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Original article

Propylene glycol liposomes for improved delivery of vancomycin to biofilms on abiotic surfaces

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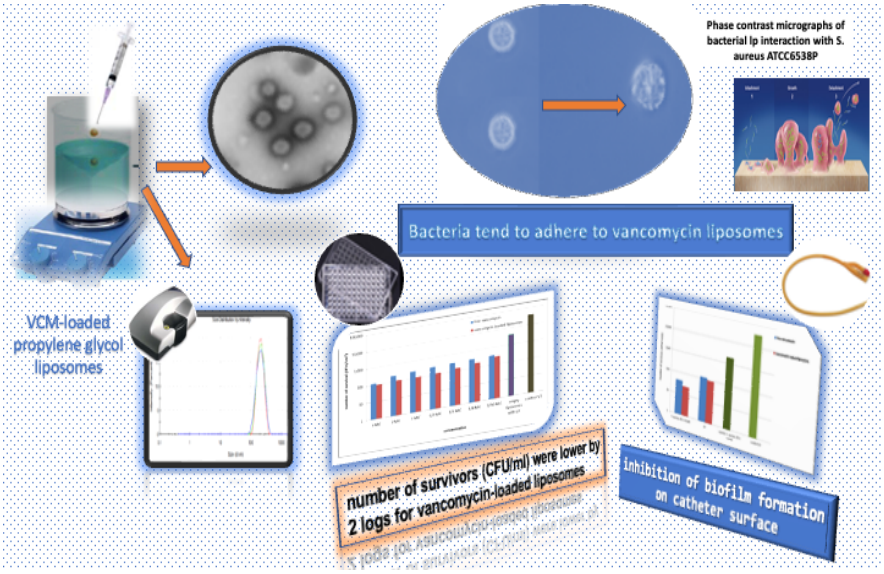
Abstract:



Patients fitted with urinary bladder and vascular catheters are subject to bacterial infections and biofilm formation. These infections are mostly caused by coagulase-positive and coagulase-negative staphylococci. Vancomycin (VCM) remains the frontline intravenous antibiotic for the treatment of catheter-related bacteremia. Liposomes are appealing drug carrier systems, especially against colonized microorganisms.

In the present study, VCM-loaded propylene glycol liposomes were prepared by the ethanol injection method The liposomes were characterized pharmaceutically and microbiologically. Pharmaceutical attributes included colloidal properties, entrapment efficiency (EE%), release, and stability. Microbiological tests included the determination of Minimum Inhibitory Concentration (MIC) by two methods, antibiofilm efficacy using the microtiter plate model including assessment

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Minimum Biofilm Inhibitory Concentration (MBIC), Minimum Biofilm Eradicating Concentration (MBEC), and biofilm formation induction by vancomycin sub-minimum inhibitory concentrations. Antibiofilm efficacy was also assessed using the catheter segments model.
VCM liposomes showed vesicle size in the nanorange (219.49 ± 20.21 nm), low PDI (0.282 ± 0.044), negative zeta potential (-4.78), and EE% (52.84±1.5%) that proved stable with no drug leakage after 3 months of storage at 4°C. It also showed a slower release profile compared to the free VCM. The antibacterial and antibiofilm efficacy of VCM liposomes compared to free VCM increased by 2-8 folds, calculated from the observed extent of reduction in MIC, MBIC, and MBEC of liposome-loaded VCM compared to free VCM. Results of catheter segment experiments indicated the potential usefulness of VCM liposomes in antibiotic lock solutions for managing biofilms on medical devices and implants.

Keywords*:* Vancomycin, liposomes, abiotic, biofilm

1. Introduction

Vancomycin remains the cornerstone antibiotic for combating biofilms on abiotic surfaces. Several published articles have tackled the issue of improving vancomycin antimicrobial and antibiofilm performance against pathogenic gram-negative and gram-positive bacteria using lipid vesicles among others as carrier systems [(1-8)].

Vancomycin niosomes proved superior to free vancomycin in inhibiting biofilm formation, eradicating surface-borne biofilms, and inhibiting biofilm growth when tested using microtiter plates as an *in vitro* model for biofilms [(1)].

Nevertheless, due to their great biocompatibility, biodegradability, immuno-genicity, lack of toxicity, and ease of modification with targeting moieties, liposomes are still one of the most promising and commonly studied nano-vehicles to carry and deliver antimicrobial agents. Moreover, the FDA has approved several liposome-based compositions for the treatment of infectious illnesses [(6)].

Biofilm formation by both Gram-negative and Gram-positive bacteria on indwelling medical devices and implants such as catheters, mechanical heart valves, pacemakers, prosthetic joints, and contact lenses poses a critical medical problem [(9)]. Among these biofilm-forming bacteria, the most common include *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus viridans*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Pseudomonas aeruginosa* [(10, 11)]. Among these biofilm-forming bacteria, *S. aureus and S. epidermidis* are most found on cardiovascular devices. It was estimated that *S. aureus and S. epidermidis* caused about 40%–50% of prosthetic heart valve infections, and 50%–70% of catheter biofilm infections [(12)].

Our research group has previously reported the development of novel propylene glycol liposomes and documented their improved physical and performance characteristics in skin drug delivery (better storage stability, higher drug EE, and enhanced skin uptake), compared to traditional liposomes, deformable liposomes, and ethosomes [(13)]. In view of the demonstrated higher efficacy of PG liposomes in topical drug delivery [(13-16)], it was deemed of interest to further investigate the potential of PG liposomes to manage catheter-related bacterial biofilms.

Managing catheter-related biofilm formation remains a medical endeavor driving continued research in this area. Interest in applying nanotechnology (including VCM liposomes) to medical biofilm control continues as attested by recent publications [(5-7, 17)].

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A clinical setting where liposomal VCM could prove more effective than free VCM in managing catheter-associated biofilms is antibiotic lock therapy, which constitutes a line of defense against catheter-induced systemic bacteremia and involves placing an effective antibiotic solution in the catheter lumen for a limited time before withdrawing it, to help eradicate the catheter-adherent biofilm, thereby eliminating the need to remove the catheter.

The present study aimed to investigate various aspects of the antibiofilm activity of an antibacterial drug, VCM, when loaded in PG liposomes compared to the free drug; biofilm formation inhibition as well as biofilm eradication were investigated. Models for abiotic surfaces used in the present study include microtiter plates [1, 18] and silicon urinary catheter segments. In designing the silicon catheter segment study, care was taken to simulate the clinical setting concerning the time of contact of the catheter segments with VCM liposomes [19, 20]. The effect of VCM liposomes possibly enhancing biofilm formation in non-biofilm forming bacteria was also investigated. The study's novelty was in investigating the potential of PG liposomes, as a nanocarrier for managing catheter-related biofilms.

2. Materials and methods

Lipoid S 100 (phosphatidylcholine (PC) from soybean lecithin), containing not less than 94%PC, its transition temperature far below room temperature was a kind gift from Lipoid GmbH (Ludwigshafen, Germany). Propylene glycol was from ADWIC, El-Nasr Pharmaceutical Chemicals Co. (Abu Zaabal, Egypt). Vancomycin hydrochloride powder used in the study was the content of Vancomycin® 500mg vials, Mylan S.A.S-France, purchased locally. All other chemicals were of analytical grade.

2.1. Bacterial isolates

A total of 21 staphylococcal isolates were used in this study. The isolates included 2 reference staphylococcal strains (*S. aureus* ATCC 6538P and *S. epidermidis* ATCC 12228) and 19 bacterial isolates obtained from different clinical specimens; 14 *S. aureus* isolates and 5 *S. epidermidis* isolates. The clinical isolates were provided by the Department of Pharmaceutical Microbiology and Immunology, Faculty of Pharmacy, and the Department of Microbiology, Faculty of Medicine, Alexandria University. Isolates were identified to species level using standard biochemical methods according to the "Identification flow charts" of Bergey's Manual of Determinative Bacteriology [22] after Gram stain. The identified stock cultures were preserved at −80°C in 15% glycerol.

2.2. Culture media

Nutrient agar, Nutrient broth, Müller-Hinton agar, and Tryptone soya broth were Oxoid-made (Oxoid Ltd; Basingostock; Hampshire, England)

2.3. Preparation of PG liposomes

Blank propylene glycol liposomes (PG liposomes) were prepared using the ethanol infusion method [21]. Lipoid S 100 (200 mg) was dissolved in a mixture of ethanol (0.3 ml) and propylene glycol (0.5 g) and added slowly with stirring (700 rpm) to distilled water (4 ml). Stirring was continued for 30 minutes. The volume was adjusted to 5 ml with distilled water. To prepare vancomycin-loaded PG liposomes (VCM liposomes), 100 mg of the antibiotic were dissolved in the aqueous medium before adding the lipoid solution [16, 21].

The formed vesicles were sonicated for a total of 45 minutes on ice (three intermittent 15-minute sessions) before extruding through two membrane filters (0.45µm nylon, Abel Industries, Vancouver BC, Canada, and 0.2µm cellulose acetate syringe filters, Sartorius, Germany) [23, 24]. Liposomes were stored at 4^{o} C.

Sonication before extrusion was necessary. In the absence of sonication, extrusion of

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liposome dispersion through a syringe fitted with membrane by hand force, proved difficult. Optimization of the size reduction process, avoiding adverse effects on drug EE, suggested sonication for 45 minutes (three 15-minute sonication sessions) before extrusion.

3. Characterization

3.1. Transmission Electron Microscopy

The microscope used was Jeol-100 CX, Japan. Negative staining was applied using 2% w/v uranyl acetate. VCM-Liposomes (initial VCM amount 100 mg per 5 ml liposome dispersion) were examined at 15000 X magnification power at 80 kV.

3.2. Vesicle size, size distribution and zeta potential

Malvern Zetasizer (Nano ZS, Malvern Instruments, Malvern, UK) was used after suitable dilutions (20 times) with filtered distilled water (no additives were used). The polydispersity index (PDI) was also determined as a measure of homogeneity.

Zeta potential was determined using the Malvern Zetasizer. Samples were placed in clear disposable ζ-cells. Filtered distilled water was used as a dilution medium.

3.3. Determination of entrapment efficiency

The method involved dialysis (Carolina ® dialysis tubing, 12,000-14,000 Da molecular weight cut off, North Carolina, USA) of the liposomes (0.5ml) for 2 h at room temperature. Bags were suspended in distilled water (75 ml, ensuring sink condition).VCM in the dialysate was determined at λ max 280nm [(1)]. A control bag containing VCM (equivalent amount to total VCM in liposome bag) was also dialyzed. Entrapment efficiency was calculated.

3.3.1. Effect of initial vancomycin amount on entrapment

The effect of increasing VCM initial amount on liposome characteristics was investigated by preparing PG-liposomes using constant phospholipid concentration (4% w/v with respect to final liposome dispersion) and variable VCM amount (70 to 300 mg/5ml of final liposome dispersion). Characteristics measured included vesicle size, PdI, and entrapment efficiency.

3.4. In vitro drug release

The release was studied using the dialysis method. Liposome dispersion (0.5 ml equivalent to 10mg VCM) was filled in dialysis bag, immersed in 75ml distilled water, and shaken (100 strokes/min) at 37^{o}C. Samples were withdrawn (and compensated) at 2, 4, 6, and 24 hr. The drug in dialysate was determined spectrophotometrically. Suitable control samples were used.

3.5. Stability testing of VCM PG-liposomes

Liposomes were stored for 12 months at 4^{o}C. The change in drug entrapment from zero time value was monitored at 1, 3, 6, and 12 months, as an indicator of possible drug leakage from vesicles. Vesicle size, PDI value, zeta potential, and VCM release profile were also examined at the same time intervals. Changes in release profiles during storage compared to release data before storage were assessed by calculating the similarity factor (f2) [(25, 26)].

3.6. Microbiological studies

3.6.1. Antimicrobial susceptibility tests

Screening for methicillin and vancomycin resistance was done by disk diffusion method based on the susceptibility of the isolates to cefoxitin (30 μg/disk) and VCM (30 μg/disk) according to the CLSI 2014 Clinical Laboratory Standards Institute guidelines for susceptibility checking (CLSI 2014) [(27)]. The zones of inhibition were determined after incubation for 24 h at 32 °C. Resistance was defined according to the Clinical Laboratory Standards Institute zone diameters [(27)].

3.6.2. MIC determination

The MICs of free VCM or in liposomal form were determined against the clinical as well as the standard strains by agar dilution method according to CLSI, 2014 [(27)]. The

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MIC was defined as the least concentration causing complete inhibition of the organism or no more than five discrete colonies per plate, under the test conditions where; susceptible, MIC ≤ 2 μg/ml; intermediate, 4–8 μg/ml; resistant, MIC ≥ 16 μg/ml). The testing MIC range of VCM was 0.156-10 μg/ml. Blank liposomes were examined for their antibacterial activity.

3.6.3. Visualization of VCM liposomes / Staphylococcus interaction by phase contrast microscope

Attempts to visualize possible interaction between VCM liposomes and two selected isolates, the standard strain S. aureus ATCC 6538P and S. aureus isolate V7, were carried out by the phase contrast microscope (Olympus model CX 41 RF)[28, 29]. Briefly, 2 ml of the bacterial suspension (~10^5 CFU/ml) in nutrient broth were incubated with 200 μl of VCM liposomes (not extruded) for 1 hr at 37ºC in a shaking water bath (GFL, Germany) at 50 rpm to allow for the interaction to take place. A drop of reaction mixture was thin layered on a glass slide and the sample was examined by phase contrast microscope at a magnification power of 100x. Bacteria alone in broth served as a control for comparison.

3.6.4. Antibiofilm studies using the microtiter plates as a model for abiotic surfaces

3.6.4.1. Screening of biofilm-forming bacteria

Quantitative biofilm measurement was done in microtiter plates as described previously [30]. Briefly, bacteria were grown overnight in tryptic soy broth fortified with 0.25% glucose (TSBG) and transferred to 96-well tissue culture plates (TPP, Switzerland) after being diluted to ~10^6 CFU/ml. Wells containing TSBG only were considered as the negative control. Following overnight incubation at 37°C, the culture medium was aseptically poured out and replaced by fresh TSBG. After incubation for another 24 hours, the culture medium was poured out. The plates were then washed three times with sterile phosphate-buffered saline (PBS, pH 7.2), and the remaining bacteria were fixed by air drying. After staining with 0.1% w/v safranin solution, the optical density of the adherent biofilm was determined at 630 nm using an enzyme-linked immunosorbent assay ELISA reader (Biotek, Highland Park, USA)[31]. The test was performed in duplicate and the values were averaged. Empty microtiter plate wells, treated with medium, fixative, and stain as described above, were used as a zero point. Biofilm-forming strains were defined as those having a mean OD_{630} greater than or equal to 0.1.

3.6.4.2. Biofilm susceptibility tests

Minimal biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC) experiments were performed as described earlier, with a serial twofold dilution of the tested VCM in Mueller-Hinton broth [30, 32]. MBIC was defined as the lowest concentration of antibiotic at which there was no observable bacterial growth in the wells containing adherent microcolonies. MBEC was defined as the minimal concentration of antibiotic required to eradicate the biofilm, that is, the minimal antibiotic concentration at which bacteria failed to regrow after antimicrobial exposure. All determinations were performed in duplicate.

3.6.4.3. Induction of biofilm formation in non-biofilm forming isolates

The lowest concentrations of free and VCM-loaded PG liposomes that induced biofilm formation in non-biofilm forming bacteria were determined (Biofilm Forming Concentration, BFC) using seven non-biofilm forming *Staphylococcal* isolates. Bacteria were incubated with serial dilutions below their corresponding MICs of free and liposomal VCM for 48 h at 37 °C. Negative and positive controls, TSBG or TSBG inoculated with the tested bacterial culture,

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respectively, were included. The biofilms developed were evaluated using the plate reader as described earlier.

3.6.5. Development of in vitro catheter-associated biofilm

Standard *S. aureus* ATCC 6538P strain and *S. aureus* isolate V7 were grown in TSBG to the early exponential phase. The urinary silicon-coated Foley catheter was cut into 1 cm segments, placed in distilled water, and sterilized by autoclaving at 121 ℃ for 15 min. Bacterial biofilms were developed on the catheter by placing individual catheter segments into tubes containing 1.0 ml of the tested bacterial suspension (~ 10^4 CFU/ml) in TSBG in the exponential phase of growth. After overnight incubation at 37 °C, two colonized catheter segments were recovered aseptically from each tested organism and rinsed with sterile PBS, to remove unbound bacteria, for quantitative analysis of biofilm development [(20)]. Control catheter segments were treated as above but without bacterial inoculum. The remaining catheter segments were used to test the ability of the tested VCM to eradicate the biofilm on the catheter segments.

3.6.5.1. Determination of the effect of free and liposomal VCM on the eradication of biofilm developed on catheter surface

Several dilutions of VCM-free solution and VCM liposomes, ranging from 4xMIC–1/32xMIC, were prepared aseptically and individual pieces of the colonized catheters were added. They were then placed in a shaking water bath at 37° C for 2 h to simulate the *in vivo* condition in terms of a possible applicable contact time in a clinical setting comparable to antibiotic lock solution. They were then transferred to warm fresh TSBG and placed in an ultrasonic bath at 50 kHz (Julabo USR3, Germany), sonicated for 5 min, and vortexed for 1 min to remove the biofilm bacteria from the support surface. The suspension of bacteria that was removed from the catheter was 10-fold serially diluted with sterile saline and 20 µl aliquots of each dilution were dropped onto the surface of dried nutrient agar plates, and incubated overnight at 37 ℃ for colony counting. The average of the viable number of colonies from two catheter segments from each dilution was recorded. The correlation between CFU counts and the corresponding VCM concentration was determined by plotting log survivors versus VCM concentration.

3.6.5.2. Testing the effect of pretreatment of catheter surface with either free VCM solution and VCM liposomes on the prevention of biofilm formation

The catheter was cut into 1 cm segments and sterilized as described earlier. Catheters were placed in tubes containing either free or liposomal VCM (concentration 4x respective MIC) for 20 minutes [(20)]. Control catheter segments were placed into tubes containing TSBG media only. Catheter segments were recovered aseptically from the VCM solutions and placed in the tested microorganisms (~ 10^4CFU/ml) in TSBG in the exponential phase of growth. The tested strains were incubated overnight at 37 ℃ to allow the growth of biofilm on catheters surfaces as described earlier. The viable count, which allows the assessment of the kinetics of biofilm formation on catheter material, was monitored as mentioned earlier and the number of colonies was recorded, and the number of survivors/catheter segment was calculated.

3.7. Statistics:

Statistical analysis was performed using Student t-test or One-way ANOVA. Differences were considered significant at a level of $p \leq 0.05$.

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4. Results and discussion

4.1. TEM, colloidal properties, release, and stability

The developed liposomes were propylene glycol liposomes (4% w/v phospholipid and 10% w/v PG calculated with respect to final liposome dispersion), in an aqueous medium. The inclusion of propylene glycol in the formulation facilitated extrusion in the size reduction step and resulted in small homogenous vesicles (vesicle size 219.49 ± 20.21 nm and PdI 0.282 ± 0.044, mean of 5 batches of VCM liposomes, n=15). **Fig. 1a** shows a TEM micrograph of a sample of the prepared vesicles. Discrete spherical vesicles are apparent.

Vesicle size distribution by intensity curve (**Fig. 1b)** provides further evidence of the monodispersity of the prepared VCM liposomes.

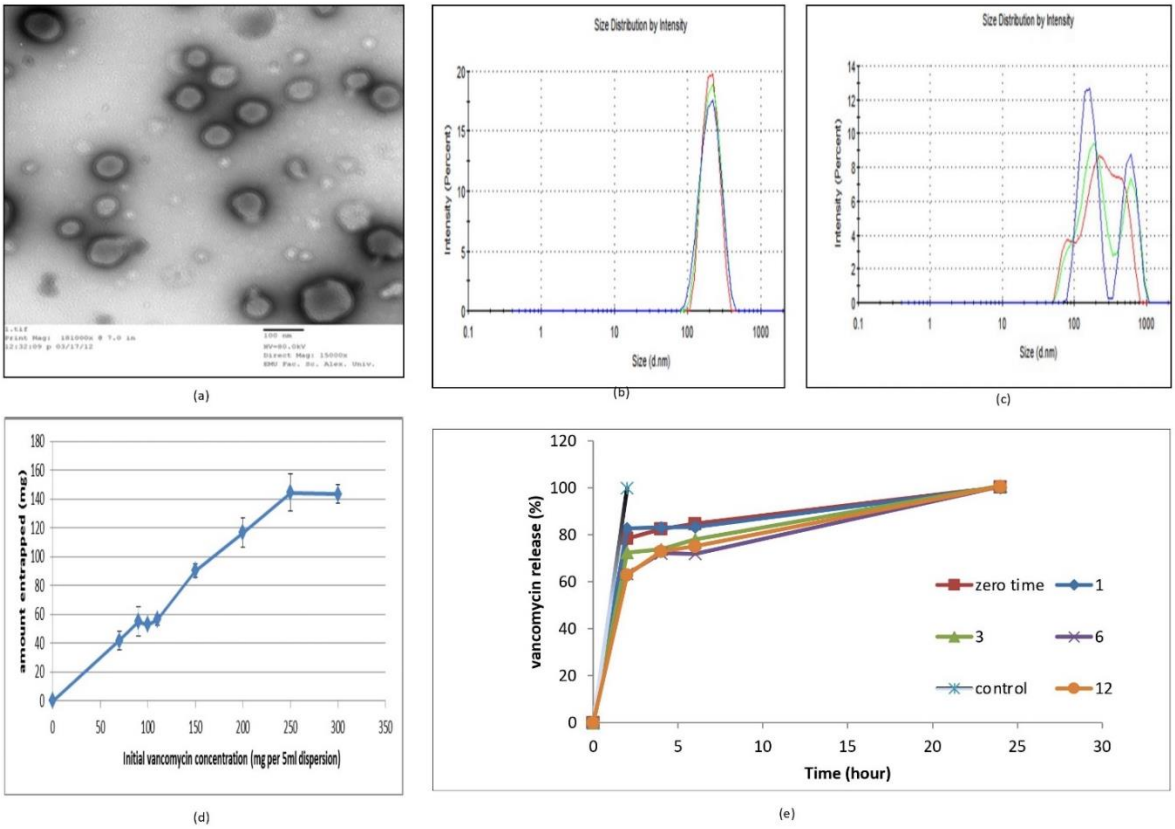

Fig. 1: (a) TEM micrographs of VCM liposomes (Magnification 15000x). (b) Vesicle size distribution curve by intensity, of VCM liposomes. (c) Vesicle size distribution curves, by intensity, of VCM liposomes stored at 4°C after 12 months of storage. (d) Amount of VCM entrapped in liposomes as a function of initial VCM amount added (values represent mean ± SD). (e) Release profile in distilled water at 37ºC of VCM liposomes determined by dialysis during storage at 4°C for 12 months (control is an equivalent amount of VCM in solution)

PG in the formulation probably contributed to the nanosize and narrow size distribution of the PG liposomes; PG interpenetrates the hydrocarbon chains allowing more flexibility of the bilayer [33]. It was shown in a previous publication that 50% of the PG added in the liposome formulation was associated with the vesicles, based on gas chromatography determination of PG in the supernatant [14].

The results presently obtained with PG liposomes, confirmed previous results reporting advantages of PG in liposomes [13,

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[34]. Another published study suggested that propylene glycol liposomes could be developed as a promising intracellular delivery carrier for therapeutic agents [(15)]. Marconi, et al., also directed attention to the possible synergic effect of glycols (including propylene glycol) and phospholipid in liposomes on the enhanced skin delivery of diclofenac [(35)].

The ethanol injection method of preparation, also known as the ethanol infusion method [(21)] was used to prepare the VCM liposomes. Compared to conventional film hydration, this method is a one-step fast method shown previously to be suitable for preparing vancomycin liposomes for nebulization (ref 30). The PG (added to the ethanolic lipid solution in the present study) also prevented drying of the phospholipid ethanolic solution during injection into the aqueous phase.

In the preparation of the liposomes, VCM was dissolved in distilled water. The use of buffer, commonly recommended in liposome preparation, was avoided; the solubility of VCM in buffers, over a range of pH, is much lower than the corresponding solubility in water as reported in an FDA report [(36)]. The report explained that the reduced solubility was partly associated with VCM degradation as observed by multiple peaks in the HPLC chromatogram. Similarly, water was used as the hydrating fluid in the preparation of VCM niosomes [(1)].

The use of buffer solution, such as phosphate-buffered saline (pH range 4-7), as a hydrating fluid, does not support the formation of vancomycin liposomes. This was evident in the preliminary work carried out in the present study and in the study of VCM niosomes [(1).] Other publications have reported poor encapsulation of VCM in liposomes. In one of these studies, phosphate-buffered saline was used as the hydrating fluid in the film hydration method. The use of buffer could partly explain the poor encapsulating reported, although this was not stated in the paper [(35)]

The presence of vancomycin as the hydrochloride salt prevented the traditional thin-layer evaporation approach (which involves only hydrating a lipid film with a buffered solution of the medication) from producing stable phospholipid vesicles [(2)]

Zeta potential values were -10.1 and -4.78 for blank and VCM liposomes respectively. Orientation of the negative groups of PC heads towards the liposome external surface was suggested to cause a negative zeta potential in blank phospholipid liposomes [(37)]. It is difficult to predict the contribution of entrapped VCM having six pKa values [(36)] to the surface charge of the liposomes. The Surface charge carried by leaked VCM molecules and possibly contributing to liposomes' surface charge will differ according to medium pH dictating ionization of specific VCM functional groups.

The mean VCM entrapment efficiency (EE%) value was 52.84±1.5% (n=5) (initial VCM amount 100mg/5 ml liposome dispersion). Liposomes were also prepared using increasing initial VCM amounts (70 to 300 mg/ 5 ml liposome dispersion). These amounts were below the saturation aqueous solubility of VCM (750 mg /5ml) [(36)]. The relation between entrapped and initial VCM amount (**Fig. 1d)**, suggested saturation of the vesicles at an initial VCM concentration of around 250 mg/5 ml. Larger vesicle size and polydispersity were also recorded for liposomes prepared with an initial VCM concentration of 300 mg (PdI values >0.4).

Release data (by dialysis) before and during storage (**Fig. 1e),** indicated diffusion of free VCM (control)into dialysate within 2 hours. The release profiles of VCM liposomes indicated some control over the release exerted by the liposome carrier compared to the VCM solution.

Release profiles **(Fig. 1e),** were assessed for similarity by calculating the similarity factor

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(f2) [25, 26]. The release profile at each month of storage was compared to the profile before storage. Calculated f2 values were 61 and 60 for release profiles after one month and three months of storage respectively suggesting similar release profiles. Corresponding f2 values at 6 months and 12 months storage were 47.5 and 49 respectively indicating changes in release during storage beyond three months.

Stability data generated for VCM liposomes during storage at 4^{o}C showed no changes in EE% during the first three months. There was evidence of VCM leakage from liposomes at 6 and 12 months (EE% decreased from 52.2% at zero time to 46.5 and 41.1% at 6 and 12 months respectively, ($p > 0.1$). A study on novel liposomal vancomycin formulations reported stability of two formulations at 4°C for 3 months [38].

Looking at changes in size distribution during storage, PdI values started at 0.33 at zero time and increased during storage to 0.73 at 12 months. Size enlargement was also evident (mean size reached 589.4±186.7 nm at 12 months). Intensity size distribution curves also suggested aggregation of some vesicles after 12 months of storage compared to zero time **(Fig. 1c).**

Zeta potential showed an increase during storage (values recorded before and after 12 months of storage were -4.78 and -13.8 respectively). The observed increase could be attributed to the association of VCM after leakage with the liposomes surface. The attachment of leaked VCM molecules to the liposomes' surface could partly explain the change in Zeta potential.

4.2. Antimicrobial susceptibility testing

The antimicrobial activities of both free and liposomal VCM were compared through the determination of their MICs against 21 Gram-positive bacterial strains; 14 *S. aureus*, 5 *S. epidermidis* clinical isolates, and two standard strains; *S. aureus* ATCC 6538P and *S. epidermidis* ATCC 12228, using the agar dilution technique [27]. Out of the 19 clinical isolates, 12 (63%) were revealed to be resistant to cefoxitin and were considered methicillin-resistant while none of the tested isolates were VCM-resistant. The VCM MIC breakpoints for *S. aureus* according to the Clinical and Laboratory Standards guidelines [27] are 2 µg/ml for susceptible, 4-8 µg/ml for intermediate, and 16 µg/ml for resistant. No VCM resistance was found among the tested isolates when using the agar dilution technique. The MICs of liposomal VCM ranged from 0.625-2.5 µg/ml while that for free VCM ranged from 1.25-5 µg/ml. The average MICs of liposomal and free VCM were 0.92 µg/ml and 2.1µg/ml, respectively, liposomal VCM showed 2.3-fold lower MICs compared to free VCM against the tested strains. Control blank liposome did not inhibit the growth of the tested isolates. Improved activity of VCM liposomes could be partly due to reported interaction between bacteria and bilayered vesicles leading to adsorption and fusion of bacteria with the vesicles and hence targeted antibiotic delivery to the organism [39].

4.3. Visualization of liposome-bacteria interaction by phase contrast microscope

Two mixed components were examined under the phase contrast microscope. The first component was bacterial cells; their size allowed them to be visible under this microscope (**Fig. 2b and 2c**). The second component was unextruded VCM liposomes; their nanosize would prevent visibility unless they were not extruded **(Fig. 2a)**.

the photos **(Fig. 2d and 2e)** indicated adherence of test bacteria to liposomes [28, 29]. These results verified the speculation that the enhanced antibacterial activity of vancomycin-loaded liposomes against Gram-positive organisms was due to the liposome-mediated targeting effect of vancomycin on the bacterial cell membrane, thus promoting its intracellular delivery. It is likely that the

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partial fusion of liposome bilayers with the bacterial cell membrane could facilitate the release of liposome content into the cytoplasm. Therefore, liposomes were able to transfer their vancomycin load through the external membrane, to the peptidoglycan wall where its antibacterial activity can be exerted.

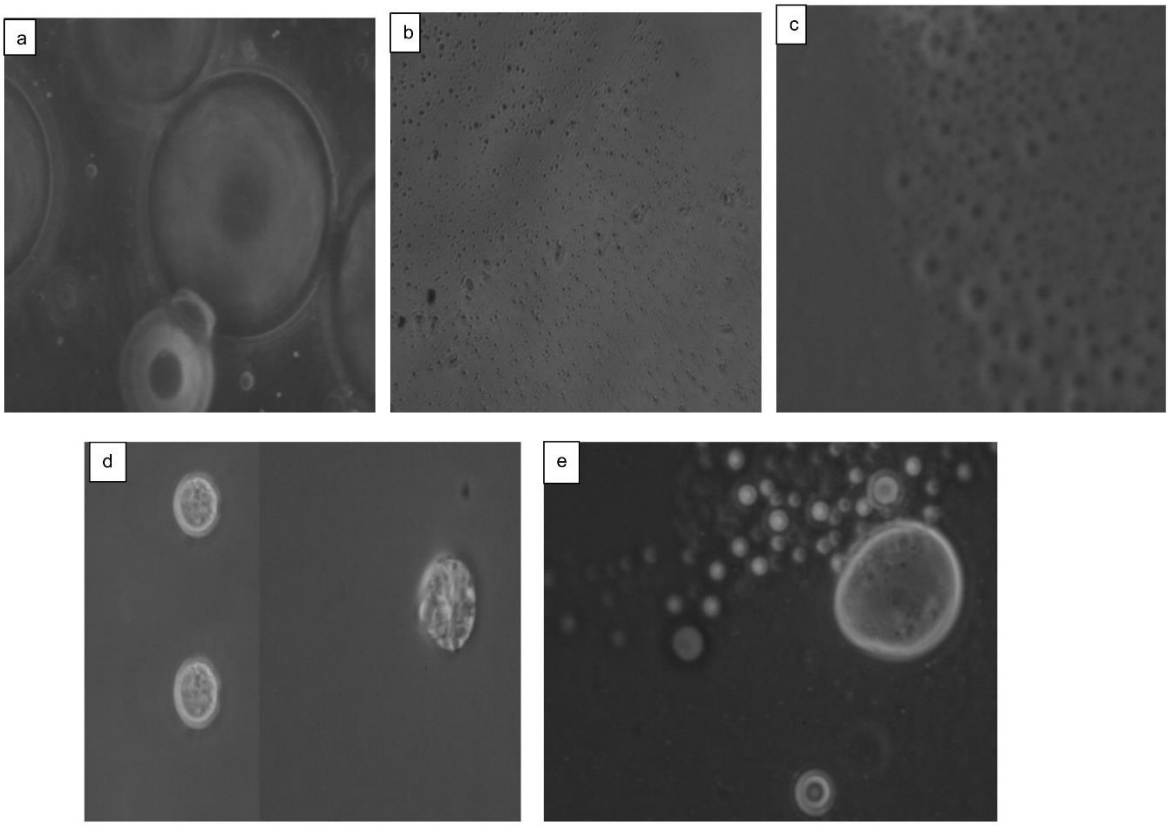

Fig. 2: Phase contrast micrographs of (a) vancomycin-loaded liposomes (unextruded), (b) V7, (c) *S. aureus* ATCC6538P and of bacterial-liposomes interaction with: (d) *S. aureus* ATCC6538P and (e) V7 (magnification X100)

It is likely that the partial fusion of liposome bilayers with the bacterial cell membrane could facilitate the release of liposome content into the cytoplasm. Therefore, liposomes were able to transfer their VCM load through the external membrane, to the peptidoglycan wall where its antibacterial activity can be exerted [40, 41].

4.4. Antibiofilm activity against biofilm-forming strains

The antibiofilm activity of both liposomal and free VCM was assessed in terms of inhibition of biofilm formation, eradication of surface-borne biofilm, and inhibition of biofilm growth following exposure to VCM. Furthermore, induction of biofilm formation in non-biofilm forming strains by sub-minimal inhibitory concentration of VCM was investigated. Blank liposomes were used appropriately as controls. Among the tested *Staphylococcal* isolates, only the standard *S. aureus* ATCC 6538P and one *S. aureus* isolate (V7) could form well-defined biofilms, hence they were the tested organisms in the biofilm experiments.

MBIC and MBEC values are reported in **Table 1** and **Fig. 3.** MBIC values were lower for liposomal VCM compared to the free

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VCM. The reduction in MBICs was matching for both tested microorganisms. Blank liposome alone also showed a biofilm suppressive effect. The optical density (OD_{630}) values of biofilms formed in the presence of blank liposomes were 50% lower than those formed in the presence of broth (positive control). Recently, Jardeleza et al. [42] reported that blank liposomes reduced the biofilm biomass of the reference strain S. *aureus* ATCC 25923 when compared to the untreated control.

Table 1: Comparative antimicrobial and antibiofilm activity of free and liposomal VCM tested on selected staphylococcal strains**.**

Microorganism		MIC [a] (µg/ ml)		MBIC [b] (µg/ ml)		MBEC [c] (µg/ ml)		BFC [d] (µg/ ml)	
		Free VCM	**Lip VCM**	**Free VCM**	**Lip VCM**	**Free VCM**	**Lip VCM**	**Free VCM**	**Lip VCM**
Biofilm-forming strains	**V7**	2.5	1.25	10	2.5	20	10	ND[e]	**ND**
	***S. aureus*ATCC 6538P**	5.0	2.5	5.0	2.5	10	5.0	ND	**ND**
Nonbiofilm-forming strains	**19**	5.0	1.25	ND	ND	ND	ND	1.25	**0.02**
	30	2.5	1.25	ND	ND	ND	ND	1.25	**0.625**
	44	2.5	0.625	ND	ND	ND	ND	--[f]	--
	47	1.25	0.625	ND	ND	ND	ND	--	--
	63	2.5	0.625	ND	ND	ND	ND	1.25	**0.156**
	67	1.25	0.625	ND	ND	ND	ND	0.625	**0.156**
	***S. epi.* ATCC 12228**	**5.0**	**0.625**	**ND**	**ND**	**ND**	**ND**	**2.5**	**0.078**

[a]MIC: Minimum Inhibitory Concentration

[b]MBIC: Minimum Biofilm Inhibitory Concentration

[c]MBEC: Minimal Biofilm Eradication Concentration

[d]BFC: Biofilm Forming Concentration; threshold concentration at which the antibiotic initiates biofilm formation in nonbiofilm forming staphylococcal strains

[e]ND: Not determined

[f]--: No biofilm induction

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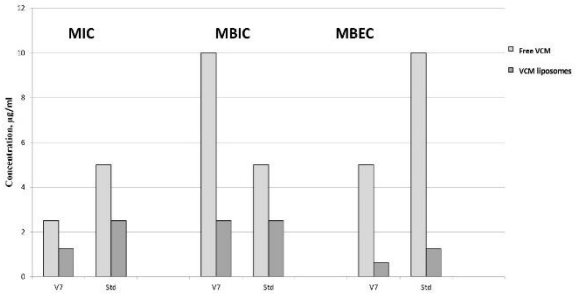

Fig. 3: Minimum inhibition concentration (MIC), minimum biofilm inhibition concentration (MBIC), minimum biofilm eradication concentration (MBEC) of biofilm- forming isolates V7 and std. *S. aureus* ATCC 6538P

For isolate V7 and *S. aureus* ATCC 6538P, the MBEC/MIC ratios were 8 and 2 for liposomal and free VCM, respectively. However, the MBEC for isolate V7 were always above the VCM susceptibility breakpoint. Biofilm bacteria can tolerate higher levels of antibiotics than planktonic bacteria as demonstrated in susceptibility assays [43].

The biofilm inhibiting effect of liposomal VCM could be explained by a dual drug-based and vesicle-based functionality. VCM exerted a direct antibacterial effect which was enhanced by liposomal encapsulation. The liposomes probably also formed a physical coating barrier on the plate surface, competing with bacterial adhesion. Liposomes can target biofilm matrix by specific attachment, allowing drug release in the vicinity of the microorganisms. A passive bacterial adhesion inhibitory effect on abiotic surfaces has been demonstrated with hydrophilic polyethylene glycol and polyethylene oxide polymer coating as well as propylene glycol [44, 45].

Different liposomal formulations of vancomycin for the in vivo eradication of MRSA and MSSA biofilms were explored by Scriboni et al. Three different forms of liposomes were examined for encapsulating vancomycin: cationic, fusogenic, and traditional big unilamellar vesicles. Fusogenic liposomal vancomycin eradicated mature biofilms to a greater extent than other liposomal formulations and the free medication, although there was no difference in MRSA and MSSA biofilm inhibition between the vancomycin-loaded liposomal formulation and the free drug [4].

4.4.1. Effect of VCM sub-minimum inhibitory concentrations (sub-MIC) on biofilm formation by nonbiofilm–forming bacteria

Biofilm induction by sub-MICs has been documented for some antibiotics including VCM as a phenomenon expressing the defensive reaction of bacteria [46]. This is of clinical relevance as bacteria may be exposed to sub-MIC antibiotic concentrations during systemic and local antibiotic therapy and antibiotic treatment of abiotic surfaces. In the present study, it was observed that sub-MIC concentrations of free and liposomal VCM induced biofilm formation by non-biofilm-forming staphylococcal strains.

The results indicated that sub-MICs of both free and liposomal VCM induced biofilm formation in six out of the seven tested strains. The biofilm-forming concentrations (BFCs) ranges were 1.25 to 2.5 µg/ml (MIC/4 to MIC/2) and 0.02 to 0.625 µg/ml (MIC/64 to MIC/2) for free and liposomal VCM, respectively (**Table 1**, **Fig. 4**). In the absence of subinhibitory concentrations of either free or liposomal VA, the controls lacked biofilms (OD_{630}<0.1) in all tested isolates.

It was also observed that, in the case of the VCM liposomes the formed biofilm by *S. epidermidis* isolate 67, was of lower optical density (OD_{630}) when compared with the biofilm formed in the presence of free VCM (**Fig. 5**). This may be due to the interference of liposomes with adhesion of planktonic cells to abiotic surface as an initial

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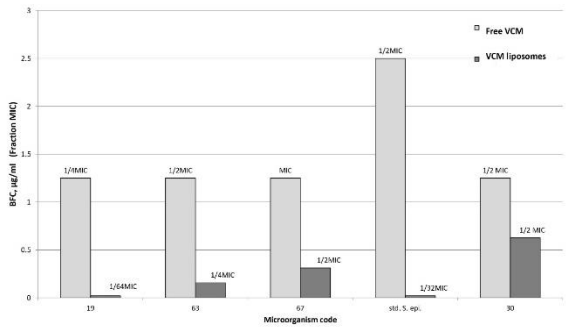

Fig. 4: Induction of biofilm formation by respective sub-MICs (Table 1) of free and liposomal VCM in non-biofilm forming *Staphylococcal* strains.

step in the induction of biofilm formation at low antibiotic concentrations. This adds to the benefits of the VCM liposomes in inhibiting biofilm formation on abiotic surfaces and other antimicrobial applications.

4.5. In vitro catheter assays

Two study designs were adopted. First, eradication of the formed biofilm on catheter segments, where different concentrations of free and liposomal VCM, were incubated overnight with the immobilized biofilm developed, followed by assessment of the remaining bacteria in biofilm by monitoring the viable count. Second, biofilm formation inhibition, where the catheter segments were pretreated with either free or liposomal VCM for twenty minutes followed by challenging with bacteria and allowing the biofilm to form. Determining the viable bacterial count then assessed the biofilm formed.

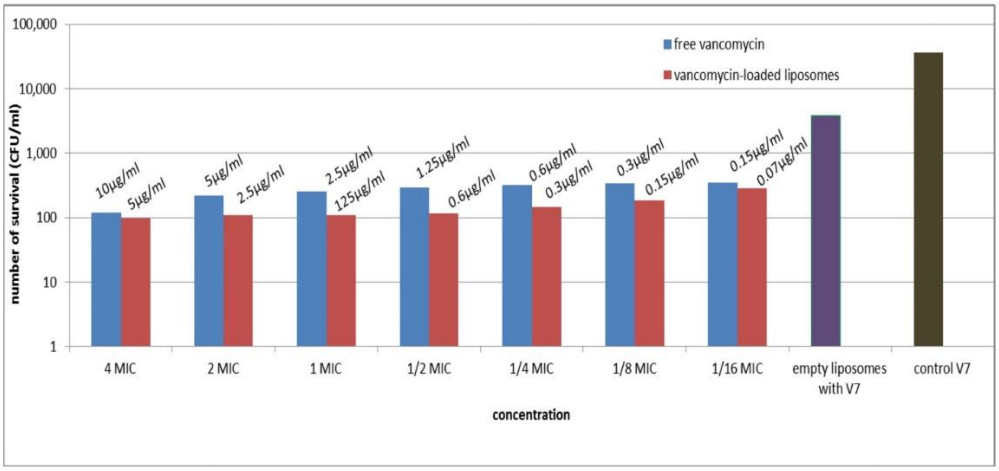

Fig. 5: Biofilm formation, expressed as optical density (OD_{630}), induced by respective sub-MICs (from Table 1) of free and liposomal determined in non-biofilm forming *S. aureus* strain 67

The ability of liposomal VCM to eradicate surface-borne biofilm developed on the catheter surface, during 2 h exposure of the formed biofilm to VCM, was compared to that of the free VCM using the surface viable count technique for determining the number of survivors (CFU/catheter segment). The results showed that the number of survivors was lower by 2 logs for free and liposomal VCM when compared to the control in both strains tested for *S. aureus* isolate V7) (**Fig. 6**) . These results confirm the MBECs results obtained with the same isolates using the microtiter plate model **(Table 1)**.

As shown in **Fig. 7**, encapsulation of the VCM in liposomes enhanced its activity relative to the free antibiotic. It has been reported that the effectiveness of the

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liposomal carrier was dependent on the fluidity of the liposome membrane and on the level of drug entrapment within the aqueous core of the vesicles [3].

The inhibition of biofilm growth on the catheter surface, pretreated with the VCM system for 20 minutes, was assessed by determining the number of survivors remaining in biofilms. Compared to the controls, the viable count decreased by 2 logs, regardless of the type of VCM system when the standard strain was tested, and by 3 logs and 2 logs when the liposomal and free VCM were tested against V7 isolate, respectively, **Fig. 7**.

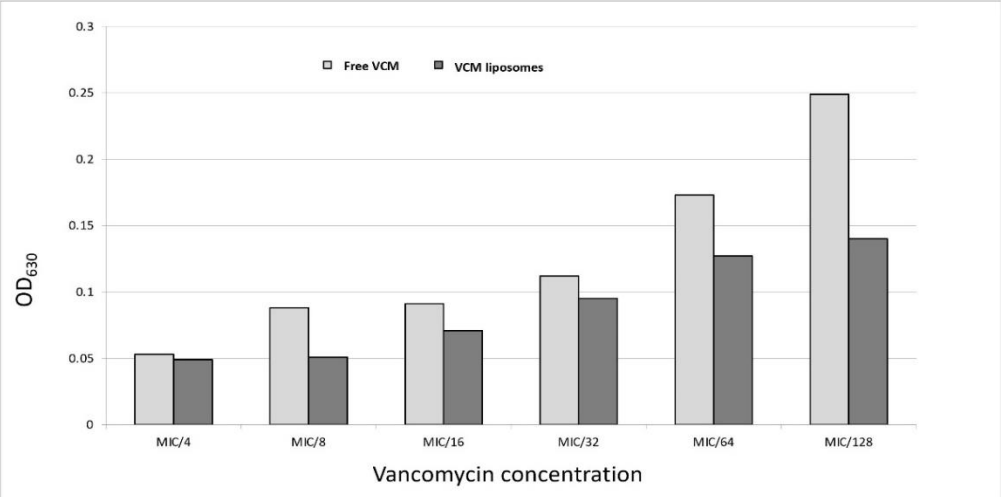

Fig. 6: Biofilm eradication of *S. aureus* isolate V7 from biofilm-coated catheter segments using different concentrations (expressed relative to respective MIC, Table 1) of either free or liposomal VCM (contact time 2 h).

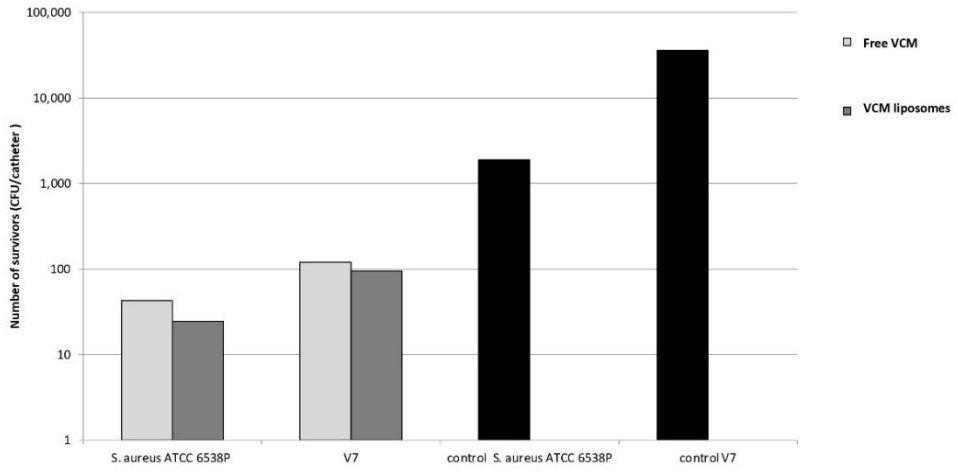

Fig. 7: Inhibition of biofilm formation by *S. aureus* isolate V7 and standard strain *S. aureus* ATCC 6538P after pretreatment of the catheter segments for 20 minutes with either free or liposomal VCM (concentration 4-fold respective MIC, Table 1).

Results obtained from this study were matched concerning the reduction in MBICs and MICs values. The enhanced activity of liposomal VCM relative to the free drug

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confirmed the potential of the vesicular system to be more effective than the free drug in antibiotic lock therapy technique used in hospitals to prevent contamination leading to infection, in central catheters as previously reported [39, 47, 48].

Antibiotic lock therapy involves placing a certain concentration of an antibiotic to which the causative microbe is susceptible in the catheter lumen. Patients could be considered to have an intraluminally colonized catheter. If these colonized catheters are left in place, patients may go on to develop a true catheter-related bloodstream infection. Therefore, if such catheters cannot be removed, antibiotic lock therapy without systemic therapy can be given through the retained catheter [19, 20].

5. Conclusions

VCM loaded propylene glycol liposomes were successfully prepared using the simple and quick ethanol injection method. The inclusion of PG in the formulation prevented drying of the lipid ethanolic solution during injection and facilitated the extrusion step in size reduction. VCM liposomes showed vesicle size in the nano range, low PDI, with slightly negative zeta potential, good entrapment efficiency, and the release profiles, determined by dialysis indicated slower release from the liposomes compared to diffusion of free VCM, and indicated maintenance of liposome integrity during the dialysis run at 37°C. Stability data generated during storage at 4°C for 12 months, indicated the absence of VCM leakage from liposomes as evident in constant EE% over the first six months of storage. Beyond six months, evidence of leakage and liposome aggregation was apparent. Microbiological assessment of the developed VCM liposomes was performed on both planktonic bacteria and bacterial biofilms in comparison with free VCM. The antibiofilm studies of VCM liposomes were performed using a model abiotic surface (microtiter plates). Antibiofilm studies were also performed using silicon urinary catheter segments under conditions simulating the clinical situation concerning the period of exposure to the antibiotic. Blank liposomes were devoid of antibacterial activity. They could, however, inhibit to some degree biofilm formation; biofilm grown in the presence of blank liposomes contained a less dense population of bacteria whether the film was grown on microtiter plates or on catheter segments. The results obtained concerning the number of folds of increase in antibacterial and antibiofilm efficacy of VCM liposomes compared to free VCM (2-8 folds) were in agreement with results obtained with VCM niosomes[1]. The results of catheter segments experiments indicated the potential of pretreatment of catheter with VCM liposomes (for 20 minutes) to inhibit the formation and growth of biofilm suggesting the possible pretreatment of the catheter with the liposome system (in concentrations lower than the free VCM) to inhibit biofilm formation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Funding

No fund

Highlights

- Drug delivery strategies have emerged as an effective approach for localized biofilm control on abiotic surfaces such as catheters.
- Entrapment of antibiotics in liposomes may enhance their antimicrobial and antibiofilm activity.
- Vancomycin-loaded propylene glycol liposomes were successfully prepared using the ethanol injection method.
- The developed liposomes showed potential for use as a substitute for VCM in antibiotic lock solutions used to prevent or treat device-related bacteremias.

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