Research Laboratories, and incubated at 37 °C under a 5% CO₂ atmosphere.

2.2 Surface tension measurements

Surface tension measurements were performed at 25 °C by using the Tensiometer K11 from Krüss GmbH. All SME samples were prepared in Phosphate Buffered Saline Solution (nominal 10 mM PBS buffer, containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.9 mM KH₂PO₄, to mimic physiological environment), pH7.4. For the concentration effect, the SME solution was diluted to the desired concentration by PBS buffer.

2.3 Vesicle preparation

Preparation of fluorophore loaded liposomes or vesicles largely followed the approaches used by Harvey et al [22] and Chen et al [23] with minor modification.

CF solution preparation After dissolving 5(6)-carboxyfluorescein (CF) in PBS buffer (PBS tablets), 2M NaOH was titrated in the solution until all the sample powder was dissolved (clear, red orange solution). Then HCl (0.1M) was titrated and the sample solution was adjusted back to pH 7.4. More PBS buffer was added to obtain the final CF concentration at 40 mM.

Vesicle preparation and CF loading The lipids (20% DOPG/DOPC (w/w)) were dissolved in chloroform at the required amount. The chloroform solution was then dried with nitrogen, following by vacuum freeze-drying for hours and this process helped remove any traces of solvent. The dry lipid multiple layers were hydrated and resuspended in the PBS buffer containing CF, and the suspension was sonicated for 5 min before undertaking liposome extrusion. The liposome extrusion was operated by the extrusion set from Avanti with which the lipid suspension passed through a 100nm pore size filter film for 31 times.

Removing external CF by columning Sephadex G50 Gel powder was dispersed in PBS buffer at about 1 g per 15 ml. The gel was preferably prepared 24 hours before loading it into the column to allow time for G50 swelling. For 5-10 ml liposome dispersion, the final packed

column height should be about 10-15 cm if the column diameter was about 2 cm. Upon loading the liposome dispersion into the column, the solvent front was soon characterized by the bright yellow colour. The liposomes containing CF had a much larger size than the individual CF molecules in the external buffer and would move to the bottom of the column quickly (see Figure S1, left). Unloaded CF molecules moved much slower and would be left behind. This fraction appeared in bright orange colour close to the top of the column and soon became well separated from the CF loaded fraction from the G50 gel size exclusion chromatography. It is clear from Figure S1 that the CF loaded liposomes look light yellow due to self-quenching at high CF concentration [23]. This fraction was collected after it had come off the bottom of the column. The structure of the liposome incorporating CF is schematically depicted in Scheme S1(ii), with the location of CF loaded being indicated.

2.4 Fluorescence measurements and detection of CF in liposome leakage tests

Fluorescence spectroscopic experiments As also evident from Figure S1, addition of surfactant such as Triton X 100 above its CMC lyses the membrane of the loaded liposomes and CF is then released. The liposome dispersion turns from yellow to fluorescently green, thus enabling direct visual observation of the colour changes upon CF release via membrane lysis. In contrast, CF release can be quantified from fluorescence measurements. This was performed on a Fluorolog-3 Spectrofluorometer (HORIBA). The instrument was operated by the software called FluorEssence at 25 °C from a 10mm pathlength Hellma (UK) QG quartz cell, oriented perpendicular to the excitation beam. Fluorescence emission spectra were collected over the range 500–550 nm from an excitation wavelength of 490 nm, a scan rate of 240 nm/min, a data interval of 0.6 nm and a response time of 2.0 s. Excitation and emission slit widths of 5 nm were used for all measurements. The emission peaks were around 515 ± 1 nm from which the values were recorded as measured data. The mean fluorescence of intact vesicles (F_0) was determined by the measurement of emission from a continually stirred 2 ml volume of vesicle suspension in buffer over a 5 min period.

The solutions of vesicles containing CF were then mixed with surfactant samples of different concentrations and equilibrated for about 1 hour. The mixed dispersions were then diluted as necessary and fluorescence readings were taken. Triton X-100 of a final bulk concentration 0.3% (w/w %) was used as surfactant to find the fluorescence reading for each sample when the liposomes were totally disrupted. Figure S2 shows an exemplar set of fluorescence readings plotted against the concentration of Triton X-100 from DOPC and 20%

DOPG/DOPC vesicles, with the two curves looking almost identical within experimental error. It shows that when working at the Triton X-100 concentration over 0.05% (w/w %) the maximum attainable leakage of encapsulated fluorescein for both charged and non-charged liposomes can be achieved. Vesicle leakage is expressed as the fraction of CF released (CF) and is calculated as:

$$CF = \frac{I_t - I_{blank}}{I_{max} - I_{blank}} \quad (1)$$

where I_t and I_{blank} are the intensity readings measured from liposome sample with and without surfactant, respectively, and I_{max} is intensity reading at the maximum attainable leakage after adding Triton X-100.

The spectrofluorometer detector could become saturated at a high fluorescence level upon the CF leakage out of the vesicles. Thus, the amount of vesicles required for each sample must be determined so that the signal would be large enough to measure with reasonable accuracy after CF release but did not saturate the detector. Figure S3 shows how the fluorescent reading changes with the CF loaded lipid concentration, with the X axis denoting the initial volume of the CF stock added (in μ l), and the Y axis denoting the real measured reading of the signal intensity. In Figure S3, I_0 indicates the signal from the liposomes before any forced leakage, and I_{total} denotes the signal after the liposomes were totally leaked. In this case, 15 μ l of the CF stock was the best choice which was in the linear area (not saturated) but well separated from the I_0 signal as evident from Figure S3.

2.5 Zein solubilization tests and BCA assay

In our zein tests, the irritants were comprised of surfactants diluted in PBS to a fixed concentration at 0.1 wt% (above their CMCs, see Table 1). A known amount of zein protein was added into each surfactant solution to make the zein solution at 1 mg/ml if fully soluble. The system was equilibrated for 24 hr under stirring to achieve the maximum dissolution at 25 °C. The dissolved solution or supernatant was then collected by centrifuging at 3,000 rpm for 60 min. The amount of zein solubilized in the supernatant was determined by the BCA (bicinchoninic acid) Assay [24]. The relative amount of zein solubilisation was used to indicate the irritation potential of the surfactant. Its principle relies on the formation of a Cu^{2+} protein complex under alkaline conditions, followed by reduction of Cu^{2+} to Cu^{1+} in solution. The amount of reduction is proportional to the amount of protein present, as cysteine,

tryptophan and tyrosine could all help reduce Cu^{2+} to Cu^{1+} . BCA formed a purple-blue complex with Cu^{1+} in alkaline environment, thus providing a basis to monitor the reduction of alkaline Cu^{2+} by proteins. The BCA assay was very sensitive and not susceptible to the presence of surfactants.

2.6 Cytotoxicity tests

The in vitro toxicities of surfactants toward 3T3 fibroblasts and HCa cells were measured by the MTT assays [25,26]. Briefly, the cells were pre-seeded in a 96-well plate at a concentration of 1×10^5 cells per mL. After 24 h of incubation, 100 μL of surfactant solutions (2-fold diluted) with different concentrations were added into the wells. Following incubation with the surfactants for 24 h, 20 μL of MTT (5 mg mL^{-1}) was added to each well and incubated for 4 h. The precipitated formazan was dissolved in 200 μL of DMSO (dimethyl sulfoxide). The absorbance at 570 nm was measured using Molecular Devices M2e. Wells without cells were used as blanks and wells without peptides were taken as negative controls.

2.7 Antibacterial tests

The antimicrobial activities of each surfactant against G+ and G- bacteria were tested using the standard microdilution method [14,15, 25-27]. *E. coli* and *S. aureus* were incubated at 37 °C to a logarithmic growth phase ($\text{OD}_{600} \sim 0.4$). Aliquots (100 μL) of bacterial suspension at 1×10^6 (CFU per mL) in culture medium were added into 100 μL of surfactant solution (2-fold serial dilutions in 10 mM PBS buffer). After incubation for 18–24 h at 37 °C, the absorbance at 600 nm was recorded using Molecular Devices M2e. The minimum inhibition concentration (MIC) was defined as the lowest concentration of peptide at which there was no visible bacterial growth. Each MIC determination was performed at least three times.

3 Results and Discussion

3.1 Critical micellar concentrations from surface tension measurements

The critical micellar concentrations (CMC) of the three sulfonated methyl ester surfactants were first determined by surface tension measurements, with the resultant surface tension plots shown in Figure 1. To mimic the physiological conditions, the pH was controlled at 7.4 using the PBS buffer containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 , 1.9 mM KH_2PO_4 (nominally 10 mM PBS), with the total ionic strength being about 160 mM. For each surfactant, the CMC value could be obtained by extrapolating two straight lines below

and above the break point from the measured surface tension plot, as demonstrated in Figure S4. The CMC values from the SME surfactants together with those from several other model surfactants are listed in Table 1. Surface tension measurements were undertaken at 25 °C and surfactant solutions were clearly soluble over the concentration range studied. However, the solution of SME-16 became slightly turbid as its concentration was above 0.1 mM. As the concentration went up the turbidity increased, but over the entire concentration studied, no precipitate dropped out, showing that the surfactant was dispersible. Increase in temperature improved the appearance and so its surface tension profile was obtained at 30 °C.

From electrical conductivity and surface tension measurements, Danov et al [16] have suggested that the SME surfactants might contain some unsulfonated methyl esters working effectively as nonionic admixtures. However, the main features of these surfactants are characterized by their surface tension responses to the addition of electrolytes, displaying the dominant features of ionic surfactants. They demonstrated that the dependence of the CMC on the alkyl chain length (n) can be approximated to

$$\ln[CMC] = 18.21 - 1.125n - 0.781\ln[C_{Na}] \quad (2)$$

The effect of the total concentration of counterions has been considered in the form of Na^+ , with the unit of CMC and C_{Na^+} being in mM. As shown in Table 1, the CMCs under the solution conditions as measured were 2.0 mM for SME-C12, 0.25 mM for SME-C14 and 4.5×10^{-2} mM for SME-C16. In contrast, application of Equation 2 leads to the calculated CMCs of 2.1 mM, 0.22 and 2.4×10^{-2} mM for the 3 surfactants, respectively, assuming that the total ionic strength is equal to the total equivalent Na^+ concentration. The consistency adds some confidence in the CMC values measured by surface tension given the suspected presence of some unsulfonated methyl esters. The slightly larger discrepancy from SME-C16 might be associated with its poor solubility as indicated earlier and the slightly higher value from our surface tension data was also consistent with the temperature of 30 °C used in this work.

Apart from the 3 SME surfactants, Table 1 also lists the CMCs from 3 anionic ones including SDS, LAS and SLES, cationic $C_{12}TAB$, zwitterionic CAPB and nonionic $C_{12}E_6$. The main observation from Table 1 is the CMC suppression by the presence of salts to both anionic and cationic surfactants, making their CMCs almost 1/10 of the corresponding values in pure water [28]. In contrast, salt addition caused little influence on the CMCs for both nonionic

C₁₂E₆ and zwitterionic CAPB. For both anionic and cationic surfactants with C12 chain, their CMCs are between 2-3 mM. LAS and Triton X 100 have equivalent alkyl chains greater than C12, and following half-logarithmic relation as shown in Equation 2, their CMCs go down further as expected. SLES contains 1-2 ethoxylates and in the presence of salt, it displays the CMC reminiscent of the dominant feature of nonionics.

Figure 1. Surface tension profiles measured from (a) SME-C12, showing the CMC around 2.0 mM, (b) SME-C14, showing the CMC around 0.25 mM, (c) SME-C16, showing the CMC around 4.5×10^{-2} mM. The continuous lines were drawn to guide the eye. The CMCs were extrapolated as indicated from Figure S4 by drawing the two straight lines just below and above the surface tension break point.

Surfactant	CMC/mM	MW/gmol ⁻¹	mM equivalent to 0.1wt%	Charge
SME-C12	2.0	316	3.1	-1
SME-C14	0.25	344	2.9	-1
SME-C16	0.045	372	2.7	-1
SLES	0.1	420	2.4	-1
LAS	0.1	348	2.8	-1
C ₁₂ E ₆	0.07	451	2.2	0
Triton X-100	0.1	650	1.5	0
CAPB	0.1	356	2.8	0

SDS	2.5	288	3.5	-1
C ₁₂ TAB	3	308	3.2	+1

Table 1. CMC values determined from surface tension measurements for the surfactant samples used in this work under PBS buffer containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.9 mM KH₂PO₄ under 25 °C (30 °C for SME-C16 to improve solubility). As protein (zein) binding was undertaken at 0.1 wt% of surfactant, the equivalent concentrations in mM are also given to indicate that they were all above respective CMCs.

3.2 Liposome leakage tests

The addition of a small amount of surfactant to a liposome solution leads to surfactant incorporation into the bilayer [29]. As surfactant concentration increases, surfactant molecules may become attached or inserted into the lipid membrane, compromising its integrity. With further increase in surfactant concentration, patches of mixed bilayer or mixed micelles may form. These surfactant-lipid aggregates may become soluble or dispersible in the aqueous phase. Thus, accompanying the structural damage, a common feature is the progressive increase in membrane permeability. Ultimately, liposomes can be completely destroyed at sufficiently high surfactant concentrations and all the lipid molecules are dispersed by surfactants [30].

Compounds such as 5(6)-carboxyfluorescein (CF) entrapped in the liposome core may leak out once the structure of the lipid bilayer is damaged. This can be used as an indication of enhanced membrane permeability [22]. The fluorescence of CF is negligible when trapped inside the liposome at a high concentration because of self-quenching [23]. When released from the liposome as a result of enhanced membrane permeability the CF concentration drops, the self-quenching disappears and the solution becomes fluorescent. The higher the amount of CF released, the more intense the fluorescence becomes.

As non-ionic Triton X-100 (also known as nonyl phenol ethoxylates with 9-10 EO groups) has been widely used to break cells by lysing their membranes, it was chosen as the reference surfactant to break CF encapsulated lipid vesicles. As described in the Experimental Section, experimental conditions were optimized with regard to the release of the CF dye and the linear detector response was adjusted to range between 0 and 100%. Thus, the fluorescence measurement system was carefully calibrated before any other surfactant was studied.

Following the checking of the fluorescence measurement from Triton X-100, we first measured the concentration-dependent vesicular lysis and release of CF from non-ionic C₁₂E₆. As evident from Figure 2, the CF release profile as shown in terms of leakage percentage is low over the low surfactant concentration range from 10⁻³ mM to 5×10⁻² mM, but as soon as the CMC is approached (0.07 mM), the percentage leakage rises sharply and reaches 100% below 0.1 mM. This feature is very much similar to the profile obtained from Triton X-100, given that both are non-ionic surfactants and have very similar CMCs as well.

The CF release profiles from the 3 SME surfactants were then measured as a function of surfactant concentration and the results are also shown in Figure 2. The initial concentration-dependent process from SME-C12 appears similar to that of the C₁₂E₆ profile in that the percentage leakage starts to rise as the CMC is approached (2.0 mM). Note that the CMC for this anionic surfactant is about 30 times higher than that of C₁₂E₆ and so the leakage onset occurs further right on the X-axis. However, the subsequent rising process is rather slow and the 100% leakage was not reached until above 30 mM. In spite of the higher CMC and slower lysing process, the basic CF releasing pattern is similar to that of the nonionic surfactant.

Figure 2. Percentage leakage of CF from vesicular encapsulation (prepared from 20% DOPG/DOPC (w/w)) as a function of the concentration of SME-C12 (●), SME-C14 (□) and SME-C16 (▲). The measurements from nonionic C₁₂E₆ (◆) and zwitterionic CAPB (■) are also shown for comparison. The lines are drawn to guide the eye.

In contrast, the CF releasing profile from SME-C14 signifies a shift from what has been observed from that of SME-C12. Around the CMC of 0.25 mM, there is a small but measurable increase in CF release, but the subsequent increase with surfactant concentration

from CMC to 30 mM of SME-C14 is relatively small, from 4% to 33% only. The subsequent increase of SME-C14 concentration to 150 mM can only achieve about 80% CF release, showing a clear trend of reduced membrane-lytic action from this surfactant.

SME-16 follows the same trend as observed from the other two SME surfactants, with the onset of the CF release starting to rise also around its CMC (0.045 mM), but the percentage release soon tends to plateau above 0.4 mM, at the saturation of CF release of some 20%. Unlike SME-C14, there is no occurrence of the second rise over the concentration range studied, but this behaviour could be well predicted from the trend of the SME-C12 and SME-C14. Thus, whilst the tendency of the CF release immediately above the CMC is the strongest from the SME-C16 due to its low CMC, its overall membrane lytic action is the weakest because of its inability to cause any further CF release over the high surfactant concentration range. As will be discussed later, SME-C16 has the weakest membrane lytic power amongst all the surfactants studied here.

Also shown in Figure 2 is the CF release profile from zwitterionic CAPB, where it can be seen that its profile closely follows that of SME-C14 in spite of the slightly lower leakage over the concentration range of 1-25 mM, showing that the two surfactants have almost comparable membrane lytic capacity.

Figure 3. The percentage leakage of CF from lipid vesicles (prepared from 20% DOPG/DOPC (w/w)) upon exposure to different types of surfactant measured against surfactant concentration, with the upturns of the leakage broadly match the CMCs of the surfactants studied: SME-C12 (●), SME-C14 (□), LAS (▲), SLES (◇) and cationic C₁₂TAB (Δ).

The percentage leakage profiles from other surfactants studied, including anionic SLES, LAS and SDS and cationic C₁₂TAB, are compared to those from SME-C12 and SME-C14 in Figure 3. Whilst the onsets of the main leakage rises broadly match the CMCs well for all of them, there are different features among them. Given different CMCs and different onsets, all 4 of the ionic surfactants as shown in Figure 3 can already cause some small but measurable leakages below their respective CMCs, indicating monomer activities. In contrast, the 3 SME surfactants displayed little measurable activities below their CMCs, showing little measurable effect from the monomers. Above CMCs, SLES and C₁₂TAB cause faster leakage rises whilst LAS and SDS show rather slower increasing leakage processes, showing different efficiencies of these surfactants in eliciting structural defects on lipid membranes. Thus, all 4 surfactants can eventually lead to the full CF leakage even though the levels to achieve the 100% leakages are different. On the other hand, given very different CMCs and charge features between SLES and C₁₂TAB, both of them produce similar increasing leakage rates, showing little influence from charge interaction. It should however also be noted that in spite of the large difference in CMC between LAS and C₁₂TAB, they tend to the full CF release almost at the same concentration, showing the much stronger membrane disruptive power from C₁₂TAB once above its CMC. In contrast, SDS achieves the full CF release at much higher concentration in spite of its CMC similar to that of C₁₂TAB, again confirming the stronger C₁₂TAB power in disrupting lipid membranes once above CMC.

Thus, the main observation is the clear rises of CF release and the respective onset points matching the CMCs of the surfactants as shown in Table 1. In addition to the main observation, there are several other features. First, whilst monomers of the conventional ionic surfactants studied here show small but measurable membrane lytic activities the 3 SME surfactants do not. Second, as the acyl chain length increases SME surfactants become less effective at causing CF release, evident from the reduced maximal leakage with *n*. Third, SLES and C₁₂TAB display sharp leakage rises whilst LAS and SDS are less effective. These differences indicate different surfactant capabilities in causing structural disruptions to lipid membranes and CF leakages. Finally, although C₁₂TAB bears opposite charge to the membrane bilayer, there is no clear indication of charge related effect in damaging the lipid membrane because the sharp leakage rise observed from C₁₂TAB can also be observed from non-ionic C₁₂E₆ and anionic SLES.

3.3 Zein solubilisation

Zein is a yellow protein extracted from corn. It is water insoluble and hydrophobic. Its insolubility and high hydrophobicity are similar to the features of keratin present in the skin and hair. Because of the similarity zein has been traditionally used as a skin protein model due to its abundance. The aim of this part of study was to investigate the irritation potential or harshness of a surfactant as an irritant to skin protein by measuring the amount of zein that could be dissolved upon exposure to a surfactant solution. The extent of zein dissolution also offers a useful estimate of the protein denaturation potential of the surfactant studied.

Following the zein tests, it was found that SDS provided the highest level of zein solubilisation. The results as shown in Figure 4 are presented in terms of relative solubility using SDS as a control. SLES and LAS are the next most powerful surfactants that achieve over 90 of SDS solubilisation, followed by SME-C14 and SME-C12. SME-C16 showed little solubility mainly because of its poor solubility itself in water (data not shown here). C₁₂E₆ and CAPB showed the lowest zein solubility (results not shown here) and the second lowest was C₁₂TAB.

Figure 4. The fraction of zein protein solubilisation (using SDS as control) studied for a range of representative surfactants showing reduced protein solubilisation.

Thus, the zein tests revealed that anionic surfactants like SDS, LAS and SLES are good protein solubilizers, whence manifesting high irritancy. Because SME surfactants are similar sulfonate surfactants, they share the characteristic feature of protein solubility, but overall they have shown less ability in protein solubilization. SME-C12 displayed a low solubilizing capacity, but with acyl chain length increasing, SME-C14 displayed a significantly higher

capacity though the solubility is still below that of LAS and SLES. As the acyl chain length further increased SME-C16 displayed little zein solubilisation capacity due to its own much reduced solubility. The reduced protein solubilisation from the SME surfactants must arise from its molecular structure that is different from any other ones used in the comparative studies. As shown in Schemes 1 and S1, although LAS, SLES and SME surfactants all carry SO_3^- groups and SDS carries SO_4^- they differ in other parts of the head group regions. Methyl esters in SME surfactants may help shield how the head groups interact with their counterions and subsequent interactions with other molecules such as proteins. Ivanova et al [17] have recently shown that ions such as Na^+ and Ca^{2+} bind differently to the head groups of LAS and SME surfactants, resulting in different surface tension and CMCs and different extent of mixing between LAS and SME. Thus, the difference arising from different chemical nature of the surfactant head groups must clearly impact their solubilizing power against proteins such as zein.

3.4 Cytotoxicity and antibacterial effect

Two mammalian cells, 3T3 fibroblast and HCa cells (human chondrocytes from articular tissue) were used to assess the cytotoxicity of the surfactants. The MTT assays clearly reveal cytotoxicity to the two mammalian cell types from the surfactants and the results are given in Figure 5, showing that under the experimental conditions used in the study, all the surfactants were cytotoxic to the growth of the two types of mammalian cells. However, the merit was to identify the relative difference between them. It can be seen from Figure 5 that SDS and C_{12}TAB are the most toxic ones and the level of toxicity from the other surfactants studied is far less; to a good approximation, the toxicity amongst the rest of surfactants is broadly similar. There is however a clear difference in the tolerance between NIH 3T3 fibroblasts and human chondrocyte cells, with greater toxicity to the latter as shown from the survival ratio in Figure 5.

The MTT assays were undertaken against surfactant concentrations in terms of $1/10\text{CMC}$, $1/5\text{CMC}$ and CMC instead of 0.1wt% as performed for zein solubilization. This means that the absolute concentrations used were hugely different, with the lowest CMCs such as C_{12}E_6 having the lowest absolute surfactant concentrations. Thus, even at $1/10\text{CMC}$, the absolute surfactant concentrations from C_{12}TAB , SDS and SME-C12 are still 3-4 times higher than that in C_{12}E_6 at its CMC. As already indicated, SDS and C_{12}TAB are clearly far more toxic than the rest of them, but due to the different CMCs between the other surfactants, the broad

similarity in toxicity actually implies different level of toxicity. For example, the relative cytotoxicity ratios to 3T3 fibroblasts at 1/10CMC and 1/5CMC are similar between C₁₂E₆ and SME-C12, but because their CMCs differ by almost a factor of 30, C₁₂E₆ is clearly far more toxic. CAPB (not shown) showed similar performance to SME-C14 and was thus amongst the least toxic surfactants studied. At CMC all surfactants caused complete death of human chondrocyte cells whilst there are some survivals from 3T3 fibroblasts. It can also be seen from Figure 5 that SME-C12 is relatively more toxic than SME-C14 on the basis of their respective CMCs. These results together show that whilst the CMC is important cytotoxicities are affected by many other factors including cell type and surfactant head group type.

Figure 5. The fraction of survival ratios obtained from MTT assays for (a) NIH 3T3 fibroblasts and (b) human chondrocyte cells measured for SME surfactants against a list of selected surfactant controls at 1/10CMC, 1/5CMC and CMC. The data were averaged from triplicate runs ($n = 3$) with standard deviations shown.

Surfactants are widely used in bactericidal formulations, but there are controversial views about the roles played by surfactants in their bactericidal actions [19]. SME surfactants together with a number of conventional surfactants have been used to examine how effective the surfactants were at killing bacteria. Again, these studies were made with respect to surfactant concentrations at 1/10CMC, 1/5CMC and CMC. As evident from Figure 6, C₁₂TAB and SDS are most effective at killing both bacterial types and non-ionic C₁₂E₆ and zwitterionic CAPB are the least effective ones. The sulphonate SME surfactants are intermediate, showing weak effects at 1/10CMC and 1/5CMC but more visible effects at CMC. These effects are however weak against the active antimicrobial agents such as C₁₂TAB. In skincare and healthcare formulations, surfactants are often used in concentrations in excess of their CMCs, the results clearly indicate the antimicrobial role of these surfactants

in addition to their other physical and biological functions, but most surfactants do not show strong bactericidal effects even above their CMCs, except C₁₂TAB and SDS. As indicated earlier, surfactants act mostly by disrupting membranes. The results from Figure 6 indicate that these surfactants interrupt membranes differently; different efficacies of bacterial killing reflect the influences from surfactant head types and intricacies of real bacterial membranes.

Figure 6. Bacterial survival ratios obtained from the microdilution assays for (a) *E. coli* and (b) *S. aureus* measured for SME surfactants against a list of selected surfactant controls at 1/10CMC, 1/5CMC and CMC. The data were averaged from triplicate runs ($n = 3$) with standard deviations shown.

The molecular structure of surfactants is characterized by a hydrophobic tail region and a hydrophilic head region. Their hydrophilic-lipophilic balance (HLB) depends on the exact size, structure and chemical nature of the two regions. The hydrophobic region is usually comprised of a hydrophobic tail and the hydrophilic region contains a head group that can be neutral, zwitterionic, positively or negatively charged. Being amphiphilic surfactants can adsorb at surfaces and interfaces. Surfactant adsorption on the surface of water tends to lower surface tension; as more surfactant is adsorbed more surface tension reduction is achieved. The CMC matches the point above which further addition of surfactant contributes to the formation of micelles in the bulk phase. The micellar forming capability equips a surfactant with the power in membrane penetration and structural disruption. Surfactants bind to membrane surface usually through electrostatic attraction. Once adsorbed, they can enter the membrane by hydrophobic interaction. This molecular process can lead to the formation of patches inside the membranes comprised of mixtures of surfactants and lipids [29,30]. These patches have distinctly different structures from the native membrane and depending on the nature and amount of surfactants mixed, the surfactant-lipid patches can become rather

soluble in aqueous phase and their detachment from the rest of membranes leads to the creation of membrane holes and exchanges of inner contents with outside, causing cell death.

Table 1 shows the CMC values for SME surfactants together with a number of other ones used as controls to reflect how different types of surfactant heads affect their CMC changes. Against reported CMC values, we see a strong effect of ionic strength to the CMC values of ionic surfactants through controlling electrostatic repulsion. Furthermore, increase in acyl chain length also has a huge influence on the reduction of the CMC of the SME surfactants. These two effects could be well incorporated using equation 2 as developed by Danov et al [15]. Thus, the CMCs were varied by a factor of 40 under the solution conditions studied, but the onsets of the membrane-lytic capacities observed from all surfactants were well linked to their respective CMCs.

Except from LAS, all other conventional surfactants studied have a dodecyl chain. Thus, changes in CMC and membrane binding capacity reflect the effects of surfactant head groups. Apart from CMC initiated onsets of membrane-lytic actions, there are two other specific features. First, conventional ionic surfactants such as SDS and C₁₂TAB show monomer concentration-dependent membrane lysis below their CMCs, with modest and measurable effects demonstrating the highest toxicity of the two surfactants. Second, all the conventional surfactants studied here display the full membrane lysis above their CMCs including SME-C12. However, as acyl chain length increases, their membrane lysis capability decreases. This is demonstrated by the progressive decline of SME-C14 and SME-C16 by displaying the reduced full lysis percentage, indicating improved biocompatibility with increasing acyl chain length.

Thus, with the monomer concentration of surfactant reaching the highest at its CMC its ability to bind lipid membrane also peaks. This feature is demonstrated in Figures 2 and 3 for all surfactants studied, but the exact style of peaking as indicated by membrane leakage differs greatly between different head groups. Such difference is evident for the SME group of surfactant against their acyl chain length, with the longer acyl chain displaying weaker membrane-lytic potency. This trend apparently contracts that observed by Morán et al from their arginine based biosurfactants [19] in which they found the increase of membrane-lytic action with acyl/alkyl chain length and a negative correlation between hydrophobicity and toxicity. However, we believe both observations are valid because changes in acyl/alkyl chain length and head group

together affect the amphiphilicity of the entire surfactant, with different combinations resulting in different membrane-lytic actions.

The concentration-dependent membrane lysis in terms of CMC is broadly consistent with the cytotoxicity observed from actions of surfactants to the two types of mammalian cells and bactericidal actions against the two representative bacteria. Whilst the consistency suggests the dominant action of membrane lysis it also shows the lack of selectivity in distinguishing between different cell types. However, it can be seen from the results of cell assays SDS and C₁₂TAB are most toxic as they illicit the highest percentage of cell death. Previous studies have reported that apart from membrane lysis these surfactants can easily deactivate the functions of mitochondria and nuclei and these biological and chemical interruptions are also associated with structural damage to the subcellular membranes causing apoptotic cell death [31]. In contrast, the SME surfactants and other conventional surfactants display similar toxicities that are lower than those from SDS and C₁₂TAB. The difference indicates either the lower chemical and biological toxicity or the reduced structural damage of these surfactants to subcellular compartments.

In addition to membrane permeation and lysis, another molecular process that can lead to compromise of biocompatibility is via protein binding. In the context of topical application, surfactant can bind to skin borne proteins resulting in structural alteration and even protein removal. Surfactant binding to zein has been developed as a model to test their affinity. Our work revealed that SDS causes the highest binding and solubilization, followed by other anionic sulphonated surfactants including SLES and LAS. The SME surfactants showed the weakest ability among the anionic ones but they showed higher zein solubility than cationic C₁₂TAB, zwitterionic CAPB and nonionic C₁₂E₆. Our previous work [32,33], those by Tanford et al [34,35] and others [36] showed that ionic surfactants can also strongly bind to proteins such as lysozyme and albumins, consistent with zein solubilization. The small solubility to zein from cationic C₁₂TAB could well be caused by its specific sequence and structure, suggesting that whilst binding is initiated by electrostatic interaction, it does not necessarily lead to full solubilization in the aqueous phase. The strong binding of C₁₂TAB to the predominantly negatively charged zein can lead to intensive precipitation that precludes the solubilization process early and as a result, the supernatant contains little zein molecules.

Conclusion

Following the previous reports of structural implications of surfactant molecules to toxicities [3,7-13,31], this work has made a more comprehensive assessment of the membrane-lytic actions involving a wide range of surfactant head groups and a group of sulphonated SME surfactants with different acyl chain lengths. All surfactants studied showed the onsets of their main membrane-lytic actions above their CMCs, but this feature does not fully explain different cytotoxicities observed. Unlike other surfactants, cationic C₁₂TAB and anionic SDS also displayed membrane-lytic activities below their CMCs, showing monomer effects combining structural damage to membranes and deactivation to mitochondria and nuclei. Whilst most surfactants could achieve the full membrane lysis at concentrations above their CMCs, the SME surfactants displayed a sharp decline of the full membrane lysis with rising acyl chain length. These physical features are broadly consistent with concentration-dependent cytotoxicities against two mammalian cell types and bactericidal actions against *E. coli* and *S. aureus*. With respect to their CMCs, the SME surfactants behave similarly to all conventional surfactants, showing the dominant effect of membrane lysis. The protein solubilisation work revealed that whilst SME surfactants could also dissolve zein like other anionic surfactants, they showed the weakest zein solubilisation capability. Thus, SME-C14 and SME-C16 showed reduced membrane-lytic and protein solubilizing capacities among the surfactants studied and these attractive benefits arise from the combined effects of head group type and acyl chain length. This work thus points to the further need to establish how structural features of surfactants such as acyl/alkyl length and head group type affect membrane-lytic processes and cytotoxicities.

Supporting Information

Supporting Information is available from the online webpage or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

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ToC

Support Information

Membrane-lytic Actions of Sulfonated Methyl Ester Surfactants and Implications to Bactericidal Effect and Cytotoxicity

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ZL and FP made equal contribution

Figure S1. Left: Separation of CF loaded liposome fraction (light yellow) from unloaded fraction (red orange) from the G50 gel size exclusion chromatography. Right: CF loaded liposome solution before (yellow) and after lysis (green fluorescence) by adding surfactant Triton X 100. The bright green fluorescence clearly indicates the release of CF due to the damage of membrane by surfactant.

Figure S2. Fluorescence readings plotted against the concentration of Triton X 100 from DOPC and 20% DOPG/DOPC vesicles.

Figure S3. Fluorescence readings plotted against the volume of stock CF loaded vesicles with and without Triton X 100 at the concentration of 0.2% (w/w) (above CMC).

Figure S4. Surface tension profile measured from SME-C1 to illustrate how the CMC is obtained by extrapolating the plots just below and around the break point as indicated by the two straight dashed lines, with the interception marking the CMC around 2 mM. Similar CMC values were obtained for other surfactants under the same solution conditions. The continuous lines were drawn to guide the eye.

Scheme S1. (i) The molecular structures of DOPC, DOPG and fluorescent dye CF and (ii) the schematic representations of a lipid vesicle in 3D and cross-section view to indicate the encapsulation of fluorescent CF dye in the vesicular interior.

(i)

(ii)

5(6)-carboxyfluorescein (CF)