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Quatsomes for the treatment of *Staphylococcus aureus* biofilm

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Abstract

The anti-biofilm effect of drug delivery systems composed of the antiseptic quaternary ammonium compound cetylpyridinium chloride (CPC) and cholesterol was evaluated in *Staphylococcus aureus* biofilm. Self-assembly of CPC/cholesterol to approximately 100 nm CPC-quatsomes was successfully accomplished by a simple sonication/dispersion method over a broad concentration range from 0.5 to 10 mg/ml CPC. CPC-quatsomes showed a dose-dependent anti-biofilm effect, killing >99% of biofilm-associated *S.aureus from* 5% mg/ml after 10 minutes exposure. Cell toxicity studies with CPC-quatsomes in Nuli-1 cells revealed no adverse effects at all tested CPC concentrations. CPC-quatsomes, therefore, have a promising potential as novel drug delivery systems with "built-in" anti-biofilm activity.

Introduction

It is now established that *Staphylococcus aureus* (*S.aureus*) biofilm is a significant contributor to numerous recalcitrant medical conditions such as wound infections, chronic rhinosinusitis, pneumonia, and endocarditis [1-4]. Through attachment to biological and non-biological surfaces biofilm is formed by sessile communities of bacteria that are embedded in a self-produced, hydrated polymeric matrix [1]. The biofilm poses a major physico-chemical obstacle for conventional antimicrobial therapies as the reduced diffusion into the polymeric matrix limits the free access of antibiotics while allowing an efficient enzymatic degradation (e.g. by lactamase) and removal of antibiotics [5]. Gradual changes in the microenvironment (e.g. oxygen, nutrients, pH) within the biofilm matrix and the existence of persister cells further complicate the treatment of bacterial biofilm [3, 5-8].

The overall protective effect of the biofilm from adverse conditions has been associated with significantly reduced sensitivity for anti-microbial compounds (up to 1000-fold) compared to the planktonic form of the same strain [5, 6]. Importantly, the biofilm matrix serves as a reservoir from which bacteria detach, causing infection relapse and persistent infections [1]. Despite the importance of *S.aureus* biofilm there is still a disparity towards the development of adequate drug delivery systems that are able to overcome the obstacles imposed by biofilm.

Nanoparticulate drug delivery systems such as liposomes have received great attention since the pioneering work of Bangham *et al.* [9]. The similarity of the phospholipid bilayer with biological cell membranes and biocompatibility has rendered liposomes a popular drug delivery option to improve the pharmacokinetic and pharmacodynamics of antimicrobial compounds [10-14]. Liposomes are typically designed to incorporate either a hydrophilic drug in the aqueous core or a lipophilic drug in the phospholipid membrane. Further manipulation of liposomes e.g. by surface decoration with nanoparticles or polymers can facilitate prolonged systemic circulation and enhance the specific interaction with cells, including biofilm [15-17]. Liposomal delivery of antibiotics has been the subject of numerous studies which have been reviewed elsewhere [18, 19]. However, drug encapsulation within liposome phospholipid membranes is generally limited as large quantities can compromise the structural integrity of the liposomes [19]. In contrast, only few reports are available on liposomes that show a 'built-in' antimicrobial effect by virtue of including antibacterial active compounds forming an

integral part of the self-assemblies structure without relying on conventional drug encapsulation. As an example, Huang *et al.* reported the development of liposomes composed of phosphatidyl choline, cholesterol and oleic acid [20]. At physiological pH the resulting anionic liposomes showed pronounced bactericidal activity against planktonic *S. aureus*. The effect on *S.aureus* biofilm was, however, not investigated.

The anti-microbial activity of quaternary ammonium compounds (QAC) such as cetyltrimethyl ammonium bromide (CTAB) and cetylpyridinium chloride (CPC) has long been recognised [21]. CPC is frequently used in the clinic and in consumer products as a disinfectant and topical antiseptic on the skin and mucosa [21, 22]. The positively charged head group and the lipophilic tail render the molecule amphiphilic facilitating the strong adsorption to the negatively charged bacterial surface [22]. The perturbation of the bacterial plasma membrane by the insertion of the hydrophobic tail initiates the gradual loss of membrane integrity leading to the leakage of metabolites critical for the survival of the bacteria which, ultimately, causes bacterial cell lysis [22].

Recently, it has been reported that equimolar amounts of cholesterol and CTAB form self-assemblies in water, termed "quatsomes" [23]. Such CTAB-quatsomes were prepared either by sonication or compressed fluid technology and were stable upon storage and dilution [23]. Similarly, equimolar mixtures of CPC and cholesterol have been shown to produce large unilamellar vesicles (LUV) [24, 25]. LUV were prepared by dissolving CPC and cholesterol in a benzene/methanol mixture followed by freeze-drying, rehydration and subsequent extrusion cycles. It is interesting to note that the individual compounds (sterol and QAC) used in these studies do not self-assemble to vesicles. In solution, the organisation of the sterol and QAC into supramolecular building blocks is thermodynamically favoured, facilitating the self-assembly of the vesicles [23, 25, 26]. Interestingly, the self-assembly is not limited to CTAB/cholesterol but was also observed for CTAB/β-sitosterol and myristyl trimethyl ammonium bromide (MTAB)/cholesterol.

In this study we synthesised and evaluated CPC-quatsomes as a potential treatment option for biofilm associated *S.aureus*. Quatsomes were prepared with different CPC concentrations by a solvent-free sonication step. The CPC-quatsomes were investigated for their antimicrobial effect on *S.aureus* biofilm and compared against conventional CPC micelle solutions. Additional toxicity studies provide guidance for the potential clinical application of CPC-quatsomes.

Materials and methods

Materials

Cetylpyridinium chloride (CPC, 99% purity), cholesterol (CHOL, 99% purity), sodium chloride (NaCl), phosphotungstic acid, and crystal violet (CV) were purchased from Sigma (Sigma-Aldrich, Sydney, NSW, Australia). Cerebrospinal fluid (CSF) was purchased from Thermo Fisher (Thermo Fisher Scientific, Scoresby, Australia) and Alamar Blue (AB) solution from Invitrogen (Invitrogen, Mulgrave, VIC, Australia). Lactate dehydrogenase (LDH) cytotoxicity assay kit was purchased from Roche (Roche Diagnostics, Indianapolis, IN, USA). Ultrapure water was obtained from a Milli-Q Direct water purification system (Millipore, Billerica, MA, USA). All chemicals were used as received unless stated otherwise.

Methods

Quatsomes preparation

Increasing amounts of CPC (final CPC concentration 0.5, 1, 2.5, 5, 10 and 20 mg/ml) and cholesterol were accurately weighed in clean screw top glass vials keeping the molar ratio of the two excipients constant at 1:1. Following the addition of 10 mL of Milli-Q water the mixture was placed in an ice bath and sonicated for 20 min (30/10 sec on/off cycle, 40% amplitude) using a QSonica probe sonicator (Newton, CT, USA). Dispersions were then stirred overnight on a magnetic stirring plate (400 rpm) at room temperature.

Preparation of micelle solutions

Formulations were prepared with the same nominal CPC concentrations as present in the CPC-quatsomes (i.e. 0.5 mg/ml to 20 mg/ml). The appropriate amounts of CPC were weighed in glass vials and dissolved in 10 ml of Milli-Q water and stirred overnight at room temperature.

Milli-Q water was used as the dispersion medium to avoid changes in the solubility and critical micelle concentration (CMC) of CPC associated with the presence of electrolytes that could adversely affect the formation of quatsomes [23, 27]. Following overnight equilibration

potential aggregates were removed from all formulations by filtration using a syringe/filter (0.45 μm pore size) assembly.

Characterisation of the colloids

Quantification of cetylpyridinium chloride

The concentration of CPC in quatsomes and micelle solutions before and after filtration was quantified by UV spectroscopy (Evolution 220, Thermo Fisher Scientific, Scoresby, VIC, Australia). The absorbance (measured at λ_{max} 259 nm) obtained for formulations after appropriate dilution in methanol was compared against a standard curve of CPC in methanol (1-100 μg/ml, R^2=0.9999).

Particle size and zeta-potential

The dispersions were diluted 1:10 with Milli-Q water (1:50 for 20 mg/ml CPC content) and the particle size and zeta potential were determined by dynamic light scattering (DLS) at 25ºC using a Zetasizer Nano-ZS particle sizer (Malvern, Worcestershire, UK). The mean hydrodynamic diameter of the colloids (reported as z-average [nm]) and the polydispersity index (PDI) were calculated from the intensity-weighed light scattering signal from three measurements using the instrument's built-in software (zetasizer software 6.34, Malvern). The zeta potential of the dispersions was determined from the same samples used for the DLS experiments by using the phase analysis light scattering (PALS) function of the nanosizer ZS. Based on the electrophoretic mobility of the colloids the mean zeta potential was computed.

Transmission electron microscopy

For transmission electron microscopy (TEM) one drop (2 μl) of freshly prepared quatsomes (0.5 mg/ml CPC) was placed on a Formvar/carbon-coated copper grid (Proscitech, Townsville, QLD, Australia) and allowed to adsorb to the grid for 1 minute. After excess fluid was removed from the grid by blotting with filter paper 2 μl of 2% phosphotungstic acid (adjusted to pH 7 with sodium hydroxide) was added to the grid for negative staining [28, 29]. Phosphotungstic acid was employed to enhance the contrast by adsorption of the tungstic anion to the positively charged quatsomes. Excess stain was removed after 1 minute and the dried grids were examined using a Jeol JEM-2100F transmission electron microscope (JEOL, Peabody, MA, USA) operated at a voltage of 200 kV.

Bacterial culture

Frozen stock of the reference strain *S. aureus* 25923ATCC (American Type Culture Collection, Manassas, VA, USA) were thawed and cultured for 24 hours on agar plates. Single bacterial colonies were transferred to sterile glass tubes containing 0.45% saline and the suspension adjusted to 1.0 ± 0.1 McFarland units. 1.2 ml of the suspension was diluted with 16.8 ml of CSF broth and 150 µl of the resulting mixture was used to inoculate individual wells of 96 well microplates for biofilm formation. For negative controls (containing no bacteria) 150 µl of plain CSF broth was added to the wells.

Minimal inhibitory concentration

The minimal inhibitory concentrations (MIC) of CPC as micelles and quatsomes were established by CSF broth dilution using standard protocols[30, 31]. Briefly, 96 well clear bottom Costar™ microplates were inoculated with an overnight culture of *S.aureus* (5 x 10^5 cfu/ml). Serial dilutions of the CPC formulations were carried out in the range of 50-0.2 µg/ml. A serial dilution of the antibiotic chloramphenicol (range 64-0.25 µg/ml) was included in each experiment as a control. Following 24 hours incubation at 37ºC the wells were visually inspected for bacterial growth. The lowest concentration at which no growth was visible was noted as the MIC.

Biofilm model

Biofilm of *S.aureus* was grown in flat 96 well clear bottom Costar™ microplates (Corning Incorporated, Corning NY, USA) at 37ºC for 48 hours on an gyratory shaker set to 70 rpm (Ratek Instruments, Boronia, VIC, Australia). The established biofilms were washed twice with sterile water (180 µl) to remove planktonic bacteria before they were exposed for 10 minutes to 180 µl of the treatments.

Viability of S.aureus biofilm

The viability of *S.aureus* in biofilm treated with CPC-quatsomes and CPC micelles was quantified in triplicate (typically 6 wells per plate and formulation) using the resazurin/resorufin (Alamar Blue, AB) assay [32]. Following treatments and a washing step (twice with 200 µl of sterile water to remove the formulations) the bacteria were allowed to recover for an additional 24 hours in 150 µl of CSF broth. The use of Milli-Q water in the

washing steps and as a solvent for the formulations was evaluated in preliminary experiments. *S. aureus* biofilms were exposed to Milli-Q water and 0.9% saline for up to one hour and showed no significant difference between Milli-Q water on *S. aureus* biofilms viability compared to saline. After the recovery step the biofilm was washed twice followed by the addition of 200 µl of Alamar Blue solution (10% AB in CSF) to each well. The plates were incubated on a rotating plate protected from light at 37ºC. The fluorescence of resorufin ($\lambda_{excitation}$ 530 nm/$\lambda_{emission}$ 590 nm, resulting from the bacterial metabolism of resazurin by viable cells) was analysed on a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany) after 30, 60, 90, 120, and 150 minutes [32]. Maximum fluorescence intensities were typically reached within 120 minutes and were used for quantification purposes. The viability was expressed as the percentage of biofilm killing (%BK) calculated from the background-corrected fluorescence intensity of the controls (I_C), i.e. biofilm not exposed to treatments, and the intensity of the treatments (I_T), according to Equation 1.

$$\% \text{ BK} = \frac{I_C - I_T}{I_C} \times 100\% \quad (1)$$

Biofilm mass

Biofilm was grown for 48 hours and treated as described in the previous section. After the 24 hour recovery step the biofilm were heat-fixed at 60ºC for 1 hour followed by staining with 180 µl of a 0.1% crystal violet solution for 15 minutes at room temperature for the quantification of total biofilm mass. Excess dye was removed by repeated rinsing (3 x) with sterile water after which the microplates were allowed to dry completely. The crystal violet stain was incubated for 15 minutes at room temperature with 200 µl of acetic acid (30% solution) to solubilise the dye before transferring the wells' content into a fresh microplate. The absorbance was measured at 595 nm in a plate reader (Model 680, Bio-Rad Laboratories, Hercules, CA, USA). According to Equation 2 the reduction in biofilm mass (%BMR) was calculated from the absorbance of background-corrected untreated controls (I_C) and the absorbance of the treatments (I_T),

$$\% \text{ BMR} = \frac{I_C - I_T}{I_C} \times 100\% \quad (2)$$

Confocal microscopy

In order to observe the interaction with *S.aureus* biofilm, CPC-quatsomes and CPC micelles were prepared containing 0.25% (w/w) of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes, Invitrogen, Willow Creek, OR, USA). This lipophilic dye has a strong affinity to membranes and shows an intense fluorescence [33]. The bacterial biofilm was grown on culture slides (BD Falcon, North Ryde, NSW, Australia) and was rinsed twice to remove planktonic cells. Following incubation with 300 µL of the DiI-labelled formulations for 10 minutes the biofilm was rinsed again and fixed with 300 µL of 5% glutaraldehyde (Sigma Aldrich, St Louis, MO, USA) for 30 minutes at room temperature [13].

Biofilm was stained using 300µL of a 5 µM solution of SYTO-9 (Invitrogen, Willow Creek, OR, USA) and incubated in the dark for 15 minutes at room temperature prior to examination by confocal scanning laser microscopy (Zeiss LSM700, Carl Zeiss, Oberkochen, Germany) with a 63×/1.4 objective and 0.5 µm laser scanning step size. The employed fixation/staining protocol was optimised in preliminary experiments, minimising the interference of the DiI and SYTO-9, and allowed the recording of the fluorescence at excitation/emission wavelengths of 555/570nm, and 488/520 nm, respectively.

Toxicity studies

The effect of CPC-quatsomes and CPC on the viability of human airway epithelial cells was determined in NuLi-1 cells (ATCC CRL-4011, Manassas, VA, USA) using the lactate dehydrogenase (LDH) assay by measuring the LDH leakage into the culture medium. Briefly, 1×10^4 cells were seeded in a volume of 100 µl per well of 96-well flat bottom plates (3×10^3 cells/mm^2) and allowed to adhere overnight. Cells were then exposed to different concentrations (0.05, 0.1, 0.25, 0.5 and 1%) of quatsomes and CPC for 10 minutes and 30 minutes, respectively. 100 µL of the media from each well was transferred to a new plate, and 100 µL of LDH reagent was added to the supernatant and incubated for 10 minutes in the dark at room temperature. The OD was measured at 490 nm on a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany).

Statistical analysis

GraphPad Prism version 6.03 for Windows (GraphPad Software, San Diego, CL, USA) was used for the statistical evaluation (Student's t-test, analysis of variance (ANOVA) followed by Holm-Sidak multi-comparisons test) of the results with a significance level of $\alpha = 0.05$.

Results and Discussion

Preparation and characterisation of quatsomes

Increasing amounts of cetylpyridinium chloride and cholesterol were used to generate a series of quatsomes that were characterized for size, polydispersity and zeta-potential (Table 1). The CPC content in all quatsomes and micelles solutions was in agreement with the theoretical values (>98% recovery, data not shown), indicating that the filtration step did not adversely affect the CPC content of the delivery systems. The CPC content of all delivery systems was above the reported CMC of 0.03 mg/ml for CPC [34].

Table 1. Hydrodynamic diameter, polydispersity index, and zeta-potential of quatsomes prepared in Milli-Q water with increasing content of cetylpyridinium chloride (CPC).

CPC content (mg/ml)	Z-average (nm)	Polydispersity (PDI)	Zeta-potential (mV)
0.5	99.9 ± 1.2	0.27 ± 0.07	70.1 ± 1.0
1	101.7 ± 2.0	0.29 ± 0.01	73.9 ± 1.3
5	98.3 ± 1.6	0.23 ± 0.02	74.5 ± 1.5
10	92.2 ± 1.9	0.28 ± 0.05	78.0 ± 1.8
20	95.6 ± 1.7	0.29 ± 0.09	75.6 ± 3.2

The increase of the CPC/CHOL concentrations was visually reflected in an increased turbidity of the dispersions, likely the result of the increasing number of light scattering quatsomes. At CPC concentrations of 20 mg/ml the packing density of quatsomes increased further to generate a viscous gel-like dispersion. It is interesting to note that upon dilution of

this concentrated dispersion in Milli-Q water, particles resulted with properties comparable to those generated at lower CPC/CHOL content (Table 1). Generally, the concentration of CPC/CHOL had little effect on the size (approximately 90-100 nm) of the CPC-quatsomes. The dispersions showed a narrow particle size distribution (PDI < 0.3) and quatsomes had a positive zeta-potential (+ 70-80 mV), in agreement with the presence of quaternary ammonium cpompound. Compared to quatsomes, the CPC micelles were relatively small (< 30 nm) independent of CPC concentrations (data not shown) which is in line with previous reports [24].

The size and size distribution of the CPC-quatsomes is in agreement with those reported previously for CTAB-quatsomes prepared under similar conditions [23]. Compared to the LUVs obtained by the lipid-film hydration/extrusion method the current sonication protocol gave rise to slightly smaller particles (approximately 100 nm *vs* 120 nm) which might be attributed to the different dispersion media used in the two studies [24]. While Milli-Q water was used in the current study, the presence of electrolytes (phosphate buffer was used for the LUV preparation [24]) affects the microenvironment of cationic surfactants [27, 35]. In the case of the self-assembly of quatsomes, electrolytes screen the positively charged head groups of CPC thereby influencing the geometry of individual amphiphile molecules required for the optimal self-assembly with cholesterol. In fact, when CPC-quatsomes were prepared using 0.9% saline in preliminary experiments the resulting dispersions were rather heterogeneous (PDI > 0.6) with larger particle size (> 130 nm).

Negatively stained samples of quatsomes were investigated under TEM. While this technique allows relatively easy and fast sample investigation, the original morphology of vesicular structures might not be conserved due to the necessary drying, application of ultra-high vacuum and interaction with the stain. In our hands quatsomes appeared as distorted vesicles with multilamellar structures (Fig. 1). These artefacts have been described previously using similar sample preparation protocols for liposomes [29, 36]. Nevertheless the TEM images provided insights in the vesicular nature of quatsomes.

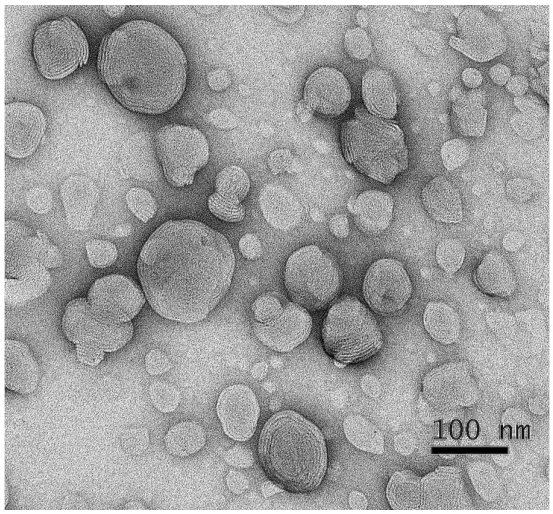

Fig. 1. Transmission electron micrograph of quatsomes (5 mg/ml CPC) after negative staining with 2% phosphotungstic acid**.**

Activity against planktonic bacteria

The minimal inhibitory concentration (MIC) for the control chloramphenicol was 8 µg/ml which is in agreement with published data (range 2-16 µg/ml) [31]. The MIC for CPC were 0.8 µg/ml (micelles) and 1.6 µg/ml (quatsomes), indicating only a slight difference in the susceptibility of planktonic *S.aureus* towards the two structurally different self-assembly types of CPC.

Anti-biofilm activity

The activity of quatsomes containing increasing amounts of CPC against *S.aureus* biofilm was evaluated and compared with micelle solutions of identical CPC concentrations. Due to the gel-like appearance of 2% CPC-quatsomes these systems were not considered for further evaluation in anti-biofilm activity. The influence of the CPC concentration on the viability (expressed as percentage of killed bacteria) of quatsomes was assessed using the Alamar Blue assay and is presented in Fig. 2. CPC micelles showed high efficacy against biofilm at relatively low concentrations (approximately 80-90% of *S.aureus* was killed at a CPC concentration of 0.05% and 0.1%). Further increase in the CPC concentration resulted in almost complete killing of the bacteria (≥ 98% above 0.25% CPC concentration). The bactericidal effect of quatsomes was dose-dependent, with a maximum of more than 99% biofilm killing with CPC concentrations of 0.5%. At these and higher CPC concentrations there was no significant difference between the biofilm killing activity of quatsomes and CPC

micelles ($P > 0.05$). At CPC concentrations lower than 0.5% the bactericidal effect of quatsomes was significantly reduced (approximately 35-60% killing) compared to the corresponding CPC micelles ($P < 0.05$).

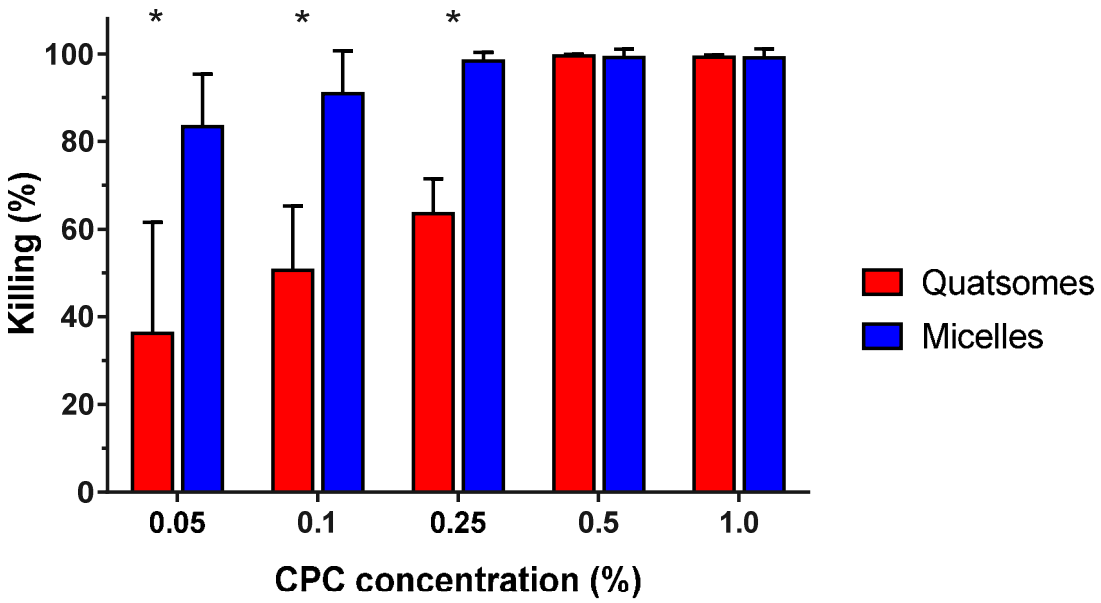

Fig. 2. The effect on *S.aureus* viability of increasing concentrations of cetylpyridinium chloride (CPC). *S.aureus* biofilm were treated with CPC quatsomes and CPC micelles for 10 minutes. The cell viability was quantified by the Alamar Blue assay. Results are shown as mean ± SD from three independent measurements with n=6. Asterisks indicate statistical differences between quatsomes and micelle treatments obtained from ANOVA/ Holm-Sidak multi-comparisons test, *$P < 0.05$.

While the Alamar Blue assay quantifies live, metabolising bacteria, crystal violet also stains extracellular matrix components that account for the majority of total biofilm mass [37]. Compared to the Alamar Blue assay the quantification of biofilm with crystal violet revealed a similar trend regarding the anti-biofilm activity of quatsomes and micelles (Fig. 3). The CPC micelles showed substantial reduction (> 80%) of the biofilm mass from 0.05% CPC. In contrast, a 40% reduction in biofilm mass by the corresponding quatsomes was observed along with a considerable variation between individual biofilms. At 0.5% CPC content of quatsomes the biofilm reduction increased to 70% and further to 85% for 1% CPC quatsomes which was comparable to the effects of the corresponding CPC micelle solutions ($P > 0.05$).

It has been shown that the bactericidal effect of CPC and other QAC is attributed to their insertion into cell membranes leading to cell leakage and, ultimately, cell death [21]. Mechanistically the bactericidal effect of QAC is driven both by electrostatic and

hydrophobic interactions. The positively charged head group of QAC can interact with anionic structures present on the surface of many bacterial biofilms (e.g. teichoic acid) while the hydrophobic tail intercalates into the cell membrane [38].

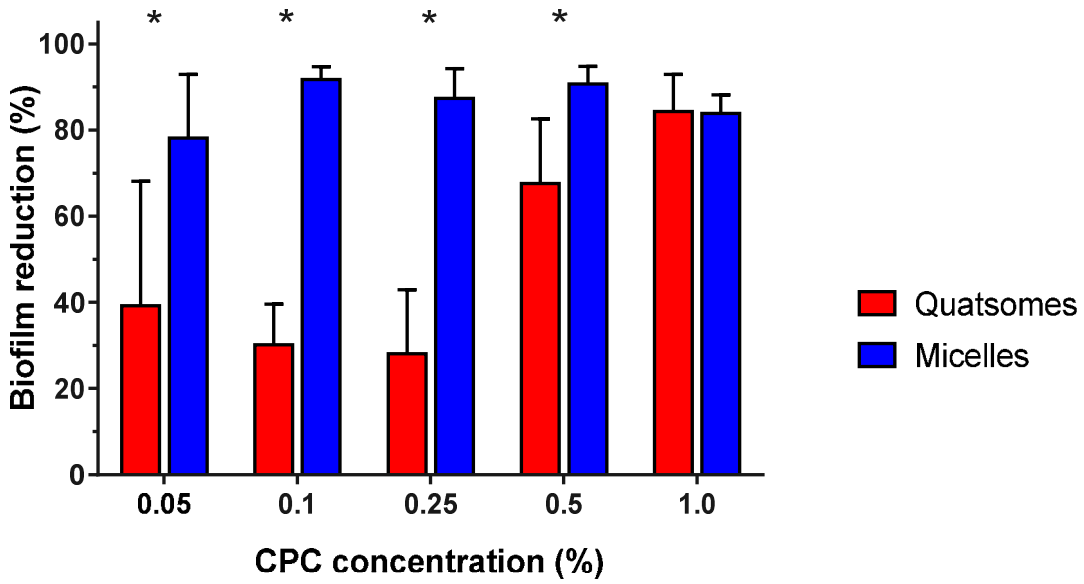

Fig. 3. The reduction in *S.aureus* biofilm mass following 10 minutes treatment with quatsomes and micelles with increasing concentrations of cetylpyridinium chloride (CPC). Biofilm mass was quantified by crystal violet staining. Results are shown as mean ± SD from three independent measurements with n=6. Asterisks indicate statistical differences between quatsomes and micelle treatments obtained from ANOVA/ Holm-Sidak multi-comparisons test, $*P < 0.05$.

The interaction of CPC micelles and CPC-containing liposomes with *Streptococcus mutans* (*S.mutans*) biofilm was studied by Cottenye *et al.* [24]. The authors reported stronger interaction of CPC micelles with the bacteria compared to cationic liposomes which corresponded to the degree of biofilm killing: following 30 minutes exposure of 24 hour *S.mutans* biofilm to treatments prepared at CPC concentrations of approximately 2%, the CPC micelles and CPC-liposomes killed biofilm-associated *S.mutans* to 80% and 50%, respectively. In the current study a similar trend was observed for the anti-biofilm efficacy, i.e. stronger anti-biofilm effect of micelles compared to CPC-quatsomes which might be attributed to steric hindrance of the larger quatsomes impairing penetration into the biofilm which has been reported previously for larger solutes and nanoparticles [39, 40]. Importantly, while only approximately 50% of *S.mutans* biofilms were eradicated at 2% CPC content [24] the current study indicated that *S.aureus* biofilms were considerably more susceptible towards CPC as almost all bacterial cells in 48 hours *S.aureus* biofilms were killed at CPC concentration of 0.5% after

10 minutes treatment time. The different susceptibilities of the two biofilms species might be attributed to a different matrix ultrastructure or cell surface properties as previously suggested[41] and warrants further studies.

Similarly to conventional antibiotics, the susceptibility of *S.aureus* biofilms towards CPC was considerably reduced, requiring higher CPC concentrations (up to 5 mg/ml) to eradicate *S.aureus* biofilms compared to the susceptibility of planktonic *S.aureus* (MIC 0.8 µg/ml and 1.6 µg/ml, respectively). The decreased susceptibility of biofilms towards antimicrobials has been commonly ascribed to limited diffusion and physicochemical interactions between antimicrobials and the matrix components[5, 42].

The anti-biofilm efficacies of the different formulations were reflected in the confocal scanning laser micrographs (Fig. 4, horizontal and vertical bars). In agreement with previous studies, it was observed that the positive charge of CPC facilitated the electrostatic interaction of micelles and quatsomes with the biofilm, which was visible by the close proximity of the red and green labelled formulations and bacteria, respectively [40]. At low CPC micellar concentrations (0.05%, upper panels, Fig. 4) the biofilm thickness appeared reduced compared to the CPC-quatsomes containing the same concentrations of CPC. At elevated CPC concentrations (0.5%, lower panels, Fig. 4) the biofilm thicknesses appeared further reduced both for micelle and quatsome-treated samples. In line with the observations of Cottenye *et al*. micelles and quatsomes were not distributed evenly throughout the biofilm [24]. While CPC micelles appeared to have access to the entire biofilm both at low and high CPC concentrations, the quatsomes were mostly found at the surface of the biofilm at low CPC concentrations. At elevated CPC concentrations the presence of quatsomes was evident mainly at the rims of the biofilms while deeper penetration into the biofilms appeared limited.

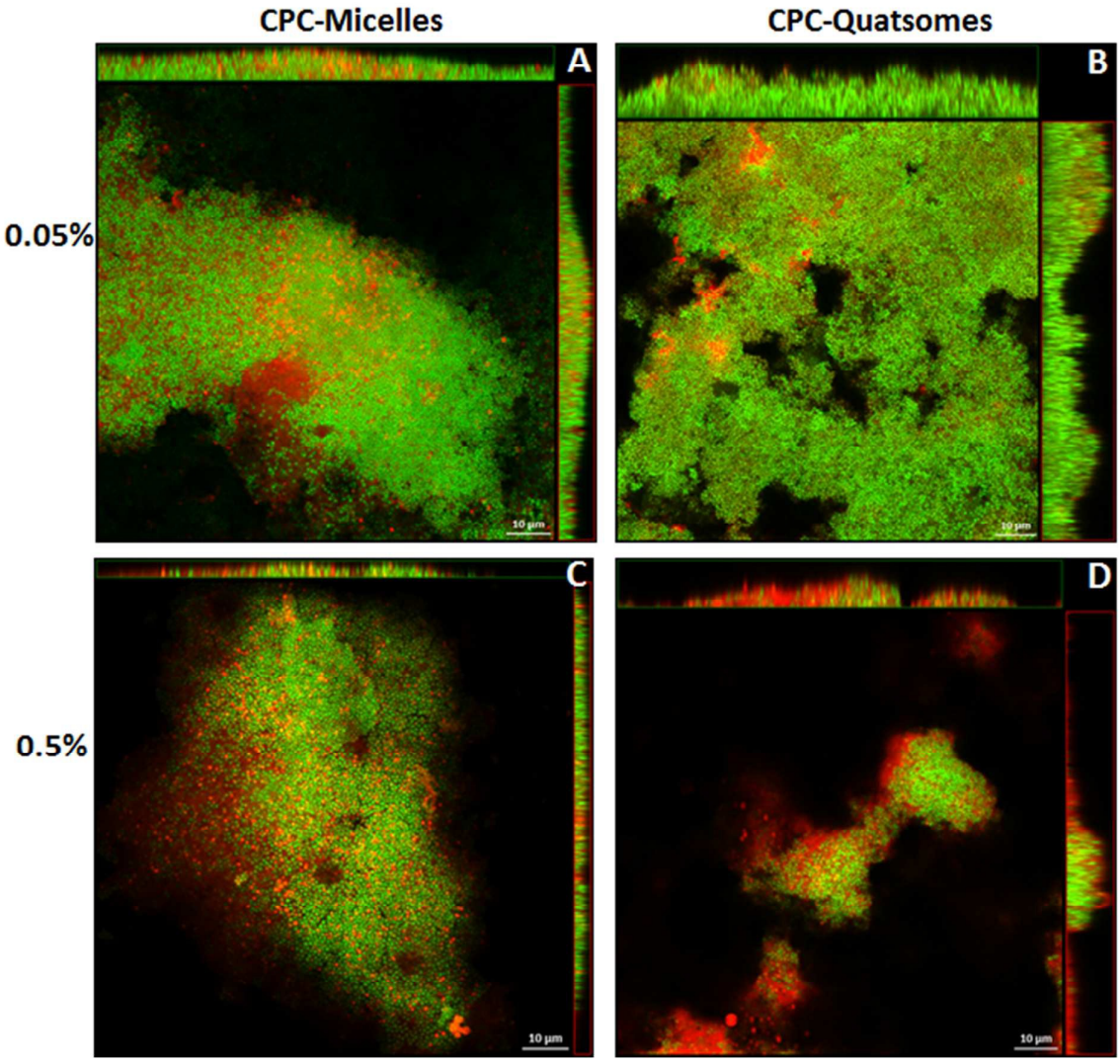

Fig. 4. Confocal laser scanning microscopy images of 48 hours *S.aureus* biofilm treated 10 minutes with cetylpyridinium chloride (CPC) micelles (A, C) and CPC-quatsomes (B, D) containing 0.05% (upper panels), and 0.5% (lower panels) CPC. Micelles and quatsome are stained red (DiI), *S.aureus* green (SYTO-9). The large centre images represent the X-Y view of the central layer from the z-stack; the small horizontal bars at the top of each image represent the X-Z view, the small vertical bars to the right show the Y-Z view of identical biofilms.

Epithelial cell toxicity

The concentration of CPC is currently limited to 0.1% (as micelles) for use in oral products due to safety concerns, although higher concentrations of up to 1% have been utilised for the treatment of meat products to control pathogen contamination [43, 44]. It should be noted that these limits refer to the micellar solutions of CPC. We hypothesised that the toxicity of CPC administered as quatsomes is reduced due its supramolecular assembly with equimolar

amounts of cholesterol. Interestingly, neither quatsomes nor CPC showed a significant toxic effect on the human airway epithelial (NuLi-1) cell line after 10 minutes at all CPC concentrations ($P < 0.05$), whereas the exposure to a solution of 3% Triton X-100 demonstrated significant cell damage (Fig. 5) compared to negative controls. In addition, the prolonged exposure (up to 30 minutes) of the cells to identical concentrations of CPC (as quatsomes and micelles) showed no detectable effect on cell viability (data not shown). Based on these results it appears that the toxicity of CPC is independent of its assembly type (i.e. in micelles or quatsomes) but rather compound specific.

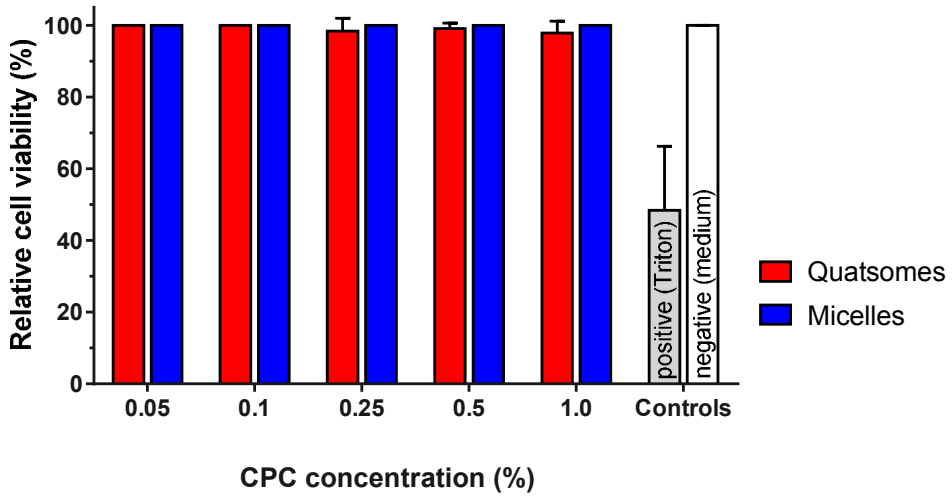

Fig. 5. Cell viability monitored by the LDH assay after 10 minutes treatment of human airway epithelial (Nuli-1) cells with various concentrations of cetylpyridinium chloride (CPC) and quatsomes. 3% Triton X-100 and media were used as positive and negative control, respectively. All values are shown as means ± SD, n = 5.

Given the recalcitrance and severity of many *S.aureus*-associated diseases such as pneumonia, endocarditis, chronic rhinosinusitis (CRS), and wound infections, novel efficient and safe treatment options are urgently needed. The results of the present study are encouraging, in particular for the use of quatsomes in topical treatments such as CRS. Although the anti-biofilm activity of quatsomes was less pronounced compared to micelles of identical CPC concentrations, this study opens promising avenues towards the development of new therapeutic formulations with multiple active pathways. More specifically, it can be hypothesized that advanced quatsome–based drug delivery systems might be advantageous since both hydrophilic and hydrophobic antibiotics can be the encapsulated in quatsomes -

analogous to conventional liposomal drug delivery- with potential synergistic anti-biofilm performance.

Conclusion

The present study has demonstrated the feasibility of quatsome preparation from equimolar ratios of cholesterol and cetylpyridinium chloride by a simple dispersion method. The anti-biofilm effect of CPC-quatsomes was confirmed in an *in vitro S.aureus* biofilm model. Compared to conventional CPC micelles, the anti-biofilm activity required a higher CPC concentration while demonstrating negligible cell toxicity at all CPC concentrations. CPC/CHOL quatsomes therefore appear a feasible, effective, and economic alternative for the treatment of *S.aureus* biofilm. Quatsomes bear great potential as nanovectors for the delivery of both hydrophilic and lipophilic drugs, similar to liposomes, but with the additional "built-in" antibacterial effects of the nanovectors .

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Graphical abstract

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