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Old drug, new wrapping – A possible comeback for chloramphenicol?

Sveinung G. Ingebrigtsen[1], Alena Didriksen[2], Mona Johannessen[2], Nataša Škalko-Basnet[1], Ann Mari Holsæter[1,*]

[1)] Drug Transport and Delivery Research Group, Department of Pharmacy, Faculty of Health Sciences, University of Tromsø The Arctic University of Norway, Tromsø 9037, Norway

[2)] Research Group of Host Microbe Interactions, Department of Medical Biology, Faculty of Health Sciences, University of Tromsø The Arctic University of Norway, Tromsø 9037, Norway

Corresponding author:

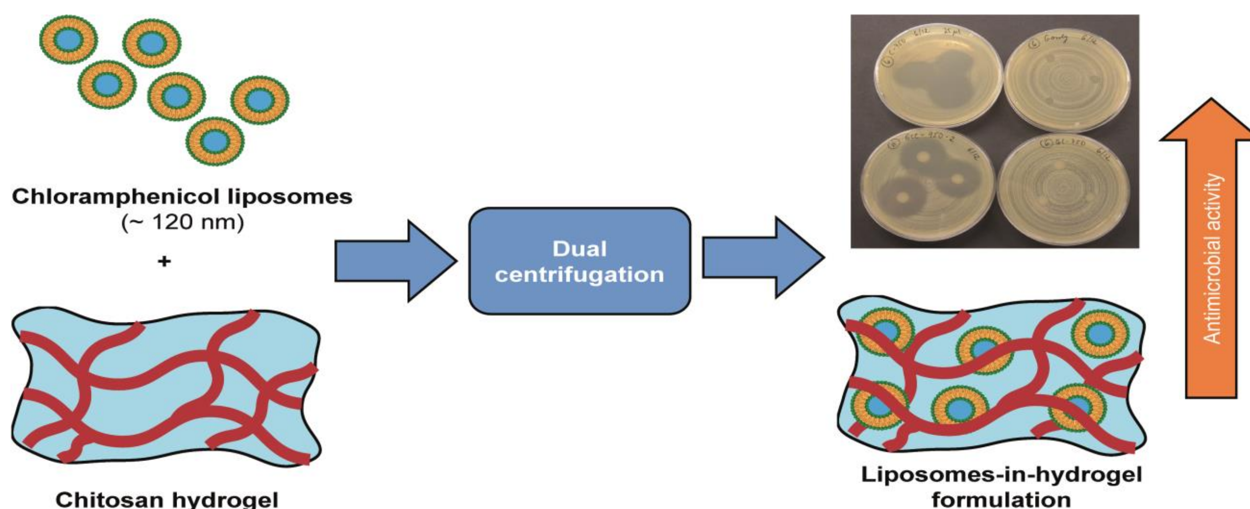
Ann Mari Holsæter

Phone: (+47) 776 46719

Fax: (+47) 77646151

E-mail: ann-mari.holsater@uit.no

Graphical abstract



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Abstract

The antimicrobial drug chloramphenicol (CAM) exhibits activity against resistant bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA). However, its use has been limited due to its toxicity. As the threat of antibiotic resistance continues to grow, a promising approach might be to increase the use of historical antimicrobial agents that demonstrate clinical efficacy, but are hampered by toxicity. We therefore aimed to prepare a liposome-in-hydrogel system for dermal delivery of CAM. Chitosan (CS) was used as the hydrogel vehicle due to its antimicrobial activity and excellent biocompatibility. All critical preparation steps were carried out by dual centrifugation (DC). The DC-method proved to be fast and simple, and organic solvents were avoided in all processing steps. Liposomes with high drug entrapment (49-56%), low polydispersity and a size of approximately 120 nm were produced. Mixing of liposomes into CS-hydrogel by DC produced a homogenous liposomes-in-hydrogel system. Bioadhesive properties were good and comparable to plain CS-hydrogel formulations. *Ex vivo* permeation studies using pig skin indicated a sustained release of CAM and limited skin permeation. The *in vitro* antimicrobial activity of CAM in the new liposome-in-hydrogel formulation was similar or better as compared to CAM in solution. Thus, the new formulation was considered highly promising.

List of abbreviations:

CAM	Chloramphenicol
CAM-Lip	Dialyzed liposomes containing entrapped chloramphenicol
CS	Chitosan
CS-CAM	Chitosan hydrogel with free chloramphenicol
CS-CAM-Lip	Chitosan hydrogel with chloramphenicol entrapped in liposomes (CAM-Lip)
CS-hydrogel	The plain 2.5% chitosan hydrogel with 10% glycerol
CS-Lip	Chitosan hydrogel with empty liposomes
DC	Dual centrifugation
dH_2O	Distilled water
HPLC	High-performance liquid chromatography
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*

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PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PI	Polydispersity index
S. aureus	*Staphylococcus aureus*
S. epidermis	*Staphylococcus epidermis*
VPGs	Vesicular phospholipid gels

Keywords: Dermal drug delivery, antibiotic, liposomes, chloramphenicol, chitosan, dual centrifugation

1. Introduction

Antimicrobial resistance represents a serious threat towards global health (World Health Organization, 2014), as the increasing resistance towards antimicrobial agents, and the emergence of so-called "super-bugs", will limit the future options in infection treatment, unless new antimicrobial treatments are developed. However, discovery and development of new antimicrobial compounds have proven to be difficult and time-consuming (Fernandes, 2015; Shlaes, 2015). Therefore, the revival of old or historical antimicrobial compounds has been suggested as an alternative approach to meet the demand for effective treatments against drug-resistant pathogens (Cassir et al., 2014; Falagas et al., 2008). Nanocarriers can be utilized to assure the efficacy and provide targeted activity of antimicrobials (Abed and Couvreur, 2014; Basnet and Škalko-Basnet, 2013), providing a new "wrapping" for "forgotten" antimicrobials.

Chloramphenicol (CAM) is a broad-spectrum antimicrobial agent, derived from the bacterium *Streptomyces venezuelae*, and introduced to the clinic in the late 1940s as the first antibiotic to be manufactured synthetically on a large scale (Ehrlich et al., 1947). However, its use has been hampered by its toxicity; systemic administration of CAM is associated with development of aplastic anemia and suppression of the bone-marrow (Feder et al., 1981). Due to this rather severe toxicity, its application has mostly been restricted to topical use for treatment of eye and ear infections. Consequently, CAM's limited use has preserved its antimicrobial activity, enabling it to exhibit activity against pathogens such as methicillin-resistant *Staphylococcus aureus* (Fayyaz et al., 2013).

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Local application of CAM for treatment of skin infections and burn wounds might be a way to utilize the preserved antimicrobial activity of CAM. The applied formulation should then be designed to deliver a high concentration of CAM to the area of administration/infection, retaining the activity of the drug at the site of action, while avoiding the potential toxicity associated with systemic uptake. Successful entrapment of CAM into liposomes has been demonstrated by our group (Hurler and Škalko-Basnet, 2012a; Ingebrigtsen et al., 2017, 2016), and CAM in liposomes has been confirmed to exhibited low cell toxicity towards HaCaT (keratinocytes) cells, when studied in clinically relevant concentrations applying the MTT assay (Ingebrigtsen et al., 2017). Therefore, in this present study, a novel wound dressing of CAM-liposomes incorporated in a hydrogel was developed.

The hydrogel vehicle should act as a liposome dispersion media, retaining the incorporated liposomes at the site of administration (Bhattarai et al., 2010). Chitosan (CS) is a natural polysaccharide polymer produced by deacetylation of chitin, a component in the exoskeleton of arthropods. The inherent biodegradability and biocompatibility of CS, and its bioadhesive properties, explains its extensive use in biomedical applications, such as in tissue engineering and drug delivery (Dash et al., 2011). However, it is the antimicrobial activity against various fungi and bacteria, such as *Stahylococcus aureus*, *Klebsiella pneumoniae*, and *Escherichia coli* (Fei Liu et al., 2001), that makes the CS hydrogel even more attractive as a vehicle for the delivery of antimicrobial agents, both in the treatment of skin infections and as a wound dressing (Kandimalla et al, 2013). CS is proposed to interact with the bacteria cell wall through electrostatic interaction and alter the bacteria cell wall permeability, which in turn results in leakage of cell material (Fei Liu et al., 2001; Kong et al., 2010; Rabea et al., 2003). The first aim of this study, therefore, was to investigate a synergy-based system, namely the antimicrobial activity of CS as a vehicle, combined with the antimicrobial activity of liposomal CAM.

The dual centrifugation method (DC-method) was applied to manufacture the liposomes-in-hydrogel delivery system, as it provides a rapid, reproducible and efficient size reduction of liposomes from the concentrated vesicular phospholipid gel (VPG) intermediate (Ingebrigtsen et al., 2016, 2017; Massing et al., 2008), and is known to enable efficient mixing of liposomes and hydrogel with removal of air bubbles and minimum risk of contamination (Ingebrigtsen et al., 2016). To further optimize the DC-method, especially in regard to potential industrial

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manufacturing, a new prototype DC-machine – the ZentriMix 380 R, was tested. With this new machine, the second aim of the study was to circumvent some of the obstacles with the previously established DC-method (Ingebrigtsen et al., 2017, 2016). The first improvement that was aimed for was to avoid the intermediate step of making a lipid-film for subsequent hydration and size reduction of the liposomes, as this would save time and eliminate the need for organic solvents during liposome preparation. Secondly, the potential of up-scaling without the need for extensive modifications of the previously developed DC-method, applying the Speedmixer DAC 150.1 FVZ-K (Ingebrigtsen et al., 2016) was investigated. The new ZentriMix 380 R centrifuge is more flexible both regarding batch sizes and number of samples/vials processed in parallel, and also has an integrated temperature controller, that allows longer run times (up to 99 hours), whereas the previously applied DC-machine- the Speedmixer, only allows run times of 5 minutes (Massing et al., 2017).

Finally, the optimized DC-method for making liposomes-in-hydrogel formulations was applied with CS as the hydrogel of choice and with CAM as the active ingredient. The novel formulation was characterized and the antimicrobial activity tested using clinically relevant bacteria in skin infections.

2. Materials and methods

2.1. Materials

Anhydrous acetic acid, ammonium molybdate, chloramphenicol (CAM), hydrochloric acid, Fiske-Subbarow reducer, glycerol 86-88%, high molecular weight (Mw = 310 – 375 kDa, degree of deacetylation > 75.0%) chitosan (CS), methanol, propylene glycol, monobasic potassium phosphate, sodium chloride and dibasic sodium phosphate dihydrate were bought from Sigma-Aldrich Chemie (Steinheim, Germany). Concentrated sulfuric acid was purchased from May & Baker Ltd (Dagenham, England), while zirconium oxide beads (Ø = 1.4 mm) was purchased from Bertin Technologies (Saint-Quentin-en-Yvelines, France). Lipoid E80 (egg lecithin, approx. 80% phosphatidylcholine (PC)), Phospholipon® 90H (hydrogenated soy lecithin, min. 90% PC), Lipoid S100 (soy lecithin, min. 94% PC), and Soluthin® S90 (soy lechitin, min. 83%, associated with calcium chloride) were kindly provided by Lipoid GmbH (Ludwigshafen, Germany). The

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pig ears used in drug release experiments and bioadhesion testing were purchased from Nortura AS (Bardufoss, Norway).

All chemicals used were of analytical or HPLC grade.

2.2. Liposome production by dual centrifugation

Liposomes were prepared in two successive steps; first, 200 mg of lipids (Lipoid E80, Phospholipon 90H, Lipoid S100 or Soluthin S90) and 20 mg CAM (for CAM-containing liposomes) were dissolved in 100 µl of propylene glycol, before adding 200 µl of distilled water (dH_2O) and 130 mg zirconium oxide beads (Ø = 1.4 mm). The mixtures were transferred to 2 ml polypropylene test tube (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). The tubes were transferred to the ZentriMix 380 R dual centrifuge (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany), with the following applied settings; run time: 5 min, speed: 2350 rpm (~ 800 × g), and temperature of 30°C. In the second step, the produced VPGs were diluted with dH_2O and vortex mixed for 15 minutes on a MS2 Minishaker Vortexer (IKA-Werke GmbH & Co. KG, Staufen, Germany) to attain diluted liposome dispersions with a total lipid concentration of 100 mg/ml and a CAM concentration of 10 mg/ml.

2.3. Liposomal drug entrapment

Free (unentrapped) CAM was removed from the liposome dispersions applying dialysis tubing with a cut-off value of 12000–14000 Da (Medicell International Ltd., London, UK). In order to assure sink conditions, the sample:dialysis medium (dH_2O) volume ratio was 1:500. Dialysis was carried out for 4 hours. The entrapment efficiency was determined by comparing the CAM concentration (as determined by HPLC, Section 2.4) in the original- and the dialyzed sample (Equation 1).

Equation 1.

$$\mathrm{EE}\ (\%) = \frac{\mathrm{C}_{\mathrm{retenate}}}{\mathrm{C}_{\mathrm{total}}} \times 100$$

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2.4. Chloramphenicol quantification by HPLC

Quantification of CAM was performed by HPLC analysis, on a Waters e2795 Separations Module, and with a Waters 2489 UV/Visible detector. A reversed phase C18 column (XTerra® RP18 5 µm, 3.9 × 150 mm column; Waters, Dublin, Ireland) was applied together with a Symmetry C18 pre-column. This method was based on a previously described method from our group (Ingebrigtsen et al., 2016). Detection wavelength was 278 nm, flow rate was 0.9 ml/min, and the mobile phase contained filtered water, methanol and anhydrous acetic acid (55:45:0.1 v/v). The retention time of CAM was 4.5 min, and total run time was set to 6 min with a 3 min injection delay between every run. Limit of quantification (LOQ) and limit of detection (LOD) for the applied HPLC-method were 0.924 µg/ml and 0.305 µg/ml, respectively.

2.5. Liposome size and zeta-potential

Liposome size, polydispersity index (PI) and zeta-potential were determined using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK). Samples were diluted 1:100 (v/v) with filtered dH_2O before the analyses of size and PI, and diluted 1:10 (v/v) with filtered dH_2O to measure the zeta potential. Analyses were performed in triplicates with an equilibration time of 180 seconds. Drive-voltage and number of runs per analysis were set automatically by the instrument software (Zetasizer software 7.11, Malvern Instruments Ltd., Malvern, UK).

2.6. Quantification of lipid content

The concentration of PC in the liposome samples was determined by a modified Bartlett assay (Bartlett, 1959), which has been described in more detail in previous publications from our group (Ingebrigtsen et al., 2017, 2016). In brief, concentrated sulfuric acid was added to diluted liposome samples and heated at 160 °C for 3 h. Then 30% water-free hydrogen peroxide was added to assist the ashing process. The samples were cooled to room temperature, and a solution of 0.22% ammonium molybdate and Fiske-Subbarow reducer were added before heating at 100 °C for 7 minutes. The concentration of phosphorus was determined by colorimetric analysis at 830 nm, to measure the amount of the blue color from 4-amino-2-naphthyl-4-sulfonic acid.

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Finally, the PC concentration was calculated based on the measured absorbance value for a pre-made stock solution of known PC concentration.

2.7. Preparation of hydrogel formulations

2.7.1. Plain hydrogels with 10 % glycerol

The hydrogel vehicle (CS-hydrogel), consisting of 2.5% (w/w) high molecular weight (Mw = 310 – 375 kDa, DD > 75.0%) CS and 10% (w/w) glycerol was prepared based on the method described by Hurler et al. (2012b). In brief, glycerol was mixed with a 2.5% (w/w) acetic acid solution in dH_2O, before adding CS. The mixture was hand-stirred for 10 minutes to assure even distribution of CS, and thereafter allowed to swell in a closed container for 48 hours at room temperature. The final pH-value of the CS-hydrogel was 4.5 ± 0.13.

2.7.2. Hydrogels containing the free or liposomally encapsulated CAM

Batches of 10 g CS-hydrogels containing 0.5 mg/g free CAM (CS-CAM) were prepared by mixing 50 µl of a 100 mg/ml CAM stock solution in propylene glycol with the CS-hydrogel. Mixing was performed for 5 minutes at 3500 rpm in a Max 40 cup (Synergy Devices Ltd., High Wycombe, UK), using the dual centrifuge Speedmixer (DAC 150.1 FVZ-K Speedmixer, Synergy Devices Ltd., High Wycombe, UK).

To prepare the CS-hydrogels with CAM entrapped in liposomes (CS-CAM-Lip), liposome dispersions with CAM were first dialyzed to remove the unentrapped drug (Section 2.3) before being mixed into the CS-hydrogel, applying the same mixing conditions as for the CS-CAM formulation. The CS-CAM-Lip formulations were prepared with a final content of 10% (w/w) liposome dispersion, formed by mixing 1 ml of the dialyzed liposome dispersion (CAM-Lip) into the CS-hydrogel, corresponding to 10 mg/g lipid and 0.5 mg/g CAM, respectively.

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2.8. Homogeneity of liposomes-in-hydrogel formulation

The homogeneity of the final liposome-in-hydrogel formulations (CS-CAM-Lip) was tested to assess the efficiency of the DC-mixing procedure. Five and three samples were withdrawn from the liposome-in-hydrogels to determine the uniformity of CAM- and lipid content, respectively. HPLC analysis (Section 2.4) was used to quantify the amount of CAM, while the lipids were quantified by performing the lipid assay (Section 2.6).

2.9. Preparation of pig skin samples

Pig skin samples were applied to study the bioadhesion of the hydrogel formulations (Section 2.10) and the drug release and skin permeation from the different formulations (Section 2.11). Pig ears were delivered frozen in bulk from the slaughter. Thus, they were thawed overnight in the refrigerator (4 °C) before excess fat and cartilage were carefully removed. Finally, the prepared skin samples were rinsed with phosphate buffered saline solution (PBS) pH 7.4 (2.98 g/l disodium hydrogen phosphate dihydrate, 0.19 g/l monobasic potassium phosphate, and 8 g/l sodium chloride). All the prepared samples had a thickness of 1.60-1.70 mm.

2.10. Determination of bioadhesion

The bioadhesion of the different hydrogel formulations (CS-hydrogel, CS-CAM and CS-CAM-Lip) to the pig skin was tested using a TA.TXT^{plus} Texture Analyzer (Stable micro systems, Surrey, UK). The method applied was a modified method developed by Hurler et al. 2012a). In brief, a piece of pig skin was fixed in a test rig (Stable micro systems, Surrey, UK). A die filled with 150 μl hydrogel formulation was lowered down to the skin and detached from the skin applying a speed of 0.1 mm/s. The applied force when the die was placed on the skin was 25.0 g and the contact time was set to 10 seconds. Bioadhesion was quantified by (1) the amount of force required to detach the die filled with hydrogel formulations from the area of application, and (2) the amount of formulation retained on the pig skin. Each formulation was tested on three different skin samples and the measurements were repeated 5 times for each skin sample.

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2.11. *Ex vivo* permeation studies

Drug release from different vehicles (CS-CAM, CS-CAM-Lip and CAM-Lip) and further permeation through the pig skin, were investigated using Franz diffusion cells (PermeGear, Bethlehem, USA) with a 5 ml acceptor chamber, and a donor diffusion area of 0.64 cm^2. The cells were connected to a heating circulator (F12-ED, Julabo Laboratechnik, Seelback, Germany), and the temperature was kept at 32 °C during the experiments. The control, a 0.5 mg/ml solution of CAM in dH_2O (CAM-Sol) was prepared from a 1 mg/ml CAM stock solution in dH_2O. A sample volume of 300 µl was applied in the donor chamber. Five ml PBS, pH 7.4, was used as the acceptor phase. Sampling from the acceptor chamber was performed every hour for 8 hours, and a sample volume of 500 µl was withdrawn at each time point. The withdrawn sample volume was immediately replaced with the fresh acceptor phase. After 8 hours, the recovery of the drug from the samples was measured considering the remaining CAM on the surface of the pig skin membrane after washing the surface with the HPLC mobile phase. CAM in the skin was carefully extracted from the skin sample with the HPLC mobile phase. All experiments were performed in triplicates. CAM was quantified by HPLC (Section 2.4). The pig skin samples were visually inspected both prior and after the experiments to assure that the integrity of the skin, and sink condition was assured in the acceptor chamber throughout the experiments.

2.12. Evaluation of antibiotic activity

2.12.1. Determination of minimum inhibitory concentration for CAM

The minimum inhibitory concentrations (MIC) of CAM in five different *Staphylococcus aureus (S. aureus)* bacteria isolates *(*ATCC25923, N315, NCTC10442, MSSA476 and SH1000) and three different *Staphylococcus epidermidis (S. epidermis)* isolates*,* were determined using MIC test strips for CAM (MIC test strip C 0.016-256 µg/ml, Liofilchem s.r.l., Roseto degli Abruzzi, Italy) as per the manufacturer's instructions.

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2.12.2. Agar diffusion testing

The zone of inhibition of CAM in different vehicles was investigated under aseptic conditions applying agar diffusion testing. In short, bacteria suspensions with a turbidity of 0.5 McFarland were prepared in 0.9 % (w/w) sodium chloride solution. Plates filled with Mueller Hinton medium were swabbed with the bacteria suspension. CAM in liposomes (CAM-Lip) and CAM in dH_2O (CAM-Sol) in two different concentrations (500 and 750 µg/ml, respectively) were tested in seven different bacterial strains; four *S. aureus* (ATCC25923, N315, NCTC10442 and MSSA476) and three clinical isolates of *S. epidermidis*. The testing was performed with duplicate agar plates, and the samples were applied onto the plates in three spots with a volume of 10 µl per spot.

The final antibiotic activity of the liposome-in-hydrogel formulation containing CAM (CS-CAM-Lip) was tested on two strains of *S. aureus* (NCTC10442 and MSSA476) and two clinical isolates of *S. epidermidis*, and compared to hydrogel formulation without CAM (CS-hydrogel), hydrogel with empty liposomes (CS -Lip), and CAM dissolved in dH_2O (CAM-Sol). All samples were tested in concentrations corresponding to CAM concentrations of 500 µg/ml and 750 µg/ml, respectively. The volume used for each spot was 25 µl.

The diameter of the inhibition zones was measured after 24 h of incubation at 37 °C for all tests performed.

2.13. Statistical analysis

Analysis of variance (ANOVA) and Student's *t*-test were performed using Microsoft Excel 2016 with the Analysis ToolPak add-in (Microsoft, Redmond, USA) to determine statistical significance. A p-value < 0.05 was considered significant.

3. Results and discussion

To revive CAM as a potent antibacterial agent, the focus in this study has been on treatment of dermal bacterial infections by developing CAM-formulations intended for topical administration, and with a limited systemic absorption of CAM, to avoid unwanted side effects. To attain the

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aimed topical antimicrobial efficacy from the new CAM-formulation, a CS-based CAM-in-liposomes-in-hydrogel formulation was chosen. For the formulation to be considered successful, a sufficient bioadhesion and antibacterial efficacy should be demonstrated, together with a sustained CAM release and retention of the drug at the site of action. Additionally, the preparation method should be as straightforward as possible, and suitable for transfer into industrial manufacturing scale.

Previously, we have reported on a novel processing method for the production of liposomes and liposomes-in-hydrogel formulations for topical skin application by a dual asymmetric centrifuge (DAC 150.1 FVZ-K Speedmixer) (Ingebrigtsen et al., 2016). The method was based on the original method developed by Massing and coworkers for preparation of liposomes for parenteral administration by dual asymmetric centrifugation (Massing et al., 2008). Since then, a prototype of a new dual centrifuge, the ZentriMix 380 R, has been developed. This centrifuge has a symmetrical sample set-up unlike the asymmetric setup of the Speedmixer; however, the principle of dual centrifugation remains the same. The ZentriMix 380 R is larger and has several sample holders, where up to 40 vials can be processed at once, making this new centrifuge attractive for pilot manufacturing and scale up. In addition to the improvements regarding the sample capacity and batch size flexibility, the ZentriMix 380 R also offers temperature control and longer run time than the Speedmixer (Massing et al., 2017). Thus, this new methodology represents a step closer to the larger scaled production that is needed in further clinical trial experiments.

3.1. Processing and characterization of liposomes

CAM is a relatively lipophilic drug, that due to its physiochemical properties; Mw of 323.1 g/mole, a log P (octanol/water) of 1.1, a pKa value of 5.5, and a solubility of 1 in 400, and 1 in 2.5 in water and ethanol, respectively, is expected to be present mainly in the lipid bilayer of liposomes, but in an equilibrium with the aqueously dissolved CAM in the aqueous core of the liposomes and outside the liposomes. Thus, highly concentrated liposome dispersion, or vesicular phospholipid gels (VPGs), favors high entrapment of the drug, and successful CAM-entrapment has been reported applying the dual centrifugation liposome preparation method (DC-method), with VPGs as an mandatory intermediate in the liposomes processing procedure (Ingebrigtsen et

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al., 2017, 2016). In these previous studies, CAM and lipids (PC) have been dissolved in organic solvents (methanol and chloroform) to form a lipid film, which assures a homogeneous mixture and a high entrapment efficiency of CAM in the liposome vesicles formed after hydration of the lipid film, and subsequent size reduction by the DC-method (Ingebrigtsen et al., 2017, 2016). Thus, to simplify the liposome processing method and to avoid the use of organic solvent, our first aim was to investigate whether lipids and CAM could be sufficiently mixed with dH_2O and propylene glycol directly in the ZentriMix.

Since the ZentriMix is a prototype centrifuge, the recommended protocol for liposome production provided by the manufacturer was our starting point for further development of the liposome preparation method. Apart from the difference in equipment, the most significant method changes made from previous publications (Ingebrigtsen et al., 2017, 2016), and that might have affected the DC-processing outcome, are summarized in Table 1. In brief, the sample containers were changed from 30 ml glass injection vial to 2 ml polypropylene tubes. The orientation of the sample holder was also different, and changed from vertical to horizontal. As homogenization aid, glass beads (50% (w/w)), were replaced by smaller, heavier and more abrasion-resistant ceramic beads (zirconium beads 25% (w/w)), in order to avoid the formation and contamination by glass dust during processing. Finally, the centrifugation speed, and the run time were adjusted to be within the recommended conditions for preparation of liposomes, given by the supplier of the ZentriMix.

With these adjustments in the processing conditions, the most pronounced improvement was that, contrary to earlier (Ingebrigtsen et al., 2016), CAM-liposomes were successfully prepared by mixing CAM, PC, propylene glycol and dH_2O directly in the ZentriMix. Thus, the intermediate lipid-film formation using organic solvents was avoided. The reason why we failed to entrap CAM without forming a lipid film, when using our previous method with the Speedmixer (Ingebrigtsen et al., 2016), but succeeded with the ZentriMix, might be explained by the smaller tubes applied in the ZentriMix. The smaller tube's surface area contributes to a more efficient homogenization compared to the larger surface of the injection vial. The horizontal position of the sample tubes in the ZentriMix, might also contribute to a higher homogenization effect (Massing et al., 2017). The increased homogenization efficiency of the ZentriMix is also reflected by the smaller liposome size and PI obtained by the new method as compared to the

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previous method, although a significant longer processing time, a higher speed, and more homogenization aid was applied by the previous method (Ingebrigtsen et al., 2017). Avoiding organic solvents in liposome manufacturing not only complies with requirement related to environmental protection and health hazards, but also eliminates the possible risks associated with the presence of trace amounts of these toxic solvents, which could also contribute to chemical instability of the final formulation.

The choice of raw materials is an important factor to consider before scale-up, concerning both cost and success of processing. The choice of lipids and its effect on liposomal characteristics, namely liposome size and drug entrapment, was therefore investigated. A one-lipid formulation was targeted, primarily for scale up reasons, but also because cholesterol, frequently applied to stabilize liposome membranes, has been reported to lower the entrapment of CAM and inactivate the antimicrobial activity of CAM in liposomes made for ocular administration (Mahmoud et al., 2008). Lipoid E80 is made from egg lecithin, frequently applied in liposomal production, and less expensive than the purer Lipoid S100 (Jaafar-Maalej et al., 2010; Li et al., 2014; Tian et al., 2010), that has been applied successfully for preparation of CAM-containing liposomes earlier (Ingebrigtsen et al., 2017, 2016). Lipoid E80 was therefore considered to be attractive for up-scaling proposes. In addition to Lipoid E80 and Lipoid S100, two lyophilized lipids, Soluthin S90 and Phospholipon 90H, were tested, as it was postulated that these fine powdery lipids might enable easier mixing with the drug than the sticky semi-solid lipids, Lipoid S100 and Lipoid E80. The processing method applied was the same for all the four lipids, and results showed that three out of the four lipids were suitable choices for successful preparation of CAM-liposomes by ZentriMix (Table 2). The Soluthin S90 was found unsuitable for the preparation of liposomes, as this lipid is associated with calcium chloride and therefore formed a foamy dispersion after dilution of the VPG with dH_2O. Regarding the three other lipids tested; Lipoid E80, Lipoid S100 and Phospholipon 90H, they all provided liposome dispersions with high CAM-entrapment (approximately 50%), with no significant difference regarding the different lipids (one-way ANOVA, $p = 0.27$). The liposomes also had a comparable size range of approximately 120-130 nm in diameter. However, the liposomes formed from Lipoid S100 were significantly larger ($p < 0.05$) and had a lower PI ($p < 0.5$), than those formed from the two others lipids (Table 2). The lower PI of the Lipoid S100 liposomes can be explained by soybean phospholipids containing more unsaturated fatty acids than the egg yolk phospholipids, present in Lipoid E80; Lipoid E80

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has a high total content (>98%) of stearic and palmitic acid, and less than 2% of unsaturated fatty acids. Phospholipon 90H contains even less flexible hydrogenated fatty acids, approximately 85% stearic acid and 15% palmitic acid, and has a relative high phase transition temperature (approximately 55 °C). Thus, a more rigid lipid bilayer seems to easy the vesicle size reduction, but also to exhibit a more heterogeneous size distribution consequently increasing the PI of the liposomes (Table 2). Thus, there was no added advantage associated with the use of lyophilized lipids for DC processing, as initially postulated. Since only small differences in the entrapment efficiency and size distributions were seen between the three lipids that formed liposomes successfully, the least expensive lipid, Lipoid E80, was preferred for further preparation of the final liposomes-in-hydrogel formulation.

As shown in Table 2, the zeta-potential measured in dH_2O was, as expected, close to neutral for both the Lipoid S100 and the Phospholipon 90H liposomes, whereas the Lipoid E80 liposomes had a zeta potential close to -30. The negative zeta-potential of the Lipoid E80 liposomes is caused by the presence of the negatively charged lipid species such as phosphatidic acid, phosphatidylinositol, phosphatidylglycerol and fatty acids. The acidic pH of the CS hydrogel formulation (pH approximately 4.5), and the positive charge of the CS-hydrogel at this condition (Dash et al., 2011), are however expected to neutralize the negative zeta-potential of the Lipoid E80 liposomes, and thus counteract the effect that the high ratio of negatively charged lipids in the human skin might have on the permeation and retention time of drugs when administered by surface charged carriers (Kirjavainen et al., 1996; Sinico et al., 2005). The CS hydrogel is also expected to provide an overall positive charge to the liposomes-in-hydrogel formulation (Andersen et al., 2015; Jøraholmen et al., 2014).

3.2. Homogeneity of liposomes-in-hydrogel formulations

To validate the efficiency of the DC mixing procedure, the homogeneity of three batches of the final liposomes-in-hydrogel formulation was tested by random sampling from different parts of the formulation. In accordance with our previous results for liposomes-in-hydrogel systems (Ingebrigtsen et al., 2016). A very homogenous distribution of both lipid (liposomes) and drug (CAM) was obtained after only 5 min mixing, with a coefficients of variation < 1.6% for the CAM content and < 8% for the lipid content. Upon visual inspection, the efficiency of the DC-

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mixing was also confirmed by the milky-white appearance of the liposomes-in-hydrogel formulation. The DC-processing also efficiently removed air bubbles from the hydrogel. Thus, the mixing procedure was found to work successfully.

3.3. *Ex vivo* permeation of CAM

The entrapment of drugs in liposomal carriers is expected to contribute to a more sustained and controlled release of the drug at the site of application. Moreover, it is also expected that incorporation of liposomes into a secondary vehicle, such as a hydrogel, will influence the release characteristics of the drug (Hurler et al., 2013; Mourtas et al., 2008, 2007). To investigate the release characteristics and skin permeation of CAM, simultaneously assessing the effect of different vehicles, a vertical Franz diffusion cell setup was applied. Pig ear skin was used as a model membrane for the experiments, as it has comparable properties to human skin (Schmook et al., 2001). It is therefore expected to better mimic the barrier properties of the *stratum corneum* and *in vivo* conditions compared to our earlier release studies using cellophane membrane (Ingebrigtsen et al., 2017).

The permeation experiments were performed for 8 hours, and the total recovery of CAM was quantified by HPLC. The total drug recovery was found to be good and of the same magnitude for all the formulations; 95.37 ± 4.61%, 93.84 ± 3.27%, 96.52 ± 5.71 and 94.26 ± 5.39%, for the CAM-Sol, CAM-Lip, CS-CAM and CS-CAM-Lip formulation, respectively. When comparing the amount of drug remaining on the skin surface after 8 hours from the different formulations; 69.76 ± 4.83%, 55.29 ± 4.04%, 44.15 ± 5.50% and 48.86 ± 2.37%, for the CAM-Sol, CAM-Lip, CS-CAM and CS-CAM-Lip, respectively, more drug was retained on the surface of the skin from the control CAM-solution (two-tailed t-test, $p < 0.05$). This was the case, although the amount of drug permeated through the skin was also significantly higher from the CAM-Sol control formulation than from the test formulations (two-tailed t-test, $p < 0.05$) (Figure 1). Both the liposome- and the CS hydrogel formulations enhanced the penetration of the drug into the skin compared to the CAM solution. However, the CS-CAM-Lip only enhanced the penetration of CAM as compared to the CAM-Lip formulation (two-tailed t-test, $p < 0.05$), and showed a higher retention of CAM on the skin surface than the CS-CAM formulation (44.15 ± 5.50%), although this difference was not significant. Thus, there seems to be no synergistic effect of the penetration

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enhancing effect of CS and liposome when mixed. This might be explained by the diffusion barrier of the hydrogel, which delay the drugs flux towards the surface of the skin, whereas the free drug diffuses faster than the larger liposomes in the gel vehicle. It appears that all the test formulations (CAM-Lip, CS-CAM, CS-CAM-Lip), although acting as the penetration enhancers for CAM, also serve to increase the drug concentration in the skin. This is a very promising result, as decreased permeation also indicates less systemic side effect from CAM and more drug at the site of action, although one should bear in mind that the compromised skin is expected to allow higher permeation than the healthy skin. The results in Figure 1 also indicate decreased skin permeation of CAM from liposome dispersion (CAM-Lip), compared to the hydrogel formulation with unentrapped CAM (CS-CAM) after 8 hours. This might be explained by the absence of a liposomal carrier in the CS-CAM formulation, as the liposomes are expected to retain the drug in the skin and improve drug deposition. However, the negative charge of CAM-Lip could also influence the permeation of CAM through the pig skin, as mentioned in Section 3.1. The liposomes-in-hydrogel formulation (CS-CAM-Lip) exhibited the most sustained release of CAM, which was expected, due to the multiple barriers to diffusion that the combined system has in terms of the lipid bilayer and the hydrogel structure. The observed sustained release from our CAM-Lip formulation could, however, according to Hurler et al. (2013), theoretically have been increased by changing the lipid composition of the liposome carrier to neutral lipids. The negative charge of Lipoid E80 applied in this CAM-Lip formulation might, in combination with the positive charge of the CS hydrogel, also cause an interaction between the polymer chains of the gel and the lipid bilayer, potentially resulting in a faster release of CAM, as previously described by Mourtas et al. (2007). However, the cost of the materials was given priority in this study, and Lipoid E80 was chosen based on its price and the similarity in liposome size and drug entrapment obtained for E80 liposomes as compared to liposomes made from more expensive lipids (Table 2).

3.5. Bioadhesiveness

Topical liposome formulations, especially wound dressings, need to remain on the skin surface for a sufficient amount of time in order to achieve their therapeutic efficiency, without the need for a too frequent dressing administration. Thus, a sufficient skin bioadhesion of the dressing is

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required (Boateng et al., 2008; Hurler and Škalko-Basnet, 2012b). Since an aqueous liposome dispersion cannot provide sufficient bioadhesion by itself, a secondary vehicle is required for optimal drug delivery (Foldvari, 1996).

The biocompatibility and bioadhesive properties of CS hydrogels combined with its inherent antimicrobial activity against several bacteria and fungi, particularly *S. aureus* (Dash et al., 2011; Fei Liu et al., 2001), makes CS hydrogels highly attractive as a secondary vehicle for liposomes targeting skin therapy. However, incorporation of liposomes into hydrogels can change the physical properties of the gel, and influences the stability and performance of the final formulation (Pavelić et al., 2001). The properties of the liposomes-in-hydrogel formulations should therefore be tested to assure that the gel exhibits the desired properties, such as sufficient bioadhesiveness.

Based on previous experiences, a 2.5% (w/w) high molecular weight CS hydrogel with 10% (w/w) glycerol was prepared, as the high molecular weight CS exhibits stronger gel hardness in lower concentration as compared to the low and medium molecular weight CS (Hurler and Škalko-Basnet, 2012a). The 10% (w/w) glycerol was included in the CS-hydrogel in order to improve the stability of the final gel formulation (Hurler et al., 2012b).

The bioadhesive properties of the final liposome-in-hydrogel formulation (CS-CAM-Lip) were compared with the plain CS-hydrogel and hydrogel containing unentrapped CAM (CS-CAM). As shown in Figure 2, there were no significant differences (one-way ANOVA, $p < 0.05$) in the detachment force or amount of formulation retained on the skin. These findings suggest that neither the addition of CAM solution nor liposomes influence the bioadhesive properties of the CS-hydrogel.

The obtained results for the CS-hydrogel also corresponds well to the data previously reported (Hurler and Škalko-Basnet, 2012), further confirming the suitability and stability of the CS-hydrogel formulation as a secondary vehicle.

3.6. Antimicrobial activity of chloramphenicol in different formulations

Initially, the minimum inhibitory concentration (MIC) for eight relevant bacterial strains from two different species of bacteria commonly found on the skin, namely *S. aureus and S.*

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epidermidis, were determined to assess their susceptibility towards CAM (Table 3). The results showed that most of the tested bacteria had a MIC-value in the concentration range of 3-6 µg/ml CAM, which is consistent with the previously reported values (Fluit et al., 2001; Neu and Fu, 1980). The only exception was *S. aureus* SH1000 that was not considered susceptible to CAM as its growth was not inhibited at the CAM concentration below 128 µg/ml.

Based on the determined MIC-values (Table 3), and the typically applied CAM concentrations in the marketed product, (0.5% or 1%), the CAM-Lip formulations were tested for their antibiotic activity applying two different CAM concentrations, 500 µg/ml and 750 µg/ml, respectively. The *S. aureus* SH1000 strain was excluded in further studies. The diameters of the inhibition zones were measured after 24 h incubation, and compared to the diameter of the inhibition zone for CAM-Sol in the given concentrations. Only small differences between the diameter of the inhibition zones were observed between the CAM-Lip and CAM-Sol formulations (Table 4), and only for the 750 mg/ml CAM-concentration samples, a significantly smaller inhibition zone for the CAM-Lip formulation when applied on the *S. aureus* N315 strain ($p < 0.05$) was detected. The small variations in diameter of the inhibition zones seen, and no trend in the results, confirmed that the entrapment and subsequent release of CAM from liposomes had no influence on its antimicrobial activity (Table 4).

In the final step of the antimicrobial evaluation, the antimicrobial effect of the CS-hydrogel (vehicle) was taken into consideration. CAM-Sol, CS-hydrogel, CS-CAM-Lip and hydrogel with empty vesicles (CS-Lip) were tested on two of the *S. aureus* strains and two *S. epidermidis* isolates. These results are presented in Table 5, and since the applied sample volume was increased from 10 µl to 25 µl in this experiment, the results presented in Table 4 and Table 5 cannot be directly compared. The CS-hydrogel showed only a minor growth inhibition of all four isolates (Table 5). This is also in accordance with previous findings by Hurler *et al.* (Hurler et al., 2012a), that reported a minor inhibitory effect of CS hydrogel against *S. aureus* ATCC 25923 and *Bacillus subtilis*. Our results also showed that the presence of the liposomal carrier had no influence on the antimicrobial activity of the liposomes-in-hydrogel formulation. The observed antimicrobial activity from the final liposome-in-hydrogel formulation is therefore a result of the inherent activity of CAM and CS. Finally, CS-CAM-Lip were able to demonstrate a similar or

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better antimicrobial effect than the CAM-Sol against the tested strains, indicating further potential of CS-based liposomes-in-hydrogel systems for delivery of CAM.

4. Conclusions

The topical liposome-in-hydrogel formulation for delivery of CAM was successfully prepared by the DC processing method, avoiding the use of organic solvents. The formulation demonstrated appropriate skin bioadhesion and assured a sustained CAM release. Finally, an improved antimicrobial activity against two clinical isolates of *S. epidermidis* as compared to CAM in solution was obtained. The manufacturing method and formulation characteristics, especially a promising antibacterial activity, confirm the promise of the proposed formulation as topical antimicrobial formulation and/or an advanced wound dressing.

Conflict of interests

The authors declare no conflict of interests.

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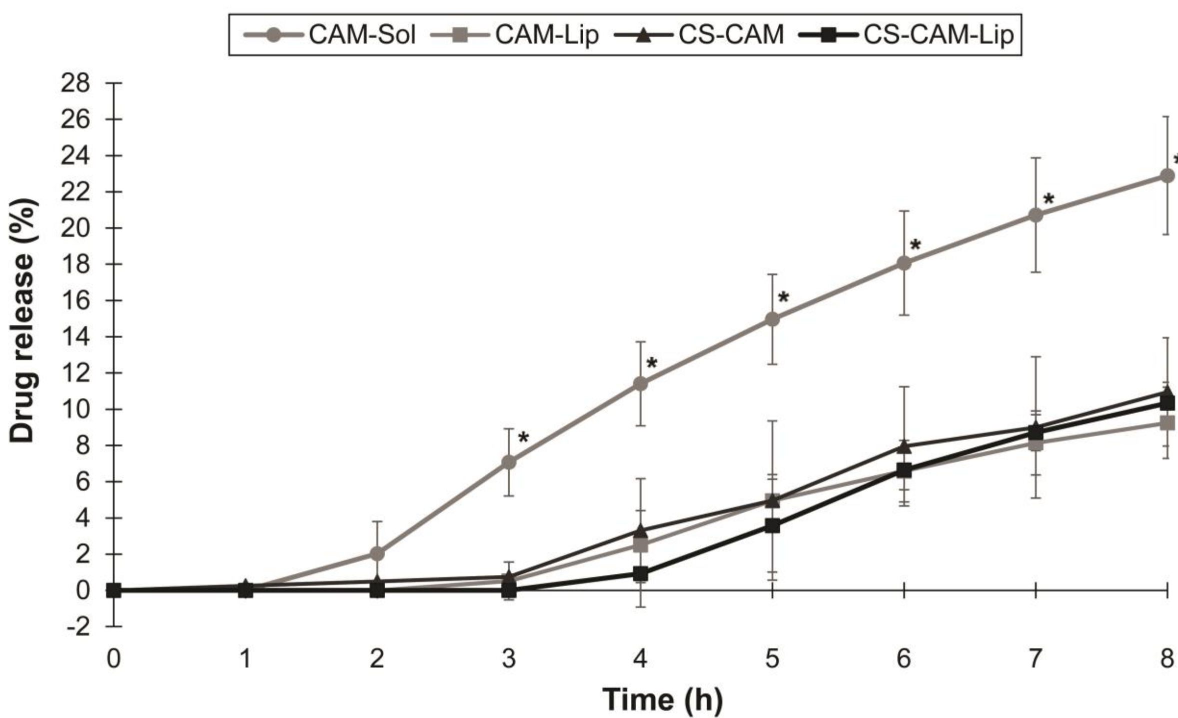
Figure legends

Figure 1. Skin permeation of CAM from solution, liposome dispersion, chitosan hydrogel and liposome-in-hydrogel ($n \geq 3$). *) The drug release is significantly higher compared to the other formulations after 2 hours (two-tailed t-test, $p < 0.05$).

Figure 2. Bioadhesiveness of three different hydrogel formulations tested on pig skin; (A) detachment force and (B) amount of formulation retained on the skin after administration ($n \geq 3$).



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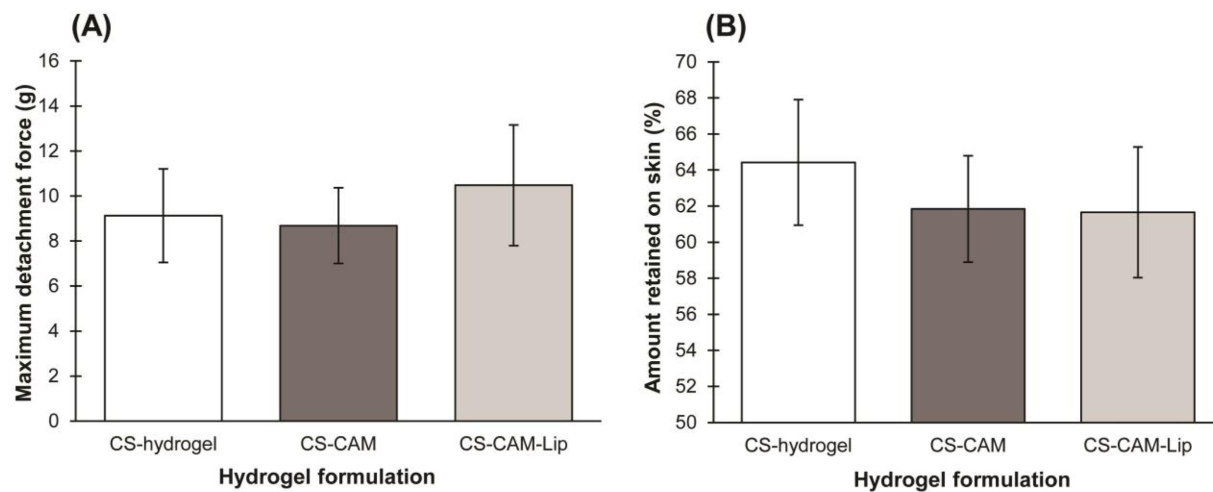

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Table 1. Differences in the method settings applied when preparing liposomes using two different DC centrifuges - the Speedmixer and the ZentriMix.

DC centrifuge	**Speedmixer***	**ZentriMix**
Sample container	30 ml glass injection vials	2 ml polypropylene test tubes
Pre-processing step	Lipid film formation	n.a.
Centrifugation time	8 x 5 min	5 min
Centrifugation speed	3500 rpm	2350 rpm
Homogenization aid	Glass beads (Ø ~ 2 mm) 50% (w/w)	Zirconium beads (Ø ~ 1.4 mm) 25% (w/w)

*) Refers to final settings applied in Ingebrigtsen et al., 2017.

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Table 2. Liposome characteristics of liposomes prepared from different lipids ($n \geq 3$).

Lipid type	Vesicle diameter (nm ± SD)	Polydispersity index (AU ± SD)	Zeta-potential (mV ± SD)	Entrapment efficiency (% ± SD)
Lipoid E80	120.9 ± 3.2	0.126 ± 0.020	-27.8 ± 1.9[c)]	55.2 ± 5.9
Lipoid S100	127.5 ± 1.9[a)]	0.088 ± 0.021[b)]	0.6 ± 4.3	49.4 ± 1.0
Phospholipon 90H	120.6 ± 2.3	0.185 ± 0.019	1.6 ± 0.1	55.8 ± 6.8
Soluthin S90	n.a.	n.a.	n.a.	n.a.

[a)]Significantly larger liposomes than liposomes formed from the two other lipids ($p < 0.05$).
[b)]Significantly lower PI than for the liposomes formed from the two other lipids ($p < 0.05$).
[c)]Significantly lower zeta-potential than for liposomes formed from the two other lipids ($p < 0.05$)

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Table 3. *S. aureus* and *S. epidermidis* susceptibility towards CAM (n = 3).

Bacteria	**Minimum inhibitory concentration (µg/ml)**
S. aureus ATCC25923	6
S. aureus N315	6
S. aureus NCTC10442	6
S. aureus MSSA476	4
S. aureus SH1000	128**
S. epidermidis 1*	3
S. epidermidis 2*	3
S. epidermidis 3*	3

*) *S. epidermidis* 1, 2 and 3 were clinical isolates from two hospitals in Norway.
**) Considered not susceptible to CAM.

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Table 4. Antimicrobial activity of CAM in solution and CAM-in-liposomes dispersions (n ≥ 3).

Bacteria	**Diameter of inhibition zone (mm ± SD)**			
	CAM-Sol (500 µg/ml)	CAM-Lip (500 µg/ml)	CAM-Sol (750 µg/ml)	CAM-Lip (750 µg/ml)
S. aureus ATCC25923	11.3 ± 1.8	11.1 ± 1.6	17.5 ± 2.2	17.1 ± 1.8
S. aureus N315	9.5 ± 2.5	9.7 ± 1.8	16.3 ± 1.2[a)]	14.8 ± 1.4[a)]
S. aureus NCTC10442	13.2 ± 1.5	13.9 ± 1.7	17.5 ± 1.5	17.9 ± 1.5
S. aureus MSSA476	11.8 ± 1.0	13.2 ± 2.3	16.3 ± 0.8	16.8 ± 1.1
S. epidermidis 1[*]	18.2 ± 1.0	18.9 ± 1.1	21.0 ± 0.6	20.7 ± 0.8
S. epidermidis 2[*]	17.8 ± 1.7	17.7 ± 1.9	22.0 ± 0.6	20.6 ± 1.7
S. epidermidis 3[*]	19.7 ± 0.8	19.2 ± 1.9	21.3 ± 1.0	22.8 ± 1.7

[*)] *S. epidermidis* 1, 2 and 3 were clinical isolates from two hospitals in Norway.

[a)] Significant difference ($p < 0.05$)

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Table 5. Antimicrobial activity of liposomes-in-hydrogel formulations (n ≥ 3).

Formulation	Diameter of inhibition zone (mm ± SD)			
	S. aureus NCTC10442	*S. aureus* MSSA476	*S. epidermidis* 1[*]	*S. epidermidis* 2[*]
CAM-Sol (500 µg/ml)	22.3 ± 0.6	21.0 ± 1.0	27.3 ± 0.6	25.0 ± 0.0
CAM-Sol (750 µg/ml)	24.7 ± 0.6	24.0 ± 0.0	27.0 ± 3.5	28.0 ± 0.0
CS-hydrogel	8.0 ± 0.0	6.7 ± 0.6	7.0 ± 0.0	8.0 ± 1.0
CS-Lip (500 µg/ml) [a]	8.0 ± 1.0	7.7 ± 0.6	7.3 ± 0.6	8.0 ± 0.0
CS-Lip (750 µg/ml) [a]	7.3 ± 0.6	7.3 ± 0.6	7.7 ± 0.6	8.0 ± 0.0
CS-CAM-Lip (500 µg/ml)	23.3 ± 1.7 [b]	22.0 ± 1.2 [b]	28.9 ± 0.8 [b, c]	29.4 ± 0.5 [b, c]
CS-CAM-Lip (750 µg/ml)	27.1 ± 1.1 [b, c]	24.6 ± 1.1 [b]	28.6 ± 1.9 [b]	29.1± 1.2 [b]

[a)] The lipid concentration equal to samples containing 500 and 750 µg/ml of CAM.
[b)] Significant difference ($p < 0.05$) compared to CS-hydrogel and CS-Lip.
[c)] Significant difference ($p < 0.05$) compared to CAM-Sol.
[*)] *S. epidermidis* 1 and 2 were clinical isolates from two hospitals in Norway.