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Original research paper

Azithromycin-loaded liposomal hydrogel: a step forward for enhanced treatment of MRSA-related skin infections

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ABSTRACT

Azithromycin (AZT) encapsulated into various types of liposomes (AZT-liposomes) displayed pronounced *in vitro* activity against methicillin-resistant *Staphylococcus aureus* (MRSA) (1). The present study represents a follow-up to this previous work, attempting to further explore the anti-MRSA potential of AZT-liposomes when incorporated into chitosan hydrogel (CHG). Incorporation of AZT-liposomes into CHG (liposomal CHGs) was intended to ensure proper viscosity and texture properties of the formulation, modification of antibiotic release, and enhanced antibacterial activity, aiming to upgrade the therapeutical potential of AZT-liposomes in localized treatment of MRSA-related skin infections. Four different liposomal CHGs were evaluated and compared on the grounds of antibacterial activity against MRSA, AZT release profiles, cytotoxicity, as well as texture, and rheological properties. To our knowledge, this study is the first to investigate the potential of liposomal CHGs for the topical localized treatment of MRSA-related skin infections. CHG ensured proper viscoelastic and texture properties to achieve prolonged retention and prolonged release of AZT at the application site, which resulted in a boosted anti-MRSA effect of the entrapped AZT-liposomes. With respect to anti-MRSA activity and biocompatibility, formulation CATL-CHG (cationic liposomes in CHG) is considered to be the most promising formulation for the treatment of MRSA-related skin infections.

Keywords: MRSA, liposomal hydrogel, azithromycin, chitosan, topical antimicrobial therapy, skin infections

INTRODUCTION

Skin and soft tissue infections (SSTIs) include a variety of pathological conditions that affect the skin (superficial SSTIs) and possibly also the underlying tissue (deep SSTIs). They are usually incited by debilitated skin defense mechanisms and consequential invasion of bacteria (2). The rising incidence of antibiotic resistance is the leading cause of poor thera-

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peutic outcomes in treating infectious diseases, including SSTIs, with methicillin-resistant *Staphylococcus aureus* (MRSA) being one of the most common drug-resistant pathogens (3). Because the emergence of antibiotic resistance is more likely with systemically administered antibiotics, an appealing alternative is localized antibiotic therapy, which is, due to the accessibility of skin, particularly suitable for topical skin infection management. Besides the ease of use and a higher degree of patient compliance, local administration of antibiotics reduces the occurrence of adverse effects and toxicity (4). Another attractive approach to overcoming antibiotic resistance relies on the implementation of nanotechnology in the development of novel and more efficient delivery systems for the existing antibiotics. Liposomes have been proven to be potent yet safe antibiotic nanocarriers, with more than a few liposomal formulations reaching clinical research and market during the last three decades (5). Although most of the commercially available liposomal antibiotics are used as systemic therapy (6), liposomes can also be administered locally onto the skin (7). However, the liquid nature of liposomal dispersions can hinder adequate retention of the formulation at the administration site, which is a prerequisite to accomplishing prolonged contact with the diseased skin and high local concentrations of antibiotics. To overcome this shortcoming, the attempts have been focused mainly on embedding liposomes into different sorts of viscoelastic vehicles, predominantly polymer-based hydrogels, which provide a platform for innovative therapeutic strategies (8). A three-dimensional network of hydrogels has been confirmed to be a suitable environment for the embedded liposomes in terms of (bio)compatibility, bioadhesiveness, increased stability of the embedded liposomes, and further prolongation of the drug release from such hybrid drug delivery systems in comparison to liposomal dispersions alone (9–13).

Our previous study (1) investigated the anti-MRSA potential of azithromycin (AZT) encapsulated in four different types of liposomes (conventional liposomes, CL; deformable liposomes, DL; propylene glycol liposomes, PGL; cationic liposomes, CATL). Compared with the free AZT-solution (AZT dissolved in water/ethanol mixture; *v*/*v* = 6/4), liposomal formulations (AZT-liposomes) demonstrated prolonged release of the drug, biocompatibility with the human skin cells *in vitro*, more pronounced retention of antibiotic within the skin (*ex vivo*), as well as significantly improved anti-MRSA and MRSA biofilm-preventing activity. All these properties of AZT-liposomes were dependent on the lipid composition and bilayer elasticity/rigidity, which allowed the selection of the optimal liposomal formulation according to the nature of the targeted skin infection (superficial or deep skin infections) (1). The present study follows up on this previous work, attempting to further explore the anti-MRSA potential of various AZT-liposomes when incorporated into chitosan hydrogel (CHG). Favorable characteristics, such as hydrophilicity, biodegradability, and bioadhesiveness, make chitosan-based hydrogels highly suitable for local antimicrobial therapy (14) and topical skin administration (15). Moreover, chitosan has been shown to exhibit intrinsic biological effects including wound healing stimulation, anti-inflammatory and antimicrobial activities (16, 17), all of which may be particularly beneficial in the development of novel AZT-loaded liposomal hydrogel (liposomal CHG). Considering all the above, interplay with CHG is reasonably expected to upgrade the therapeutical potential of the incorporated AZT-liposomes in the localized topical treatment of MRSA-related skin infections. High molecular weight (HMW) chitosan used in the present work is known to form robust hydrogels, which could be beneficial for the topical skin application of liposomes. Moreover, HMW chitosan was proven to be more successful in promoting

skin healing than medium molecular weight (MMW) and low molecular weight (LMW) chitosan, which is a very useful feature in the context of skin infection management (18).

Only a few studies focusing on the incorporation of elastic liposomes into chitosan hydrogels have been published so far (17, 19). Therefore, one of the objectives of the presented work was to evaluate and compare liposomal CHGs, comprising various types of AZT-liposomes, on the grounds of antibacterial activity against MRSA, as well as AZT release profiles, cytotoxicity, texture, and rheological properties. Furthermore, to the best of our knowledge, this is the first research to address topical delivery of AZT *via* chitosan-based liposomal hydrogel aimed to combat MRSA-related skin infections.

EXPERIMENTAL

Materials

Soy lecithin (Lipoid S75) and dipalmitoylphosphatidylcholine (DPPC) were gifts from Lipoid GmbH (Germany). Azithromycin (AZT) in the form of dihydrate was generously donated by PLIVA Croatia Ltd. (Croatia). Sodium deoxycholate (SDCh), dimethyldioctadecylammonium bromide (DODAB), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (USA). All organic solvents (methanol, ethanol, and acetonitrile) were of HPLC grade, purchased from BDH Prolabo (UK). Müller-Hinton broth (MHB), Müller-Hinton agar (MHA), and tryptic soy broth (TSB) were obtained from Merck (Germany). HMW chitosan was a product of Fluka Chemie GmbH (Switzerland). All other chemicals or solvents used in this study were of analytical grade and purchased from Kemika (Croatia) or Sigma-Aldrich (USA). Phosphate buffered saline (PBS, 0.01 mol mL^{-1}) was prepared by dissolving KH_2PO_4 (1.3609 g) in 1000 mL of purified water, whereas an appropriate pH of 7.5 was adjusted by adding 10 mol L^{-1} KOH. PBS was filtered through cellulose nitrate membrane filters (0.45 μm pore size) purchased from Sartorius AG (Germany). Commercially available phosphate buffer with pH 7.4 (Gibco, Thermo Fisher Scientific, UK) was used for *in vitro* antimicrobial assays.

Preparation and characterization of liposomes

The film hydration method was employed to prepare four different types of AZT-liposomes: CL, DL, PGL, and CATL (1). Their composition is summarized in Table I. Physicochemical characterization of AZT-liposomes included particle size, size distribution, and zeta potential measurements, which were performed on Zetasizer 3000 HS (Malvern Instruments, UK). The encapsulation efficiency of a particular liposomal formulation was determined by HPLC analysis, following the separation of the encapsulated from the free drug by the ultracentrifugation method (1).

Preparation of liposomal CHGs

CHG was used as a vehicle for the preparation of liposomal CHGs. Briefly, 2.5 g of HMW chitosan was dispersed in 37.5 g of 3.5 % lactic acid solution (*m*/*m*) and 10 g of propylene glycol at room temperature, applying vigorous stirring by hand and ultrasound sonication bath (Branson 1210, Branson Ultrasonics, USA) for 60 minutes. Finally, demineralized

Table I. Composition of AZT-liposomes

	AZT-liposome formulation			
Component (mg)	CL	DL	PGL	CATL
Lipoid S75	100	85	100	–
SDCh	–	15	–	–
PG	–	–	1500	–
DPPC	–	–	–	85
DODAB	–	–	–	15
AZT	15	15	15	15

AZT, azithromycin; CATL, cationic liposomes; CL, conventional liposomes; DL, deformable liposomes; DODAB, dimethyldioctadecylammonium bromide; DPPC, dipalmitoylphosphatidyl-choline; Lipoid S75, soy lecithin; PG, propylene glycol; PGL, propylene glycol liposomes; SDCh, sodium deoxycholat. The volume of all liposomal dispersions was 5 mL.

water was added to obtain a final mass of 100 g and a final chitosan concentration of 2.5 %. CHG was allowed to swell at room temperature for 48 hours (covered with parafilm).

In the following step, AZT-liposomes (CL, DL, PGL, CATL), previously separated from the free drug by the ultracentrifugation method (1), were evenly distributed within the CHG at a concentration of 30 % (*m*/*m*) (AZT-liposomes/liposomal CHG) by hand stirring for 5 minutes. Four different liposomal CHG formulations were prepared: CL-CHG, DL-CHG, PGL-CHG, and CATL-CHG. Control hydrogel (control-CHG) was prepared by entrapping an equivalent amount (30 %, *m*/*m*) of AZT-solution into the CHG.

The amount of lactic acid was carefully optimized during the CHG preparation procedure to ensure both the complete dissolution of chitosan and the final hydrogels' pH as close to 5 as possible. A pH meter equipped with an electrode for semisolid formulations (Mettler-Toledo, Switzerland) was used to measure the pH of CHG, before (pH 4.80 ± 0.02) and after the addition of AZT-liposomes (pH 4.89 ± 0.05) or AZT-solution (pH 4.98 ± 0.02). Three consecutive measurements were performed for each sample at 25 °C (19).

Rheological evaluation of liposomal CHGs

Empty CHG (before the addition of liposomes) and CHG after the addition of liposomes (liposomal CHG) or AZT solution (control-CHG), were rheologically characterized with respect to flow behavior and viscoelasticity. All the rheological measurements were performed on a Modular Compact Rheometer MCR 102 (Anton Paar GmbH, Austria), using a parallel-plate (PP25) and measuring gap set to 1 mm. Rotational tests were performed in the shear rate range from 0.1 to 1000 s^{-1}, whereas oscillatory amplitude sweep tests were carried out applying an angular frequency of 10 s^{-1} in the shear strain range of 0.1–1000 %. All tests were performed in triplicate, at two different temperatures (25 and 34 °C), with samples being equilibrated for 10 min at the corresponding temperature before the measurements. Data were documented and analyzed by rheometer software RheoCompass TM Light (Anton Paar GmbH).

Texture analysis of liposomal CHGs

Texture properties (cohesiveness, adhesiveness, and hardness) of CHG, before and after the addition of AZT-liposomes (liposomal CHGs) or AZT-solution (control-CHG), were tested on texture analyzer TA.XT Plus (Stable Micro Systems LTD, UK). According to the method developed by Hurler *et al.* (20), the cylindrical container was filled with around 50 g of each hydrogel formulation, then a probe disk (40 mm) was compressed 10 mm into it (1 mm s^{-1}) and removed. Each sample was tested in quintuplicate, under the same experimental setup.

In vitro *AZT release studies*

The *in vitro* drug release study was performed on a Franz cell diffusion system (surface area of 3.14 cm^2), under sink conditions, using cellulose membranes (Sartorius AG, Germany). The receptor chamber was filled up with 15 mL of PBS (pH 7.5), and then continuously stirred with a small magnetic stirrer (200 rpm), and the temperature was set at 32 ± 1 °C to imitate the physiological skin surface temperature conditions of approximately 32 °C in the donor chamber. Each sample of liposomal CHG and control-CHG was carefully and evenly spread onto the surface of the cellulose membrane in the donor chamber, in the amount that corresponds to approximately 1 mg AZT (the exact amount of sample was weighed for each experiment). The donor chamber was then tightly sealed by parafilm. At precise time intervals (1, 2, 3, 4, 5, 6, and 24 h) 500 μL aliquots of the receptor medium were removed and immediately replaced with an equal amount of buffer thermostated at 32 °C. Quantification of the drug in all collected samples was performed by HPLC (1). The experiments were performed in triplicate.

In vitro *antibacterial assays*

Antibacterial activity of AZT-liposomes (liquid liposomal dispersions), liposomal CHGs, control-CHG, and empty CHG was studied against *S. aureus* (ATCC 29213) and five different clinical isolates of *S. aureus* MRSA (MFBF 10674, MFBF 10676, MFBF 10677, MFBF 10679, MFBF 10680) originating from the microbial collection of the Department of Microbiology, Faculty of Pharmacy and Biochemistry, University of Zagreb (Croatia). To this purpose, an agar well diffusion method was applied. Briefly, inoculums of tested bacterial strains in MHB (1×10^8 CFU) were prepared as previously described (1). Inoculums were then mixed with saline solution (0.9 % NaCl) in a 1:10 dilution ratio (*v*/*v*) and thereafter evenly dispersed within the molten MHA kept at 37 °C. 20 mL of MHA inoculated with bacteria was poured into sterile Petri dishes (100 × 15 mm) on a flat surface and left to cool down. After the inoculated MHA solidified, on each Petri dish 6 wells with a diameter of 6 mm were punched aseptically, utilizing a sterile steel ring.

100 mg of each liposomal CHG formulation, empty CHG, or control-CHG was introduced into the wells in the agar plates. Agar plates were then placed in the refrigerator for 60 minutes (2–8 °C) and incubated aerobically under 37 °C (Sanyo, MIR-553, Japan). After a 24-hour incubation period, confluent bacterial growth was established on agar plates, with clearly recognizable growth inhibition zones. The diameter of inhibition zones was measured in millimeters, with post-measurement correction being made with respect to the exact concentration of AZT in each sample. The exact same procedure was employed

to assess the antibacterial activity of AZT-liposomes, unloaded liposomes and AZT-solution (100 μL of each sample was introduced into the wells of agar plates). Tests were conducted in triplicates.

In vitro *cytotoxicity assessment*

In vitro cytotoxicity of liposomal CHGs was tested on the human keratinocyte cell line HaCaT (Cell Line Services, Germany), cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Invitrogen, UK) supplemented with 10 % fetal bovine serum (Gibco, Thermo Fisher Scientific, USA) and a mixture of penicillin, streptomycin and amphotericin B (Lonza, Switzerland). After being seeded with HaCaT cells at a density of 10^4 cells/well, 96-well plates were incubated for 48 h at 37 °C and 5 % CO_2, until reaching confluence.

To obtain samples with final AZT concentrations of 0.25, 1, 4, 16, and 64 μg mL^{-1}, respectively, liposomal CHGs were diluted with non-supplemented DMEM at proper ratios. Likewise, dilution with DMEM at the corresponding ratios was applied on empty CHG and control-CHG. Immediately before the treatment with tested samples, the cell culture medium was carefully aspirated, and the cells were washed with PBS. 100 μL of each sample was transferred into the wells with HaCaT cells. After a 24-hour incubation period, the treating agents were carefully removed. The cells were washed twice with PBS to remove all the treating samples before adding 100 μL of fresh DMEM (supplemented) as a proliferation medium for the cells in the subsequent 24-hour incubation period. The next day colorimetric MTT assay was employed, following the previously reported procedure (1). Since the cell viability of the control group (treated with non-supplemented DMEM) was set as a benchmark, the viability of the samples (CL-CHG, DL-CHG, PGL-CHG, CATL-CHG, empty CHG, and control-CHG) was expressed relative to the control (%). No interference between the samples and the MTT assay was noticed. Each sample was tested in quadruplicate.

Statistical analyses

The data were reported as means ± SD. Statistical data analyses were performed using the GraphPad 5 Prism program (GraphPad Software Inc., USA). Student's *t*-test was applied for the comparison of two groups, whereas for the comparison of three or more groups one-way analysis of variance (ANOVA) with post-hoc Tukey's test was applied. The statistical significance level in all tests was set at 5 %.

RESULTS AND DISCUSSION

Physicochemical characterization of AZT-liposomes

Since physicochemical properties of liposomes (size, size distribution, zeta potential, drug content, and drug loading capacity) are well known to play a crucial role in their efficacy as drug delivery systems (6, 21, 22). AZT-liposomes were characterized with respect to the mean diameter, size distribution, zeta potential, and entrapment efficiency, as summarized in Table II.

Table II. Physicochemical characterization of AZT-liposomes

AZT-liposomes	Mean vesicle diameter (nm)	Polydispersity index	Zeta potential (mV)	Entrapment efficiency (μg AZT/mg lipid)
CL	162 ± 5	0.13 ± 0.03	−40.4 ± 0.8	15.0 ± 0.9
PGL	138 ± 2	0.15 ± 0.04	−42.6 ± 0.3	16.5 ± 0.6
DL	150 ± 4	0.10 ± 0.03	−49.3 ± 1.7	19.2 ± 1.2
CATL	223 ± 4	0.41 ± 0.20	+60.1 ± 1.9	13.5 ± 0.3

AZT – azithromycin, CATL – cationic liposomes, CL – conventional liposomes, DL – deformable liposomes, PGL – propylene glycol liposomes

During liposomes' size optimization, it is important to achieve a balance between drug loading capacity and the size of the vesicles, according to the features of the targeted route of administration. Additionally, the size of liposomes can also have an impact on their antibacterial activity (23). Previous studies report that liposomes of a mean diameter of up to 300 nm are optimal for skin drug delivery (16, 17, 24). In this study, all liposomes were shown to be within the targeted size range. CATL were somewhat larger than CL, DL, and PGL, however, still appropriate for dermal drug delivery, particularly for the retention in the upper layers of the skin (1). Polydispersity indexes (PDI) indicated the formation of homogenous liposomal dispersions (Table II). PDIs were less than 0.15 for CL, DL and PGL. However, PDI was significantly higher for CATL. Regarding AZT entrapment efficiency, it was shown to be in the range of approximately 13.5–19.3 μg of the entrapped AZT per mg of lipids. Higher entrapment efficiencies of DL and PGL in comparison to CL and CATL are most probably the result of the presence of surfactant (SDCh) or co-solvent (propylene glycol) increasing encapsulation of the drug (1).

Zeta potential data obtained for each liposomal formulation was relative to the liposome membrane composition. Namely, the main lipid component of CL, DL, and PGL was soy lecithin (Lipoid S75), exerting a negative net charge. On the other hand, positively charged CATL comprised DODAB, cationic lipid. Zeta potential closer to neutral could be more favorable in the context of entrapping liposomes into the CHG, since previous studies show that it could improve release properties and textural properties of the final liposomal hydrogel formulation (11). On the other hand, cationic liposomes were shown to possess more pronounced anti-MRSA activity than negatively charged liposomes of similar size in our previous study (1), probably due to stronger electrostatic interaction with negatively charged bacterial cell surface (25). Cationic liposomes could also exhibit stronger interaction with the negatively charged *stratum corneum* (26), enabling retention of the encapsulated AZT on the skin surface and in the upper layers of the epidermis, whereas anionic liposomes demonstrated better AZT deposition into the skin (1).

Rheological evaluation of liposomal CHGs

Preparation of chitosan hydrogels may be achieved through the physical gelation mechanism exploited herein or chemical crosslinking of the chitosan polymer chains.

Chemically cross-linked hydrogels of chitosan display excellent mechanical properties. However, the potential toxicity of the chemicals typically used as covalent crosslinking agents represents a safety issue (27), which is particularly relevant for the hydrogels aiming at diseased skin with disrupted barrier function. Taking into consideration the higher degree of biocompatibility of physical chitosan hydrogels, they seemed a more appealing option in the development of liposomal hydrogels targeting infected skin. On the other hand, physical chitosan hydrogels are generally recognized as less robust than chemical chitosan hydrogels (28). Since the addition of liposomes into the hydrogels may affect the rheological properties of hydrogels (17, 19, 29), rheological evaluation of the developed liposomal hydrogels was important to ensure proper formulation quality and applicability of liposomal CHGs.

All tested liposomal CHGs demonstrated pseudoplastic flow with shear-thinning behavior characteristics for hydrogels (30, 31). Possible differences in viscosity of the liposomal CHGs were tested at two different temperatures tuned to imitate typical storage temperature (25 °C; Fig. 1a) and peak physiological skin temperature conditions (34 °C; Fig. 1b). All tested formulations exhibited insignificantly lower viscosity at higher temperature ($p > 0.05$) with analogous flow behavior tendency being maintained. Liposomal CHGs and control-CHG revealed a notable decrease of viscosity ($p < 0.05$) in comparison to empty CHG (Fig. 1). Such findings agree with the recent study by Čačić *et al.* (19) and a previous study by Kaplan and colleagues (32). On the contrary, Hemmingsen and co-workers (15) did not observe differences in the viscosity of chitosan hydrogel before and after the addition of liposomes. Differences in the composition and surface charge of AZT-liposomes incorporated into CHG do not seem to have a significant influence on the flow behavior of the final formulation, since all the tested samples showed very similar viscosity curves ($p > 0.05$). Assumedly, initial chitosan polymer concentration and the amount of liposomal dispersion embedded into the chitosan hydrogel (liposome/hydrogel ratio) seem to be more relevant parameters influencing viscosity of the final formulation than the composition and surface charge of the embedded liposomes. Namely, the aforementioned studies (15, 19, 32) exploited chitosan hydrogels of different initial chitosan concentrations (*m*/*m*): 4.5 % (15), 4 % (19), and 2 % (32) *vs*. 2.5 % used in the present study. Furthermore, the amount of liposomes embedded into chitosan hydrogels was 10 %, 30 %, and 10 % *vs*. 30 %, respectively. Most importantly, when the final chitosan concentration (after the addition of liposomes) in all of the proposed liposomal hydrogels is analyzed, the following concentrations are deduced (in the order given): 4.05 %, 2.80 %, and 1.80 % *vs*. 1.75 %. Apparently, the final concentration of chitosan in the liposomal hydrogel proposed by Hemmingsen and co-workers (15) was substantially higher as compared to other proposed formulations, which could be the most plausible explanation as to why no difference in viscosity between empty/liposomal hydrogel was detected in their study. This unalike rheological behavior might also be due to the electrostatic interactions involving positively charged chitosan polymer chains and liposomal surfaces of the corresponding charge. Whereas Čačić *et al*. (19) and Kaplan *et al*. (32) investigated negatively charged liposomes, Hemmingsen *et al*. (15) embedded positively and neutrally charged liposomes into the chitosan hydrogel network. However, in the present study the addition of both positively (CATL) and negatively charged liposomes (CL, DL, PGL) induced a similar reduction of the viscosity of the initial CHG ($p > 0.05$). We hypothesize that either the liposome surface charge has a minor impact on the viscosity of the final liposomal hydrogel, in comparison to the concentration of liposomes embedded into the hydrogel, or this effect was not detected at particular experimental conditions.

Noteworthy, to the best of our knowledge, no systematic study investigating the effect of embedding different surface charges and concentrations of liposomes into the chitosan-based hydrogels has been conducted up to date, that we could refer to.

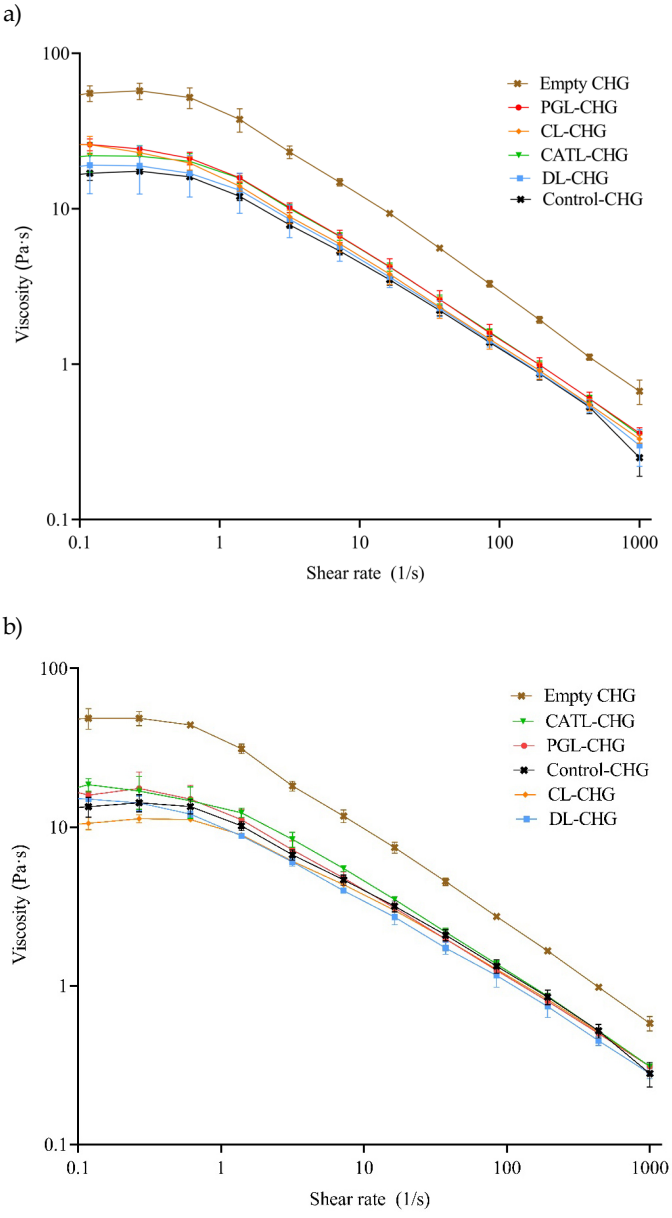


Fig. 1. Viscosity curves (logarithmic plot) of liposomal CHGs at: a) 25 °C and b) 34 °C.

Viscoelasticity describes materials that can behave both like elastic solids and viscous fluids. Viscoelastic properties correlate with the physical appearance of hydrogels and patients' experience during the application, as well as the therapeutic efficiency of the semisolid formulations (33). Oscillatory sweeping was conducted to monitor the values of storage modulus (G′) and loss modulus (G″) upon increasing oscillatory stress. During oscillatory tests, liposomal CHGs exhibited linear viscoelastic region (LVR), which is valuable information since samples with broad LVR are considered well-dispersed and stable (33). LVR of all the tested samples was characterized with storage modulus (G′) higher than

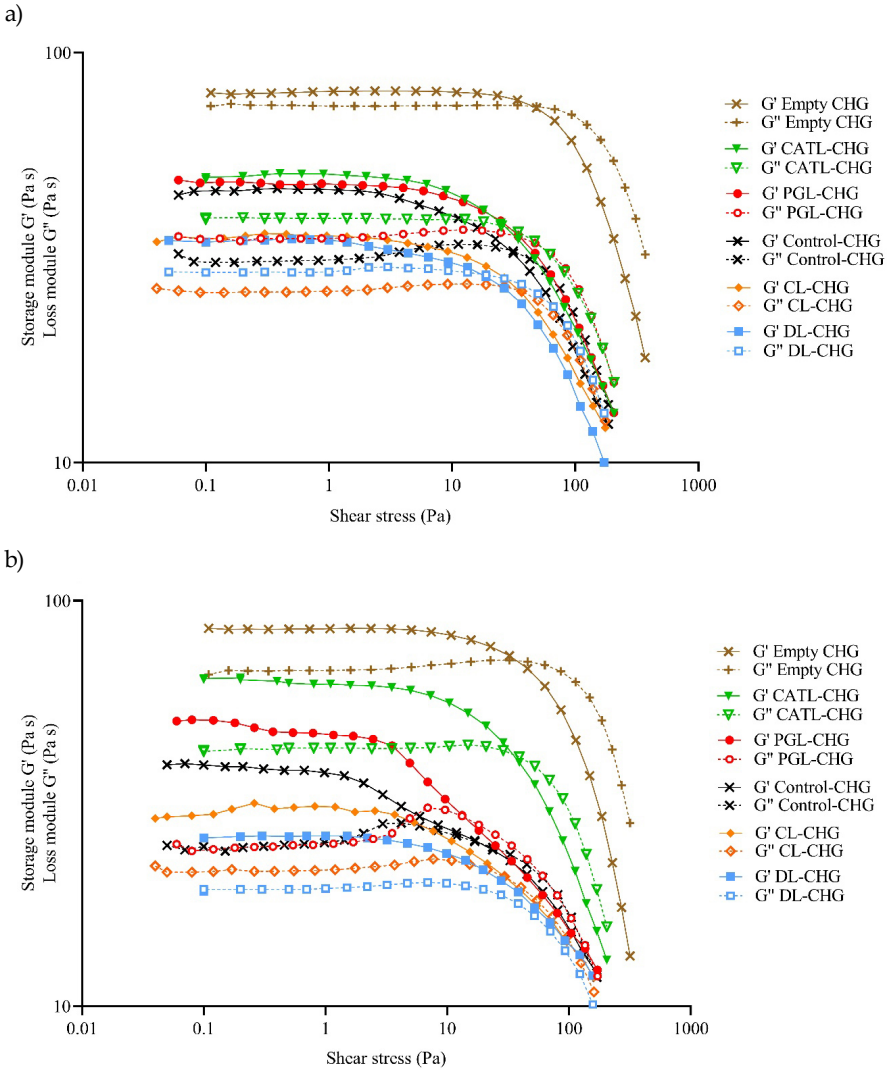


Fig. 2. Amplitude sweep curves (logarithmic plot) of liposomal CHGs at: a) 25 °C and b) 34 °C.

loss modulus (G″), thus corroborating dominantly elastic nature of the samples both at 25 °C (Fig. 2a) and 34 °C (Fig. 2b). The obtained amplitude sweep curves confirmed that even though the viscosity of CHG is decreased to some extent after the addition of AZT liposomes or AZT-solution, its gel-like structure remained preserved.

As shown by Fig. 2, the discrepancy between G′ and G″ diminishes above the determined LVR values probably because of the intense extension of the polymer chains and partial breakage of hydrogen bonds inside the hydrogel matrix (33). The value at which G″ and G′ equalize is considered to be the point at which the gel-like structure is disrupted and after which formulation starts to behave more like viscous fluid (G″ > G′). If this cross-over value is higher, then the system is relatively more stress-resistant (33). G″/G′ cross-over values for each liposomal CHG were deduced from the amplitude sweep curves (Fig. 2) and graphed in Fig. 3. As expected, for most samples, G″/G′ cross-over values were lower at 34 °C in comparison to values obtained at 25 °C. The exceptions were DL-CHG and CATL-CHG, displaying higher G″/G′ cross-over values at 34 °C. Furthermore, according to G″/G′ cross-over values at 25 °C, the most durable sample was empty CHG, followed by PGL-CHG, whereas the most prone to deformation were CATL-CHG and DL-CHG (Fig. 3a). Oppositely, at 34 °C, which corresponds to the temperature attained on the skin surface, CATL-CHG and DL-CHG were shown to be the most durable to mechanical stress (Fig. 3b). Such results indicate that the interaction between various liposomes incorporated into the hydrogel and CHG may differ at different temperatures.

Texture properties of liposomal CHGs

Texture properties of semisolid formulations, *i.e.*, hardness, cohesiveness, and adhesiveness, can be related to the easiness of application onto the skin, extrusion of the prepa-

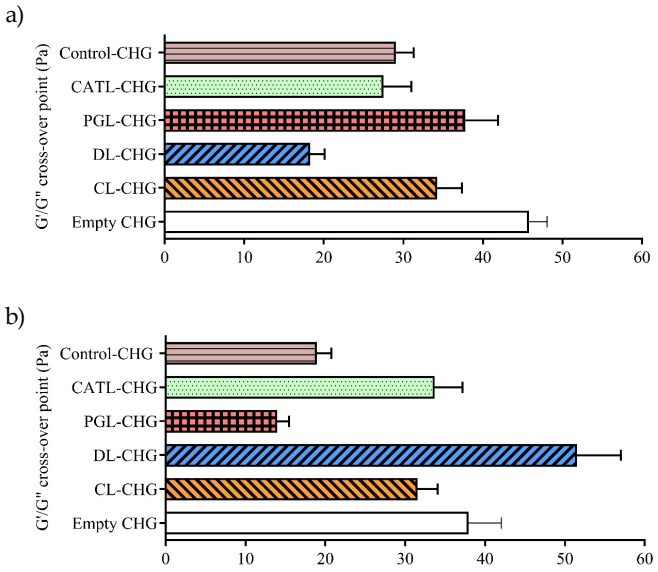


Fig. 3. G″/G′ cross-over values of liposomal CHGs at: a) 25 °C and b) 34 °C.

ration from the container, and retention at the site of application, respectively (20, 34). Moreover, these parameters tend to reflect on the efficacy of the treatment and the level of patients' compliance (17). Therefore, texture analysis of empty CHG, control-CHG, and liposomal CHGs was performed to provide information on the texture properties of these formulations and to investigate how they are affected by various types of AZT-liposomes. The results revealed that the incorporation of AZT-liposomes significantly affects the original CHG (empty CHG) texture properties (Fig. 4). The observed decrease in adhesiveness, cohesiveness and hardness is supposed to be generated primarily by the considerable amount of the fluid mixed into the CHG (30 %, *m*/*m*), causing fair dilution of the CHG,

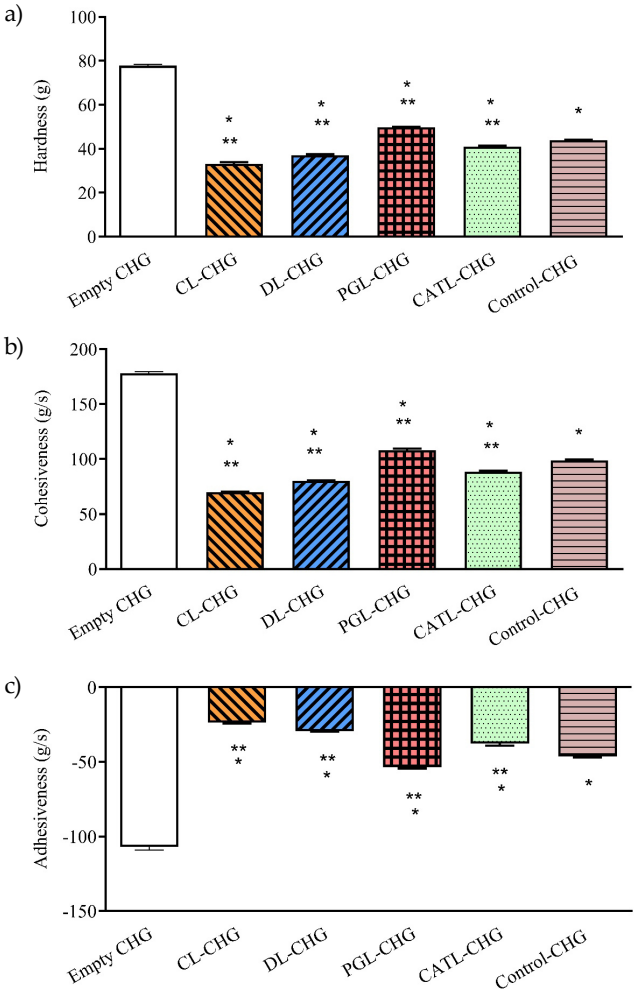


Fig. 4. Texture properties of the different CHG: a) hardness, b) cohesiveness and c) adhesiveness. The presented values are the mean ± S.D. ($n = 5$). * Significantly different compared to empty CHG ($p < 0.05$) ** Significantly different compared to control-CHG ($p < 0.05$).

because the same effect was detected after the addition of AZT-solution into the CHG as well. The extent of the decrease in texture parameters did not seem to be influenced by the different surface charges of the incorporated AZT-liposomes but rather by their composition. Namely, the addition of all AZT-liposomes altered the texture properties of CHG to a greater extent ($p < 0.05$) than the addition of free AZT solution (control-CHG), except for PGL, which could be attributed to the presence of propylene glycol in the preparation. In particular, propylene glycol and similar co-solvents are known to have a beneficial effect on the texture properties of hydrogels (17, 35, 36).

The results presented in Fig. 4 agree with the previous research dealing with liposomal hydrogels. Namely, a significant decrease in hardness and cohesiveness of vaginal chitosan hydrogel after the addition of AZT-loaded liposomes or AZT-solution was confirmed by Čačić *et al*. (19), as a result of the high portion of the liquid phase (30 %, *m*/*m*) added into the original hydrogel. Ternullo and collaborators (17) investigated chitosan hydrogel as a vehicle for the incorporation of curcumin-loaded deformable liposomes of different surface charges in the development of novel wound dressings. Incorporated liposomes (15 %, *m*/*m*) weakened the hydrogel texture properties to a greater extent than the free drug solution. The surface charge of the embedded liposomes in their research was also not recognized to be the dominant inducer of changes in the texture properties of the vehicle. Conversely, Jøraholmen *et al*. (36) detected the opposite behavior of chitosan hydrogel after the addition of liposomes: hardness, adhesiveness, and cohesiveness were increased for hydrogel containing 10 % or 20 % (*m*/*m*) of liposomes, demonstrating stabilizing effect of liposomes on the hydrogel network.

The abovementioned studies were performed with liposomal hydrogels differing in molecular weight and concentration of chitosan in the hydrogel matrix, as well as the lipid composition of the embedded liposomes. Furthermore, the amount of the incorporated liposomal dispersions was in range from 10 % to 30 % (*m*/*m*). Noteworthy, all the liposomes embedded into chitosan hydrogels were prepared as dispersions in PBS, except in a study by Jøraholmen *et al*. (36), in which distilled water was used.

In vitro *release of AZT from liposomal CHGs*

Interactions between the hydrogel and embedded liposomal nanocarriers are anticipated to influence the pharmacokinetic properties of the entrapped drug (37–40). Hence, the polymeric network of CHG was expected to slow down the release of the liposomal AZT, ensuring the desired sustained or prolonged drug release. Results of the *in vitro* release study (Fig. 5) confirmed that the AZT release from liposomal CHGs, regardless of the type of AZT-liposomes entrapped into the CHG, is significantly slower than the AZT release from the control-CHG. In other words, by the incorporation of AZT into the CHG, a prolonged release of the antibiotic is achieved, and this effect is even more pronounced when liposomal AZT and CHG are combined. Furthermore, the release of AZT from liposomal CHGs was prolonged and more controlled in comparison to the corresponding AZT-liposomes characterized in our previous study (1).

The findings in this research correspond to the results reported by Jøraholmen *et al*. (36) and Hemmingsen *et al*. (34), in which the authors investigated liposomal chitosan hydrogels as a delivery system for polyphenols and chlorhexidine, respectively. In these studies, *in vitro* release experiments supported significantly prolonged release of the

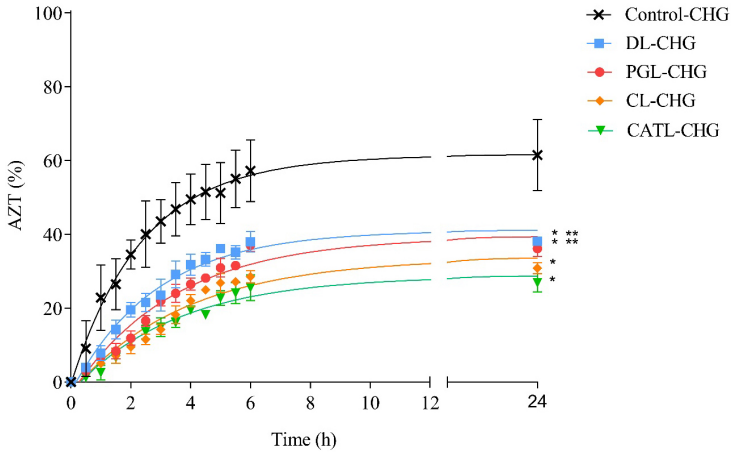


Fig. 5. Cumulative *in vitro* release of AZT from liposomal CHGs. The values represent the mean ± S.D. ($n = 3$). * Significantly different compared to control-CHG at the 24 h-time point ($p < 0.05$). ** Significantly different from CL-CHG at the 24-hour time point ($p < 0.05$).

encapsulated drugs from the liposomal hydrogels in comparison to plain liposomal dispersions. Such release behavior was also documented in the most recent study, focusing on the AZT-loaded liposomal chitosan hydrogel designed for vaginal administration (19). Accordingly, the release of AZT from liposomal gels in the latter study during the first 8 hours of *in vitro* release experiments was much slower than the *in vitro* release from free AZT-solution incorporated into the chitosan hydrogel. Even though the authors in all the above-mentioned studies used MMW chitosan for the preparation of chitosan hydrogels (2.5–4.5 %) (19, 34, 36), in contrast to HMW chitosan used herein (2.5 %), release profiles and cumulative release of the entrapped drug were quite similar.

Finally, it is important to point out that the cumulative amount of AZT released from liposomal CHGs (Fig. 5) was above the established minimal concentration of AZT that is expected to impair the growth of the targeted bacteria (1), assuring that the proposed drug delivery system attains appropriate concentration of the antibiotic at the administration site over a certain period of time, which is beneficial for the desired clinical outcome and also from the resistance issue point of view.

In vitro *anti-MRSA activity of liposomal CHGs*

We have previously reported the anti-MRSA effects of all AZT-liposomes (CL, DL, PGL, and CATL), showing that the bacterial growth was strongly inhibited *in vitro*, where all the AZT-liposomes were found to be more potent than free AZT (1). However, the anti-MRSA properties of AZT-liposomes incorporated into CHG have not yet been reported. The principal objective in using CHG as a vehicle for the AZT-liposomes was to boost their anti-MRSA activity. To test this hypothesis, the antibacterial activity of liposomal CHGs against different clinical isolates of MRSA was assessed by the *in vitro* agar-diffusion method.

As shown in Fig. 6, all the liposomal CHGs successfully inhibited the growth of MRSA isolates and were more effective than the control-CHG. The exception was CL-CHG, which

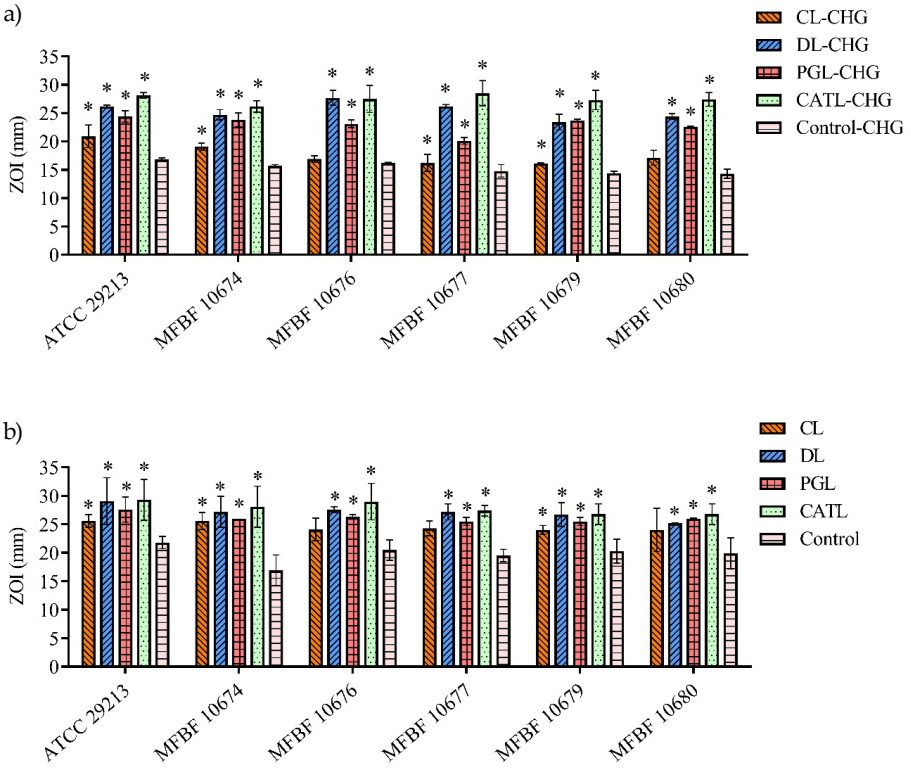


Fig. 6. *In vitro* anti-MRSA activity of different: a) liposomal CHGs and b) AZT-liposomes (AZT concentration was 3.3-fold higher compared to the corresponding liposomal CHGs). Results are presented as the diameter of the inhibition zone (ZOI; mm) including well diameter of 6 mm (mean ± SD, $n = 3$). * Statistically significant difference (*t*-test, $p < 0.05$) compared to control-CHG.

was insignificantly more effective than control on the majority of the tested MRSA isolates. Furthermore, CATL-CHG and DL-CHG demonstrated similar anti-MRSA activity against most tested MRSA isolates. CATL-CHG was more effective than PGL-CHG against all tested MRSA isolates ($p < 0.05$), whereas DL-CHG was more effective than PGL-CHG only against two MRSA strains (MFBF 10674 and MFBF 10676). Accordingly, CATL-CHG was concluded to possess the strongest anti-MRSA activity of all liposomal CHGs.

Even though some of the previous studies indicated the intrinsic antibacterial activity of chitosan hydrogels (12, 19, 41, 42), the employed *in vitro* test in this research did not detect an inhibitory effect of the empty CHG on the growth of tested MRSA isolates. This could be attributed to different bacterial strains tested, different experimental methods of assessing the antibacterial activity of the formulation and/or different characteristics of the chitosan used for the preparation of the hydrogel (molecular weight, deacetylation degree, chitosan concentration), which are known to affect the biological activities of the chitosan (43). Nevertheless, even if the empty CHG does not possess direct anti-MRSA activity in this particular experimental setup, the polymeric chitosan network of CHG was

presumed to contribute indirectly to the anti-MRSA potential of the liposomal CHGs. To investigate this indirect impact of CHG on the overall anti-MRSA activity of the liposomal CHGs, the agar-diffusion method with exactly the same procedure was applied to assess the growth inhibition of MRSA isolates by AZT-liposomes in the form of plain liposomal dispersions. Growth inhibition zone diameters (ZOI) of AZT-liposomes obtained (Fig. 7) were at first sight greater than the ZOI of the corresponding liposomal CHGs (Fig. 6). However, considering the fact that the AZT concentration in liposomal CHGs was 3.3 times lower than in the corresponding AZT-liposomes (original AZT-liposomes were diluted in the CHG at 30 %, *m*/*m*), incorporation into the CHG was proved to boost anti-MRSA activity of AZT-liposomes as well as AZT-solution. This effect could be ascribed to the three-dimensional structure of the CHG which enables prolonged retention of the formulation at the application site as well as prolonged and controlled release of the liposomal AZT, all of which assure high and less variable antibiotic concentration at the application site (14).

In addition, the results of the *in vitro* anti-MRSA experiments affirm that the ZOI determined for AZT-liposomes against all of the tested MRSA isolates were significantly larger than the ZOI determined for control (AZT-solution), which is in agreement with our previous study (1), in which the different *in vitro* method (a two-fold microdilution assay) was used for the evaluation of the antibacterial activity of the same types of AZT-liposomes. Furthermore, although CATL was significantly more effective than CL against the bacterial isolates MFBF 10676 and MFBF 10677 and more effective than PGL against the isolate MFBF 10676 (ANOVA, $p > 0.05$) (Fig. 6b), it follows that no significant difference in anti-MRSA effect was observed among different types of AZT-liposomes (ANOVA, $p > 0.05$). Such results dispute our previous findings (1), where the anti-MRSA effect was significantly influenced by the composition of the particular liposomal nanoformulations. In other words, it seems that the agar-diffusion method has a lower capacity to differentiate the effect of the physicochemical properties of AZT-liposomes with respect to their antibacterial potential in comparison to the previously reported microdilution method (1). Interestingly, the corresponding unloaded liposomes (without the entrapped AZT) did not produce growth inhibition zones, complying with the results obtained by the microdilution test and corroborating that the particular liposomal formulation itself does not exhibit anti-MRSA effects or does not display anti-MRSA activity at the tested concentrations.

In vitro *cytotoxicity assessment*

Topical antimicrobial formulations are required not only to be effective but also non-toxic to the skin. The potential cytotoxicity of various liposomal CHGs was evaluated *in vitro* on human keratinocytes (HaCaT). This cell line was exposed to the different liposomal CHGs for 24 hours at AZT concentrations ranging from 0.25 to 64 μg mL^{-1}, which was 16–256 fold higher than the minimal inhibitory concentration (MIC) previously reported for particular AZT-liposomes (1).

At AZT concentrations up to and including 16 μg mL^{-1} none of the liposomal CHGs induced any cytotoxic effect on keratinocytes, nor did empty CHG or control-CHG (Fig. 7). Furthermore, no significant difference in biocompatibility was established among the different liposomal CHGs ($p > 0.05$), as well as in comparison to empty CHG. However, the viability of HaCaT cells was significantly lower when treated with control-CHG than after the treatment with liposomal CHGs ($p < 0.05$). This observation is in agreement with our

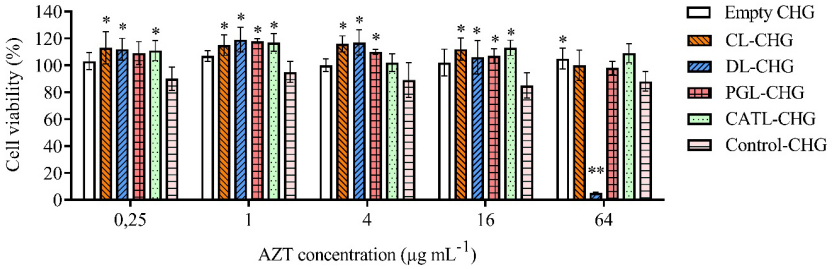


Fig. 7. Viability of HaCaT keratinocytes after 24-hour incubation with different liposomal CHGs, empty CHG and/or control-CHG at 37 °C. Results are presented as mean ± SD (n = 4). **Cell viability < 70 %; * Significantly different compared to control-CHG at the corresponding AZT concentration.

previous research (1), verifying liposomal entrapment of AZT as favorable from a biocompatibility point of view.

As shown by Fig. 7 DL-CHG induced a major decrease in the cell viability at the highest tested concentration of AZT (64 μg mL^{-1}), whereas all other liposomal CHGs were proved to be biocompatible with the HaCaT cells *in vitro* (viability ≥ 88 %). Interestingly, the biocompatibility assessment of AZT-liposomes (1) did not demonstrate any cytotoxic effect of DL on HaCaT cells at the corresponding AZT concentration. In the same study (1) free AZT solution demonstrated a plausible cytotoxic effect at 64 μg mL^{-1}, whereas in the present work control-CHG (AZT-solution in CHG) was biocompatible with HaCaT cells at the same AZT concentration tested. Since chitosan hydrogels are generally considered safe and recognized as biocompatible with human cells (44–47), it was reasonable to anticipate the enhanced biocompatibility of the drug after the incorporation into the CHG, as was indeed demonstrated for free AZT. In contrast, the results obtained for DL-CHG do not follow this concept and one of the possible explanations could be inopportune electrostatic interaction between the cationic chitosan in the CHG and anionic surfactant sodium deoxycholate included in DL formulation. Namely, chitosan is known to form ionic complexes with oppositely charged surfactants, resulting in the enhanced solubility of chitosan and conjugation with other polymers onto chitosan/surfactant complexes (48, 49), which can possibly cause interferences with relevant macromolecules at the cell level.

CONCLUSIONS

CHGs loaded with 4 different types of AZT-liposomes were prepared and evaluated for localized topical treatment of MRSA-related skin infections. All liposomal CHGs demonstrated prolonged release of AZT and exhibited desirable pseudoplastic flow behavior and texture properties suitable for topical skin application. Moreover, the incorporation of AZT-liposomes into CHG was also demonstrated to be beneficial in terms of enhanced anti-MRSA activity and high biocompatibility with skin cells, verifying this strategy as a promising trend in topical skin drug delivery. By exploring the influence of the incorporated liposomes′ composition, the development of liposomal CHGs for controlled dermal delivery of AZT can be tailored and effective therapy assured, which remains to be validated *ex vivo*/*in vivo*. Taken together, CATL-CHG formulation was proved to be the most promising formulation for further *ex vivo*/*in vivo* investigation.

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Authors contributions – Conceptualization, Z.R., Ž.V., M.Š.K. and N.Š.-B.; methodology, Z.R., Ž.V., M.Š.K., M.W.J., N.Š.-B., D.B.M. and P.G.G.; investigation Z.R., M.W.J., D.B.G. and I.F.; writing-original draft preparation, Z.R., Ž.V., M.Š.K., M.W.J. and N.Š.-B.; writing-review and editing, Z.R.; Ž.V., N.Š.-B., M.Š.K., and M.W.J.; supervision, Ž.V. and M.Š.K. All authors have read and agreed to the published version of the manuscript.

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