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Dual function surfactants for pharmaceutical formulations: The case of surface active and antibacterial 1-tolyl alkyl biguanide derivatives

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ABSTRACT

One interesting field of research in the view of developing novel surfactants for pharmaceutical and cosmetic applications is the design of amphiphiles showing further bioactive properties in addition to those commonly displayed by surface-active compounds. We propose here the chemical synthesis, and characterization of 1-o-tolyl alkyl biguanide derivatives, having different lengths of the hydrocarbon chain (C3, C6, and C10), and showing surface active and antibacterial/disinfectant activities toward both Gram-positive and Gram-negative bacteria. Both surface active properties in terms of critical micelle concentration (CMC) and surface tension at CMC (γCMC), as well as the antimicrobial activity in terms of minimum inhibitory concentrations (MICs), were strongly dependent on the length of the hydrocarbon chain. Particularly, the C6 and C10 derivatives have a good ability to decrease surface tension (γCMC <40 mN/m) at low concentrations (CMC < 12 mM) and a satisfactory antibacterial effect (MIC values between 0.230 and 0.012 mM against *S. aureus* strains and between 0.910 and 0.190 against *P.aeruginosa* strains). Interestingly, these compounds showed a disinfectant activity at the tested concentrations that was comparable to that of the reference compound chlorhexidine digluconate. All these results support the possible use of these amphiphilic compounds as antibacterial agents and disinfectants in pharmaceutical or cosmetic formulations.

1. Introduction

Biguanide is an interesting molecule whose chemical moiety can be found in the structure of several compounds with different therapeutic activities such as antidiabetic, antiviral, antimalarial, antibacterial, antimycotic, and anti-HIV (Barbieri et al., 2019; Bharatam et al., 2005; Grytsai et al., 2021a). Biguanide derivatives are currently employed in clinics as orally administered agents and have also been proposed for the treatment of several other conditions (e.g. polycystic ovary syndrome, cancer, as well as bacterial, viral, fungal, and protozoan infections) (Di Magno et al., 2022; Kathuria et al., 2021). Regarding antimicrobial activity, several biguanide derivatives are known to be active against a wide range of Gram-positive and Gram-negative bacteria as well as fungi, yeasts, and viruses (Badea et al., 2021; Chen et al., 2018). Indeed, a cationic bisbiguanide as chlorhexidine (and its salt form chlorhexidine digluconate) is one of the most used topical disinfectants and antiseptic agents for skin and mucosa (Shi et al., 2019; Van den Poel et al., 2022). Chemical research about the biguanide moiety led to the production of oligomers as polyhexamethylenebiguanide (PHMB), which consists of 7–10 biguanide moieties connected by a hexamethylene linker. PHMB shows a broad range of antimicrobial activity and finds applications for different purposes (e.g. disinfectant, preservative, biocide) (Hirsch et al., 2011; Niro et al., 2023; Rippon et al., 2023). Despite the large interest in biguanides derivatives, no detailed studies have been conducted so far for the development of novel surface-active molecules bearing this chemical moiety. Indeed, only a few studies can be found in the literature, in which a biguanide moiety was linked to alkyl chains of different lengths to produce amphiphilic molecules (Ne Fortun and Schmitzer,

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2018; Song et al., 2012). Single-chain alkyl-biguanides and alkyl-biguanidium chlorides (C6-C12) were synthesized as surfactants to develop a "greener" Suzuki − Miyaura cross-coupling reaction in a micellar environment (Ne Fortun and Schmitzer, 2018). Moreover, in another work, two series of alkyl chain guanidine surfactants as mono-alkylguanidine (C8-C12) and dimethylalkylguanidine (C8-C12) were synthesized to investigate their self-aggregation and antimicrobial activity. These surfactants showed a good ability to decrease air–water surface tension and were effective as antimicrobials against Gram-negative, and Gram-positive bacteria and fungi at the concentration of 25–100 μg/mL (Song et al., 2012).

According to all these premises, biguanide represents a versatile chemical moiety to be exploited for the design of new surfactants with a dual activity as surface-active molecules and antimicrobial agents, potentially employed for the preparation of topical formulations intended for pharmaceutical and cosmetic use.

Amphiphilic compounds having biological properties (e.g. antimicrobial) in addition to the intrinsic surface activity are proposed in this work as a novel class of surfactants, which can be employed for the optimization of pharmaceutical and cosmetic formulations. As such, a new series of amphiphilic compounds derived from the alkylation of 1-(*o*-tolyl)biguanide (TB) was synthesized and characterized in terms of surface tension, aggregation properties, and antibacterial activity. TB moiety has been selected as polar head, since this biguanide derivative has never been employed so far to develop novel surfactants. Therefore, a screening in a wide range of alkyl chains (C3, C6 and C10) was performed to understand which carbon length is the more suitable to prepare amphiphilic compounds with good surface activity together with antimicrobial properties.

2. Materials and methods

2.1. Materials

o-Toluidine (≥99.5 %), sodium dicyanamide (96 %), hexylamine (99 %), copper (II) sulfate pentahydrate (98 %), decylamine (95 %), tetrahydrofuran (≥99.5 %), propylamine (98 %), EDTA-Na_2 (99.0–101.0 %), anhydrous 1-butanol (99.8 %), 2-propanol and 1-(*o*-Tolyl)biguanide (TB) were purchased Sigma-Aldrich® (Steinheim, Germany). Ammonium hydroxide solution (~25 %), sodium sulfate (Na_2SO_4), and concentrated hydrochloric acid were obtained from Honeywell/Fluka™ (Germany). Ethanol 96 %, dichloromethane (DCM), chloroform ($CHCl_3$) and methanol (CH_3OH) were obtained from Carlo Erba Reagents (Cornaredo, Italy). Chlorhexidine digluconate was purchased from A.C.E.F. (Fiorenzuola d'Arda, Italy).

2.2. Methods

2.2.1. Chemistry

2.2.1.1. General methods. Melting points were taken in glass capillary tubes on a Büchi SMP-20 apparatus and were uncorrected. ^{1}H NMR spectra were recorded with a Bruker 500 Ascend (Bruker BioSpin Corporation, Billerica, MA, USA), and chemical shifts (ppm) were reported relative to tetramethylsilane. Abbreviations used in the analyses of the NMR spectra: s = singlet, d = doublet, dd = double doublet, t = triplet, m = multiplet, br = broad. IR spectra were recorded on a PerkinElmer 297 instrument and spectral data (not shown because of the lack of unusual features) were obtained for all compounds reported and are consistent with the assigned structures. Electrospray Ionization Mass Spectra (ESI-MS) were recorded in positive- (ESI-MS(+)) or negative-ions (ESI-MS(−)) mode on a Waters Micromass ZQ Spectrometer equipped with a single quadrupole (Waters Corporation, Milford, MA, USA). All reactions were monitored by thin-layer chromatography using silica gel plates (60 F254; Merck), visualizing with ultraviolet light. Chromatographic separations were performed on silica gel columns (Kieselgel 40, 0.040 − 0.063 mm, Merck) by flash chromatography. Elemental analyses (C, H, N, S) were performed in-house with Fisons THERMO Fisher Flash 2000 instrument.

2.2.1.2. Synthesis of N-(o-tolyl)cyanoguanidine (AS1). A solution of di *o*-toluidine (1 g, 9.35 mmol) in H_2O (5 mL) was added to a solution of 6 N HCl (2 mL) and sodium dicyanamide (1 g, 11.2 mmol) and the reaction mixture was stirred for 6 h at 60 °C. After cooling, a precipitate was formed, which was filtered and washed with H_2O to get AS1 as a grey solid: 1.24 g, 76.1 % yield (m.p. = 132–134 °C). ^{1}H NMR (500 MHz, DMSO d-6): δ = 2.19 (s, 3H), 6.92 (s, 2H), 7.12–7.28 (m, 4H), 8.51 (s, 1H). ESI-MS (major positive ions, CH_3OH), *m/z* (%): 197 (27 %) $[M + Na]^+$. ESI-MS (major negative ions, CH_3OH), *m/z* (%):173 (100 %) $[M - H]^-$.

2.2.1.3. Synthesis of 1-(o-tolyl)-5-propyl-biguanide hydrochloride (TBC3). $CuSO_4{\cdot}5H_2O$ (0.57 g, 2.32 mmol) and propylamine (0.81 g, 13.7 mmol) were added to a solution of AS1 (0.6 g, 3.44 mmol) in THF (4.12 mL) and H_2O (3.44 mL) at 25–30 °C, and the reaction mixture was stirred overnight at 40 °C. After evaporation of THF, an aqueous solution of 12 N HCl (1.7 mL) in H_2O (3.4 mL) was added, and the mixture was stirred for 30 min at r.t. Then, an ammonia solution of sodium ethylenediaminetetraacetate (EDTA-Na_2) (2.75 mL H_2O, 1.3 mL 30 % NH_4OH, 1.2 g EDTA-Na_2) was added and the resulting mixture was stirred for 30 min at r.t. and extracted with $CHCl_3$ (4 x 20 mL). The organic layer was dried over anhydrous Na_2SO_4. The evaporation of the solvent under reduced pressure afforded a residue that was purified by flash chromatography, eluting with $CHCl_3/CH_3OH$ (9:1), to give a white solid, which was crystallized from 2-PrOH: 0.18 g, 33.2 % yield (m.p. = 216–218 °C). ^{1}H NMR (500 MHz, DMSO d-6): δ = 0.84 (t, 3H), 1.43 (m, 2H), 2.26 (s, 3H), 2.99 (m, 2H), 7.03–7.40 (m, 10*H*). ESI-MS (major positive ions, CH_3OH), *m/z* (%): 234 (100 %) $[M + H]^+$. Elemental Analysis (%) calculated for $C_{12}H_{19}N_5$.HCl: C 53.43, H 7.47, N 25.96; found: C 53.06, H 7.25, N 25.63.

2.2.1.4. Synthesis of 1-(o-tolyl)-5-hexyl biguanide hydrochloride (TBC6). This compound was prepared starting from AS1 and hexylamine following the procedure described for TBC3: a white solid was obtained (48 % yield, m.p. = 193–194 °C). ^{1}H NMR (500 MHz, DMSO d-6): δ = 0.86 (t, 3H), 1.26 (m, 6H), 1.35 (m, 2H), 2.25 (s, 3H), 3.01 (dd, 2H), 6.72–7.43 (m, 10*H*). ESI-MS (major positive ions, CH_3OH), *m/z* (%): 276 (100 %) $[M + H]^+$. Elemental Analysis (%) calculated for $C_{15}H_{25}N_5$.HCl: C 57.77, H 8.40, N 22.46; found: C 57.41, H 8.72, N 21.99.

2.2.1.5. Synthesis of 1-(o-tolyl)-5-decyl-biguanide hydrochloride (TBC10). This compound was prepared starting from AS1 and decylamine following the procedure described for TBC3: a white solid was obtained (47.2 % yield, m.p. = 185–187 °C). ^{1}H NMR (500 MHz, DMSO d-6): δ = 0.87 (t, 3H), 1.25 (m, 14H), 1.35 (m, 2H), 2.26 (s, 3H), 3.01 (dd, 2H), 7.04–7.40 (m, 10*H*). ESI-MS (major positive ions, CH_3OH), *m/z* (%): 332 (100 %) $[M + H]^+$. Elemental Analysis (%) calculated for $C_{19}H_{33}N_5$.HCl: C 62.02, H 9.31, N 19.03; found: C 62.21, H 9.40, N 18.71.

2.2.2. Thermal characterization at solid state

Thermogravimetric analysis (TGA, simultaneous thermal analyzer STA 6000, Perkin-Elmer Inc., Waltham, MA, USA) was performed from 30 °C to 700 °C at a rate of 10 °C/min under nitrogen flow. Differential scanning calorimetry (DSC, DSC 8500, Perkin Elmer) was conducted from 20 °C to 230 °C at 10 °C/min using aluminum pans in a nitrogen environment.

2.2.3. Surface properties and self-aggregation

The air–water surface tension of TB and synthesized alkyl biguanide

surfactant solutions at different concentrations in ultrapure water was measured using the "Du Noüy ring" method (DCA-100 force tensiometer, First Ten Angstroms) at 25 °C. Recorded surface tension values were the average of three consecutive measurements. Data are the mean ± standard deviation of three independent measurements. CMC and γCMC values were calculated from the fitting of the experimental plots surface tension *vs* concentration using the segmental linear regression model (GraphPad Prism 6 software). The Gibbs surface excess (Γ_{MAX}) was calculated from the following equation (Eq. (1):

$$\Gamma_{MAX=}\frac{1}{2.303nRT}\left(\frac{\delta\gamma}{\delta LogC}\right) \quad (1)$$

where R is the gas constant (8.314 J/mol K), T the absolute temperature, *C* the surfactant concentration (M), and n the number of the species in the solution. δγ/logC was calculated from the maximum slope of the plot surface tension (γ) *vs* Log surfactant concentration in the linear region before CMC.

The area per surfactant molecule at the air–water interface (A_{min}) ($Å^2$) was calculated from the Γ_{MAX} through Eq. (2):

$$A_{min} = \frac{10^{20}}{N\Gamma_{Max}} \quad (2)$$

where N is the Avogadro number.

The standard free energy of micellization (ΔG°_{mic}) and adsorption (ΔG°_{ads}) are given by:

$$\Delta G^{\circ} mic = RT\,LnCMC \quad (3)$$

$$\Delta G^{\circ} ads = \Delta G^{\circ} mic - 0.6023\,\Pi_{CMC}A_{min} \quad (4)$$

where Π is the surface pressure (γ of pure solvent − γ of surfactant solution at CMC).

2.2.4. Dynamic light scattering (DLS)

DLS analyses were performed at 25 °C by setting up the measurement at a constant cell position of 4.65 and an attenuator of 11 using a Malvern Zetasizer Nano S (Malvern, Worcestershire, UK). Counts (Kcps), which are a measure of the scattering light intensity to the detector, were recorded at different concentrations of the compound solutions and the CMC value was determined by the straight-line interception method as previously reported (D. R Perinelli et al., 2016; Topel et al., 2013). Particle size and distribution are expressed as hydrodynamic diameter (nm; from volume distribution %) and width (nm; width of the distribution at half height), respectively. All measurements were performed in triplicate.

2.2.5. Conductivity measurements

Specific conductivity (μS/cm) of TB and synthesized alkyl biguanide surfactant solutions, at the same concentrations in ultrapure water as for surface tensiometry, was measured at 25 °C using a MicroCM 2200 conductometer (Crison, Spain). All concentrations were measured three times. Data were the mean ± standard deviation of three independent measurements. CMC values were determined from the minimum point of the second derivative curve calculated from each conductivity *vs* surfactant concentration plot (OriginPro8 software) (Perinelli et al., 2020).

2.2.6. Cell viability assay

Immortalized human keratinocytes cell line (HaCaT) was furnished by IFOM (Institute of Molecular Oncology, Rome, Italy) and was cultured in DMEM supplemented with 10 % fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU mL^{-1} penicillin/streptomycin and maintained at 37 °C with 5 % CO_2 and 95 % humidity. For the preparation of the stock solutions, 10 mg of chlorhexidine digluconate, TB, TBC3, and TBC6, were dissolved in a mixture of DMSO 20 % v/v in water, while 10 mg of TBC10 were dissolved in 1 mL of DMSO, due to solubility issue. Then, all stock solutions were diluted in the cell medium until the tested concentration. $3x10^4$ cells/mL were seeded in 96-well plates in a final volume of 100 μL/well. After one day, compounds or vehicles were added and six replicates were used for each treatment. After 72 h, cell viability was investigated by adding 0.8 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) (Sigma Aldrich) to the media. After 3 h the supernatant was removed, and the pellet of salt crystals was solubilized with 100 μL/well of DMSO. The absorbance of the sample against a background control was measured at 570 nm using an ELISA reader microliter plate (BioTek Instruments, Winooski, VT, USA). All experiments were repeated three times.

2.2.7. Antibacterial activity

The antibacterial activity of TB and synthesized alkyl biguanide surfactants, in comparison to Chlorhexidine digluconate as reference compound, was evaluated against the Gram-positive species *Staphylococcus aureus* (two strains: ATCC 6538 and ATCC 25923) and *Bacillus subtilis* (one strain: ATCC 6633) and the Gram-negative species *Pseudomonas aeruginosa* (two strains: ATCC 15442 and ATCC 9027) and *Escherichia coli* (one strain: ATCC 25922).

Strains were cultured at 35–36 °C in Mueller-Hinton-Broth (MHB) (Oxoid). A 20 mg/mL stock solution of each sample was prepared in a mixture of DMSO and water at the ratio of 1:4. These solutions were then diluted 1:10 serially in the culture medium using a 96-well plate.

Minimum Inhibitory Concentrations (MIC) were determined by the broth microdilution method as recommended by EUCAST (European Committee on Antimicrobial Susceptibility Testing) (ISO 20776–1:2019). Additionally, Minimal Bactericidal Concentration (MBC) was determined as the concentration able to decrease the bacterial cell viable counts of the bacterial culture (expressed as colony forming units − CFU) by at least 4 Log. Tests were conducted in duplicate, using two replicates for each experiment.

2.2.8. Disinfectant activity

Tests were conducted according to the ISO 1040:2005 specification. 20 mg/mL stock solutions (DMSO to water 1:4) of each sample were serially diluted in water.

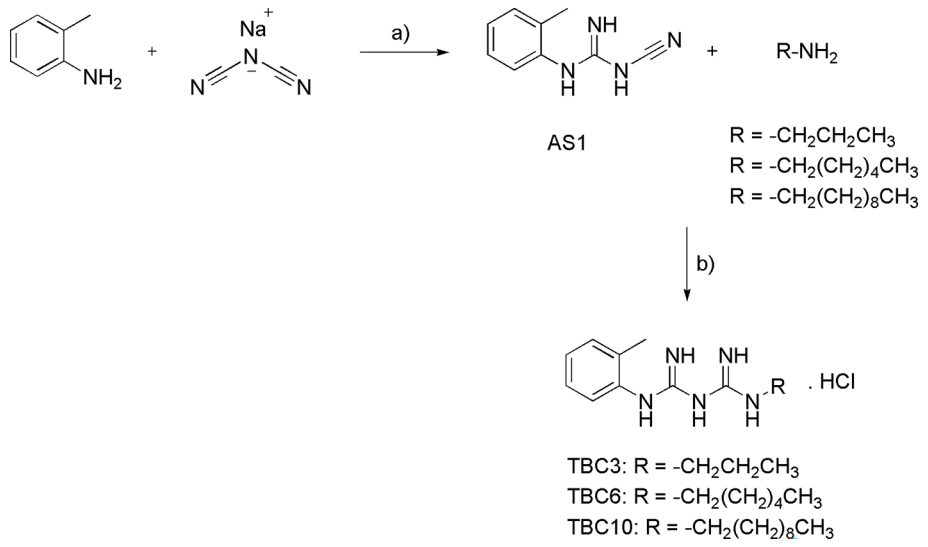
Bacterial suspensions were prepared by dispersing *S. aureus* or *P. aeruginosa* into the diluent (Tryptone 1.0 g/L, NaCl 8.5 g/L) until obtaining a cell density of 0.5 McFarland (~108 CFU/mL). 20 μL of the bacterial suspension were added to each sample solution and incubated at room temperature for 2 min. Compounds were tested at the concentrations of 0.5 % and 0.05 % w/v. Sterile water was used as a non-active control. For the neutralization step, 20 μL from the test tubes were added to 20 μL of sterile water and 160 μL of neutralizing agent (tryptone 1.0 g/L, NaCl 8.5 g/L, and polysorbate 80 5 ml/L) and incubated for 5 min at room temperature. An appropriate dilution of these mixtures was plated onto the agar medium and incubated for 20 h at 37 °C. Then, the colony forming unit (CFU) was counted for each treatment to assess the bacterial cell viability.

Tests were conducted in duplicate, using two replicates for each experiment.

3. Results

3.1. Synthesis

Biguanide derivatives 1-tolyl-5-propyl-biguanide (TBC3), 1-tolyl-5-hexyl-biguanide (TBC6), and 1-tolyl-5-decyl-biguanide (TBC10) were synthesized according to the procedure reported in Scheme 1. The reaction of *o*-toluidine with sodium dicyanamide in an acidic condition gave the intermediate *N*-(*o*-tolyl)-cyanoguanidine (AS1), which was reacted with the suitable amine (propylamine, hexylamine or decylamine) in the presence of copper sulfate pentahydrate, followed by treatment with EDTA-Na_2 solution to afford the desired final products



Scheme 1. Synthetic procedure for biguanide derivatives TBC3, TBC6, and TBC10. Reagents and conditions; (a) 6 N HCl, 60 °C, 6 h; (b) 1. $CuSO_4 \cdot 5H_2O$, THF/H_2O, 40 °C overnight, 2. 4 N HCl, r.t., 30 min, 3. 30 % NH_4OH, EDTA-Na_2, r.t., 30 min.

(TBC3, TBC6 and TBC10, respectively) as hydrochloride salts.

3.2. Thermal properties at solid state

Thermal analysis has revealed that both the degradation temperature (from TGA analysis, Fig. 1) and the melting temperature (from DSC, Fig. 2) of the synthesized alkyl biguanide derivatives were strongly dependent on the length of the alkyl chain. Specifically, the weight (%) *vs* temperature plots show two inflections, corresponding to the two minima observed in the first derivative signal (Fig. 1). This indicates that a two-step thermal decomposition reaction occurs for TB and the synthesized alkyl biguanide derivatives (TBC3, TBC6, and TBC10). The two degradation temperatures, calculated from the minima of the first derivative signal are reported in Table 1. Specifically, the degradation process occurring at a higher temperature and referred to as the second degradation event seems much more affected by the length of the alkyl chain than the other one. Indeed, the calculated second degradation temperature decreased from ~ 334 °C for TBC10 to 326 and 296 °C for the other derivatives with shorter alkyl chains (TBC3 and TBC6, respectively). The multi-step degradation of biguanide derivatives has already been reported, and thermal events associated with the decomposition (starting from 130 °C) have been assigned to amino group loss, leading to ammonia and melamine formation, which further decomposes at higher temperatures (generally above 250 °C) (Bell et al.,

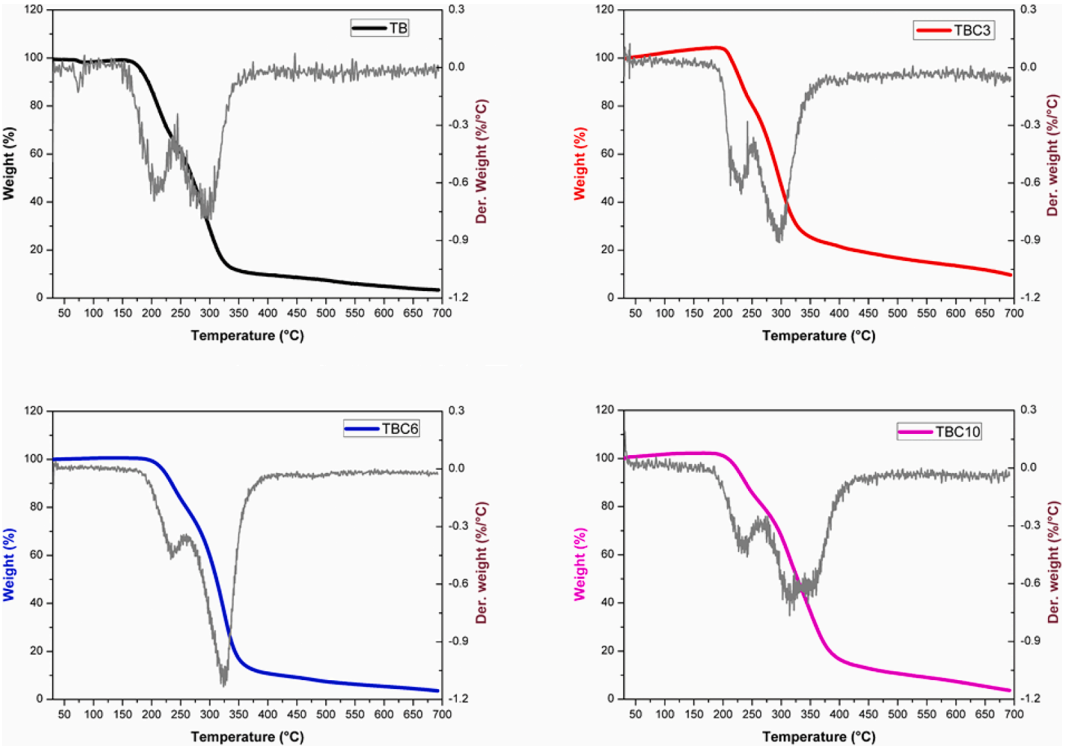


Fig. 1. TGA analysis of for 1-(*o*-tolyl)biguanide (TB) and alkyl 1-(*o*-tolyl)biguanide derivatives (TBC3, TBC6 and TBC10). The plots show the weight (%) *vs* temperature (°C) traces and their first derivative signal.

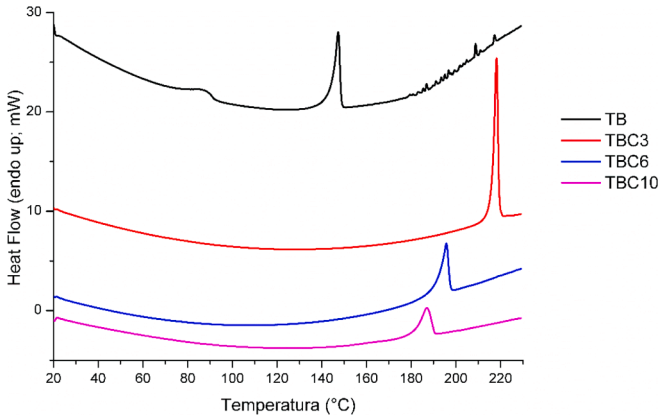


Fig. 2. DSC traces for 1-(*o*-tolyl)biguanide (TB) and the synthesized alkyl 1-(*o*-tolyl)biguanide derivatives (TBC3, TBC6 and TBC10).

Table 1
Degradation temperature (°C) values as calculated from TGA analysis and melting temperature (°C) and enthalpy (J/g) as calculated from DSC for 1-(*o*-tolyl)biguanide (TB) and the synthesized alkyl 1-(*o*-tolyl)biguanide derivatives (TBC3, TBC6 and TBC10).

	TGA Degradation temperature (°C)		DSC Melting temperature (°C)	Enthalpy (J/g)
	1° event	2° event		
TB	205.36 ± 0.66	296.81 ± 0.55	147.01 ± 0.55	86.58 ± 0.71
TBC3	231.45 ± 0.35	296.96 ± 0.48	214.23 ± 0.84	123.67 ± 0.49
TBC6	235.80 ± 0.68	326.23 ± 0.32	195.83 ± 0.09	69.04 ± 0.90
TBC10	234.78 ± 0.24	334.06 ± 0.33	187.21 ± 0.28	46.42 ± 0.39

1977; Grytsai et al., 2021b). Loss of adsorbed water below 100 °C (~2% of the initial weight) was only observed for commercial TB and not for the synthesized derivatives.

Differently from the degradation temperature, an inverse relationship was found between the length of the alkyl chain and the thermal parameters associated with the melting process (melting temperature, Tm, and melting enthalpy ΔH). Indeed, all thermograms showed a narrow endothermic event recognized as the melting of the compounds. Specifically, the calculated Tm value was around 140 °C for TB and decreased from 218 °C to 187 °C moving from TBC3 to TBC10. As regards ΔH, values decreased from 126 J/g to 46 J/g by lengthening the alkyl chain of the synthesized compounds, while an intermediated value (~86 J/g) was calculated for the reference compound TB. TB thermogram also showed an additional endothermic event at around 80 °C, related to the loss of adsorbed water, as also evidenced by the slight weight loss observed on TGA trace at the same range of temperatures. The decrease of the melting point of biguanide derivatives as a function of the length of the alkyl chain has already been reported for nitroguanidine derivatives and it was attributed to the steric effect of the alkyl chain in destroying the strong intermolecular forces (i.e. hydrogen bonds) between guanidine groups into the crystals (Chen et al., 2020).

3.3. Air-water surface behavior and CMC determination

The aggregation properties in water of the commercial biguanide TB and the synthesized 1-(*o*-tolyl) alkyl biguanide derivatives (TBC compounds) were investigated by force tensiometry, dynamic light scattering, and conductivity measurements.

As regards surface tension analysis, all tested compounds were able to decrease the air–water surface tension as a function of their concentration, although to a different extent (Fig. 3). The less surface active compound resulted to be the commercial biguanide TB, since it did not induce a decrease of surface tension below ~ 60 mN/m, thereby not configuring itself as a surfactant. The slight effect in reducing surface tension for this compound may be related to its chemical structure bearing a hydrophobic tolyl group linked to the polar biguanide moiety. The synthesized 1-(*o*-tolyl) alkyl biguanides, instead, were able to decrease surface tension at a greater extent (below ~ 60 mN/m), configuring them as surface active molecules. Indeed, the derivatization of the 1-(o-tolyl) biguanide with alkyl chains exerted a strong effect in terms of surface activity, since the decrease of the surface tension over concentrations as well as the minimum achieved surface tension were strongly dependent both on the alkylation itself and on the length of the alkyl chain. Specifically, surface tension values of of ~ 55 mN/m for TBC3, of ~ 39 mN/m for TBC6 and of ~ 28 mN/m for TBC10 were reached when the air–water interface has been saturated by surfactants, confirming the strong effect exerted by the length of the alkyl chain on their adsorption properties.

All surface tension plots showed an inflection at a certain concentration generally recognized for amphiphilic molecules as the CMC. However, to confirm the formation of surfactant micelles above these concentrations at which the air–water interface is saturated, dynamic light scattering analyses were performed.

Fig. 4A shows the counts (kCps) *vs* concentration plot for TB, TBC3, and TBC6, collected at a fixed attenuation and position. TBC10 was not possible to be analyzed since the concentrations above the calculated CMC were nor sufficiently clear. It can be observed that only for TBC6 the measured scattered light to the detector, expressed as counts (kCps), increased at a surfactant concentration slightly higher than the CMC value calculated from surface tension measurements. This increase in counts can be attributed to the formation of micelles and/or aggregates that can act, differently from surfactant unimers, as scattering points when the laser light passes through the samples. On the other side, a particle size distribution in the nanometric range compatible to that of micelles formed by surfactants (hydrodynamic diameter 10.8 ± 1.6 nm and width 3.7 ± 1.4 nm from volume distribution %) was recorded for TBC6 at 35 mM, differently from TB and TB3 at 40 mM for which the mean estimated particle size was below 1 nm (Fig. 4B). These results suggest that micelles can form only for TBC6 and homologs with longer alkyl chains, despite further studies are required to confirm this.

Therefore, according the DLS results, CMC values can be reliably calculated only for TBC6 and TBC10 (Table 2). Specifically, they decreased from 12.6 mM for TBC6 to 3.3 mM for TBC10, thereby

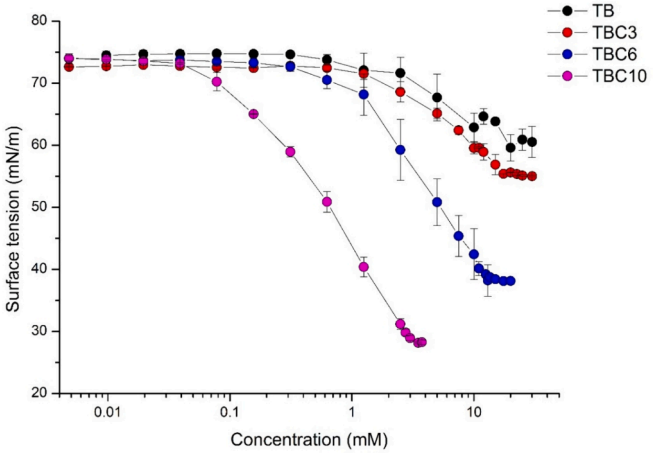


Fig. 3. Surface tension *vs* concentration plots at 25 °C for 1-(*o*-tolyl)biguanide (TB) and the synthesized 1-(*o*-tolyl)biguanide derivatives (TBC3, TBC6 and TBC10).

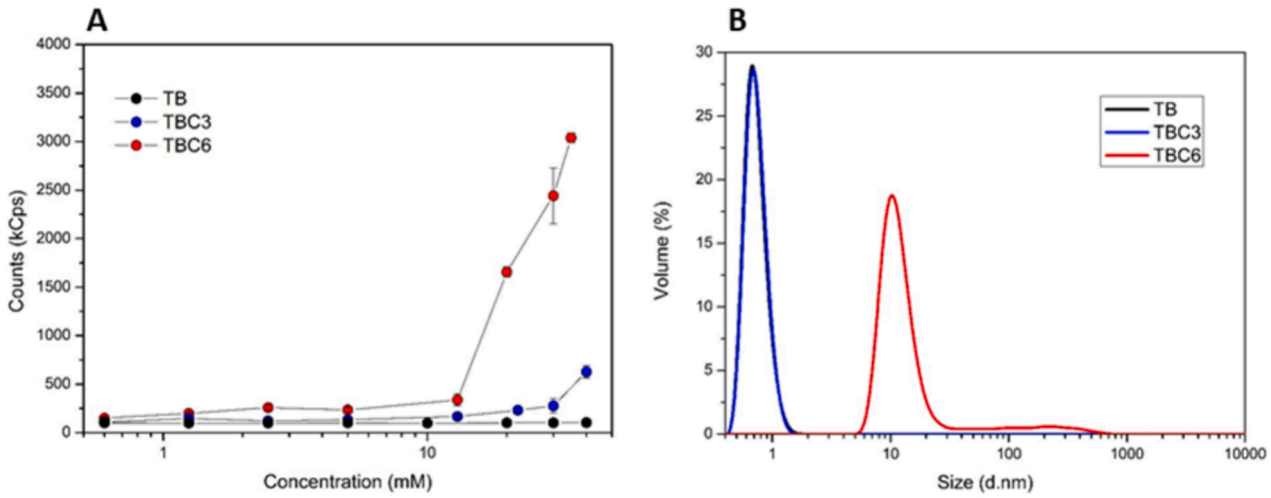


Fig. 4. Counts (kCps) *vs* surfactant concentration (mM) plot (A) and particle size distribution (volume %) obtained from DLS measurements.

Table 2
Surface parameters from tensiometry and conductivity measurement at 25 °C of the synthesized alkyl 1-(*o*-tolyl) biguanide surfactants. Calculated IC_{50} values on HaCaT cells for the synthesized alkyl 1-(*o*-tolyl) biguanide surfactants in comparison to chlorhexidine digluconate.

	Tensiometry						Conductivity	MTT assay
	CMC (mM)	γCMC (mN/m)	$10^6\ \Gamma_{max}$ (mol/m^2)	A_{min} (Å^2)	$\Delta G°_{mic}$ (kJ/mol)	$\Delta G°_{Abs}$ (kJ/mol)	CMC (mM)	IC_{50} (mM)
TB	*	*	*	*	*	*	*	> 0.52
TBC3	*	*	*	*	*	*	*	0.242 ± 0.007
TBC6	12.59 ± 1.15	39.48 ± 0.94	5.32 ± 1.23	32.7 ± 3.4	−10.67 ± 0.22	−16.94 ± 0.40	10.90 ± 1.18	0.016 ± 0.006
TBC10	3.41 ± 0.06	28.14 ± 0.03	5.60 ± 0.30	29.7 ± 1.7	−13.86 ± 0.04	−21.69 ± 0.05	3.02 ± 0.26	0.003 ± 0.001
Chlorhexidine digluconate	–	–	–	–			–	0.011 ± 0.002

* TB and TBC3 compounds do not form micelles/aggregates detectable by DLS analysis in the nanometric range. An inflection point for TBC3 in concentration *vs* surface tension plot was observed in any case at a concentration of 18.37 ± 0.97 mM and a surface tension of 55.75 ± 0.06 mN/m.

evidencing a clear effect of the elongation of the alkyl chain as also observed for other classes of surfactants (Mousavi et al., 2022; D. R Perinelli et al., 2016). Table 2 also reports the maximum surface excess, Γ_{max}, calculated from Gibb's adsorption equation, which can be considered a tendency of surfactants to be adsorbed at the air–water surface at concentrations close to CMC and the lower area per surfactant molecule at the interface (A_{min}). Γ_{max} was found to be in the range from 5 to 6 × 10^{-6} mol/m^2, while A_{min} was in the range 30–33 Å^2 for all the synthesized 1-o(tolyl) alkyl biguanide, evidencing a slight effect of the length of the alkyl chain on these surface parameters. The calculated standard free energy of micellization ($\Delta G°_{mic}$) and adsorption ($\Delta G°_{ads}$) became more negative moving from TBC6 to TBC10, indicating that the elongation of the alkyl chain favored the adsorption of the amphiphilic molecules at the air–water interface and the self-aggregation process (Table 2).

A dependence of specific conductivity (μS/cm) over concentrations was observed for TB and the 1-(o-tolyl) biguanide derivatives. Being hydrochloride salts, the synthesized amphiphiles can be considered as cationic surfactants that behave as electrolytes in water and can affect the conductivity of solutions in a different manner as a function of their aggregation state (Fig. 5). An increase in conductivity over concentration was also observed for the commercial compound TB, despite it is a free base and not a hydrochloride salt (pH of its solution is 11.8 at a concentration of 35 mM). The effect on conductivity can be explained by considering that biguanides have high pKa value (reported in the range 10.3–13.4) (Dubey and Bharatam, 2023), therefore they undergo a partial protonation in water (Bridges et al., 2016; Ne Fortun and Schmitzer, 2018). Specifically, two linear segments can be recognized in the raw data, which can be considered indirect evidence that these compounds are present in water as unimers or as self-assembled aggregates. All plots do not show clearly evident inflections in the change of electrical conductivity over concentrations, which can be probably related to the low contribution of the inclusion of counterions within the micelles and/or surfactant aggregates in affecting conductivity values, as previously reported in the literature (Durand-Vidal et al., 2020; Perinelli et al., 2020). Therefore, the slope of the two linear segments are quite similar, and the breakpoint, recognized as the CMC, has been visualized as the minimum of the first derivate of the conductivity signal.

The calculated CMCs are reported in Table 2 and a good agreement was found between values obtained from conductivity measurements with respect to those from tensiometric analysis for TBC6 and TBC10.

Homologues with a longer alkyl chain as the C14 derivative (TBC14) were also synthesized and the variation of surface tension and conductivity over concentrations was measured (Figure SF1). This derivate had a very poor solubility in water and it started precipitating when the CMC was approached. Therefore, no reliable measurements of surface tension and conductivity were acquired at higher concentrations, not allowing the calculation of CMC values.

3.4. Cell cytotoxicity

The cytotoxicity of the synthesized alkyl 1-(*o*-tolyl) biguanide surfactants in comparison to the commercial compound 1-(*o*-tolyl) biguanide (TB) and chlorhexidine digluconate as controls was assessed preliminarily using MTT assay toward HaCaT cell lines. This cell line was selected as an *in vitro* model to acquire information regarding the possible cytotoxic effect of these new compounds on cells through

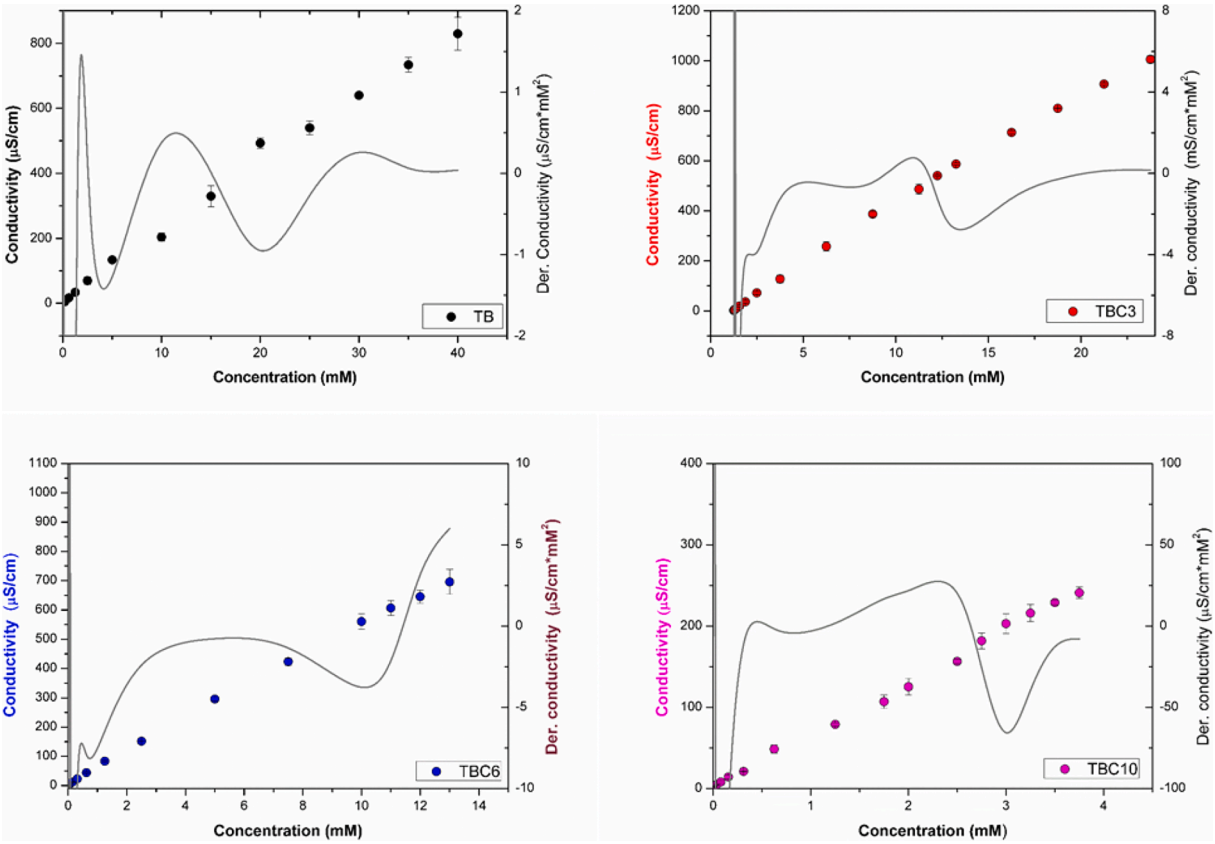


Fig. 5. Conductivity (μS/cm) *vs* concentration (mM) plots at 25 °C for TB and the synthesized 1-(o-tolyl)biguanide derivatives (TBC3, TBC6, and TBC10). Lines refer to the first derivate of conductivity *vs* concentration plots.

topical application on the skin. At the tested concentrations, all compounds show a marked decrease in cell viability (%), from values close to that of vehicle (~100 % cell viability) to values close to zero, with the exception of the TB compound resulting the less cytotoxic (Fig. 6). The calculated concentrations that cause 50 % of cell death, named as inhibitory concentration (IC_{50}) are reported in Table 2. As for other homologous series of surfactants, IC_{50} values resulted to be strongly dependent on the length of the hydrophobic tail, ranging from ~ 0.242 mM for TB-C3 and ~ 0.003 mM for TB-C10, likewise observed for CMC values. Specifically, the calculated IC_{50} for chlorhexidine digluconate was ~ 0.011 mM, which was only slightly lower but not statistically different from that of TB-C6 (~0.016 mM).

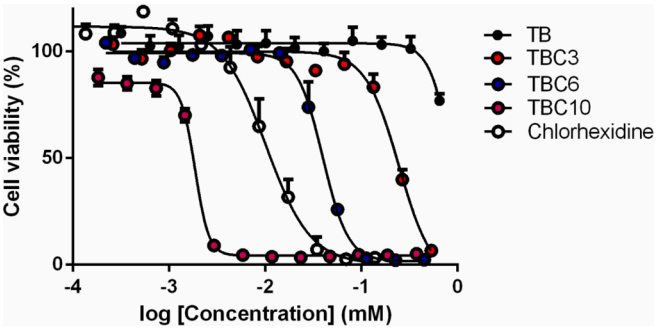


Fig. 6. Cell viability (%) *vs* alkyl 1-(*o*-tolyl) biguanide surfactants concentration (mM) plots as determined from MTT assay on immortalized human keratinocytes (HaCaT) cell line. Data are presented as mean ± SD.

3.5. MIC determination and disinfectant activity evaluation

The MIC and MBC values of TB and 1-(*o*-tolyl) biguanide derivatives against *S. aureus* and *B. subtilis* (Gram-positive species) and *P. aeruginosa* and *E. coli* (Gram-negative species) are shown in Table 3 and Table 4. All compounds, from TBC3 to TBC10, showed a higher activity against Gram-positive bacteria than against Gram-negative ones. Among the Gram-negative species, *E. coli* was more susceptible than *P. aeruginosa* to

Table 3
Minimum inhibitory concentration (MIC) and Minimum Bactericidal Concentration (MBC) values (mg/mL) for the synthesized alkyl 1-(*o*-tolyl) biguanide surfactants and the reference compound chlorhexidine digluconate against strains of Gram-positive species.

Compound	MIC mg/mL (MBC mg/mL) [mM]		
	S. aureus		*B. subtilis*
	ATCC 6538	ATCC 25923	ATCC 6633
TB	>1.0 (>1.0) [>5.23 (5.23)]	>1.0 (>1.0) [>5.23 (5.23)]	>1.0 (>1.0) [>5.23 (5.23)]
TBC3	0.5–1.0 (1.0) [2.14–4.28 (4.28)]	1.0 (1.0) [4.28 (4.28)]	1.0 (1.0) [4.28 (4.28)]
TBC6	0.0625 (0.0625) [0.227 (0.227]	0.0625 (0.0625–0.125) [0.227 (0.227–0.454]	0.0625 (0.0625) [0.227 (0.227]
TBC10	0.002–0.004 (0.004–0.008) [0.006–0.012 (0.006–0.012)	0.002–0.004 (0.004–0.008) [0.006–0.012 (0.006–0.012)	0.002 (0.002) [0.006 (0.006)
Chlorhexidine digluconate	0.00024–0.00048 [0.0003–0.0005]	N.D.	N.D.

Table 4
Minimum inhibitory concentration (MIC) and Minimum Bactericidal Concentration (MBC) values (mg/mL) for the synthesized alkyl 1-(*o*-tolyl) biguanide surfactants and the reference compound chlorhexidine digluconate against strains of Gram-negative species.

	MIC mg/mL (MBC mg/mL) [mM]		
Compound	*P. aeruginosa*		*E. coli*
	ATCC 15442	ATCC 9027	ATCC 25922
TB	>1.0 (>1.0) [>5.23 (5.23)]	>1.0 (>1.0) [>5.23 (5.23)]	>1.0 (>1.0) [>5.23 (5.23)]
TBC3	>1.0 (>1.0) [> 4.28 (4.28)]	>1.0 (>1.0) [> 4.28 (4.28)]	>1.0 (>1.0) [> 4.28 (4.28)]
TBC6	0.125–0.250 (0.250) [0.454–0.908 (0.908]	0.250 (0.250) [0.908 (0.908]	0.125 (0.125) [0.454 (0.454)]
TBC10	0.0625–0.125 (0.125) [0.188–0.377 (0.377)]	0.0625 (0.0625) [0.188 (0.188)]	0.0078 (0.0078) [0.023 (0.023)]
Chlorhexidine digluconate	0.0039–0.0078 [0.0043–0.0087]	N.D.	N.D.

TB derivatives with longer hydrocarbon chains with $MIC_{P.\ aeruginosa}/MIC_{E.\ coli}$ of 0.5 for TBC6 and $MIC_{P.\ aeruginosa}/MIC_{E.\ coli} = 8$ for TBC10. TBC10 worked at the lowest MIC values, despite being more than ten times less active than chlorhexidine digluconate, which was used as the reference biguanide-derivative compound. A preliminary study was also carried out to assess whether the measured activity was bacterial strain dependent. The results showed that two different strains of *S. aureus* (ATCC 6538 and ATCC25923) and two different strains of *P. aeruginosa* (ATCC 15,442 and ATCC 9027) were comparably susceptible. Overall, the activity of the biguanide derivatives was bactericidal as all MBC values were less than or equal to four times the respective MIC values. This conclusion could not be reached for TBC3 against Gram-negative species since MICs were not defined. Their values were higher than the upper concentration of the molecule that was tested, i.e. 1 mg/L (Table 4).

In view of possible applications of the biguanide derivatives, the antibacterial activity study was extended by the determination of the disinfectant activity. This was assessed by measuring the log reduction of the CFU count after 2 min of contact of the biguanide derivatives with the bacterial suspension (Table 5). This investigation has been limited to one strain of *S. aureus* and one strain of *P. aeruginosa* as per the requirements of the ISO 1040:2005. As in the case of MICs, the disinfectant activity was greater for derivatives with longer hydrocarbon chains (TBC6 and TBC10) than for TBC3, at both concentrations tested (0.5 % w/v and 0.05 % w/v). In particular, the log CFU reduction (≥5 units) for TBC6 and TBC10 was similar to that of chlorhexidine digluconate at both concentrations tested against *S. aureus* and at the concentration of 0.5 % w/v against *P. aeruginosa*.

4. Discussion

Surfactants are a large class of amphiphilic compounds with a broad range of applications in all technological fields (Le Guenic et al., 2019; Myers, 2020; Shaban et al., 2020). In pharmaceutical and cosmetic products, surfactants are generally employed as solubilizing agents or stabilizers (e.g. emulsifiers) for disperse systems in both traditional and advanced formulations (Ceresa et al., 2021; Katz et al., 2022; Rodríguez-López et al., 2018; Vinarov et al., 2018). Moreover, the interest in surfactants also relies on their ability to interact or to be portioned inside biological or artificial bilayers and membranes, thereby attracting much interest as permeability enhancers across skin or mucosa and for the design of functional vesicles and nanocarriers (Cavanagh et al., 2022; Chen et al., 2019; McCartney et al., 2021; Perinelli et al., 2017).

Table 5
CFU counts Log reduction after 2-min-contact at 20 °C for the synthesized alkyl 1-(*o*-tolyl) biguanide surfactants and chlorhexidine digluconate at the concentration of 0.5 % and 0.05 % w/v.

CFU counts Log Reduction				
	S. aureus ATCC 6538		*P. aeruginosa* ATCC 15442	
Compound	0.5 %	0.05 %	0.5 %	0.05 %
TB C3	0	0	<1	0
TB C6	≥5	≥5	≥5	1
TB C10	≥5	≥5	≥5	1
Chlorhexidine digluconate	≥5	≥5	≥5	<2

However, the chemical features of surfactants as amphiphilic compounds consisting of a hydrophilic portion (polar head) linked to one or more hydrophobic tails, provide great versatility in modulating the surfactant properties. It is well known that self-aggregation and surface absorption of surfactants are mainly driven by the hydrophobic interactions between the hydrophobic tails (Ghosh et al., 2020). Indeed, surfactant homologs bearing longer hydrocarbon chains display poor solubility in aqueous media, together with lower CMC and γCMC values (Hu et al., 2020; D. R Perinelli et al., 2016). On the other side, the characteristics of the polar head impart an ionic, non-ionic, or zwitterionic character to the surfactant, thereby affecting their use for different technological applications (Oremusová et al., 2019).

The chemical features of the polar heads can also be specifically designed to develop novel amphiphilic molecules endowing additional functional properties in addition to the intrinsic surface active ability, which is distinctive of any surfactant. According to this, novel surfactants with dual functional properties can be synthesized starting from water-soluble compounds possessing biological activity via alkylation or acylation. As such, hydrochloride derivatives of the commercial product 1-(*o*-tolyl) biguanide having a C3, C6, or C10 hydrocarbon chain were synthesized using a two-step approach.

The amphiphilic properties of the 1-(*o*-tolyl) biguanide surfactants were found to be markedly affected by the length of the hydrocarbon chain as for other homologous series of surfactants bearing the same polar head (Kuznetsova et al., 2021; D. R Perinelli et al., 2016). Specifically, TBC3 due to the shorter alkyl chain showed a poor surface activity, since the measured surface tension was not below ~55 mN/m and the formation of micelle was not detectable by DLS. On the other side, a pronounced decrease in surface tension occurred for derivatives having a 6-length or a 10-length hydrocarbon chain. Specifically, TBC6 and TBC10 showed γCMC values (between 30 and 40 mN/m) comparable to those of commonly-employed surfactants (Liu et al., 2016; Szymczyk et al., 2018).

A direct comparison of the surface ability of these compounds with the literature is tricky due to the poor data available on this class of amphiphilic compounds, especially on the derivatives of the 1-(*o*-tolyl) biguanide. A comparison can be only performed with the derivatives of the biguanide without the tolyl group. In one study, CMC values in the range of 0.22 mM to 2.3 mM were determined for the hydrochloride salts of the hexyl chain (C6) and the dodecyl chain (C12) biguanide derivatives. These values are slightly lower than those calculated for our compounds (Ne Fortun and Schmitzer, 2018).

Relevantly, biguanide derivatives with surface active properties have never been tested for their antimicrobial activity toward Gram-positive and Gram-negative bacteria, despite the antimicrobial effect of the biguanide moiety exerted by different compounds (e.g. chlorhexidine) (Abu-obaid et al., 2019; Mensitieri et al., 2023), polyelectrolytes (Zaki et al., 2016), inorganic complexes (Nuţă et al., 2020), and polymers (e.g. poly hexamethylene biguanide) (Bueno and Moraes, 2018; Xing et al., 2019), is well known. The antibacterial activity against Gram-negative and Gram-positive bacteria and fungi has been reported only for the alkylguanidium salts having surface active properties. It was demonstrated that guanidine surfactants having a C8-C12 alkyl chain showed a decrease of the microbial viability of about 90 % at the concentration of 25–100 μg/mL and the effect was found mainly dependent on the hydrocarbon chain length (Song et al., 2012). A marked effect on the hydrocarbon chain length was also found for the biguanide surfactant

series (C3, C6, and C10), as resulted from the lowering of the determined MIC values over the elongation of the alkyl chain. Moreover, as previously observed for many classes of cationic compounds including surfactants with antibacterial properties (Silva et al., 2022), a higher susceptibility was found towards Gram-positive bacteria (e.g. *S. aureus*) with respect to Gram-negative bacteria (e.g. *P. aeruginosa*). The lower antibacterial effect against Gram-negative can be explained by considering the presence and peculiar composition of the lipid outer membrane in terms of fatty acids, lipopolysaccharides, and efflux proteins (Epand et al., 2016). The mechanism of action of the series of surfactants has still not been elucidated. However, a review of the literature reveals a substantial body of evidences indicating that other molecules with similar structural and/or functional properties, including quaternary ammonium compounds and other biguanides such as polyhexamethylene biguanides, impair the structural and functional properties of membranes (Sowlati-Hashjin et al., 2020).

A more straightforward comparison can be made by plotting CMC, $IC_{50,}$ and MIC values as a function of the hydrocarbon chain length (Figure SF2). A linear relationship between the CMC and the hydrocarbon chain length has been found. As regards, IC_{50} and MIC the linearity can be observed using semi-log plots, instead. Another way to compare the obtained results is to plot the ratio between the calculated parameters (CMC/MIC, CMC/$IC_{50,}$ and IC_{50}/MIC) and the hydrocarbon chain length (Figure SF3). From CMC/MIC plots, it has been evidenced that as the hydrocarbon chain becomes longer, the antibacterial activity against the Gram-positive *S. aureus* increases, since MIC values become much lower than CMC. This effect was not observed for the antimicrobial activity of these compounds toward the tested Gram-negative *P. aeruginosa* for which the CMC/MIC ratio remains almost constant. The same trend in comparison to CMC, was observed for IC_{50} values. Indeed, the concentration causing 50 % of cell death becomes increasingly lower than CMC over the elongation of the alkyl chain, configuring them as more cytotoxic compounds.

As regards the IC_{50}/MIC ratio, also known as selectivity index, values around 0.1 were found for the Gram-positive *S. aureus* indicating that the concentrations at which bacterial growth is inhibited occurs is ten times higher than the concentrations causing 50 % of HaCaT cells death, at least at the experimental conditions tested (sample exposure time of 72 h for HaCaT cells). On the contrary, the calculated selectivity index for chlorhexidine digluconate at the tested conditions was much higher than 1 and equal to 27.5, despite this commercially available antiseptic and disinfectant also showing intrinsic cell cytotoxicity (IC_{50} value in the micromolar range). These results could have a possible impact on the safe use of these amphiphilic compounds when applied onto the skin, although further *ex vivo/in vivo* tests should be carried out to finally assess whether these compounds can be used for the preparation of topical formulations for pharmaceutical and cosmetic use. However, it should be also highlighted that the promising results regarding the disinfectant activity, which were compared to those of chlorhexidine digluconate ($\geq$5 CFU counts Log reduction) were achieved after a contact time of 2 min, thereby requiring further investigation on the toxic effect of these compounds after a short time of exposure. On the other side, from the obtained results it also emerges that the addition of an alkyl chain into the 1-(*o*-tolyl) biguanide moiety increased both the surface properties as well as the potential cytotoxicity toward mammalian cells and antibacterial activity (especially against Gram-positive), probably related to the ability of the amphiphilic compounds to be inserted into the cytoplasmic membrane. Therefore, the presence of an alkyl chain may improve the biological properties of compounds bearing the biguanide moiety, despite a direct comparison with chlorhexidine digluconate cannot be made due to structural differences (bis-biguanide compounds, presence of chlorine).

5. Conclusions

Alkyl 1-(*o*-tolyl)biguanide derivatives were synthesized and characterized as novel surfactants and antimicrobial or disinfectant compounds, potentially employed in pharmaceutical and cosmetic formulations by exploiting the advantages of their dual functional activity. The most effective molecule was TBC10 (C10-alkyl chain), highlighting the relevant effect exerted by the length of the alkyl chain both on surface tension, aggregation, and antibacterial activity toward the Gram-positive and Gram-negative bacteria. Notably, the low calculated MIC values for TBC10 (between 0.006 and 0.012 mM against the Gram-positive *S. aureus* and between 0.188 and 0.377 mM against the Gram-negative *P.aeruginosa*) are promising for their use as antimicrobial agents or preservatives in pharmaceutical and cosmetic formulations. Moreover, the observed disinfectant activity against the Gram-positive *S. aureus* and the Gram-negative *P. aeruginosa* comparable to that of the reference compound chlorhexidine digluconate suggests their applications in antiseptic products. Further investigations are needed to assess the antimicrobial efficacy and applicability of these dual-function surfactants in aqueous-based (e.g hydrogels) or dispersed (e.g. creams) formulations as preservatives or topically applied disinfectants that require limited contact time onto the skin.

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CRediT authorship contribution statement

Diego Romano Perinelli: Writing – original draft, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Fabio del Bello:** Writing – original draft, Methodology, Investigation. **Luca Agostino Vitali:** Methodology, Investigation. **Massimo Nabissi:** Methodology, Data curation. **Marco Cespi:** Visualization, Data curation. **Wilma Quaglia:** Writing – review & editing, Data curation. **Cristina Aguzzi:** Investigation. **Veronica Lupetti:** Investigation. **Martina Giangrossi:** Investigation. **Giulia Bonacucina:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2024.124388.

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