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Liposomes-in-chitosan hydrogel boosts potential of chlorhexidine in biofilm eradication *in vitro*

Lisa Myrseth Hemmingsen [a], Barbara Giordani [b], Ann Kristin Pettersen [a], Beatrice Vitali [b], Purusotam Basnet [c,d], Nataša Škalko-Basnet [a,*]

[a] Drug Transport and Delivery Research Group, Department of Pharmacy, University of Tromsø, The Arctic University of Norway, Universitetsvegen 57, 9037, Tromsø, Norway
[b] Molecular and Applied Microbiology, Department of Pharmacy and Biotechnology, University of Bologna, Via San Donato 19/2, 40127, Bologna, Italy
[c] IVF Clinic, Department of Obstetrics and Gynecology, University Hospital of North Norway, Sykehusvegen 38, 9019, Tromsø, Norway
[d] Women's Health and Perinatology Research Group, Department of Clinical Medicine, University of Tromsø, The Arctic University of Norway, Universitetsveien 57, 9037, Tromsø, Norway

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ABSTRACT

Successful treatment of skin infections requires eradication of biofilms found in up to 90 % of all chronic wounds, causing delayed healing and increased morbidity. We hypothesized that chitosan hydrogel boosts the activity of liposomally-associated membrane active antimicrobials (MAA) and could potentially improve bacterial and biofilm eradication. Therefore, liposomes (~300 nm) bearing chlorhexidine (CHX; ~50 μg/mg lipid) as a model MAA were incorporated into chitosan hydrogel. The novel CHX-liposomes-in-hydrogel formulation was optimized for skin therapy. It significantly inhibited the production of nitric oxide (NO) in lipopolysaccharide (LPS)-induced macrophage and almost completely reduced biofilm formation. Moreover, it reduced *Staphylococcus aureus* and *Pseudomonas aeruginosa* adherent bacterial cells in biofilm by 64.2–98.1 %. Chitosan hydrogel boosted the anti-inflammatory and antimicrobial properties of CHX.

1. Introduction

Antimicrobial resistance is currently a serious medical threat, especially because of the decelerated and unsuccessful pipeline of antimicrobial candidates (Hall et al., 2020). Although bacterial resistance often derives from genetic mutations, the biofilm formation and increased inflammatory cascades are also known to be strong contributors (Balaure & Grumezescu, 2020; Cepas et al., 2018; Romana-Souza, Santos, Bandeira, & Monte-Alto-Costa, 2016). Biofilms are found in between 60 and 90 % of all chronic wounds, delaying healing and leading to increased morbidity and costs (Kadam, Shai, Shahane, & Kaushik, 2019; Matos de Opitz & Sass, 2020). Novel approaches for biofilm eradication and efficient wound therapy are urgently needed as skin and soft tissue infections are among the most common infections in humans (Poulakou, Lagou, & Tsiodras, 2019). These infections exhibit a polymicrobial nature and cleaver, novel strategies to eradicate multiple bacteria are necessary for their treatment.

Among the most common bacteria embedded in wound matrices are *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Balaure & Grumezescu, 2020; Kadam et al., 2019). These bacteria display multiple mechanisms of resistance, rendering their eradication particularly challenging. Treating wound biofilms requires innovative approaches. Novel drug delivery systems comprising cationic polymers (Guo et al., 2018) offer potential solutions. Chitosan has attracted a lot of interest due to its broad range of beneficial effects and biodegradability (Ambrogi et al., 2017; Islam, Shahruzzaman, Biswas, Nurus Sakib, & Rashid, 2020). The intrinsic antimicrobial and anti-inflammatory properties are highly relevant for wound therapy (Islam et al., 2020). Several antimicrobial mechanisms are proposed for chitosan; its interaction with negatively charged bacterial membranes leading to possible

Abbreviations: BHI, Brain Heart Infusion Broth; CHX, chlorhexidine; DMEM, Dulbecco's Modified Eagle Medium; EE, entrapment efficiency; FBS, fetal bovine serum; LPS, lipopolysaccharide; MAA, membrane active antimicrobials; MLC, minimal lethal concentration; MW, molecular weight; NB, Nutrient Broth; NO, nitric oxide; PBS, Phosphate Buffer Saline; PI, polydispersity index; RPMI, Roswell Park Memorial Institute; SWF, Simulated Wound Fluid; TEM, transmission electron microscopy.

* Corresponding author.
E-mail address: natasa.skalko-basnet@uit.no (N. Škalko-Basnet).

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lysis being the main mechanism (Matica, Aachmann, Tøndervik, Sletta, & Ostafe, 2019). Other mechanisms include interruption of microbial protein synthesis, chelation of metal ions and formation of an envelope on the microbial surface (Matica et al., 2019). In addition, chitosan can influence the haemostasis, inflammatory stage and proliferation as well as accelerate wound healing (Liu et al., 2018; Moeini, Pedram, Makvandi, Malinconico, & Gomez d'Ayala, 2020). Chitosan hydrogel's three-dimensional network contributes to high water-retaining properties and gas-exchange capacity (Tavakoli & Klar, 2020). However, hydrogels often exhibit rapid drug release from the gel matrix (Peers, Alcouffe, Montembault, & Ladavière, 2020).

Combining hydrogel with a lipid-based carrier, such as liposomes, could prevent this rapid drug release (Grijalvo, Eritja, & Díaz, 2020; Peers et al., 2020). Studies report considerably slower release from liposomes-in-hydrogels compared to plain hydrogels (Jøraholmen, Basnet, Tostrup, Moueffaq, & Škalko-Basnet, 2019). The release will be also influenced by the type of entrapped drug. Antiseptics, such as chlorhexidine (CHX), are commonly used in local treatment of wound infection and reasonable candidates for new topical formulations (Smith, Russo, Fiegel, & Brogden, 2020). CHX acts as a membrane active antimicrobial (MAA) on both the dormant and active bacteria (Hubbard, Coates, & Harvey, 2017). The resistance is generally lower than for more target-specific compound due to the rapid and broad bactericidal effect of MAAs (Hubbard et al., 2017). Their mechanism of action could, in synergy with chitosan, enhance eradication of resistant bacteria.

Several researchers reported promising results on the antimicrobial effects of chitosan hydrogel and hydrogels containing carriers or particles. Anjum et al. evaluated a cotton dressing coated with chitosan, polyethylene glycol and polyvinyl pyrrolidone gel loaded with tetracycline against *Escherichia coli* and *S. aureus* (Anjum, Arora, Alam, & Gupta, 2016). In another study, the authors showed effect in the same species of both chitosan and a multi-network hydrogel based on chitosan (Zou et al., 2018). Masood and collaborators reviled antimicrobial activity in several species of silver nanoparticles loaded in chitosan and polyethylene glycol hydrogels (Masood et al., 2019). In addition, moxifloxacin entrapped in niosomes loaded into chitosan hydrogel demonstrated an improved antimicrobial activity in *P. aeruginosa* (Sohrabi, Haeri, Mahboubi, Mortazavi, & Dadashzadeh, 2016). The antimicrobial effects of chitosan are often greater in gram-positive bacteria (Moeini et al., 2020). However, a study on *S. aureus* and *P. aeruginosa* co-existing in biofilm exposed increased vulnerability of *P. aeruginosa*. The finding that bacterial membrane exhibited higher fluidity in the presence of MAAs could lead to novel target in biofilm treatment of wounds (Orazi, Ruoff, & O'Toole, 2019). Since these bacteria are often highly abundant in wounds, we aimed to exploit this vulnerability and create a system with synergic effects on the bacterial membrane. The activity of chitosan on the bacterial membrane is often associated with the molecular weight (MW) of the polymer (Tao, Qian, & Xie, 2011), and to achieve the strongest membrane activity on these bacteria in biofilms, we utilized chitosan with higher MW. The action of chitosan combined with the membrane activity of CHX could promote eradication of *S. aureus* and *P. aeruginosa* biofilms.

We therefore hypothesized that chitosan hydrogel can boost the activity of liposomally-associated MAAs. We aimed, for the first time, to investigate potential synergy between CHX, a model MAA, associated with liposomes and chitosan hydrogel against *S. aureus* and *P. aeruginosa* biofilms.

2. Materials and methods

2.1. Materials

Chitopharm™ M - Medium MW chitosan from shrimp (average of 350–600 kDa; and degree of deacetylation of >70 %) was a gift from Chitinor (Tromsø, Norway) and Lipoid S100 (phosphatidylcholine content >94 %) a gift from Lipoid GmbH (Ludwigshafen, Germany). Methanol (≥99.9 %) was purchased from VWR (Fontenay-sous-Bois, France). Acetic acid ≥99.8 %, chlorhexidine >99.5 %, glycerol solution (86–89 %), sodium nitrite, Kollisolv® PEG E 400, Cell Counting Kit – 8, Trizma base, calcium chloride, sodium phosphate dibasic dehydrate, potassium dihydrogen phosphate, sodium chloride and crystal violet were acquired from Sigma-Aldrich (St. Louis, USA). Ortho-phosphoric acid ≥85 % was obtained from Kebo Lab Ab (Oslo, Norway). Uranyless was procured from Electron Microscopy Sciences (Hatfield, PA, USA). Lipopolysaccharide (from *Escherichia coli* 055:B5), sulfanilamide ≥98 % and N-(1-Naphthyl)ethylenediamine dihydrochloride ≥98 % were acquired from Sigma Life Science Norway AS (Oslo, Norway). Roswell Park Memorial Institute (RPMI) medium 1640, bovine serum albumin, penicillin-streptomycin and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (Steinheim, Germany). Nutrient Broth (NB) and Brain Heart Infusion broth (BHI) were supplied by Becton Dickinson and Company (Sparks, MD, USA). Dulbecco's Modified Eagle Medium (DMEM) high glucose w/ L-glutamine and sodium pyruvate was acquired from Biowest (Nuaillé, France). Murine macrophage RAW 264.7 cells, *S. aureus* ATCC29213 and *P. aeruginosa* ATCC10145 were delivered by ATCC (Manassas, VA, USA). *S. aureus* SO88 is a clinical isolate (Ospedale Sant'Orsola-Malpighi, Bologna, Italy) (Giordani et al., 2019). HaCaT cell line (immortalized human keratinocytes) was purchased from CLS Cell Lines Service GmbH (Eppelheim, Germany).

2.2. Preparation of CHX-liposomes and liposomes

CHX-liposomes were prepared by the film method as previously described (Hurler & Škalko-Basnet, 2012). Briefly, Lipoid S100 (200 mg) and CHX (10 mg) were dissolved in methanol. A lipid film was formed after methanol evaporation on a rotavapor (Büchi rotavapor R-124 with vacuum controller B-721, Büchi Vac® V-500, Büchi Labortechnik, Switzerland) at 60 kPa and 45 °C for at least 1 h, and re-suspended in 10 mL distilled water to form CHX-liposomes. Liposomes (without CHX) were made of lipid alone.

The size of CHX-liposomes was reduced by manual extrusion through polycarbonate membranes (Nuclepore Track-Etch Membrane, Whatman House, Maidstone, UK) with average diameter of 0.8 μm and 0.4 μm three and five times, respectively. Liposomes were extruded under same conditions including additional extrusions twice through a 0.2 μm membrane to be of comparable size.

2.3. Characterization of CHX-liposomes and liposomes

The size was measured on a NICOMP Submicron particle sizer (NICOMP Particle Sizing System, Santa Barbara, California, USA) as described by Ternullo, Gagnat et al. (2019). Liposomal suspensions diluted to an attain intensity of 250–350 KHz were measured (weight-intensity distribution) in three cycles of 10 min. The zeta potential was determined with a Zetasizer Nano Zen 2600 (Malvern, Worcestershire, UK, Jøraholmen et al., 2019). The liposomal suspension was diluted with deionized water. The pH of liposomal suspensions was measured using sensION + PH31 pH benchtop meter (Hach, Loveland, Colorado, USA).

CHX entrapped in the liposomes was separated from the unentrapped CHX using dialysis at 24 ± 1 °C (tube cut-off for MW at 12–14 kDa; Medicell International Ltd., London, UK). The CHX entrapment efficiency was determined using Tecan Spark M10 multimode plate reader (Tecan Trading AG, Switzerland) at 261 nm. A standard curve of CHX was prepared in the concentration range of 1.25–40 μg mL^{-1} for the analysis ($R^2 = 0.999$).

2.4. Preparation of hydrogels

2.4.1. Preparation of hydrogel and liposomes-in-hydrogel

Chitosan hydrogel was prepared as described previously (Hurler, Engesland, Kermany, & Škalko-Basnet, 2012). In brief, 4.5 % chitosan

(w/w) was dispersed in 2.5 % acetic acid (w/w) and 9% glycerol (w/w) and hand-stirred stirring at 24 ± 1 °C for 5 min to form hydrogel. The hydrogel was bath sonicated in an ultrasonic bath (Branson, Ultrasonic cleaner 5510E-MT, Danbury, USA) for 30 min (degassed) and permitted to swell at room temperature for 48 h. Glycerol-free hydrogel was prepared in the same manner without glycerol.

CHX-liposomes or liposomes without drug (10 % w/w) were incorporated into hydrogel comprising 5% (w/w) chitosan and 10 % (w/w) glycerol by hand-stirring at 24 ± 1 °C for 5 min to prepare respective liposomes-in-hydrogel. The concentrations of chitosan and glycerol in hydrogels after incorporation of liposomes were 4.5 % and 9% (w/w), respectively.

2.4.2. Preparation of CHX-hydrogel

CHX was dissolved in 2.5 % acetic acid (w/w) and glycerol by mechanical stirring for 2 h. Chitosan (4.5 %, w/w) was dispersed in the CHX solution and hand-stirred stirring at 24 ± 1 °C for 10 min to form hydrogel. The final concentrations of chitosan and glycerol were 4.5 % (w/w) and 9% (w/w), respectively.

2.5. Characterization of hydrogel

Texture properties were evaluated by utilizing a backward extrusion rig set using a Texture Analyser TA.XT Plus (Stable Micro Systems Ltd., Surrey, UK; Hurler et al., 2012a). Hydrogel (65 g) was transferred to the rig set container. A 35 mm disk was fixed to the texture analyser, and compressed into the hydrogel, and redrawn to starting position. The speed was 4 mm s^{-1} and starting position right above the hydrogel surface. The distance and trigger force were 10 mm and 10 g, respectively.

The pH of hydrogels was evaluated using Accumet ® portable pH meter kit AP115 (Fischer Scientific, Massachusetts, USA).

2.6. Release of CHX

The CHX release from formulations (liposomes and hydrogels) and free CHX were evaluated in a Franz cell diffusion system (PermeGear, Hellertown, PA) utilizing cellophane membrane and acceptor cell of 12 mL (1.77 cm^2) as previously described (Jøraholmen et al., 2019). To address low water solubility of CHX base, 10 % polyethylene glycol (v/v) in distilled water was added to the acceptor chamber. The system was kept on constant heating (32 °C) and mechanical stirring. The formulation (600 μL) was added to the donor chamber and samples were withdrawn after 24 h. CHX from both the acceptor chamber and membrane was quantified on a UV–vis plate reader (SpectraMax 190 Microplate Reader, Molecular Devices, CA, USA) at 261 nm. The hydrogels were weighted before and after each experiment to adjust for fluid exchange.

2.7. Bioadhesion studies

The bioadhesive properties of hydrogel formulations were evaluated using the mucoadhesion rig on the Texture Analyser TA.XT Plus (Stable Micro Systems Ltd., Surrey, UK; Hurler & Škalko-Basnet, 2012). The full thickness human skin was obtained from patients undergoing abdominal plastic surgery. Consent from all patients was obtained prior to every surgical procedure. Skin slices (1.26 ± 0.04 mm) were rinsed in Phosphate Buffer Saline (PBS, pH 7.4, 2.98 g L^{-1} $Na_2HPO_4{\cdot}2H_2O$, 0.19 g L^{-1} KH_2PO_4, 8 g L^{-1} NaCl) and stored in −20 °C. Prior to each experiment, the skin slice was thawed in distilled water for 30 min and rinsed thoroughly. Excess liquid was removed and the skin slice was mounted to the mucoadhesion rig. The hydrogel (150 μL) was applied to the die and the die weighed. The die with hydrogel was pressed onto the skin slice for 10 s with a force of 25 g. The speed was set to 1.0, 0.5 and 0.1 mm s^{-1} for the pre-test, test and post-test, respectively. The die was immediately weighed after the test (Ternullo, Schulte Werning, Holsæter, & Škalko-Basnet, 2019).

2.8. Anti-inflammatory activity

The anti-inflammatory activity was expressed through inhibition of NO production in lipopolysaccharide-induced RAW 264.7 murine macrophages (Basnet, Hussain, Tho, & Škalko-Basnet, 2012). CHX-liposomes-in-hydrogel, hydrogel, CHX-liposomes and liposomes were evaluated. Cell suspension (5×10^5 cells mL^{-1}, 1000 μL, in RPMI supplemented with glutamine, 10 % FBS, penicillin and streptomycin) was added to 24-well plates and incubated in humidified 5% CO_2 at 37 °C for 24 h. The medium was removed and 990 μL of LPS (1 μg mL^{-1}) in supplemented RPMI added to each well. The 10 μL of diluted liposomal suspensions (concentrations corresponding to 1, 10 and 50 μg mL^{-1} lipid content, respectively) and hydrogels (corresponding to liposomal suspensions) were added in triplicates. Supplemented RPMI (100 %) and LPS in medium served as normal and controls, respectively. The cells were incubated in humidified 5% CO_2 at 37 °C for 24 h. After incubation, NO released in medium was measured as nitrite concentration using Griess reagent (1:1, v/v) and quantified on a UV–vis plate reader (Tecan Spark M10 multimode plate reader, Tecan Trading AG, Switzerland) at 540 nm.

2.9. Antimicrobial evaluation

2.9.1. Inhibitory activity against planktonic cultures

The free and formulated CHX (liposomes and hydrogels, 2.5 mL) were diluted in water, mixed with an equal volume of Simulated Wound Fluid (SWF, pH 7.4, bovine serum albumin 2% w/v; $CaCl_2$ 0.02 M; NaCl 0.4 M; Trizma base 0.05 M, Cerchiara et al., 2020, final CHX concentration of 0.005 mg mL^{-1}) and inoculated with microbial suspensions (*S. aureus* ATCC239213, *S. aureus* SO88 and *P. aeruginosa* ATCC10145) prepared from a broth culture in log phase of growth (inoculum concentration: 10^6 CFU mL^{-1}). Non-treated suspensions served as a control. Counts of viable cells were carried out on NB (*S. aureus* strains – gram-positive) or BHI (*P. aeruginosa* – gram-negative) plates at the inoculum time and after 3, 6, 8 and 24 h of incubation at 37 °C. Results are expressed as viability (log CFU mL^{-1}) of microorganisms over time in presence of different formulations.

2.9.2. Anti-biofilm activity

The anti-biofilm activity of formulations was assessed against *S. aureus* ATCC239213, *S. aureus* SO88 and *P. aeruginosa* ATCC10145 as described by Giordani et al. (2019). Briefly, two different mechanisms of action were targeted, the inhibition of biofilm formation and eradication of pre-formed biofilm. For the inhibition assay, 100 μL of bacterial suspension (10^6 CFU mL^{-1}) in NB (*S. aureus* strains) or BHI (*P. aeruginosa*) were incubated in 96-multi-well plates together with 100 μL of free or formulated CHX (liposomes and hydrogels). For the eradication assay, the biofilm was first formed for 48 h (200 μL of bacterial suspension 10^6 CFU mL^{-1}) and then treated with 200 μL of free or formulated CHX (liposomes and hydrogels) for 24 h. Crystal violet is a dye commonly employed in microbiology field because it allows staining of the bacterial cell walls. To quantify the biofilm after treatments, the cells adherent to the wells were stained with crystal violet (0.41 %, w/v), dye exhibiting an absorbance peak at 595 nm when dissolved in ethanol. Thus, the higher absorbance (OD_{595}, EnSpire 217 Multimode Plate Reader, PerkinElmer Inc., Waltham, MA) corresponds to a larger number of bacterial cells adherent to the wells of 96-multi-well plates (37 °C, 100 rpm). Results were expressed in percentage relative to the untreated control accordingly to the following equation (Eq. (1), Giordani et al., 2019):

$$\text{Inhibition of biofilm formation/Eradication of pre-formed biofilm (\%)} = [1\text{-}(\text{mean } OD_{595} \text{ sample / mean } OD_{595} \text{ control})] \times 100 \quad (1)$$

2.10. Statistical analyses

Statistical significance was evaluated by one-way ANOVA followed by Turkey's correction or student's *t*-test. The results are expressed as means ± SD.

3. Results and discussions

The treatment of infected wounds requires careful tailoring of formulation properties to optimize the treatment outcome (Malaekeh-Nikouei, Fazly Bazzaz, Mirhadi, Tajani, & Khameneh, 2020; Tottoli et al., 2020). Moreover, the formulation should offer mechanical stability, moisture maintenance, protection from environmental exposure, promotion of tissue regeneration and biocompatibility (Anjum et al., 2016). Importantly, infected wounds require high local concentrations of antimicrobials over prolonged periods, therefore, formulations increasing the antimicrobial and anti-inflammatory effects while reducing chances for resistance development are preferred (Lam et al., 2018). Liposomes-in-hydrogel for antimicrobials is a promising strategy in treatment of infected wounds. The synergy between formulations and antimicrobials create a potential therapeutic advantage.

3.1. Characteristics of CHX-liposomes and liposomes

Liposomes are extensively used in studies targeting skin therapies. These lipid-based vesicles provide sustained and controlled release of associated antimicrobial compounds from a safe carrier (Filipczak, Pan, Yalamarty, & Torchilin, 2020; Gonzalez Gomez & Hosseinidoust, 2020; Ibaraki et al., 2020). They provide a reservoir for compounds within the skin layers increasing the local concentration (Lai et al., 2020; Peers et al., 2020). The optimal size considering a depot effect should be around 200−300 nm (Ternullo, Schulte Werning et al., 2019). Generally, the size of liposomes is very likely influencing the antibacterial activity (Martin et al., 2015). Indeed, it has been reported that conventional liposomes that were effective in delivery of antimicrobials to biofilms had a size significantly below 500 nm (Rukavina & Vanić, 2016). For example, amikacin-loaded liposomes with a mean diameter of approximately 300 nm penetrated readily into *P. aeruginosa* biofilm and infected mucus (Meers et al., 2008). Similarly, azithromycin-loaded liposomes of about 400 nm were able to reduce the growth of *P. aeruginosa* in biofilm (Solleti, Alhariri, Halwani, & Omri, 2015). However, the liposomes in our study were incorporated in hydrogels and not applied as suspensions; therefore, the effect of their size should be rather limited. We generated liposomes of a homogenous size distribution exhibiting a relatively low polydispersity index (PI, Table 1). The size of CHX-liposomes was confirmed with transmission electron microscopy (TEM, Fig. S1). Moreover, the images showed spherical liposomes with well-defined surfaces. The liposomes, in addition to all other formulations presented in this study, are summarized in Table S1.

The zeta potential of empty liposomes was neutral due to the high content of neutral phosphatidylcholine (>94 %), however, CHX-liposomes exhibited zeta potential of almost 50 mV (Table 1), making them highly cationic due to presence of CHX. The physiochemical nature of CHX and its biguanide groups would point to CHX being incorporated within and onto the bilayer of liposomes, which could further increase potential interactions with bacterial membranes (Farkas, Zelkó, Török, Rácz, & Marton, 2001). Recently, Ibaraki and colleagues reported increased damage to bacterial biofilms by cationic compared to anionic liposomes (Ibaraki et al., 2020). The amphipathic nature of CHX allows its distribution stretched towards the core in the inner layer and the surface and within the bilayer resulting in a considerably high entrapment (Hassan et al., 2013).

We evaluated the stability of liposomal dispersions over 4 weeks and did not detected any significant changes in their characteristics (data not included).

3.2. Characteristics of hydrogel

Liposomes require secondary vehicles such as hydrogels to remain on the skin. A key feature of hydrogels in skin therapy is to offer mechanical stability. Hydrogel′s texture properties are often measured as an indicator of mechanical stability. Texture analyses provide both in-process controls and information on stability (Hurler et al., 2012a). The relevant parameters are hardness, cohesiveness and adhesiveness. The hardness describes the skin applicability of the formulation (Ternullo, Schulte Werning et al., 2019). The cohesiveness defines the recovery of the structural network within hydrogel after application and level of deformation (Amasya, Inal, & Sengel-Turk, 2020). The adhesiveness represents the ability to remain on the skin (Hurler et al., 2012a). These features define the applicability and user-friendliness of hydrogels.

The texture properties are influenced by plasticisers and polymer concentrations and the addition of glycerol increased the hydrogel hardness (Fig. 1). This was evident from the results of the glycerol-free hydrogel and the CHX-liposomes-in-glycerol-free hydrogel. CHX, in liposomes-in-hydrogel or free in the hydrogel, did not affect the hydrogel hardness. The cohesiveness did not follow the same pattern as the hardness; all hydrogels demonstrated similar cohesiveness, except from the glycerol-free hydrogel (4.5 %, Fig. 1) that exhibited a significantly higher cohesiveness compared to other formulations. Glycerol was added to hydrogels to improve the long-term texture properties (Hurler et al., 2012a) as well as the level of deformation within the hydrogel, the cohesiveness. Glycerol could create three hydrogen bonds with the amino sugar unit and subsequently increase the mobility (Chen et al., 2018). The adhesiveness decreased when CHX-liposomes were incorporated into the hydrogel as compared to liposome.

Our results cannot be directly compared to other studies since we applied higher polymer concentrations and modified set-up. For example, Ternullo and colleagues observed reduced texture properties upon addition of liposomes, whereas Jøraholmen and colleagues observed the opposite (Jøraholmen et al., 2019; Ternullo, Schulte Werning et al., 2019).

The pH of hydrogels was also investigated (Table S3) to assure an appropriate pH of the final formulation. Although the CHX-liposomes demonstrated a higher pH, liposomes were merely the primary formulation since the CHX-liposomes were further incorporated within a chitosan hydrogel network. The pH of the hydrogels was significantly lower than the pH of the CHX-liposomes, assuring that externally applied formulation does not elevate the issue of high pH; on the contrary, it acts on lowering pH.

Table 1
Characteristics of liposomes and CHX-liposomes.

Sample	Size (nm)	PI[a]	Zeta (mV)	pH	EE (%)[b]
Liposomes	271 ± 16	0.33 ± 0.07	−0.4 ± 0.3	6.9 ± 0.2	–
CHX-liposomes	318 ± 9*	0.24 ± 0.03*	45.5 ± 1.3*	8.0 ± 0.1	94.7 ± 0.7

Results are expressed as means with their respective SD (n = 3, * n = 6).
[a] Polydispersity index.
[b] Entrapment efficiency (%).

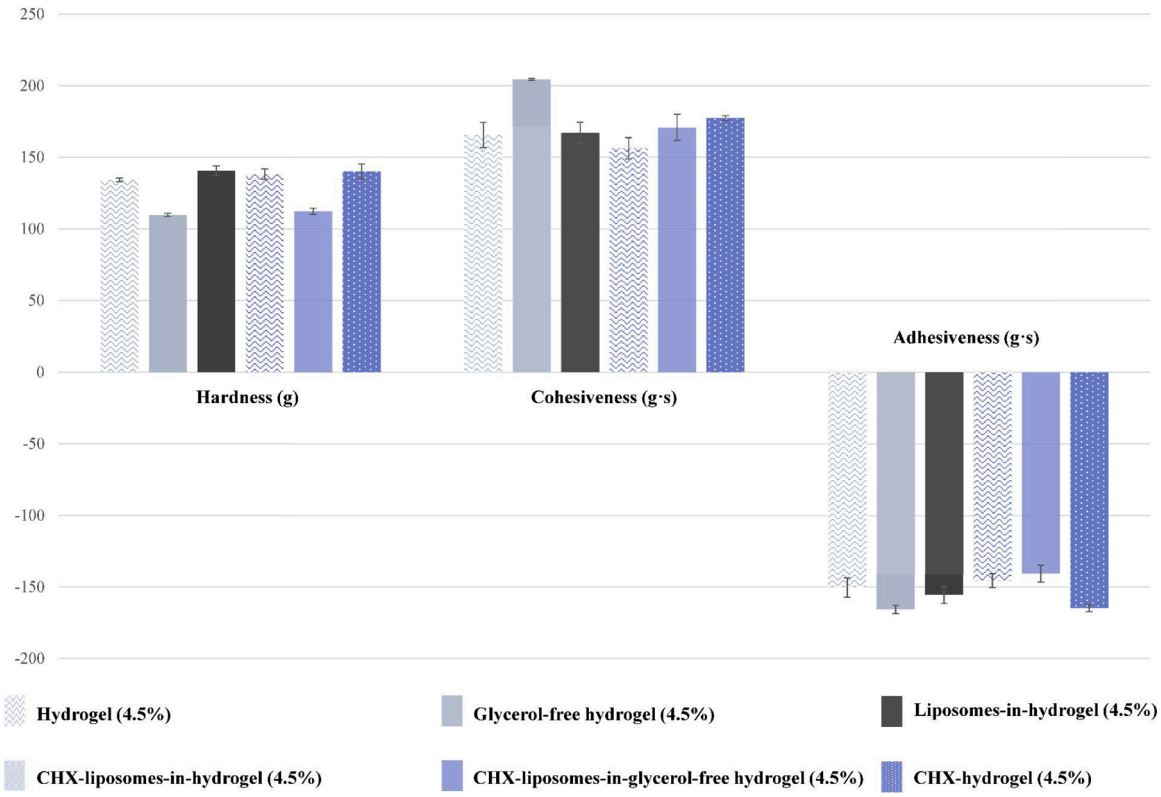

Fig. 1. The texture properties of chitosan hydrogels measured as hardness, cohesiveness and adhesiveness. The values are expressed as mean of three replicates with their respective SD.

3.3. Stability of chitosan hydrogels

The stability testing expressed as the changes of the hydrogels' texture properties (Table S2) indicates that the cohesiveness and adhesiveness of hydrogels comprising glycerol and liposomes remained more stable over a period of 4 weeks than plain hydrogel of the same concentration, probably due to stabilizing effect of both glycerol and liposomes. Additionally, the CHX-liposomes-in-hydrogel exhibited satisfying stability for the evaluation period. This stability could be attributed to the charge of both CHX-liposomes and chitosan, creating repulsive effects through electrostatic forces (Ternullo, Schulte Werning et al., 2019). The liposomal bilayer is electrostatically stabilized and the repulsions might decelerate the release of CHX into the hydrogel network (Grijalvo et al., 2020). The basic CHX is only slowly released into the network, and this would possibly lead to a stable pH in the network. Since liposomes most likely preserve their original size without aggregation, the network mobility would be more stable. In addition, Hurler and colleagues studied the effects of zeta potential of liposomes on the properties of the liposomes-in-hydrogel formulations. The authors postulated that liposomes bearing a positive charge seem to stabilize the chitosan hydrogel network better than neutral or negatively charged liposomes (Hurler et al., 2013). The pH of hydrogels (Table S3) remained stable over four weeks. However, liposomes-in-hydrogels, both with and without CHX, exhibited a small increase in pH.

3.4. Release of CHX

Considering skin therapy, liposomes-in-hydrogel should provide sustained CHX release assuring bacterial eradication without regrowth, reduced administration frequency and improved patient compliance (Smith et al., 2020). Additionally, antimicrobial-liposomes-in-hydrogel formulations prevent burst release from polymeric networks or liposomes alone (Grijalvo et al., 2020).

In Fig. 2, the CHX release (24 h) is presented. CHX-liposomes exhibited sustained release compared to the permeating free CHX ($p < 0.1$). However, incorporation of CHX-liposomes into chitosan hydrogel significantly sustained the release ($p < 0.05$). No differences in release between liposomes-in-hydrogels with and without glycerol were observed. Chitosan hydrogels without liposomes failed to reduce CHX release indicating that liposomes were crucial to provide a sustained release of CHX. Similarly, CHX release from montmorillonite and chitosan composite was reported to be controlled and sustained as compared to free CHX (Onnainty et al., 2016).

These results emphasize the importance of including both systems, liposomes and hydrogel, in the final formulation. In addition to the 24 h endpoint release, we performed preliminary studies on the release profile from CHX-liposomes, CHX-liposomes-in-hydrogel, CHX-liposomes-in-glycerol-free hydrogel and free CHX (Fig. S2). The same patterns as observed in the 24 h endpoint measurements (Fig. 2) were confirmed in hourly release profile; however, we noticed the fluid exchange between the donor and acceptor chamber. Therefore, we proceeded with the endpoint measurement of the release.

3.5. Bioadhesive properties

The efficacy of formulations destined for skin administration is influenced by their bioadhesive properties. Hurler and colleagues developed a method to determine the amount of hydrogel that remained on the skin after compression rather than measuring force of detachment (Hurler & Škalko-Basnet, 2012). The bioadhesive properties of tested hydrogels are found in Fig. 3. All hydrogels were bioadhesive; however, liposomes-in-hydrogel displayed the highest bioadhesive properties.

Most often, reports on bioadhesion refer to mucosal adhesion; however, mechanisms for skin adhesion are also reported (Horstmann, Müller, & Asmussen, 1999; Venkatraman & Gale, 1998). The adhesion to skin is, unlike to the mucosa, more dependent on the surface structure

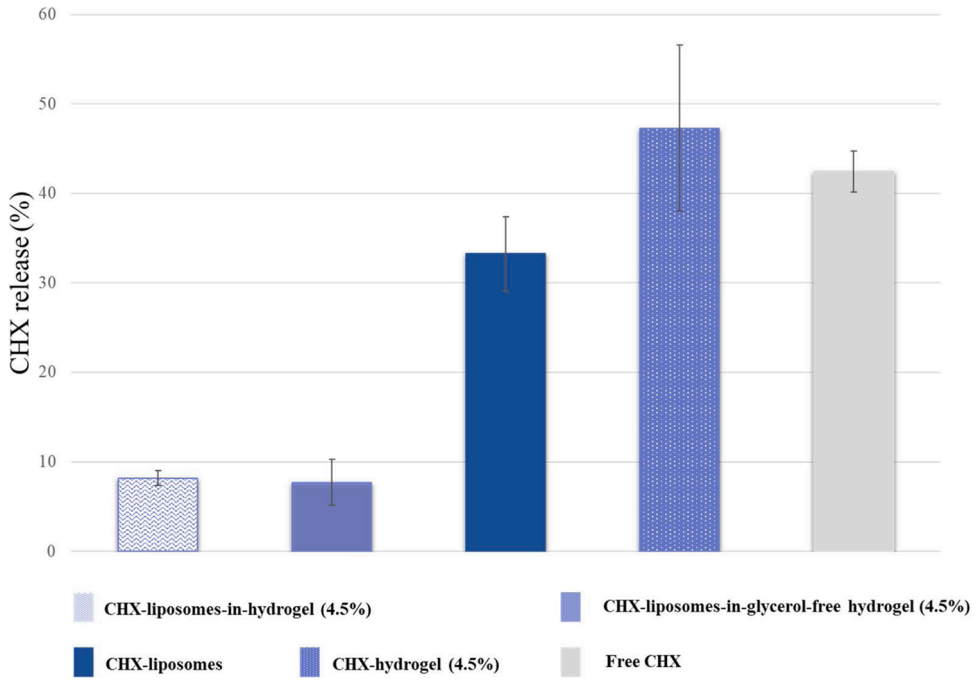

Fig. 2. CHX release or permeation over 24 h at 32 °C. Results are represented as percentage of CHX released compared to the initial concentration. Results are expressed as mean of three replicates with their respective SD.

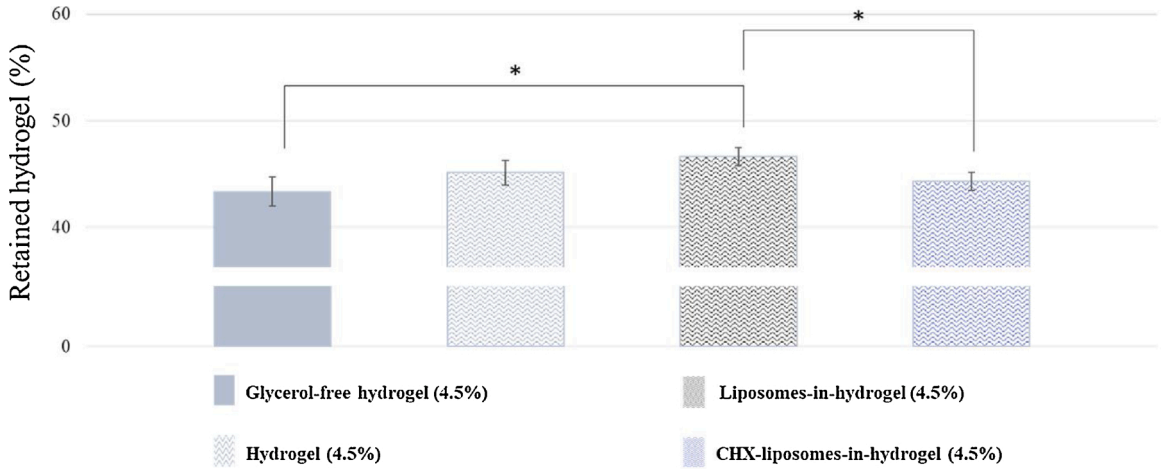

Fig. 3. Bioadhesive properties of chitosan hydrogels and liposomes-in-hydrogel. The bioadhesion is presented as the amount of hydrogel retained on the skin (%) after compression compared to the original amount applied to the die in the rig set to the Texture Analyser. The results are presented as the mean of three replicates with their respective SD.
*) Significantly different ($p < 0.05$).

and size of the surface area (Horstmann et al., 1999). Moreover, the bioadhesion could potentially be affected by interpenetration between the polymer and biological surface (Palacio & Bhushan, 2012), moreover the plasticizer could increase bioadhesion (Horstmann et al., 1999).

3.6. Anti-inflammatory evaluation

Macrophages play an important role in skin infections and inflammations by regulating the production of NO (Kloc et al., 2019). The reduced NO level has been considered beneficial after the inflammatory phase of wound healing. We evaluated the anti-inflammatory effects of chitosan formulations and CHX; CHX is expected to have a mild effect on inflammation in this experimental set-up by neutralizing LPS through binding (Zorko & Jerala, 2008).

Our results confirmed a dose-dependent reduction in NO production (Fig. 4). Even though the difference in production between 1 and 10 μg mL^{-1} was not statistically different except for the CHX-liposomes-in-hydrogel, a clear tendency could be observed. All formulations demonstrated a significant reduction in NO production between 10 and 50 μg mL^{-1}. Furthermore, all formulations at every concentration, except liposomes (1 μg mL^{-1}), demonstrated a significant reduction in NO production compared with the control (non-treated LPS-induced macrophages). The reduction of NO production in the cells treated with hydrogels either with or without CHX liposomes demonstrated a significantly reduced NO production.

Chitosan-based formulations have previously been reported to reduce inflammatory response in macrophages; however, the results were often dependent on MW of chitosan (Chang, Lin, Wu, Huang, & Tsai, 2019). Our group has reported anti-inflammatory activity of liposomes-in-hydrogel formulation with polyphenols (Jøraholmen et al.,

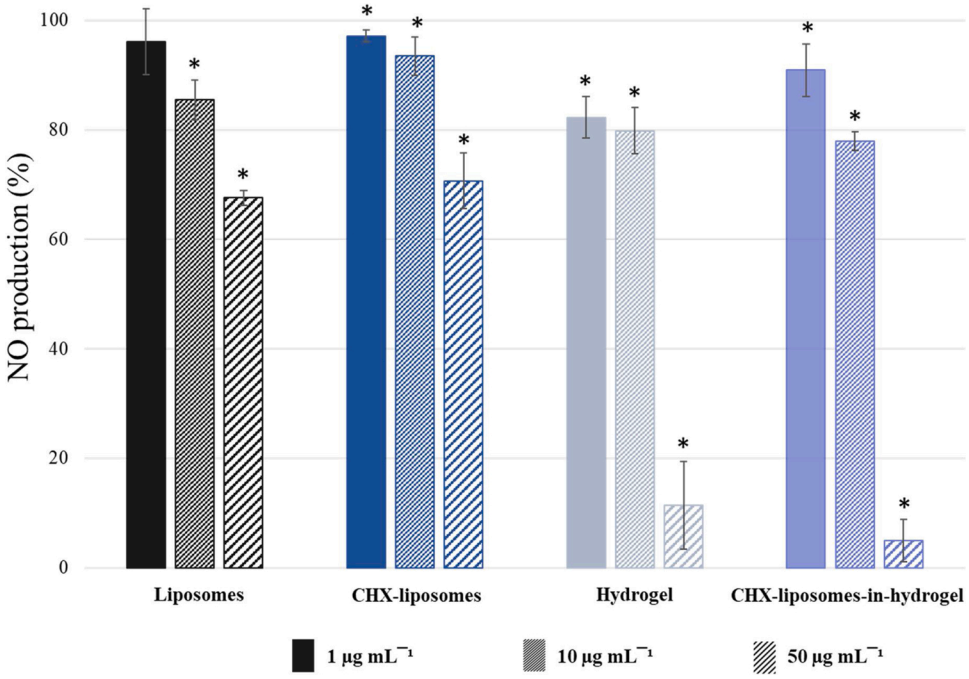

Fig. 4. Anti-inflammatory evaluation on murine macrophages (RAW 264.7). Concentrations refer to the lipid concentration or corresponding concentration where lipids are not present. The NO production is presented as the percentage compared to the NO production in non-treated LPS-induced macrophages (100 %). All results are expressed asmeans of three parallels with their respective SD.
*) Significantly different compared to non-treated LPS-induced macrophages ($p < 0.05$).

2019). In their study, the anti-inflammatory response was lower than we observed, however, the chitosan concentration used in this study was significantly lower than in our study. Since we used higher chitosan concentrations, the differences may indicate that chitosan concentration plays a role in modulating anti-inflammatory response. However, we measured nitrites as an indicator for NO. Due to the anionic nature of nitrites, chitosan might neutralize these inorganic anions (Il'ina & Varlamov, 2016) leading to lower concentration of available nitrites for the Griess reaction. Consequently, the values may be an overestimation of the anti-inflammatory effect and should be further investigated. The most important characteristic to verify is that new formulations for wound therapy do not induce an additional inflammation response, which would lead to impaired healing. Pettinelli and colleagues demonstrated that chitosan hydrogel did not induce any inflammation response in macrophages (Pettinelli et al., 2019). In addition to the evaluation of NO production in macrophages, we evaluated the cell viability of immortalized human keratinocytes treated with CHX-liposomes (Fig. S3). The results demonstrated improved viability of cells treated with CHX-liposomes.

3.7. Antimicrobial evaluation

3.7.1. Minimal lethal concentration (MLC) of formulated and free CHX

The treatment of wound infections is challenging due to the growing problem of antibiotic resistance and tendency of *S. aureus* and *P. aeruginosa* to form biofilms (Serra et al., 2015). Chitosan demonstrates antimicrobial properties towards gram-positive bacteria (Hamedi, Moradi, Hudson, & Tonelli, 2018; Moeini et al., 2020). Since infected wounds comprise polymicrobial manifestation, we aimed to take advantage of the synergic effects of chitosan and CHX to ameliorate the available antibacterial therapies against both bacteria.

We investigated the MLC of free CHX and formulated CHX (liposomes and hydrogels with glycerol) for three bacterial strains (Table S4). As expected, free CHX exerted strong antimicrobial activity against both *S. aureus* and *P. aeruginosa*. The effects were retained in CHX- liposomes, whilst empty liposomes were inactive. Importantly, the incorporation of CHX-liposomes in chitosan hydrogel improved the CHX antibacterial effect, as demonstrated by the lowering of MLC for all tested strains. In addition, hydrogel (4.5 %) revealed an antibacterial activity itself, since it inhibited *S. aureus* ATCC29213, *S. aureus* SO88 and *P. aeruginosa* ATCC10145 at dilutions corresponding to chitosan concentrations of 0.056 %, 1.125 % and 2.25 %, respectively.

3.7.2. Inhibitory activity against planktonic cultures

Considering topical application for the treatment of wounds, the antimicrobial activities towards planktonic culture were evaluated in SWF. The viability of *S. aureus* and *P. aeruginosa* in SWF was compared in the presence of free or formulated CHX (Fig. 5). The untreated bacteria and bacteria exposed to liposomes retained viability. In presence of free CHX, no viable cells were found after 3 h.

CHX-liposomes and CHX-hydrogels both retained antimicrobial activity against planktonic cultures but with delayed effects, due to sustained release of CHX. In particular, CHX-hydrogel completely abolished viability after 9 h, while CHX-liposomes and CHX-liposomes-in-hydrogel required 24 h to achieve a complete depletion of bacterial cells. Interestingly, after 6 and 8 h the antimicrobial effects against all tested microorganisms were more marked for CHX-liposomes-in-hydrogels than for CHX-liposomes ($p < 0.05$), even if release studies showed a reduced release of CHX from CHX-liposomes-in-hydrogels. However, in all cases no viable bacteria were found after 24 h of incubation with formulated CHX (liposomes or hydrogels), as well as with free CHX. It is worth noting that hydrogel without CHX reduced *S. aureus* viability by 1.27–1.55 log CFU after 24 h, while cell viability of *P. aeruginosa* decreased by 1.14 log CFU. This is in agreement with MLC data and suggests that chitosan-based wound treatment improves the antimicrobial potential of CHX.

3.7.3. Anti-biofilm activity

Considering that *S. aureus* and *P. aeruginosa* biofilms are often associated with chronic infections and decreased susceptibility to antimicrobial treatments (Roy, Tiwari, Donelli, & Tiwari, 2018), we focused on anti-biofilm activity of free and formulated CHX against *S. aureus* ATCC29213, *S. aureus* SO88 and *P. aeruginosa* ATCC10145 by means of dispersal and biofilm formation inhibition assays (Fig. 6). Free CHX revealed a moderate anti-biofilm activity, reducing the biofilm formation of all tested microorganisms (inhibition of 42–51 %) and partially eradicating pre-formed biofilm (eradication rate of 29–43 %).

Contrary to what was observed for planktonic cultures, the

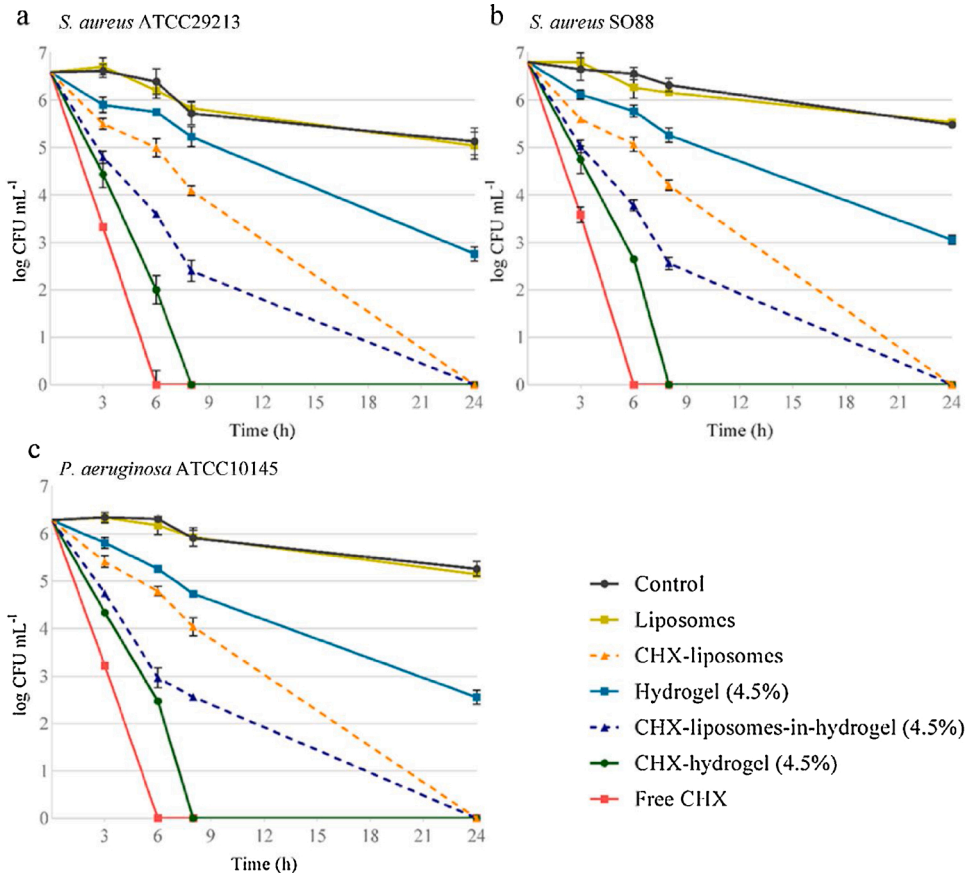

Fig. 5. Viability (log CFU mL^{-1}) of microorganisms over the time (up to 24 h) in presence of different formulations. The results are presented as the mean of three replicates with their respective SD.
a: *S. aureus* ATCC29213; b: *S. aureus* SO88; c: *P. aeruginosa* ATCC10145.

incorporation of CHX inside liposomes significantly enhanced its ability both to counteract biofilm formation (inhibition of 53–63 %) and to disperse pre-formed biofilm (eradication rate of 40–53 %, $p < 0.05$). The increased anti-biofilm activity of CHX-liposomes might be due to a more efficient penetration of positively charged liposomes into the extracellular matrix of biofilms (Rukavina & Vanić, 2016). Our finding is in agreement with data on incorporation of vancomycin (Scriboni et al., 2019) and gentamicin (Alhariri et al., 2017) in liposomes, which improved the penetration of *S. aureus* and *P. aeruginosa* biofilms, respectively. Moreover, reports show that cationic formulations could potentially extend the penetration of active compounds into bacterial biofilm, which are probably negatively charged (Drulis-Kawa et al., 2009; Robinson, Bannister, Creeth, & Jones, 2001). Nevertheless, the composition of external matrix of biofilm is very complex and heterogeneous and can be influenced by numerous factors including surface properties, nutrient availability and microbial species that form the biofilm (Flemming & Wingender, 2010). Thus, we can only make assumptions. However, different authors reported that cationic vesicles showed better performances compared to free active compounds or negatively charged vesicles in targeting biofilm of *Staphylococcus spp*., including *S. aureus* (Rukavina & Vanić, 2016). Actually, our results support the hypothesis that liposomes and cationic formulations may improve the delivery of active compounds to biofilms.

As expected, liposomes showed no anti-biofilm activity, while hydrogels without CHX exhibited a mild anti-biofilm effect ($p < 0.05$). Consequently, CHX-hydrogel improved activity as compared to free CHX ($p < 0.05$).

Notably, the best anti-biofilm profile was observed for CHX-liposomes-in-hydrogels, and was significantly better than CHX-liposomes. In particular, CHX-liposomes-in-hydrogels almost completely inhibited the formation of *S. aureus* and *P. aeruginosa* biofilm, while the eradication rate was higher for *S. aureus* (82–98 %) than for *P. aeruginosa* (64 %). These results suggest that the insertion of CHX-liposomes inside chitosan hydrogel is a promising and innovative strategy to treat wound infections.

4. Conclusion

To address the challenges related to both treatment of chronic wounds comprising biofilms and increased antimicrobial resistance, we developed novel antimicrobial chitosan-based system. The system comprising liposomally-associated CHX in chitosan hydrogel exhibited superior anti-biofilm activities while maintaining properties relevant for skin administration. In addition, we observed anti-inflammatory effects of the chitosan hydrogel, an important feature considering wound therapy.

CRediT authorship contribution statement

Lisa Myrseth Hemmingsen: Conceptualization, Data curation, Formal analysis, Methodology, Validation, Writing - original draft, Writing - review & editing. **Barbara Giordani:** Data curation, Formal analysis, Methodology, Writing - original draft, Writing - review & editing. **Ann Kristin Pettersen:** Data curation, Formal analysis, Writing - review & editing. **Beatrice Vitali:** Methodology, Writing - original draft, Writing - review & editing. **Purusotam Basnet:** Data curation, Methodology, Writing - review & editing. **Nataša Škalko-Basnet:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing - original draft, Writing - review & editing.

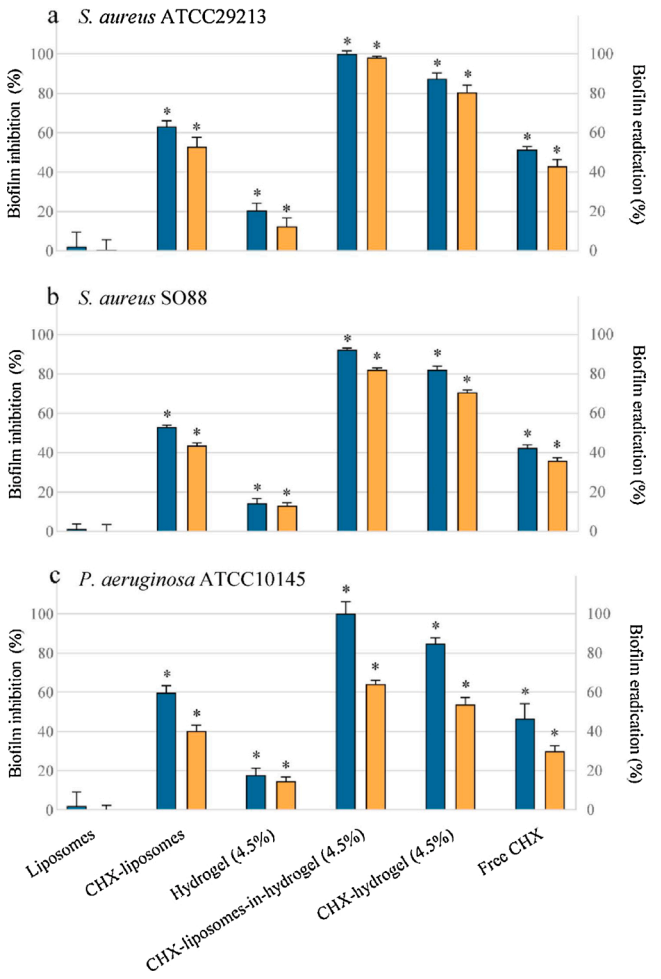

Fig. 6. Anti-biofilm activity: inhibition of biofilm development (%) on left Y-axis (petrol bars), eradication of pre-formed biofilm (%) on the right Y-axis (orange bars). The results are presented as the mean of three replicates with their respective SD.
*) Significantly different from untreated control ($p < 0.05$).
a: *S. aureus* ATCC29213; b: S. aureus SO88; c: P. aeruginosa ATCC10145.

Declaration of Competing Interest

The authors report no declarations of interest.

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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.carbpol.2021.117939.

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