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Synergistic effect of hen egg white lysozyme and lysosomotropic surfactants on cell viability and membrane permeability

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ABSTRACT

The interactions between two types of quaternary ammonium surfactants (*N,N,N*-trimethyl-2-(dodecanoyloxy) ethaneammonium bromide (DMM-11) and *N,N,N*-trimethyl-2-(dodecanoyloxy)propaneammonium bromide (DMPM-11)) and hen egg white lysozyme were studied through several techniques, including isothermal titration calorimetry (ITC), circular dichroism (CD) and fluorescence spectroscopy, and surface tension measurement. The average number of surfactants interacting with each molecule of lysozyme was calculated from the biophysical results. Moreover, the CD results showed that the conformation of lysozyme changed in the presence of DMM-11 and DMPM-11. The studies drew a detailed picture on the physicochemical nature of interactions between both surfactants and lysozyme. Both DMM-11 and DMPM-11, with and without lysozyme were studied against three target microorganisms, including Gram-negative (*Escherichia coli*) and Gram-positive (*Enterococcus hirae* and *Enterococcus faecalis*) bacteria. The results revealed a broad spectrum of antibacterial nature of surfactant/lysozyme complexes, as well as their effect on the membrane damage, hence providing the basis to further explore DMM-11 and DMPM-11 combined with lysozyme as possible antibacterial tools.

1. Introduction

Lysosomotropic surfactants are an interesting group of therapeutic agents characterized by amphiphilic properties, that are known to be used as antibiotics and anticancer drugs [1]. Quaternary ammonium compounds (QACs), that contain a moderately basic amino group, are able to passively diffuse across cell membranes and act primarily on lysosomes [2]. The biological activity of synthetic long-chain QACs is known [3], as well as their activity against several human tumor cell lines [4,5] and some pathogenic bacterial strains [6,7]. Since the toxicity of long-chain QACs against normal mammalian cells is very high, their adverse effects can be avoided by the use of soft analogues of QACs. Indeed, several soft cationic QACs showed also satisfactory antimicrobial [8] and antitumor [9] properties. Despite extensive research into the antimicrobial effects of QACs [10], the development of new types of antimicrobial lysosomotropic surfactants is still an unmet need.

Hen egg white lysozyme is a globular protein with a molecular weight of 14 305 Da, containing 129 amino acid residues. Lysozyme contains four disulfide bonds, 17 positively and 9 negatively charged residues (including 6 Lys, 11 Arg, 7 Asp and 2 Glu) and is characterized by an isoelectric point of 11.2 [11]. Important active site residues are located at the positions Glu35, Asp52, Trp62, Trp63, Asp101 and Trp108, of which the most important in substrates binding are Glu35 and Asp52, located opposite each other at the active site. Lysozyme contains two domains (α and β); β-domain consists mostly in antiparallel β-sheet, while α-domain consists of 3_{10}-helix and α-helices (A–D) [12]; both domains share the active site.

Lysozyme monomer, i.e. its basic form, exhibits antibacterial properties, mainly against Gram-positive bacteria. The antimicrobial action of lysozyme is mediated through its muramidase activity, which catalyzes the hydrolysis of β-1,4-glycosidic bonds in the peptidoglycan layer of cell walls in Gram-positive bacteria [13]. The lytic activity of lysozyme against Gram-negative bacteria is reduced due to the content of additional polypeptides and lipopolysaccharides that are present in the structure of the cell wall. Lysozyme does not exhibit toxicity in humans [14] and does not affect the physical properties of food products. Worldwide, it is commonly used as a preservative in several food products, such as wine, cheese, sausage and meat, and as an ingredient in pharmaceutical products [15]. However, it has been reported that several bacteria are resistant to its action [16]. Nevertheless, it has also been shown that the antimicrobial effect of lysosome can be enhanced by both synthetic and naturally occurring antimicrobials [17]. Chen

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and co-workes [18] reported a synergistic effect of a cathelicidin LL-37-lysozyme complex against *Staphylococcus aureus* and *Escherichia coli*. These studies put forward the hypothesis that lysosomotropic surfactants in combination with lysozyme may be useful antimicrobial treatments.

Many studies focused on the biomedical applications of lysozyme immobilized onto natural-based materials. Li and co-workers [47] showed that lysozyme-coated cellulose nanofibrous mats exhibit antibacterial activity against both Gram-negative E. coli and Gram-positive S. aureus. Additionally, these nanofibrous mats presented excellent biocompatibility with L929 fibroblasts. In another study it was reported that immobilized lysozyme on the N-[(2-hydroxy-3-trimethyl-ammonium) propyl] chitosan chloride (HTCC) shows better antibacterial effects compared with the free lysozyme [48].

Two quaternary ammonium surfactants (*N,N,N*-trimethyl-2-(dodecanoyloxy)ethaneammonium bromide (DMM-11) and *N,N,N*-trimethyl-2-(dodecanoyloxy)propaneammonium bromide (DMPM-11)) have been chosen to study the occurrence of synergistic antibacterial activity against *Enterococcus faecalis*, *Enterococcus hirae* and *E. coli* whenever combined with lysozyme. The membrane lysing properties of these compounds were additionally studied through fluorescence microscopy. To the best our knowledge, this is the first assessment of the antimicrobial efficacy of the above mentioned lysosomotropic surfactants, with and without lysozyme. Moreover, in order to get a deeper insight into the nature of the interactions between DMM-11/DMPM-11 and hen egg white lysozyme, a classical protocol for the study of interactions of small molecules with proteins was used including isothermal titration calorimetry (ITC), circular dichroism (CD), fluorescence spectroscopy and surface tension studies. The binding mechanism, the number of binding sites, the binding forces as well as conformational changes induced by DMM-11 and DMPM-11 in lysozyme were investigated in detail and discussed herein.

2. Experimental section

2.1. Chemicals

The synthesis of DMM-11 and DMPM-11 was described in earlier studies [19,20]. These compounds were synthesized by Dr. Jacek Łuczyński from the Department of Chemistry, Wroclaw University of Science and Technology, Poland. The structures of DMM-11 and DMPM-11 are shown in Supplementary Fig. S1. Hen egg white lysozyme (purity ⩾ 98%), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt (Hepes) (purity ⩾ 99.5%) and sodium chloride (purity ⩾ 99.5%) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). All stock solutions were prepared in Hepes buffer (5 mM Hepes, 150 mM NaCl, pH 7.4). The concentration of lysozyme was calculated by dividing the absorbance at 280 nm by the molar extinction coefficient (ε_{280} = 37 970 $M^{-1}\,cm^{-1}$).

2.2. Surface tension analysis

The surface tension (γ) measurements were performed according to the du Noüy's ring method described elsewhere [21] using a Krüss K20 Tensiometer (Krüss GmbH, Hamburg, Germany). Ultra-pure water was used to calibrate the tensiometer before the measurements. The values of surface tension were recorded at 25 °C. All the measurements were performed in three independent experiments.

2.3. Fluorescence measurements

The fluorescence spectra of lysozyme were carried out using a spectrofluorometer (Cary Eclipse, Varian, NC, USA) at 37 °C. The excitation wavelength was set at 280 nm. The excitation and emission slits were set at 5 nm. The emission spectra were recorded in the wavelength range 300–430 nm. The lysozyme solution (7 μM) was titrated with various concentrations of surfactants, ranging from 0.1–5 mM. The samples were allowed to equilibrate for 2 min before the spectra were recorded. Signal intensities of lysozyme samples were corrected against the intensity of both surfactants and inner filter effect [22].

2.4. Circular dichroism (CD) studies

Far-UV circular dichroism experiments were recorded in a 5 mm quartz cuvette on a Jasco J-1500 spectropolarimeter (Jasco Corporation, Tokyo, Japan) at 37 °C. The CD spectra were measured in the range between 200 and 260 nm. Each spectrum was the average of nine scans and the scan speed was 50 nm per minute. Background contributions from the Hepes buffer were subtracted.

2.5. Isothermal titration calorimetry (ITC)

ITC experiments were carried out using a Nano ITC calorimeter (TA Instruments) with a standard volume of 1.0 mL at 37 °C. All the solutions were prepared in buffer solution and deionized water (> 18Ω). All the solutions used to fill both the cell and the syringe were degassed before analysis. The reference cell was filled with deionized water. The titrations were performed in experimental mode in which the protein was an analyte and the surfactant was a titrant (DMM-11/DMPM-11 [~43 mM]→ lysozyme [~0.13–0.15 mM]). Each time freshly prepared solution of titrant was taken up in a 250 μL injection syringe and titrated into freshly prepared protein solution. A total number of 25 or 50 injections (4 μL each) were added after the calorimeter finalized the primary equilibration, with 200–300 s interval between the injections, leaving 200 s at the beginning of the experiment without injection. The stirring rate was set at 300 rpm. The calorimeter was operated using Nano ITC Run software and all the data obtained were analyzed with NanoAnalyze v. 3.1.2 program provided by the manufacturer. An 'independent model' was used to evaluate the results. Control experiments were performed in each case; the enthalpies of reagents dilution and demicellization were subtracted from the enthalpies of lysozyme-surfactant interactions. Heat of lysozyme dilution was negligible. Each ITC data was collected by at least two independent measurements and reproducible data was employed.

2.6. In vitro antibacterial activity

Two Gram-positive bacteria (*Enterococcus faecalis* ATCC 29212 and *Enterococcus hirae* ATCC 10541) and one Gram-negative bacteria (*Escherichia coli* ATCC 10536) were used. These strains were stored at −80 °C until sub-cultured onto Luria-Bertani (LB; 10 g L^{-1} bacto-tryptone, 5 g L^{-1} bacto-yeast extract, 10 g L^{-1} NaCl) agar plates for further studies.

The minimum inhibitory concentration (MIC) values of the lysosomotropic surfactants and lysozyme were determined using the microdilution broth method in 96-well microplates (Sarstedt, Nümbrecht, Germany) [23]. The serial dilutions of DMM-11 (0-0.15 mM), DMPM-11 (0–0.15 mM) and lysozyme (0–1000 μg mL^{-1}) were dissolved in Mueller–Hinton broth (Merck, Germany). The inoculum (2.5 μL) of each strain (10^8 CFU mL^{-1}) was placed into each well containing 200 μL of serial dilutions of the tested compounds. Negative and growth control wells did not contain the tested compounds. After 24 h of incubation at 37 °C, the optical density at 600 nm of each well was measured using a TECAN Spark 10 M (Tecan Group Ltd., Männedorf, Switzerland) microplate reader. The MIC was defined as the lowest concentration of the tested compounds that completely inhibited visible bacterial growth.

To evaluate the synergistic effect of cationic surfactants with lysozyme against *E. faecalis*, *E. hirae* and *E. coli*, bacterial suspensions were incubated with DMM-11 and DMPM-11 at sublethal concentrations (0.05 mM) with or without lysozyme (250 and 500 μg mL^{-1}). The inoculum (2.5 μL) of each strain (10^8 CFU mL^{-1}) was placed into each

well of 96-well microplates containing 200 µL of LB medium supplemented with the corresponding antimicrobial compounds. After 24 h of incubation, the antibacterial activity, expressed as viability (%), was calculated using the following formula: viability (%) = (OD_{600} in the presence of antimicrobial agents/OD_{600} of growth control) × 100.

2.7. Microscopic evaluation of bacterial membrane permeabilization

E. faecalis, *E. hirae* and *E. coli* cells were collected from liquid cultures by centrifugation (4500 × *g*), washed, and adjusted to 10^6 CFU mL^{-1} in sterile Hepes buffer solution (pH 7.4). Briefly, 3 µL of the reagents from the Live/Dead BacLight bacterial viability stain (L-7007, Invitrogen) was added to the cells previously treated with lysosomotropic surfactants (0.05 mM), lysozyme (250 µg mL^{-1}) or the surfactant/lysozyme complexes (0.05 mM/250 µg mL^{-1}) for 2 h (untreated cells were used as control). The bacteria were viewed using a fluorescence microscope (Axio Scope A1, Zeiss, Jena, Germany) using 40X objective lens. Images were acquired and analyzed using Carl Zeiss ZEN 2.3 lite software for quantification of live (green fluorescence) and dead (red fluorescence) cells. Bacterial cells with intact cytoplasmic membranes are stained green by SYTO-9 that enters the cells, while bacterial cells with compromised membranes are stained red by propidium iodide (PI). The experiments were repeated three times.

2.8. Cytotoxicity assay

Normal Human Epidermal Keratinocytes (NHEK) (PromoCell GmbH, Heidelberg, Germany) were cultured in Keratinocyte growth medium (KGM-GoldTM from Lonza, Basel, Switzerland), supplemented with 10% fetal bovine serum (FBS) and 1X antibiotic-antimycotic mix (Gibco Thermo Fisher Scientific, India) at 37 °C and 5% CO_2. The cells were plated in 96 well plates (1×10^4 cells per well) and incubated overnight. Cell viability was tested in the presence of DMM-11 and DMPM-11 at various concentrations (0-0.5 mM) with or without lysozyme (250 and 500 µg mL^{-1}) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [24]. The amount of MTT formazan product was determined by measuring the absorbance at 570 nm using a microplate reader. All the measurements were made in three independent experiments, each performed in triplicate.

3. Results and discussion

3.1. Surface activity of the surfactant-protein systems

In order to understand the surfactant-protein interactions it is necessary to know the micellization process of the surfactants. The dependence of surface tension (γ) on ln[C] for the surfactants with or without lysozyme is shown in Fig. 1. In the case of pure DMM-11 and DMPM-11, a sharp break point at the critical micelle concentration (CMC) is found. For the pure DMM-11 and DMPM-11 in Hepes buffer solution, the CMC values are 0.75 mM and 0.6 mM, and the surface tension values at the CMC (γ_{cmc}) are 26.4 mN m^{-1} and 28.1 mN m^{-1}, respectively. The surface tension of buffered surfactants solutions containing lysozyme is lower than the pure surfactants. The surface tension curves in the presence of lysozyme exhibit one break point that corresponds to the critical micelle concentration in the presence of protein (CMC*). Compared with the pure surfactant solutions, the CMC* values were found to be lower, namely 0.55 mM and 0.45 mM for DMM-11/lysozyme and DMPM-11/lysozyme, respectively. The results reported by Green et al. [25] showed the CMC* < CMC trend, which is an unusual feature rarely reported in literature. In this case, the CMC for sodium dodecyl sulfate (SDS) in the mixed system with lysozyme was found to be lower when compared with the pure surfactant, thus suggesting that SDS micellization was facilitated by the polypeptide fragments of lysozyme [24].

The number of surfactant molecules (*n*) bound to the protein was estimated using Eq. (1) [26]:

$$n = \frac{CMC - CMC^*}{C_p} \quad (1)$$

where CMC is the critical micelle concentration of the surfactants in the absence of lysozyme, CMC* is the critical micelle concentration in the presence of lysozyme, and C_p is the lysozyme concentration. The molar ratios were 29:1 for DMM-11 and 21:1 for DMPM-11 in the presence of lysozyme. It was found that the hydrophobic interactions of DMM-11 and DMPM-11 with lysozyme play a key role in the binding ratio of lysosomotropic surfactants to lysozyme. This observation was confirmed by the ITC analysis, as it will be further detailed.

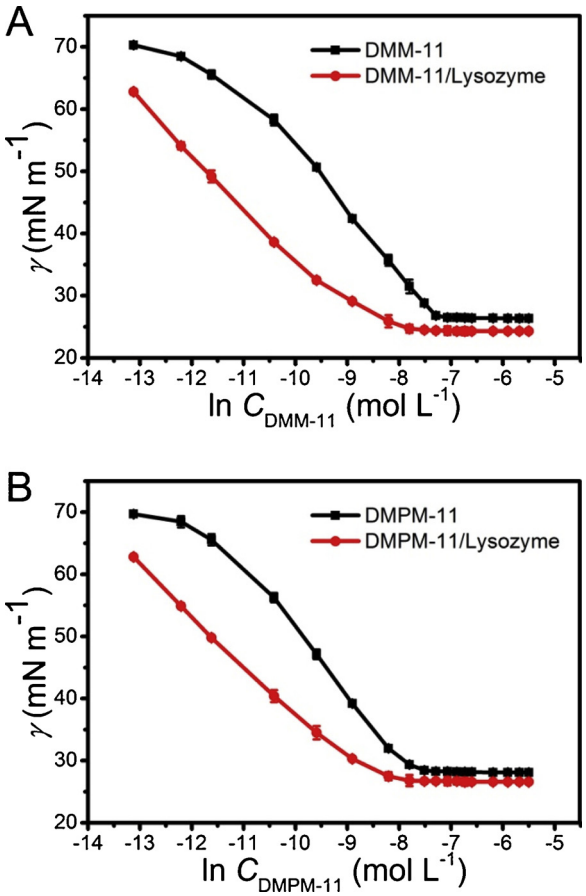

Fig. 1. Surface tension of DMM-11 (A) and DMPM-11 (B) solutions in the absence and presence of lysozyme ($C_{lysozyme}$ = 7 µM). The measurements were performed at 25 °C. The values represent the mean of triplicates ± SD.

3.2. Fluorescence measurements

Fluorescence spectroscopy is a technique widely used to monitor the intermolecular interactions, conformational changes and dynamics between proteins and other molecules. The presence of fluorophores, namely phenylalanine (Phe), tryptophan (Trp), and tyrosine (Tyr) is responsible for the intrinsic fluorescence of proteins. Hen egg white lysozyme contains 6 tryptophan residues, and steady state fluorescence data have shown that 80% of the fluorescence in the native protein comes from Trp62 and Trp108 [27]. To explore the surfactant-induced fluorescence change of lysozyme, fluorescence measurements were performed at an excitation wavelength of 280 nm [28]. Supplementary Fig. S2 shows the fluorescence spectra of lysozyme at increasing surfactants concentrations. The effect of adding DMM-11 and DMPM-11 at concentrations up to 5 mM causes an increase of fluorescence intensity. Upon the interaction of DMM-11 and DMPM-11 with lysozyme, the

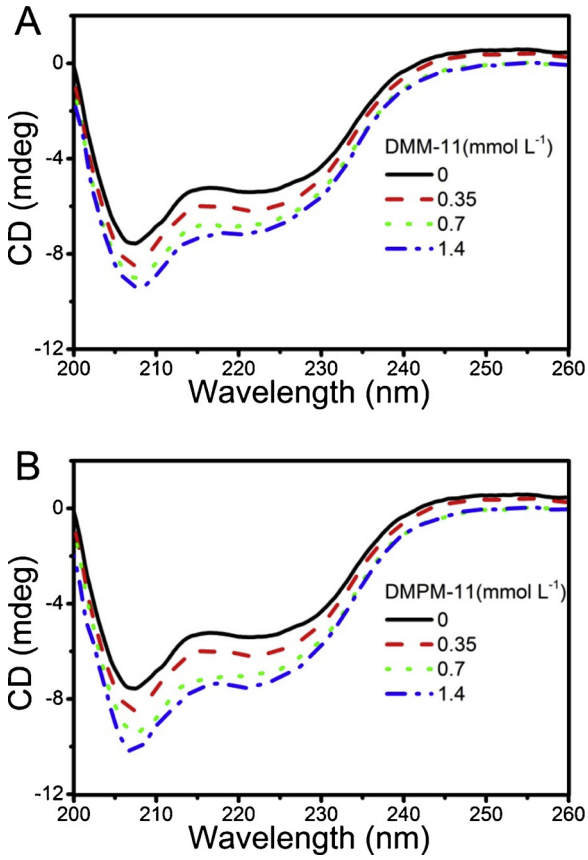

Fig. 2. Far-UV CD spectra of lysozyme as a function of increasing concentrations of (A) DMM-11 and (B) DMPM-11 at 37 °C. $C_{lysozyme}$ = 7 μM.

increase in fluorescence intensity occurred due to the unfolding of the lysozyme tertiary structure caused by hydrophobic interactions. Lysozyme is positively charged due to the presence of 17 protonated basic residues and 9 deprotonated acidic residues at pH 7.4, and DMM-11 and DMPM-11 are also positively charged; consequently, only hydrophobic interactions are possible. A similar unfolding behavior of cytochrome c (isoelectric point, pI = 9.6) was also recorded when treated with DMM-11 and DMPM-11 [29]. This observation was confirmed by the Far-UV CD results at these concentrations.

3.3. Circular dichroism (CD) studies

CD spectroscopy provides information on the structure of proteins and nucleic acids, as well as their ligand bound states [30,31]. The binding of a given ligand to the protein can change its secondary structure. In the Far-UV region of the spectrum, different forms of regular secondary structures found in proteins lead to characteristic CD spectra. Therefore, CD spectroscopy is used to investigate the structural changes in proteins. The Far-UV CD spectra presented in Fig. 2 evidenced two negative bands at 208 and 222 nm that are characteristic of the α-helical structure of the protein [32,33]. The K2D3 web server [34] was used to estimate the secondary structure contents of lysozyme with and without DMM-11 and DMPM-11 via analyzing the CD spectra. The α-helical content of pure lysozyme was found to be ~43%, which is in good agreement with the results obtained by Sethuraman et al. [35]. As the concentration of DMM-11 and DMPM-11 increases, the α-helicity of lysozyme also increases, as suggested by the increase of the negative ellipticity at 208 and 222 nm. It was observed that, as a result of the addition of DMM-11 (Fig. 2A) in the concentration range from 0.35 mM to 1.4 mM, the α-helical content of lysozyme increased from 43.25%–58.25%. In the case of DMPM-11 at the same concentrations (Fig. 2B), the α-helical content of lysozyme increased from 43.25%–62.80%. The percentages of α-helix content are presented in Table S1. The increase in the α-helical content can be elucidated due to the formation of hydrophobic linkages between the hydrophobic chains of lysosomotropic surfactants and non-polar residues present in lysozyme. The same behavior was observed in the case of interactions of cationic single-chain and gemini surfactants with human serum albumin [36], and between hemoglobin and single-chain and gemini surfactants [37].

3.4. Isothermal titration calorimetry (ITC) studies

ITC was used to evaluate the energy involved in the surfactant-protein interactions. ITC results for the interaction of both anionic [38–41] and cationic [39,41–44] surfactants with lysozyme were reported in the literature, and the binding of cationic surfactants to lysozyme was proven to occur despite the electrostatic repulsion of positively charged molecules. The nature of such binding is complex since it involves conformational changes of the protein, and in the case of cationic surfactants it has been reported to be driven mainly by hydrophobic interactions, involving different folded protein states [42]. It is also known that the thermodynamics of the interaction is strongly influenced by different factors (e.g., pH, temperature, presence and concentration of background electrolyte, concentration of substrates among others) [44,45]. Fig. 3 and Supplementary Fig. S3 show the ITC titrations of DMM-11 and DMPM-11 into lysozyme, as well as into buffer solution (since the data needs to be interpreted together with the demicellization process of the surfactant, which occurs in parallel). The latter titrations allowed the determination of the thermodynamic parameters of the micellization of both studied surfactants ($\Delta H_{mic} = -\Delta H_{demic}$), as well as the estimation of the CMC under the applied conditions (Supplementary Table S2, Fig. S4, Fig. S5). An energetically favorable demicellization process ($\Delta G < 0$) clearly dominates over the interaction of both surfactants with lysozyme. The changes on the lysozyme-present enthalpograms are very small. However, subtracting the curves it can be observed that there is a minor interaction between both DMM-11 and DMPM-11 and lysozyme. The heat flow points can be divided into regions, characterized by different thermodynamics, which was also observed for other cationic surfactants (*e.g.,* DTAB (dodecyl trimethyl ammonium bromide) [42], TTAB (tetradecyl trimethyl ammonium bromide) [42], and CPB (n-cetylpyridinium bromide) [44]). The first one, an exothermic and relatively steep region, which we were not able to fit; and the second, endothermic, present in a concentrated solution, that represents the interaction of numerous surfactant molecules with lysozyme (n_{DMM-11} = 31, $n_{DMPM-11}$ = 22). The association is very weak ($K_{ITC\ DMM-11/lysozyme} = 2.81 \times 10^3\ M^{-1}$, $K_{ITC\ DMPM-11/lysozyme} = 1.10 \times 10^3\ M^{-1}$) and since both enthalpy and entropy changes are positive ($\Delta H > 0$, $\Delta S > 0$) it seems to be favorably driven ($\Delta G < 0$) by hydrophobic interactions (Table 1). However, the calorimetric results need to be treated cautiously because 1) the obtained K_{ITC} values, especially for DMPM-11, fall practically below the range available for ITC measurements ($\sim 10^3 < K_{ITC} < 10^8$) [46]; and 2) very low enthalpy changes were detected ($\Delta H \sim 0.9$ kJ mol^{-1}). According to these limitations, the interaction detected cannot be considered as binding to the protein.

3.5. Study of antibacterial activity in vitro

The antibacterial activities of DMM-11, DMPM-11 and lysozyme against *E. faecalis*, *E. hirae* and *E. coli* were evaluated. The antibacterial activities of these agents were found to be dose-dependent. As presented in Table 2, DMM-11 and DMPM-11 were more effective against Gram-positive bacteria than against *E. coli*. The most susceptible strain to the compounds tested was *E. faecalis*. Consistent with previous reports, lysozyme did not show inhibitory activity against *E. coli*; however, some antibacterial activity of lysozyme against both Gram-

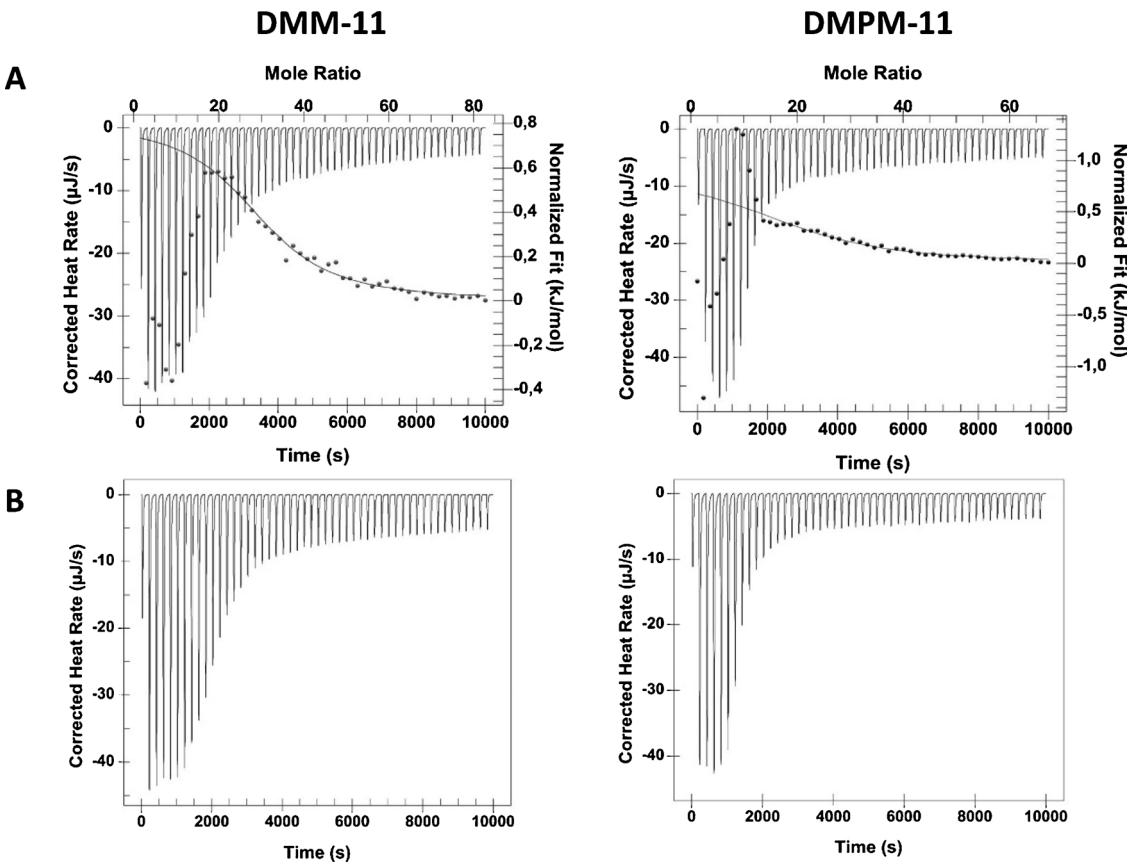

Fig. 3. Representative ITC data: left column (A) DMM-11 [43.5 mM]→lysozyme [0.13 mM]; (B) DMM-11 [43.5 mM]→buffer; right column (A) DMPM-11 [43.2 mM]→lysozyme [0.15 mM]; (B) DMPM-11 [43.2 mM]→buffer. All the experiments were performed in 5 mM Hepes, 150 mM NaCl solution with pH 7.4 at 37 °C.

positive bacteria was observed, although the MIC values could not be determined (Table 2).

Furthermore, the synergistic effect of the antibacterial agents under study against pathogenic bacteria at neutral pH was evaluated. The concentrations at which DMM-11, DMPM-11 and lysozyme did not exhibit significant antibacterial activity when used alone were determined, and these concentrations were further used to evaluate synergistic effects. When DMM-11 and DMPM-11 were combined with lysozyme at 250 and 500 µg mL^{-1}, an enhancement of their antibacterial activities against *E. faecalis*, *E. hirae* and *E. coli* was observed (Fig. 4). While lysozyme was more effective against the Gram-positive bacteria, in the presence of DMM-11 and DMPM-11 it helped to disrupt the outer lipopolysaccharide-containing *E. coli* layer, thus resulting in a rapid lysis.

To our knowledge, such synergistic activities of a mixture of different, but structurally similar, lysosomotropic surfactants with lysozyme have not been previously reported. Our data suggest that cationic surfactants in combination with lysozyme exhibit a higher antibacterial activity, and therefore they may have a substantial ability to enhance the efficacy of lysozyme against pathogenic bacteria. Besides, lysozyme and lysosomotropic surfactants use different mechanisms of action, which make them effective against resistance pathogens. Lysosomotropic surfactants in combination with lysozyme can possibly be used as antimicrobial agents in medical applications against microorganisms responsible for diseases and infections, thus making them a suitable alternative to conventional antibiotics.

Table 2
Antibacterial activity of DMM-11, DMPM-11 and lysozyme against pathogenic bacteria.

Strains	MIC[a] DMM-11 (mM)	DMPM-11 (mM)	Lysozyme (µg mL^{-1})
E. faecalis ATCC 29212	0.080 ± 0.003	0.075 ± 0.001	ND[b]
E. hirae ATCC 10541	0.095 ± 0.002	0.080 ± 0.004	ND[b]
E. coli ATCC 10536	0.110 ± 0.003	0.110 ± 0.002	ND[b]

[a] Minimum inhibitory concentration.
[b] Not determined.

Table 1
Thermal transition parameters of the systems studied.

Run	syringe*	cell*	n	K_{ITC} [M^{-1}]	ΔH_{ITC} [kJ mol^{-1}]	ΔS_{ITC} [Jmol^{-1} K^{-1}]	ΔG_{ITC} [kJ mol^{-1}] (kcal mol^{-1})
DMM-11→lysozyme	43.5	0.13	31	2.81×10^3	0.806	68.92	−20.57 (-4.89)
DMPM-11→lysozyme	43.2	0.15	22	1.10×10^3	0.879	60.72	−17.95 (-4.29)

*Initial concentration of reagent [mM].
[n]molar ratio at which the inflection was observed on the titration curve.

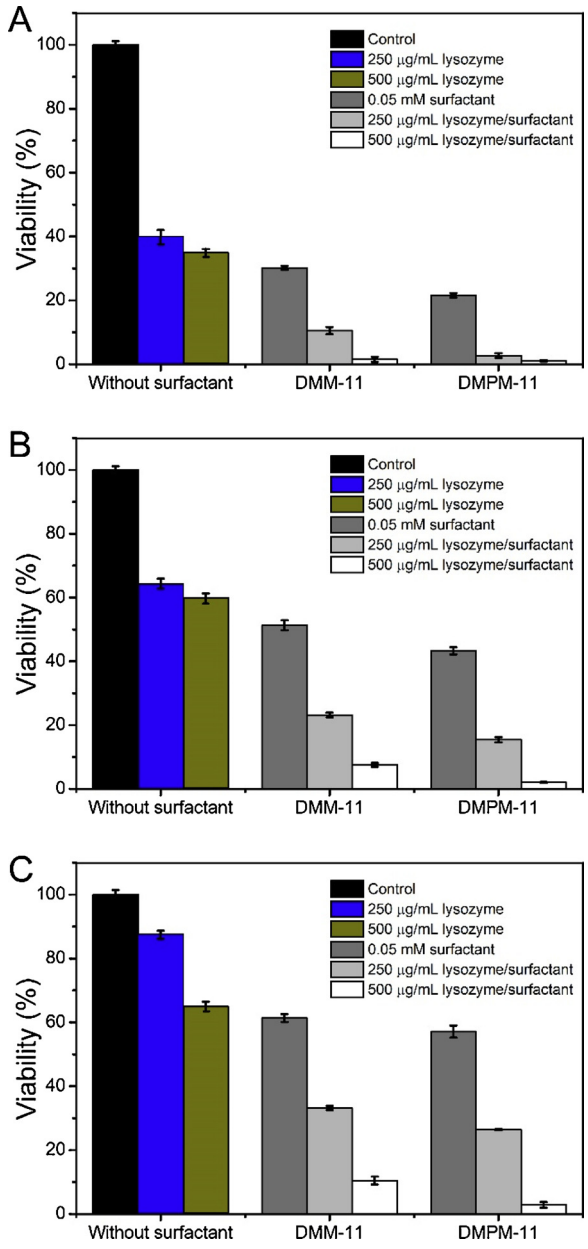

Fig. 4. Antibacterial activity of DMM-11, DMPM-11, lysozyme, DMM-11 + lysozyme and DMPM-11 + lysozyme against *E. faecalis* ATCC 29212 (A), *E. hirae* ATCC 10541 (B), and *E. coli* ATCC 10536 (C). The bacteria were grown in the presence of 0.05 mM DMM-11, 0.05 mM DMPM-11, 250 or 500 μg mL^{-1} lysozyme, and combinations of surfactant and lysozyme for 24 h at 37 °C. The antibacterial activity was expressed as viability (%). Data represent the mean ± SD of three independent experiments.

3.6. Study of the bacterial membrane permeabilization by fluorescence microscopy

Fluorescence images revealed the cell viability after treatment with the combination of DMM-11 and DMPM-11 with lysozyme. The permeability of the cell membrane was visualized after staining the cells with two fluorescent nucleic acid stains, SYTO-9 and PI. In the control assays (Fig. 5) it can be observed mainly the green color, which indicates a large number of live cells. The treatment with the combination of DMM-11 and DMPM-11 with lysozyme (Fig. 5) led to an almost complete red color, which suggests that surfactants and lysozyme synergistically kill cells. The combinations DMM-11/lysozyme and DMPM-11/lysozyme (0.05 mM/250 μg mL^{-1}) were equally effective against *E. faecalis*, *E. hirae* and *E. coli*. The percentage of live (green fluorescence) and dead (red fluorescence) cells was quantified from the microscopic images. Fig. S6 shows the increase in PI staining (red fluorescence) due to the surfactant/lysozyme (0.05 mM/250 μg mL^{-1}) exposure. Between 80 and 100% killing was observed even when a high bacterial density (10^6 CFU mL^{-1}) was used. The interaction of lysozyme with the peptidoglycan structure of the bacterial cell wall may be facilitated by the cationic surfactants, which neutralizes the anionic charges and favors its association with the membrane head groups. Despite the evidence of cell membrane damage, this study does not exclude additional bactericidal mechanisms potentially exerted by the surfactant/lysozyme complexes.

3.7. Study of cytotoxic activity

The cytotoxic effect of DMM-11 and DMPM-11 was investigated on NHEK cells by MTT assay. The concentrations used were in the range of those tested in the antimicrobial assays. After 24 h of exposure to the highest surfactant concentrations tested, the cell viability decreased to values around 30% for DMM-11 and 25% for DMPM-11 (Supplementary Fig. S7). When DMM-11 and DMPM-11 were combined with lysozyme, similar decreases in the viability of NHEK cells as compared to the surfactants alone was observed, hence meaning that the lysozyme has a residual effect on cell viability (Supplementary Fig. S7). The cytotoxic effect of the cationic surfactants could be due to their amphiphilic nature by which they have the capacity to penetrate the cell membrane easily. There was no significant increase in necrotic cells in either control or surfactant-containing wells at concentrations between 0.01 and 0.1 mM, thus suggesting that both surfactants with or without lysozyme are safe for application on human cells at those concentrations.

4. Conclusions

In the current work, several advanced biophysical tools were used to investigate the nature of the binding of two cationic surfactants (DMM-11 and DMPM-11) to lysozyme. It was found that both surfactants can interact with lysozyme mainly through hydrophobic interactions. From the Far-UV CD and fluorescence spectroscopy results, a gain in secondary structure due to the DMM-11/lysozyme and DMPM-11/lysozyme interactions was determined. Moreover, the Gram-positive bacteria *E. faecalis* and *E. hirae* were found to be more sensitive to both studied surfactants than the Gram-negative *E. coli* possibly due to a stronger disruption of the Gram-positive cell walls by these compounds after their incorporation. In addition, fluorescence microscopy was used to confirm the bacterial cells membrane damages after exposure to DMM-11 and DMPM-11 combined with lysozyme. The results herein gathered show the broad-spectrum antibacterial nature of both surfactants, and their effect on the bacterial cells membrane. They prove the existence of a synergistic effect of hen egg white lysozyme upon being complexed with lysosomotropic surfactants, that can be considered as a starting point for a foundation of a novel antimicrobial tool. We believe that these results will be useful to further develop and improve surfactant/lysozyme systems towards their application for biomedical purposes.

Authors contributions

Conceived, designed the experiments and wrote the paper: TJ. Performed the experiments: TJ (circular dichroism and fluorescence spectroscopy, and biological studies), EG (surface tension) and JB (isothermal titration calorimetry studies). Reviewed the manuscript: LR and JB. All authors read and approved the manuscript.

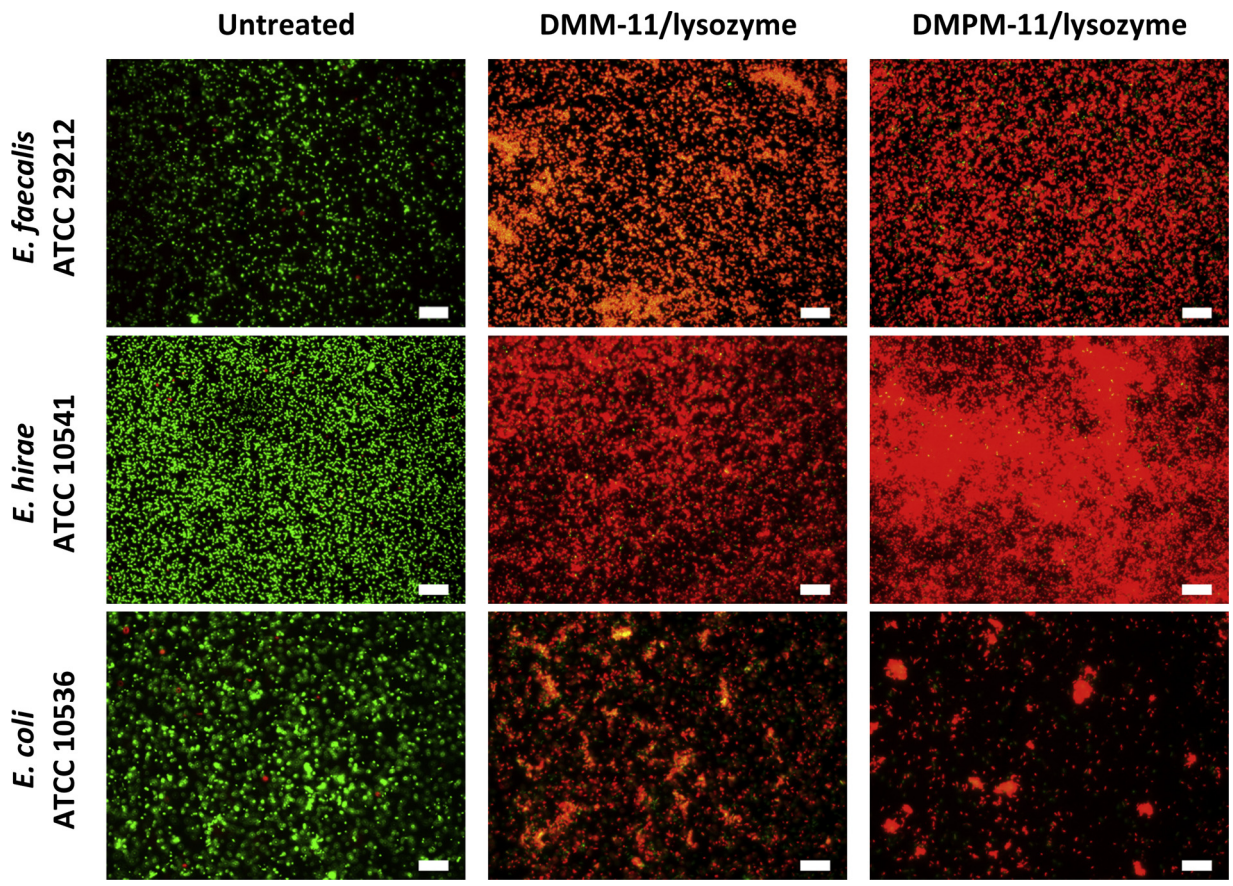

Fig. 5. Fluorescence microscopy assays for the study of the viability of *E. faecalis*, *E. hirae* and *E. coli* treated with surfactant/lysozyme mixtures. The bacteria were exposed to the surfactants (0.05 mM) in combination with 250 μg mL^{-1} of lysozyme, or Hepes buffer (negative control) for 2 h at 37 °C. Scale bar - 20 μm. Bacteria in green indicates live/healthy cells, whereas red are indicative of dead or membrane damaged bacteria. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Declaration of Competing Interest

There are no conflicts to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.colsurfb.2019.110598.

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