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Enzyme responsive copolymer micelles enhance the anti-biofilm efficacy of the antiseptic chlorhexidine

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ABSTRACT

Staphylococcal biofilms cause many infectious diseases and are highly tolerant to the effects of antimicrobials; this is partly due to the biofilm matrix, which acts as a physical barrier retarding the penetration and reducing susceptibility to antimicrobials, thereby decreasing successful treatment outcomes. In this study, both single and mixed micellar systems based on poly vinyl caprolactam (PCL)-polyethylene glycol (PEG) copolymers were optimised for delivery of chlorhexidine (CHX) to S. aureus, MRSA and S. epidermidis biofilms and evaluated for their toxicity using Caenorhabditis elegans. The respective polyethylene glycol (PEG) and poly vinyl caprolactam (PCL) structural components promoted stealth properties and enzymatic responsive release of CHX inside biofilms, leading to significantly enhanced penetration (56%) compared with free CHX and improving the efficacy against Staphylococcus aureus biofilms grown on an artificial dermis (2.4 log reduction of CFU). Mixing Soluplus-based micelles with Solutol further enhanced the CHX penetration (71%) and promoted maximum reduction in biofilm biomass (> 60%). Nematodes-based toxicity assay showed micelles with no lethal effects as indicated by their high survival rate (100%) after 72 h exposure. This study thus demonstrated that bio-responsive carriers can be designed to deliver a poorly water-soluble antimicrobial agent and advance the control of biofilm associated infections.

Keywords

Biofilm; penetration; nanoparticles; Micelles; Efficacy

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1. Introduction

Tolerance and resistance to antimicrobials are the most important features of microbial biofilms preventing successful infection treatment (Brauner et al., 2016). Skin infections, chronic wound infections, surgical-site infections, and biomaterial-associated infections are frequently caused by bacterial biofilms formed by *Staphylococcus aureus* (*S. aureus*) and *Staphylococcus epidermidis* (*S. epidermidis*) (Costerton, 1999; Fux et al., 2003; Gomes et al., 2014; Høiby et al., 2015; Otto, 2008). These surface attached bacteria organise themselves within a matrix of self-produced extra-polymeric substances (EPS) that increases tolerance towards antimicrobials and other environmental stresses compared to the non-attached planktonic cells (Bridier et al., 2011; de la Fuente-Núñez et al., 2013; Mah and O'toole, 2001; Xavier and Foster, 2007). The EPS matrix acts as a physicochemical barrier affecting penetration of a biocidal by electrostatic, hydrophilic or enzymatic interactions ultimately promoting resistance due to potential for sublethal levels of antimicrobials (Albayaty et al., 2018; Flemming et al., 2016; Jefferson et al., 2005). For instance, sub-inhibitory concentrations of several antibiotics induced an increased expression of polysaccharide intercellular adhesin (PIA), a virulence component of *S. epidermidis* biofilm matrix (Rachid et al., 2000).

Antimicrobial diffusion through biofilms is affected by its maturity, matrix thickness and composition, physicochemical properties of an agent as well as dose and duration of exposure (Billings et al., 2015; Estela and Alejandro, 2012). For example, the limited penetration of the positively charged aminoglycoside tobramycin through *P. aeruginosa* biofilms has been attributed to the interaction with biofilm components such as negatively charged alginate and cDNA (Tseng et al., 2013).

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The recalcitrance of biofilm-related infections increases the demand for antiseptics and disinfectants to decolonise solid surfaces where Staphylococci typically adhere (Koljalg et al., 2002; Tomic et al., 2004). Chlorhexidine (CHX) is a commonly used topical antiseptic agent in hospitals and in the community to prevent and control transmission of pathogens including methicillin resistant *Staphylococcus aureus* (MRSA) (Bonez et al., 2013; McDonnell and Russell, 1999).

Since 1954 when CHX was described for the first time, its antimicrobial activity has been well documented against a range of microorganisms (Davies et al., 1954). By displacing divalent cations, CHX causes defects in the bacterial membrane ultimately leading to the loss of cell viability (Gilbert and Moore, 2005). The free base of CHX is characterised by low water solubility (0.08 mg mL^{-1}at 20°C) and is cationic in charge (*pKa*= 11.73); this significantly interferes with its efficacy against microbial biofilms (Albayaty et al., 2018; Block, 2001; Jeffrey, 1995; Meiering et al., 2005). These properties of CHX base explain the use of CHX soluble salt forms in numerous studies rather than its non-soluble free base (Scholz et al., 2017; Teixeira et al., 2015; Vestby and Nesse, 2015; Yun et al., 2014). However, CHX base is important and responsible for antiseptic activity against pathogens. Thus, it is necessary to establish improvements in the efficacy of CHX against biofilms and this can be achieved by using a novel drug delivery system (Barbour et al., 2013; Lboutounne et al., 2002; Wang et al., 2015).

The graft copolymer polyvinyl caprolactam (PCL)–polyvinyl acetate (PVA)–polyethylene glycol (PEG) Soluplus® (Sol) forms polymeric micelles (PMs) that have been successfully employed as carriers for drugs with low water solubility (Dian et al., 2014; Linn et al., 2012). This amphiphilic polymer has been used alone or in combination with other polymers to improve the solubility of poorly water-soluble agents. For example, Sol-based micelles have been shown to improve the solubility of quercetin, clarithromycin, carvedilol,

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osthole, and itraconazole (Dian et al., 2014; Shamma and Basha, 2013; Takahashi et al., 2017; Yun et al., 2014; Zhang et al., 2013). Moreover, Sol has been blended with Solutol (ST) to form mixed micelles, that have been shown to improve lornoxicam's solubility and therapeutic efficacy (Bhuptani et al., 2016). Recently, it was demonstrated that Sol exerts antibacterial effects against *S. epidermidis* biofilms and it was shown that it has significant binding ability to the bacterial biofilm, which enhances antibiofilm activity (Takahashi et al., 2015). Interestingly, the structure of Sol reveals the presence of a biodegradable caprolactam ring which is susceptible to lipases/esterases produced by important pathogens such as *S. aureus*, *P. aeruginosa* and *Candida spp*.(Elsässer et al., 2013; Novotný et al., 2015). The degradation of the nanocarriers by bacterial lipases is hypothesised to facilitate a targeted release of antimicrobials within biofilms. To the best of our knowledge there are no reports describing the use of Sol/ST as a carrier for CHX free base and evaluating its efficacy against bacterial biofilms. Consequently, in this study polymeric micelle carriers based on Sol and ST were developed, characterised and evaluated for their toxicity using a nematode-based whole animal model assay. The efficacy of CHX solubilised in micelles was investigated in vitro using different planktonic (i.e. MIC and MBC) and antibiofilm susceptibility assays (i.e. CV assay, colony biofilm assay, and 3D artificial dermis assay) of *Staphylococci species.*

2. Materials and methods

2.1 Materials

Soluplus® was kindly supplied by BASF (Ludwigshafen, Germany), Solutol HS 15 (Polyethylene glycol 15-hydroxystearate) (ST) was purchased from Sigma-Aldrich, Munich, Germany. Chlorhexidine (CHX) and chlorhexidine digluconate (CHXG) were purchased from Sigma-Aldrich, Castle Hill, NSW, Australia. Organic solvents including acetone and methanol were procured from Merck, Darmstadt, Germany. Tryptic soy broth (TSB), tryptic

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soy agar (TSA), phosphate-buffer saline (PBS) and dimethyl sulphoxide (DMSO) were purchased from BD Biosciences, North Ryde, NSW, Australia. Hyaluronic acid (1.20–1.80 MDa) and collagen were from Lifecore Biomedical, Chaska, MN and Corning Incorporated, Corning, NY, respectively. Milli-Q grad water purified through a Direct Water purification system (Millipore, Massachusetts, USA) was used throughout this study.

2.2 Preparation of CHX-loaded micelles.

Polymeric micelles based on Soluplus®, Solutol and Sol:ST were prepared using a modified emulsion solvent diffusion (ESD) method to form CHX-loaded Sol (CHX-Sol), CHX-loaded ST (CHX-ST) and mixed CHX-loaded micelles (CHX-Sol:ST), respectively (Takahashi et al., 2015; Wegmann et al., 2017). Briefly, the polymers (Sol and ST, 10 mg mL^{-1}) along with CHX (1 mg mL^{-1}) were dissolved in 1 mL acetone, followed by dispersion of the organic solution into aqueous phase (Milli-Q water). The emulsion was stirred at 100 rpm for 24 h at room temperature to remove the organic solvent from the mixture by evaporation. The resulting micellar systems were centrifuged at 4499 × g (Haake Z36HK, Wehingen, Germany) for 15 min and filtered to remove any aggregates using 0.45 µm filter membrane (Filtropur S 0.45, Sarstedt Technology Park, SA, Australia). The micellar solution was kept at 4°C until further studies. Blank micelles were also prepared using the same method but without CHX. The resulting three different blank micelles are abbreviated as following B-Sol, B-ST and B-Sol:ST.

2.3 Optimization of polymeric micelle synthesis

During the preparation of CHX-loaded micelles, the effects of CHX concentrations, solvent volumes and different weight ratios of polymers (Sol/ST) were investigated and

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optimised to produce small, uniform micelles with high drug load and encapsulation efficiency.

2.3.1 Effect of CHX and Soluplus concentration

The following preparation parameters were evaluated for their effect on particle size, drug load (DL%) and encapsulation efficiency (EE%) of polymeric micelles: CHX concentration (i.e. 0.5, 1 and 2) mg mL^{-1} and Sol concentration (i.e. 10, 20 and 30) mg mL^{-1} while the magnetic stirrer speed, stirring time and aqueous as well as organic solvent ratio were kept constant.

2.3.2 Effect of organic solvent

Different organic solvents (acetone, methanol, ethanol, chloroform) were used to prepare polymeric micelles while the remaining parameters were kept constant. The desired organic solvent was further used in different volumes (0. 1, 1.5, 2. 2.5, 3) mL and its effect on developing CHX-loaded Sol micelles was determined.

2.3.3 Effect of the Sol:ST ratio

Soluplus® and Solutol HS 15 of different ratios (1:0, 1:0.5, 1:1, 1:1.5, 1:2 and 0:1) (wt:wt) (Sol:ST) and CHX were co-dissolved in acetone, followed by dispersion into Milli-Q water to form mixed micelles, while the other optimised preparation parameters were kept constant.

2.4 Physicochemical characterisation

2.4.1 Particle size and size distribution

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The mean particle size, and size distribution (PDI) of the prepared micellar systems were measured using a Malvern Zetasizer NanoZS90 (Malvern Instruments, Worcestershire, UK). The mean particle size of three independent replicates are reported as z-average.

2.4.2 Drug loading and encapsulation efficiency

Chlorhexidine-loaded polymeric micelles were dissolved in methanol and the amount of encapsulated CHX was determined by using UV/VIS spectroscopy at 255 nm against an established standard curve of known CHX concentrations (1-20 μg mL^{-1}). The absorbance of a methanolic solution of blank micelles was determined at 255 nm.

2.4.3 In vitro release study

Chlorhexidine release profiles from both Soluplus micelles and Soluplus:Solutol mixed micelles were determined using the dialysis bag method as described previously (Liu et al., 2016; Zhou et al., 2016). Briefly, a dialysis bag (molecular weight cut-off: 12−14 kDa) was loaded with 2 mL of a freshly prepared CHX-loaded micellar system and subsequently immersed in 20 ml of acidified Milli-Q water (pH 5.5) and 10 mM PBS (pH 7.4) and kept in a shaker incubator (100 rpm) at 37°C. At specific sampling points (every 10 min for the first 10 samples and then every 30 min for the next 4 samples and every hour for the remaining time up to 400 min), 1 mL of dialysis medium was collected and replaced with the same volume of fresh medium to maintain sink conditions during the experiment.(Musial et al., 2012) The concentration of CHX in collected samples was quantified by UV/VIS spectroscopy as described above (Farkas et al., 2007). The effect of bacterial lipase on CHX release was studied by incubation of the micellar systems with 1 mg mL^{-1} of *Pseudomonas* lipase (Sigma-Aldrich, St. Louis, MO, USA) in the dialysis bag.(Xiong et al., 2012)

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2.4.4 Stability study

The polymeric micelle solutions were stored in plastic vials at 4°C. Particle sizes and encapsulation efficiencies were assessed weekly over 8 weeks and then every month for up to 6 months and compared with freshly prepared formulations (Lin et al., 2003).

2.5 Microbiology

2.5.1. Bacterial strains

Biofilm forming *S. aureus* (ATCC 25923), MRSA (ATCC 335911) and *S. epidermidis* (ATCC 14990) were used in this study. These strains were kept as frozen stock at -80°C and cultured on TSA. Planktonic overnight cultures were grown in TSB at 37°C for 16-18 hours. The overnight culture was subsequently diluted with PBS to an absorbance at 600 nm (OD_{600} = 0.2) (corresponding to approximately 3×10^8 CFU. mL^{-1}) using a UV/VIS spectrophotometer (Jenway 6305, John Morris, Wayville, SA, Australia) and used as inoculum to form bacterial biofilms.

2.5.2 Planktonic susceptibility test

The lowest concentration that showed no visible growth of microorganisms (minimum inhibitory concentration, MIC) was determined for each sample of CHX and blank micelles (B-Sol, B-ST, B-Sol:ST) after 24 h incubation using the broth microdilution method described previously (Wiegand et al., 2008). Briefly, stock solutions of CHX were prepared (12.5 – 12,800) µg mL^{-1} with DMSO followed by dilution (1/100) with TSB to a concentration range of (0.125 – 128) µg mL^{-1} (Rodriguez-Tudela et al., 2003). Blank micelles, B-Sol, B-St and B-Sol:ST were prepared at 3,000-100,000 µg mL^{-1}, 100-20,000 µg mL^{-1} and 3,000-20,000 µg mL^{-1} with TSB, respectively. 0.1 mL of each formulation was added to the wells of a sterile microtiter plate, followed by inoculation of each well with 0.1

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mL of bacterial suspension containing 10^5 CFU mL^{-1}. Subsequently, the plate was incubated at 37°C for 24 h. To determine the minimum biocidal concentration (MBC), 0.01 mL of each sample with no visible growth in the MIC plate was cultured on a fresh TSA plate and incubated at 37°C for 24 h and observed for microorganism growth. The assay was performed in triplicate.

2.5.3 Biofilm susceptibility test

The minimum biofilm eradication concentration (MBEC) (e.g. the concentration at which biofilm-embedded bacteria are killed) was determined using a previously described method with minor modification (Dosler and Mataraci, 2013; Hasan et al., 2017). Briefly, 200 µl of bacterial suspension containing 1 × 10^8 CFU mL^{-1} in TSB was transferred to 96-well plates and incubated at 37°C for 24 h using an orbital shaker at 40 rpm to grow biofilms. Following the removal of non-adherent cells by gentle washing with sterile PBS, CHX and blank micelles were added to the washed wells. After further incubation for 24 h at 37°C, the liquids were gently removed, rinsed with PBS, and refilled with 200 µL TSB. The wells were carefully scraped and vigorously pipetted to detach and disperse biofilms from the well surface. The MBEC was identified by observing the lowest concentration at which no bacteria grow on an agar plate that was inoculated with 100 µL of samples. The assay was performed in duplicate.

2.5.4 Biofilm biomass assay

The effect of CHX-loaded polymeric micelles on the total biofilm biomass was determined and compared with free CHX using the crystal violet assay (Thomas et al., 2015). Briefly, 200 µL of bacteria suspension (*S. aureus* or *S. epidermidis*) previously adjusted to ~3×10^8 CFU mL^{-1} and diluted with TSB to ~ 10^6 CFU mL^{-1} was added to a sterile 96 – well

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plate and incubated at 37°C for 24 h with continuous shaking to grow biofilms. Next, non-adherent cells were removed by washing with PBS and the remaining biofilm was exposed to the test formulations. Following incubation for 24 h at 37°C, the microtiter plates were washed twice with PBS and dried for 1 h at 60°C. The remaining dried biofilm layer was stained with 150 µL of crystal violet (CV 0.1%) and incubated for 20 min at room temperature. The plates were washed and then air dried at room temperature. 150 µl of 33% acetic acid was added to solubilize the dye before quantification by a microtiter-plate reader at 595 nm (Bio-Rad, Hercules, CA, USA). The experiment was performed in triplicates.

2.5.5 Colony biofilm assay

The colony biofilm assay was performed to study the effect of CHX and CHX-loaded polymeric micelles on bacterial biofilms grown on polycarbonate membrane filters (0.22 µm) (Thermo Fisher Scientific, Massachusetts, USA) on TSA plates (Anderl et al., 2000; Merritt et al., 2005). 30 µl of overnight cultures of *S. aureus*, MRSA and *S. epidermidis* bacteria (~10^8 CFU ml^{-1}) were separately used to inoculate sterile membrane, followed by 24 h incubation at 37°C on TSA agar plates. The growing biofilms were exposure to the free CHX and CHX-loaded micelles. After 24 h incubation at 37°C, bacteria were extracted from membranes by immersion in fresh TSB medium and alternating sonication and vortex mixing for 10 min, followed by serial dilution in 0.9 % saline. The diluted samples were spotted (10 µL) on TSA plates and after 24 h incubation at 37°C, the viable cells (CFU) were enumerated (Harriott and Noverr, 2009). The experiment was performed in triplicates

2.5.6 Biofilms penetration to CHX

The extent of CHX penetration through all Staphylococcal biofilms was evaluated using the method described by Souli and Giamarellou (Souli and Giamarellou, 1998). Briefly,

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biofilms of *S. aureus* and *S. epidermidis* were grown on a filter membrane substrate (0.2 µm pore size; 25 mm, diameter) placed on TSA for 48 h at 37°C. The biofilms were harvested using a sterile loop. The harvested biofilms were then mixed with CHX-loaded polymeric micelles and transferred into punched holes of TSA agar plates seeded with Staphylococci (~ 10^6 CFU mL^{-1}). A chlorhexidine solution in 1% DMSO served as control. The ability of CHX to penetrate biofilms was quantified by measuring the inhibition zones after 24 h incubation. The experiment was performed in triplicate.

2.5.7 Antibiofilm study using 3D artificial dermis model

A mixture of hyaluronic acid (1.20–1.80 MDa, Lifecore Biomedical, Chaska, MN, USA) and collagen (Corning Incorporated, Corning, NY, USA) was used to prepare an artificial dermis following a previously described method (Richter et al., 2017b). The prepared dermis was nourished with a mixture of lyophilized bovine plasma (Sigma, St. Louis, MO, USA), Bolton broth (19 mL), horse blood (1 mL), and heparin (10 IU) and then infected with 10 µl of *Staphylococci* overnight cultures diluted to 1×10^6 CFU mL^{-1}. Following 24 h incubation at 37°C, the grown biofilms were treated with 100 µL of free CHX, CHX-loaded micelles, and blank micelles. Subsequently, the treated biofilms were transferred into 10 mL of 0.9 % saline and detached from the dermis using alternating cycles vortexing and sonication (3 × 30 s). The dispersed biofilms were serially diluted followed by CFU enumeration on TSA. The assay was performed in duplicate.

2.5.8 Distribution of micelles within Staphylococcal biofilms

To study the distribution of the micelles within bacterial biofilms, micelles were loaded with the fluorescent dye Nile red at the same ratio as used for CHX (1:10 (wt:wt), (dye:polymer) using a Nile red stock solution in acetone (5mg. mL^{-1}). Staphylococcal

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biofilms were grown at 37°C for 48 h in an 8-well chamber slide using 300 µL of above prepared inoculum (~3 × 10 ^{8} CFU. mL–1). Following washing with physiological saline (NaCl 0.9%) to remove loosely attached bacteria from the surface of biofilms. Washed biofilms were incubated with Nile red-loaded micelles for 3 h at 37°C. After exposure, biofilms were washed three times with NaCl 0.9%, fixed with 6% formaldehyde and stained for 30 min with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St Louis, MO, USA). The stained biofilms were subjected to three washing steps to remove excess dye. The samples were subsequently analysed by a Zeiss LSM 700 confocal scanning laser microscope (Zeiss, Thornwood, NY, USA) equipped with a 63x-oil immersion objective, using excitation/emission wavelengths of 358/461 nm and 552/636 nm for DAPI and Nile red, respectively. Confocal images were acquired and processed using ZEN 2009 software.

2.5.9 *Toxicity study*

Caenorhabditis elegans AU37 (glp-4; sek-1) were used as a whole animal model to investigate the uptake and toxicity of the developed micelles. The nematodes were maintained and synchronized using previously described method (Richter et al., 2017a; Tampakakis et al., 2008). The study was carried out using equivalent aged worms (L4 stage) generated from synchronized nematodes. The worms were transferred into nematode growth medium broth (M9 buffer (95%), brain heart infusion broth (5%) supplemented with cholesterol (10 µg mL^{-1})). Both Nile red loaded Soluplus and Nile red loaded Soluplus:Solutol micelles at three different concentrations (i.e. 5, 10 and 20 mg mL^{-1}) were evaluated for their toxicity. Each well of a 96-well plate was loaded with 75 µL of test solution and 25 µL of nematode growth medium containing about 20-30 worms. The worms were exposed to the tested solutions for 72 hr at 20°C using six replicate wells for each test and control samples (i.e. worms with only growth medium as a negative control and worms

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with toxic $CuCl_2$ (63 µg mL^{-1}) as a reference positive control). The uptake of Nile red loaded micelles and their impact on worm viability were observed using fluorescence microscopy and bright field microscopy, respectively.

2.5.10 Statistical analysis

The generated data from the biofilm biomass assays, colony biofilm assays and the artificial dermis model are presented as mean ± standard deviation (SD). Data were analysed by two-way analysis variance (ANOVA) with Tukey's multiple comparisons test using GraphPad Prism 7.02 (La Jolla, CA, USA). Data from toxicity study represent the average number of life/ dead worms of six replicate monitored by brightfield microscope using 2x objective lens. Viability percentage of treated worms was calculated from the viability percentage of the control (untreated worms) which is considered 100%.

3. Results

3.1 Optimization and characterization of polymeric micelles

3.1.1 Effect of CHX and polymer concentration

The effects of the CHX concentration on the particle size, size distribution, drug load and encapsulation efficiency of polymeric micelles are shown in Table 1. CHX at 1 mg mL^{-1} produced micelles with smallest diameter (62.9 nm) and high drug load (7.36% w/w) and encapsulation efficiency (81.4%). Both lower and higher CHX concentrations (0.5 mg mL^{-1} and 2 mg mL^{-1}) reduced the drug load to 3.35% and 4.66%, respectively translating to lower encapsulation efficiencies between 60.5% and 30.0%, respectively. As shown in Table 1, the DL% and EE% decreased with increasing Soluplus concentrations while the effect on the size of CHX-loaded micelles was less pronounced.

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3.1.2 Effect of organic solvent

CHX-loaded Sol micelles were prepared using different organic solvents (i.e. acetone, ethanol, methanol and chloroform). The DL% and EE% increased (i.e. 7.25 ± 0.5 and 83.75 ± 0.6, respectively) with acetone compared to other organic solvents (Table 2). The use of ethanol in the preparation process increased the size of CHX-PMs to 75.6 ± 1.8 nm while no considerable effects were observed with the other solvents (sizes between 61-65 nm). The effects of solvent volume (0, 0.5, 1, 1.5, 2, 2.5 and 3 mL acetone) during the fabrication process on the size, DL% and EE% of CHX-PMs are shown in Table 2. No significant effect on particle size was observed when increasing the volume of acetone up to 2.5 mL, while the DL% and EE% of CHX-PMs were increased (7.6 and 85.8%, respectively) for 1 mL acetone.

3.1.3 Effect of polymer blend (Soluplus:Solutol)

CHX-mixed micelles were prepared with different ratios of Soluplus® and Solutol HS 15 polymers. Table 1 shows that mixed micelles formed with a size of 63.2 nm and high drug load (4.70%) as well as high encapsulation efficiency (98.1 %) at a 1:1 ratio of Sol:ST. Moreover, blending of Solutol HS 15 with Soluplus® remarkably reduced the micelle size from 531 nm to 63 nm while increasing the loading efficiency for CHX 2-fold. Polymer blends produced micelles with higher encapsulation efficiencies compared to the use of single polymers (85.8 % and 27.5 % for CHX-Sol and CHX-ST, respectively).

3.1.4 In vitro drug release

CHX release kinetics from polymeric micelles are shown in Fig. 1. Faster release of CHX was observed from both micellar systems within the first 100 min of the experiment with a subsequent sustained release for up to 400 min. Cumulative releases profile of CHX from Sol micelles and Sol:ST mixed micelles was comparable at different pH values. CHX

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release from both micellar systems was further elucidated in the presence of bacterial lipase at pH 5.5 and 7.4 (Fig. 1). For Sol-micelles, the cumulative release of CHX in the presence of lipase was significantly increased after 100 min incubation at both pH values (P=0.03 at pH 5.5 and P=0.02 at pH 7.4) with about 40% of CHX remaining inside the Sol-micelles after 7 hour release period (Fig. 1A and 1B). Interestingly, mixed micelles in presence of lipase demonstrated lower cumulative release of CHX within the first 100 min of the release study at both pH (4% at pH 5.5 and 7% at pH 7.4, respectively) than in the absence of the enzyme (Fig. 1C and 1D). Due to the undesired physicochemical characters associated with CHX-ST micelles (e.g. large size, low DL% and EE %), the release profile of these micelles was not included in this study.

3.1.5 Stability studies

The physicochemical properties of CHX-loaded micelles were evaluated over 6 months storage at 4°C. No visible precipitates were observed during the storage period. The size and PDI of micelles after one-month storage increased from 62.4 nm and PDI = 0.09 to 73.1 nm and PDI = 0.16 for single micelles, and from 61.6 nm and PDI= 0.12 to 70.5nm and PDI = 0.18 for mixed micelles. However, no further significant changes in the micellar properties were observed after 6 months storage. The encapsulation efficiency of both micelles during the study period was comparable and more than 90% of initial loaded CHX were recovered from the micelles after 6 months storage.

3.2 Antimicrobial activity against planktonic and biofilm bacteria

The activities of chlorhexidine and blank micelles against planktonic bacteria and biofilms of selected bacterial strains are reported in Table 3. Interestingly, the MIC and MBC of Sol-ST mixed blank micelles were lower compared to those observed for individual blank

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polymeric micelles (Sol and ST). The anti-biofilm activity was also assessed with an MBEC of 32 μg mL^{-1} (*S. aureus*), 32 μg mL^{-1} (MRSA), and 8 μg mL^{-1} (*S. epidermidis*) for CHX and > 100,000 μg mL^{-1} (all strains), and > 10,000 μg mL^{-1} (all strains) for Sol blank micelles and ST blank micelles, respectively.

3.3 Biofilm biomass

The anti-biofilm efficacy of various treatment was assessed against *S. aureus* and *S. epidermidis*. The crystal violet assay for the quantification of biofilm biomass revealed that CHX-loaded micelles were significantly more effective in reducing bacterial biofilms compared to CHX alone (Fig. 3). Statistically significant differences were observed with all CHX-loaded micellar systems ($P<0.0001$) against *S. aureus biofilms* compared to control (untreated biofilm), whereas an increased biofilm biomass was noted with CHX alone. The biofilm biomass of *S. aureus* was reduced to 34% and 38% with CHX-Sol micelles and CHX-Sol:ST mixed micelles, respectively (Fig. 3A). Similarly, significant anti-biofilm effects were evident for CHX-Sol micelles and CHX-Sol:ST mixed micelles against *S. epidermidis* with a reduction in the biofilm biomass up to 57% and 38%, respectively (Fig. 3B).

3.4 Colony biofilm assay

The blank micellar systems showed no detectable activity against biofilms in a colony assay while there were significant anti-biofilm effects by CHX-loaded micelles. As shown in Fig. 4A**,** the number of viable bacterial cells of *S. aureus* biofilms following exposure to CHX-Sol was significantly reduced ($P=0.005$, while free CHX exhibited no anti-biofilm effects. Interestingly, a synergistic effect was observed with Sol:ST mixed micelles against *S. aureus* biofilms that promoted the activities of both individual micellar systems (e.g. CHX-

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Sol and CHX-ST) and further reduced the viability of biofilm-embedded bacteria Fig. 4A. This synergy was confirmed using the previously established Bliss Independence Model (Thangamani et al., 2015). The following equation was used to calculate synergy (S):

$$S = \left(\frac{fA0}{f00}\right)\left(\frac{f0B}{f00}\right) - \left(\frac{fAB}{f00}\right) \qquad \text{Equation1}$$

where fA0, f0B and fAB refer to CFU mL–1 of the bacteria in the presence of Soluplus, Solutol and Sol-ST respectively; and f00 refers to the CFU mL^{-1} of the control (untreated biofilm). The calculated S value (S>0) of the combined polymers (mixed micelles) indicated synergy between the polymers. Moreover, CHX-mixed micelles were more effective against MRSA biofilms than single micelles as shown in Fig. 4B. The effects of CHX- loaded micellar systems against *S. epidermidis* were also evaluated and the results indicated that CHX-Sol and CHX- mixed micelles substantially reduced the number of viable bacterial cells of *S. epidermidis* ($\log_{10}$ reduction of 3.16 for CHX-Sol and 2.56 for CHX-mixed micelles) (Fig. 4C).

3.5 Biofilm penetration

The penetration ability of CHX-Sol micelles, CHX-Sol:ST mixed micelles, CHX and its soluble form chlorhexidine gluconate (CHXG) through biofilms of *S. aureus* and *S. epidermidis* were examined and represented as inhibition zone diameters (Table 4). Penetration (%) of each formulation through biofilms was calculated from the zone diameter of growth inhibition produced by respective control (CHX without biofilms) on TSA plates which was considered as 100% penetration. According to the Table 4, Sol micelles and Sol:ST mixed micelles were remarkably increased the penetration extend of CHX through *S. aureus* biofilms (56.8 % for Sol and 71.6 % for Sol:ST) as well as *S. epidermidis* biofilms

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(75.6% for Sol and 85.4 % for Sol:ST). While biofilms of *S. aureus* displayed substantial inhibitory effects on CHX and CHXG, completely hindered their penetration, the biofilm of *S. epidermidis* showed less inhibitory effects on CHX and CHXG penetration (56.1% for both CHX and CHXG). An improvement in CHX penetration percentage was observed with the increased concentration of Sol from 10 mg mL^{-1} to 20 mg mL^{-1} (56.8% to 62.2%). While CHX penetration % through *S. epidermidis* biofilms was similar at different concentrations of Sol (Table 4).

3.6 Antimicrobial activity in artificial dermis

CHX-Sol micelles and CHX-mixed micelles were further evaluated for their anti-biofilm activity in an *in vitro* 3D artificial dermis model infected with MRSA and *S. epidermidis*. The biofilms treated with blank micelles demonstrated no anti-biofilm effect as evidenced by the same bacterial growth observed in untreated biofilms. CHX-Sol and CHX-mixed micelles reduced the CFU of MRSA by 2.4 and 2.1 log respectively, while 1.3 and 1.7 log reductions were observed in *S. epidermidis* biofilms (Fig. 5). In comparison, a 0.9 log reduction in the CFU was observed for free CHX against MRSA and *S. epidermidis* biofilms.

3.7 Distribution of micelles within biofilms

To demonstrate micelle distribution within biofilms of DAPI stained Staphylococci biofilms were treated with Nile red loaded micelles followed by image analyses. While untreated biofilms (biofilm without micelles) generated blue-fluorescent images (Fig. 6A), Sol micelles treated biofilms showed visible purple-fluorescent images indicating an interaction and accumulation of micelles within biofilms. (Fig. 6B). Similarly, Sol:ST mixed micelles showed considerable affinity towards Staphylococcal biofilms (Fig. 6C).

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3.1.6 Toxicity study

The survival rate of *C. elegans* after 72 h exposure to micelles was 100% at all investigated concentrations and comparable to untreated controls growth medium (Fig 7A). In contrast, no surviving worms were observed with positive control ($CuCl_2$) (Fig. 7B). Results indicated no toxicity effects of micelles in worms. The uptake of micelles by *C. elegans* was confirmed by the presence of the fluorescent dye in in the lumen of the digestive system of the worms. (Fig. 7C).

4. Discussion

Staphylococcus aureus and *Staphylococcus epidermidis* biofilms are highly tolerant to antimicrobials, challenging successful treatment and causing recalcitrant infections (Brauner et al., 2016; Otto, 2008). This tolerance is to some extent attributed to biofilm penetration failure of existing antimicrobials. Therefore, reformulation of antiseptics such as chlorhexidine opens up new opportunities in the prevention and control of biofilm infections (Bonez et al., 2013; Tomic et al., 2004).

While a number of studies have investigated the efficacy of the water-soluble form of CHX (i.e. CHX gluconate) against the planktonic and biofilm mode of growth of pathogenic bacteria, the current study is the first to evaluate the efficacy of CHX as poorly water-soluble free base and as CHX-loaded polymeric micelles against Staphylococci biofilms (Bonez et al., 2013; Hendry et al., 2009; Vestby and Nesse, 2015).

Commercially available Soluplus® and Solutol HS 15 were chosen as polymers to form micellar carriers to incorporate and enhance the delivery of CHX to bacterial biofilms. Soluplus® was chosen due to its amphiphilic properties that self-assemble into micelles in aqueous phase and its competency in loading poorly soluble drugs and formation of stable mixed micellar systems with Solutol HS 15 (Bhuptani et al., 2016; Linn et al., 2012).

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Parameters that affected the preparation method of polymeric micelles were evaluated to produce micelles with high load and encapsulation efficiency of CHX. These micelles were further evaluated for their ability to form mixed micelles with Solutol HS 15 that could improve the physicochemical properties and antibiofilm efficacy of single micelles.

In agreement with previous studies, single and mixed micelles were comparable with regard to particle size, (~63 nm) and a narrow size distribution (PDI ~0.1) (Bhuptani et al., 2016). The comparable size of both micellar systems can be attributed to the small volume fraction that is occupied by ST due to its small molecular weight (963.25 g $mol^{-1)}$ in comparison to Sol (90,000-140,000 g mol^{-1}) when mixed at a 1:1 ratio (Bhuptani et al., 2016; Grotz et al., 2017; Paaver et al., 2014). The observed decrease in CHX loading with increasing ST content is attributed to changes in the hydrophobicity of the micelle caused by the incorporation of an increased amount of hydrophilic polymers into the structure of mixed micelles as has been proposed for other Sol-based mixed micellar systems (Bernabeu et al., 2016). The characteristic large size, low DL% and EE% exhibited by pure ST micelle can be attributed to the proportions of the polymer and drug used in the formulation and to its low solubilisation capacity for CHX in comparison to the mixed micelles. Similar phenomenon have been reported in encapsulation of hydrophobic anticancer agent paclitaxel using pure ST micelles (Hou et al., 2016).

It was hypothesised that the release of CHX from polymeric micelles can be enhanced by bacterial lipase. Consequently, Sol-micelles and Sol:ST -mixed micelles loaded with CHX were incubated with lipase and the release profiles evaluated. As shown in Fig. 1A and B an improvement in the release of CHX from Sol -micelles was facilitated by the activity of lipase, most likely due to cleavage of the polycaprolactam ring in Sol. Micelles with lipase sensitive polymers were considered an effective strategy in delivery and accumulation of drug locally in biofilms where the lipase-producing bacteria are embedded (Elsässer et al.,

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2013; Liu et al., 2016). In contrast, the incubation of mixed micelles with lipase resulted in a significant reduction in the release of CHX (Fig. 1C and D) that can be attributed to the accumulation of non-soluble products of Solutol during of the degradation of the polymer by lipase (Fig. 2) (Minkov et al., 2005).

Chlorhexidine is a considered gold standard in many clinical applications such as surgical hand scrub (Ankola et al., 2008). However, CHX is less effective against the biofilm mode of growth of microorganisms in comparison to planktonic cells due to restricted penetration through mature biofilms (Shen et al., 2011; Yamaguchi et al., 2013). The limited activity of unformulated CHX against biofilms highlights the need to develop novel carrier systems to enhance the penetration of CHX in biofilms. *S. aureus* and *S. epidermidis* biofilms are associated with many acute and chronic biofilm related infections (Otto, 2008). Therefore, unformulated CHX and CHX polymeric micelles were evaluated for their ability to penetrate different staphylococcal biofilms. Interestingly, the developed CHX-micelles demonstrated high efficiency in delivering CHX through bacterial biofilm in comparison to unformulated control (free CHX) (Table 4). In line with the enhanced penetration of CHX-micelles, the confocal images of treated biofilms demonstrated a high affinity of the micelles towards biofilms likely due to their small size and stealth properties (Fig. 6) in line with previous studies (Liu et al., 2016; Takahashi et al., 2015).

In order to confirm that the penetration of CHX was due to the positive charge of the antiseptic rather than its low water solubility, the penetration of CHXG through biofilms was also assessed (Table 4**).** The limited penetration of the water-soluble CHXG through bacterial biofilm indicated that the charge of the CHX cation was the major cause for its retarded biofilm penetration. It is well known that biofilms have a sequestering effect towards many antimicrobial agents that render them less effective against bacteria located in deeper layers of the biofilm (Estela and Alejandro, 2012; Flemming et al., 2016). For example, about 80%

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of chlorine was retarded by mixed cultured biofilm composed of *Pseudomonas aeruginosa* and *Klebsiella pneumoni* (De Beer et al., 1994). The enhanced penetration through formulation of CHX as polymeric micelles in the current study can be attributed to the stealth properties of CHX polymeric micelles, facilitated by the polyethene glycol chains that prevent non-specific binding of positively charged CHX to biofilms. The stealth phenomenon has previously been described to enhance the penetration of the antiseptic triclosan when encapsulated in PEG-PCL di-block polymeric micelles (Liu et al., 2016).

The data from planktonic and biofilm *in vitro* susceptibility tests demonstrated that a much higher concentration of CHX was required to eradicate Staphylococcal biofilms compared to non-surface-attached planktonic bacteria (Table 3) consistent with previous reports (Forier et al., 2014; Høiby et al., 2010). An improvement in the efficacy of Soluplus in the form of mixed micelles against planktonic cells and biofilms of Staphylococci can be attributed to the ideal characteristics exhibited by incorporation of nonionic surfactant ST as being reported in numerous studies (Hou et al., 2016; Wang et al., 2018; Zhang et al., 2014). Indeed, mixing ST with other polymers leads to homogenised, small size micelles with large surface area that often more stable and competent than micelles made of single polymer (Ke et al., 2017). It should be mentioned that an increased attention towards mixed micellar systems has been evolving recently due to their attractive features, however; most of the available binary systems have mainly been used as carriers to deliver anticancer drugs. Therefore, this study takes an advantage in utilising mixed micelles to deliver antimicrobial agents.

Further validation of the antibiofilm efficacy of CHX-micellar systems was carried out in a 3D artificial dermis model. The collagen matrix employed in this model resembles the *in vivo* wound bed closer than a polystyrene substrate for biofilm growth used in more simple multi-titer plate-based models (Brackman and Coenye, 2016). In comparison with the

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results obtained from the colony biofilm assay, CHX-loaded micelles were more efficient against MRSA biofilms on an artificial dermis. The variation in the biofilm susceptibility between colony biofilm and artificial dermis assays can be explained by the variation in the surfaces on which biofilms are established and by the differences in the nutrient source as well as its availability to the bacteria producing biofilms (Amato et al., 2014). For instance, the reduction of *P. aeruginosa* in biofilms established on soft tissue and treated with silver containing specimens was more efficient in comparison to biofilms on a solid surface (Hakonen et al., 2014). This reduction was attributed to the effective release of silver particles in the soft tissue model due to the availability of water as a release medium. A similar variation in the susceptibility of *S. aureus* biofilms between different models was also noticed in another study, in which biofilms were grown on different surfaces (Brackman et al., 2011).

The simplicity and efficiency of *C. elegans* in the evaluation of various substances for their toxicity make them an attractive model to be used in this study to investigate the toxicity of micelles at their effective concentration (Brackman et al., 2011; Richter et al., 2017a; Tampakakis et al., 2008). The compatibility and biodegradability nature of Sol and ST explain the high survival rate of nematodes in the present of micelles as a singular as well as mixed (Wang et al., 2018). Sol and ST safety has been reported in the literature using different cell lines which confirmed non-toxic nature of such micelles in the studied model. Potentially, this study emphasized the effectiveness of the developed system in reducing biofilm embedded bacteria compared to free CHX offering potential advantages for the treatment of clinically relevant biofilms of highly resistance bacteria.

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5. Conclusion

Soluplus and Solutol based polymeric micelles were successfully developed to enhance the delivery of chlorhexidine to Staphylococcal biofilms. Owing to their stealth properties these carriers prevented nonspecific binding of the encapsulated positively charged antiseptic CHX to the negatively charged biofilm components resulting in enhanced penetration of CHX in biofilms. Overcoming biofilm penetration limitations is a crucial step to improve the antibiofilm efficacy of CHX which translates into broader applications in the clinic and food industries. Bio-responsive carriers can enhance the activity of antimicrobials against biofilms promoting major advance towards controlling biofilm associated infections.

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Declaration of interest

The authors reports no conflicts of interest

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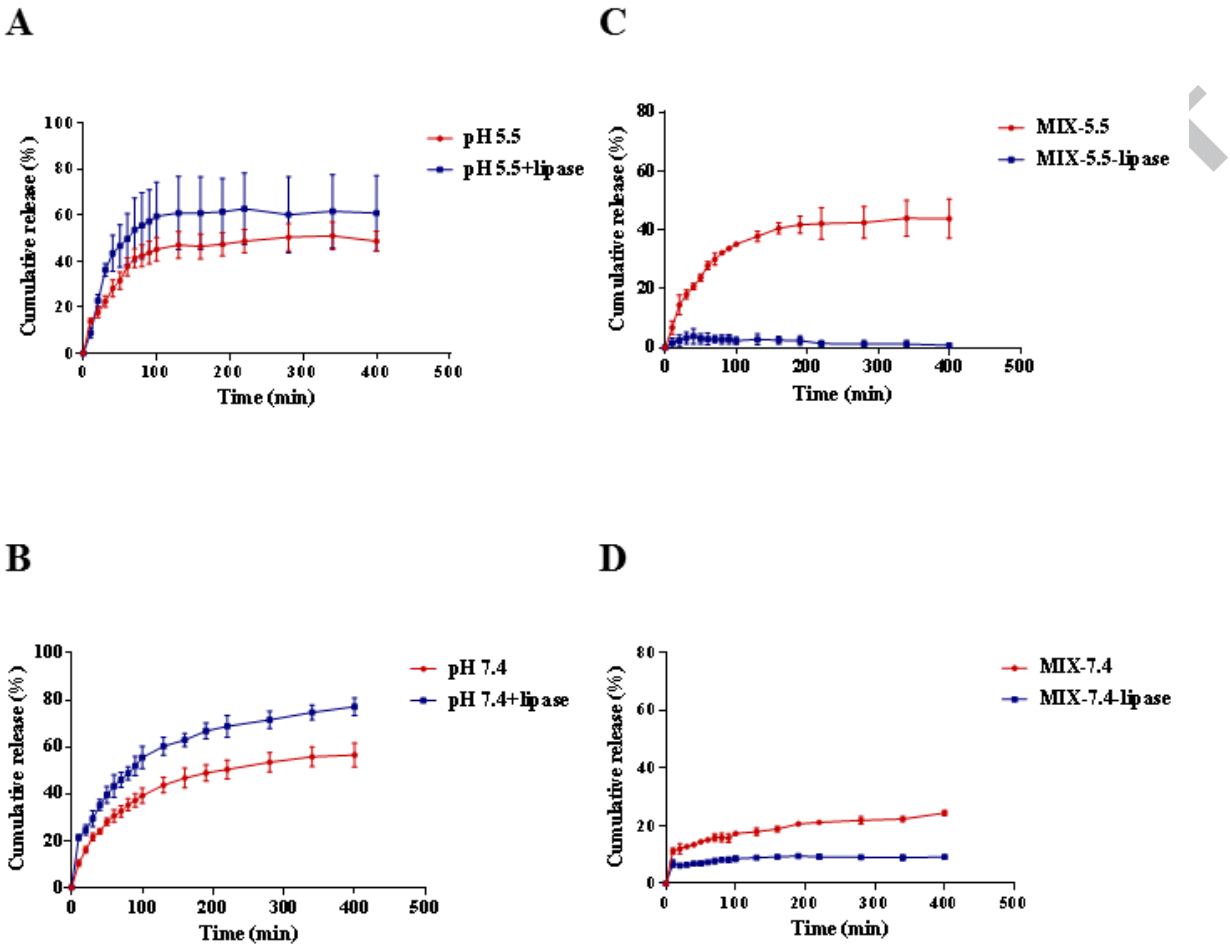


Fig. 1. Chlorhexidine (CHX) release from micelles in the presence or absence of lipase at different pH. CHX cumulative release from Soluplus micelles pH 5.5 (A) and 7.4 (B). CHX cumulative release from Soluplus:Solutol mixed micelles at pH 5.5 (C) and 7.4 (D).. Data represents mean ± SD, n=3.

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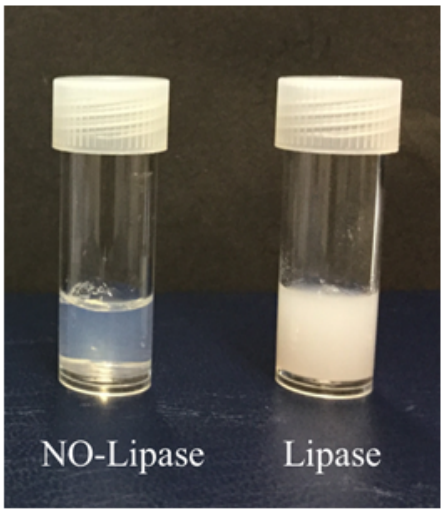


Fig. 2. Mixed micelles in the absence or presence of lipase after the release study.

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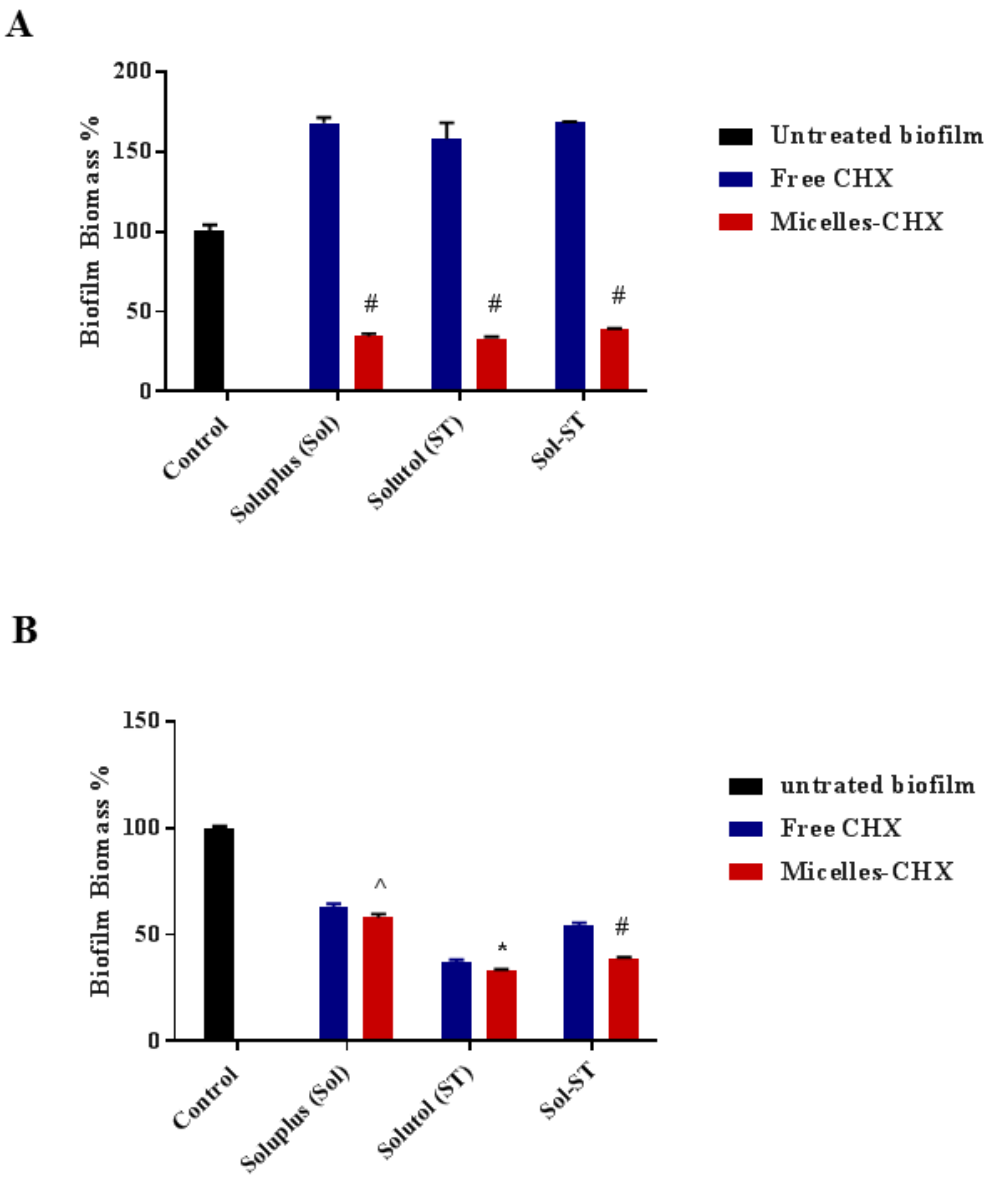


Fig. 3. Biofilm biomass reduction in S.aureus (A) and S. epidermidis (B) by chlorhexidine (CHX) alone and CHX-loaded micelles after 24 h exposure. Data represent the mean ± SD of three replicates. Statistical comparison to free CHX. *, $P < 0.05$; ^, $P < 0.01$; #, $P < 0.0001$

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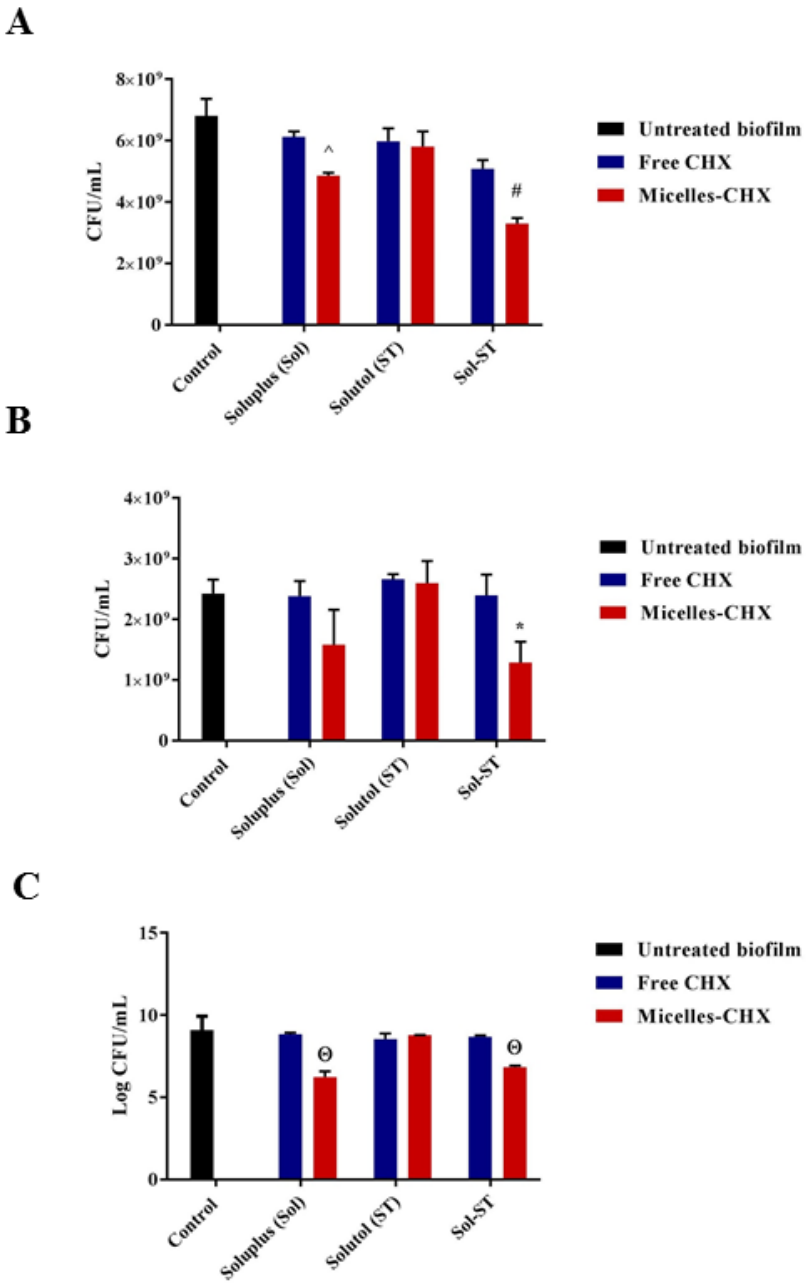


Fig. 4. Reduction in colony forming units (CFU) of biofilm-embedded microorganisms. (A) CFU/mL of S. aureus biofilms after 24 h exposure to different treatments. (B) CFU mL−1 of MRSA biofilms after 24 h exposure to different treatments. (C) Log CFU mL−1 of S. epidermidis biofilms after 24 h exposure to different treatments. Data represent the mean ± SD of three replicates. Statistical comparison to free CHX. *, $P < 0.05$; ^, $P < 0.01$; Θ, < 0.001; #, $P < 0.0001$

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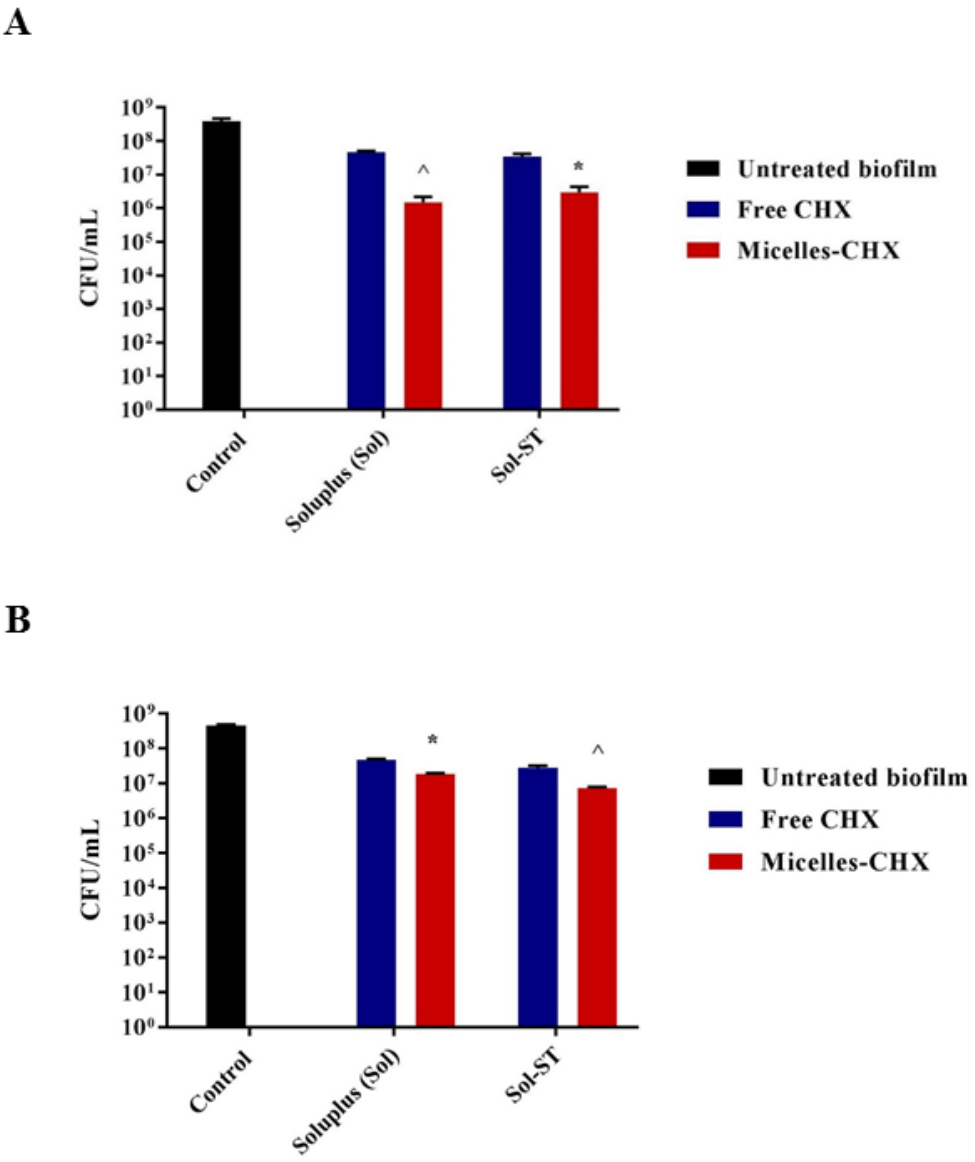


Fig. 5. Antibiofilm effects of CHX-loaded micelles vs free CHX using 3D artificial dermis model. CFU/ml reduction of MRSA (A) and *S. epidermidis* (B) biofilm after 24 h exposure to CHX-Sol micelles, CHX- Sol:ST mixed micelles and free CHX. Data represent the mean ± SD of two replicates. Statistical comparison to free CHX. *, < 0.05; ^, < 0.01

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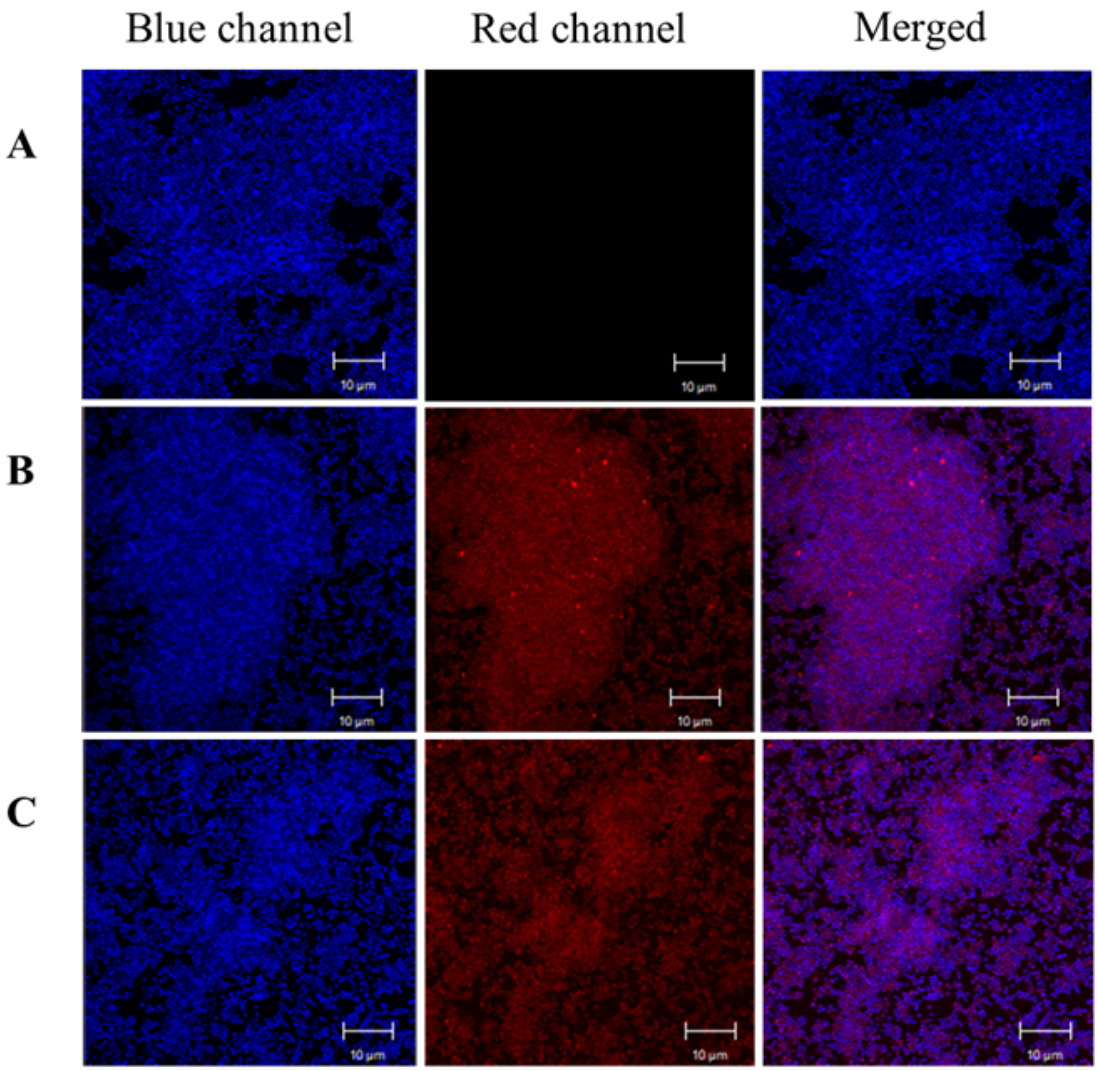


Fig. 6. Distribution of Nile red loaded polymeric micelles in a *Staphylococcus aureus* biofilms using CLSM. Biofilm embedded bacteria were stained with DAPI (blue) and micelles stained with Nile red (red). Fluorescent images were taken for control biofilms (A), biofilms treated with Nile red-loaded Sol micelles (B), biofilms treated with Nile red loaded Sol:ST mixed micelles (C).

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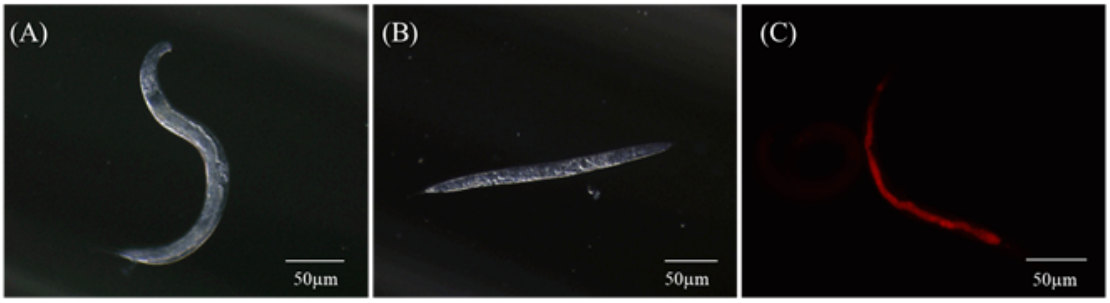
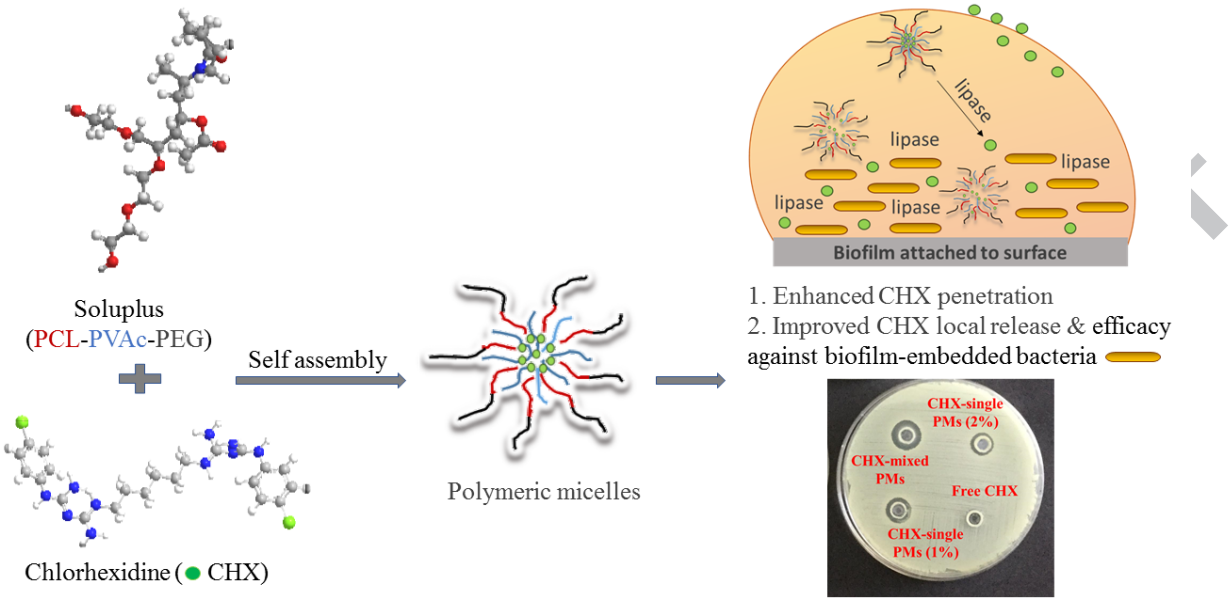


Fig. 7. C. elegans based toxicity assay shows bright field images of viable worms (A) and dead worms (B). Red fluorescent images show Nile red loaded polymeric micelles uptake by nematodes (C). Scale bars are 50 µm (A, B and C).

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Table 1 Effect of CHX, Soluplus® and Sol/ST concentration ratio on CHX-loaded micelles

Agents	Concentration (mg mL^{-1})	Physicochemical properties			
		size (nm)	PDI	DL %	EE %
CHX	0.5	59.6 ± 2.0	0.08 ± 0.01	3.35 ± 0.9	60.5 ± 4
	1	62.9 ± 0.1	0.09 ± 0.01	7.4 ± 0.2	81.4 ± 1.1
	2	73 ± 0.8	0.24 ± 0.03	4.7 ± 0.2	30 ± 1.5
Soluplus®	10	62.4 ± 0.6	0.09 ± 0.01	7.42 ± 0.04	83 ± 0.2
	20	59 ± 0.5	0.14 ± 0.02	6.54 ± 0.4	78.8 ± 4.8
	30	59.53 ± 0.2	0.09 ± 0.02	2.26 ± 0.2	64.6 ± 2.2
Sol/ST blend	1/0	61 ± 1.6	0.08 ± 0.01	7.6 ± 0.5	85.8 ± 1.8
	1/0.5	65.6 ± 0.5	0.13 ± 0.02	5.6 ± 0.2	90.4 ± 1.3
	1/1	63 ± 0.8	0.12 ± 0.01	4.7 ± 0.2	98 ± 1
	1/1.5	63 ± 1.2	0.11± 0.01	3.7 ± 0.4	95.8 ± 1.6
	1/2	65.3 ± 0.5	0.09 ± 0.01	2.6 ± 0.5	81 ± 2
	0/1	531 ± 1.4	0.6 ± 0.07	2.5 ± 0.7	27.48 ± 1.7

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Table 2 Effect of solvent type and volume on CHX-loaded micelles

Agent	Type/volume (mL)	Physicochemical properties			
		Size (nm)	PDI	DL %	EE %
Solvent	Acetone	61.6 ± 1.3	0.07 ± 0.01	7.25 ± 0.5	83.8 ± 0.6
	Ethanol	75.6 ± 1.8	0.14 ± 0.01	3.19 ± 0.3	41.5 ± 1.4
	Methanol	64.3 ± 1.8	0.09 ± 0.01	6.15 ± 0.8	67.5 ± 1.3
	Chloroform	65 ± 0.6	0.17 ± 0.02	2.08 ± 0.1	22.4 ± 1.5
Acetone	0	65.4 ± 1.3	0.21 ±0.02	5.15 ± 1.2	56.6 ± 1.1
	1	61 ± 1.6	0.08 ± 0.01	7.6 ± 0.4	85.8 ± 1.8
	1.5	63.3 ± 0.5	0.1 ± 0.01	4.6 ± 1.1	49 ± 3
	2	64.5 ± 0.8	0.14 ± 0.02	5.14 ± 1.1	56.3 ± 1.1
	2.5	68.6 ± 0.7	0.2 ± 0.02	5.2 ± 0.3	58 ± 1.3
	3	68.8 ± 0.1	0.2 ± 0.02	6.2 ± 1.1	58.8 ± 1.6

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Table 3 The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and the minimum biofilm eradication concentration (MBEC) for chlorhexidine and investigated blank micellar systems

Agent	*S. aureus* *ATCC 25923*			MRSA ATCC 33591			*S. epidermidis* *ATCC 14990*		
	MIC	MBC	MBEC	MIC	MBC	MBEC	MIC	MBC	MBEC
	($\mu g\ mL^{-1}$)			($\mu g\ mL^{-1}$)			($\mu g\ mL^{-1}$)		
CHX	2	4	32	1	2	32	2	4	8
Soluplus® (Sol)	25,000	$>100\times10^3$	$>100\times10^3$	50,000	$>100\times10^3$	$>100\times10^3$	25,000	50,000	$>100\times10^3$
Solutol HS15 (ST)	5,000	10,000	> 10,000	10,000	> 10,000	> 10,000	2,500	5,000	>10,000
Sol-ST	12,500	5,000	> 10,000	5,000	10,000	> 10,000	2,500	5,000	>10,000

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Table 4 Penetration of CHX-loaded micelles and CHX alone through S. aureus and S. epidermidis biofilms.

Agent	*S. aureus* biofilms		*S. epidermidis* biofilms	
	Zone diameter (mm)	% penetration	zone diameter (mm)	% penetration
CHX	< 6	0.00	11.5 ± 0.7	56.1 ± 3.5
CHXG	< 6	0.00	11.5 ± 0.7	56.1 ± 3.5
CHX-Sol (10 $mg.mL^{-1}$ polymer)	10.5 ± 0.7	56.8 ± 3.8	15.5 ± 0.7	75.6 ± 3.4
CHX- Sol (20 $mg.mL^{-1}$ polymer)	11.5 ± 0.7	62.2 ± 3.8	15.5 ± 0.7	75.60 ± 3.4
CHX- Sol:ST	13.3 ± 0.4	71.6 ± 1.9	17.5 ± 0.7	85.4 ± 3.5

Penetration (%) was calculated from the zone diameter of growth inhibition produced by respective control (free CHX) on TSA plates which was considered as 100% penetration. Detection limit was 6 mm equivalent to the size of the punched wells set into TSA plates.

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Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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