

Efficacy of neutral and negatively charged liposome-loaded gentamicin on planktonic bacteria and biofilm communities

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Abstract: We investigated the efficacy of liposomal gentamicin formulations of different surface charges against *Pseudomonas aeruginosa* and *Klebsiella oxytoca*. The liposomal gentamicin formulations were prepared by the dehydration–rehydration method, and their sizes and zeta potential were measured. Gentamicin encapsulation efficiency inside the liposomal formulations was determined by microbiologic assay, and stability of the formulations in biologic fluid was evaluated for a period of 48 h. The minimum inhibitory concentration and the minimum bactericidal concentration were determined, and the in vitro time kill studies of the free form of gentamicin and liposomal gentamicin formulations were performed. The activities of liposomal gentamicin in preventing and reducing biofilm-forming *P. aeruginosa* and *K. oxytoca* were compared to those of free antibiotic. The sizes of the liposomal formulations ranged from 625 to 806.6 nm in diameter, with the zeta potential ranging from –0.22 to –31.7 mV. Gentamicin encapsulation efficiency inside the liposomal formulation ranged from 1.8% to 43.6%. The liposomes retained >60% of their gentamicin content during the 48 h time period. The minimum inhibitory concentration of neutral formulation was lower than that of free gentamicin (0.25 versus 1 mg/L for *P. aeruginosa* and 0.5 versus 1 mg/L for *K. oxytoca*). The negatively charged formulation exhibited the same bacteriostatic concentration as that of free gentamicin. The minimum bactericidal concentration of neutral liposomes on planktonic bacterial culture was twofold lower than that of free gentamicin, whereas the negatively charged formulations were comparable to free gentamicin. The killing time curve values for the neutral negatively charged formulation against planktonic *P. aeruginosa* and *K. oxytoca* were better than those of free gentamicin. Furthermore, liposomal formulations prevent the biofilm-formation ability of these strains better than free gentamicin. In summary, liposomal formulations could be an effective lipid nanoparticle to combat acute infections where planktonic bacteria are predominant.

Keywords: drug delivery, stability, antibacterial activity, biofilm

Introduction

Antibiotic resistance of bacterial pathogens has become a serious public health problem since the introduction of the first antibiotics, such as sulfa drugs and penicillin, into clinical use.^{1,2} Multidrug-resistant pathogens present a serious challenge in the treatment of infectious diseases; they are classified as the second leading cause of mortality globally and the third leading cause in developed countries.^{2–4} To avoid and prevent the emergence and spread of resistant bacteria, it is important to modify conventional antibiotics and develop new antibacterial agents.^{2,5,6}

It has been reported that many strains of *Staphylococcus aureus* are already resistant to all antibiotics, except vancomycin. However, some strains did exhibit resistance

to vancomycin, defining the problem of multi-antibiotic resistance.⁷ A Gram-negative bacterium *Pseudomonas aeruginosa* has already evaded most existing antibiotics.⁸ The multidrug-resistant strains of *P. aeruginosa* are often isolated from nosocomial infections of patients who are admitted to intensive care units.^{9,10} Moreover, *Acinetobacter* species showed increased antimicrobial resistance levels in the past decade, and *Acinetobacter baumannii* is recognized as a multidrug-resistant species that is difficult to control and treat.^{11,12} The clinical isolates of *Klebsiella oxytoca* is an active K1 β -lactamase enzymes which can cause resistance to cephalosporin and aztreonam antibiotics.^{13,14} In addition, *Proteus vulgaris* has the capacity to resist antibiotic treatment due to the inducible expression of chromosomal β -lactamases,¹⁵ while *Listeria monocytogenes* showed resistance to antibiotic treatment that could be acquired through self-transferable plasmids, mobilizable plasmids, and conjugative transposons.¹⁶

Biofilm formation is an important factor in bacterial virulence. It follows the adherence of the bacteria to abiotic or biotic surfaces, and increases the ability of bacteria to survive environmental stress and enhances protection against bactericidal effects.¹⁷ In addition, biofilm formation is crucial for bacteria to resist antibiotic chemotherapy.¹⁸ *P. aeruginosa* and *K. oxytoca* biofilms are negatively charged; therefore, binding of the positively charged antibiotics to the negatively charged biofilm matrix polymers prevents the penetration of antibiotics such as gentamicin into the bacteria.^{19–22}

Gentamicin, a member of aminoglycoside antibiotics, is commonly used for treating severe nosocomial infection caused by Gram-negative bacteria.²³ Aminoglycosides exert their effect on bacteria by increasing the cell membrane permeability through irreversible binding of aminoglycosides to divalent cations and inhibiting normal protein synthesis through binding to the highly conserved A-site of bacterial 16 S subunit of 30 S rRNA.²⁴ This binding leads to disruption of proofreading in the protein synthesis process, as a result, bacterial virulence factors production including biofilm can be disrupted.^{25,26} The disruption results in the instability of the membranes, greater penetration of bactericidal agents, and finally cell death.²⁷ Aminoglycosides can be used synergistically with different types of antibiotics such as β -lactams to provide a broad coverage for bacterial infections.²⁸ The use of aminoglycosides in clinical settings has been challenged by their high toxicity and emergence of drug-resistant strains.²⁹ The use of novel liposomal formulations appears to be a viable option in combating this universal problem.

Liposomes are spherical lipid nanoparticles composed of one or more phospholipid bilayers.³⁰ A diverse group of

biologic molecules such as proteins, DNA, RNA, drugs, antioxidants, as well as vitamins can be encapsulated within the liposome bilayers.³¹ Similar to a cellular membrane, liposomal formulations can incorporate hydrophilic and hydrophobic molecules and are biocompatible and biodegradable.³² The protective nature of liposomes is another attractive feature of this nanoparticle. Encapsulated drugs and molecules within liposome compartments are protected from the biologic milieu and destructive host defense molecules, such as interleukins and the proteases of the complement system.³³ Liposomal particles are characterized by slow and continued release of encapsulated drugs from their compartment, ultimately improving their bioavailability and distribution in targeted tissues.³² Liposome-encapsulated bactericidal agents can be delivered to the lung for direct effect on pulmonary infection.³⁴ Liposomes are an attractive option for delivery of bactericidal agents to the lung, since they can be prepared from ingredients compatible to the lung.³⁵

The loading capacity of the therapeutic agent inside the liposomal nanoparticles is critical for achieving an effective and sustainable drug release at the target site. Therefore, the objective of the study was to make and test the effectiveness of neutral and negatively charged liposomal formulations. We found that the neutral formulation has a potent activity against planktonic bacteria, whereas the negatively charged liposomes exhibit a potent antibiofilm activity.

Materials and methods

Microorganisms

P. aeruginosa ATCC 27853, *Escherichia coli* ATCC 25922, *A. baumannii* BAA747, *K. oxytoca* 700324, *P. aeruginosa* BAA1744, *P. aeruginosa* 9721, *Pr. vulgaris* 6380, *S. aureus* BAA976, *S. aureus* BAA977, *L. monocytogenes* BAA751, and *S. aureus* ATCC 29213 were kindly provided by Dr Essam Alyamani at the National Center for Biotechnology, King Abdulaziz City for Science and Technology. These organisms were stored at -80°C in a Mueller–Hinton broth supplemented with 50% (v/v) glycerol. For experimentation, these strains were inoculated onto Mueller–Hinton agar plates and incubated for 18 h at 37°C .

Chemicals

The negatively charged phospholipid 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol) (DMPG), cholesterol (Chol), and the neutral phospholipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Triton

X-100, chloroform, and methanol were purchased from Sigma-Aldrich (St Louis, MO, USA). Gentamicin sulfate was purchased from Amresco (Solon, OH, USA). The Difco Mueller–Hinton agar and broth and Luria-Bertani agar were purchased from BD (Sparks, MD, USA).

Preparation of liposomal formulations

Different formulations of negatively charged and neutral lipids and Chol in different molar ratios with a total of 20 mg/mL of lipid content, that is, 7.5 mg of DPPC:10.5 mg of DMPG:2 mg of Chol in a molar ratio 2:3:1 (NELG-1), 6.3 mg of DPPC:12 mg of DMPG:1.7 mg of Chol in a molar ratio 2:4:1 (NELG-2), and 15.8 DPPC:4.2 mg of Chol in a molar ratio 2:1 (NLG), were prepared through the dehydration–rehydration vesicle method.³⁶ Lipids were weighed and placed in round-bottomed flasks. An appropriate amount of chloroform:methanol (2:1) was added, until the lipids were completely dissolved. Then, the organic solvents were evaporated in a rotary evaporator (BÜCHI Rotavapor R-114) at 45°C, until a thin layer of lipid was formed. High-grade nitrogen gas was used to flush out any trace of chloroform, and the flasks were left at room temperature overnight to ensure that there was no trace of chloroform. The lipid film was then rehydrated with 1 mg/mL gentamicin dissolved in PBS at pH 7.4 or rehydrated with PBS to form empty liposomes without antibiotics. The samples were sonicated (Model 3000 ultrasonic homogenizer; Biologics, Inc., Manassas, VA, USA) for 5 min (40 s run, 20 s pause) in order to homogenize liposomal particle size before lyophilizing the preparation in a freeze dry system (FreeZone Triad Cascade Benchtop; Labconco Corporation, Kansas City, MO, USA). The lyophilized liposomes were kept at –80°C until use.

For rehydration, 10% of PBS was added at 10% of the original volume and vortexed. This step was repeated three times before adding more PBS to bring the solution to its original volume. Excess unencapsulated gentamicin was removed by three rounds of washing with cold PBS using an Eppendorf centrifuge at 16,000×g for 20 min at 4°C (5804R; Eppendorf, San Diego, CA, USA). Size and z-potential were measured in triplicate using a Zetasizer Nano ZSP (Malvern, Worcestershire, UK).

Encapsulation efficiency (EE) of gentamicin

The quantity of encapsulated gentamicin was measured through an agar diffusion assay using *S. aureus* (ATCC 29213) as a reference microorganism according to the Clinical and Laboratory Standards Institute standards.³⁷ Briefly, the overnight bacterial culture was adjusted to

1.5×10^8 colony forming units (CFU)/mL (equivalent to 0.5 McFarland standard). The bacteria were then suspended in 300 mL warm (45°C) Mueller–Hinton agar, poured into a sterilized disposable square culture plate (45×45 cm), and left to solidify at room temperature. Using a vacuum puncher, wells of 5 mm in diameter were made on the agar. Standard curves of diluted gentamicin as well as samples of liposomes were prepared. Liposome samples were lysed with 0.2% Triton X-100. This level of Triton X-100 has no effect on the assay performance as tested. Triplicate samples (50 µL) were transferred into the agar plate holes. The plate was covered and incubated for 18 h at 37°C. The inhibition zones were measured, and the average of the triplicate measures was used in the data analysis. The standard curve was utilized to calculate the concentration of encapsulated gentamicin that was released from the liposomes by 0.2% Triton X-100. The EE was calculated as a percentage of encapsulated gentamicin in the liposome relative to the initial concentration of gentamicin. Measuring the EE was performed in triplicate.

EE (%)

$$= \left(\frac{\text{Concentration of encapsulated gentamicin}}{\text{Initial concentration of gentamicin}} \right) \times 100$$

Biologic fluids collection

Sputum

Sputum samples were obtained from the patients following informed consent, after the regular daily routine laboratory tests were performed by the Microbiology Laboratory at King Abdulaziz Medical City National Guard Hospital Riyadh, Saudi Arabia (protocol approved by King Abdullah International Medical Research Center [KAIMRC] Institutional Review Board: SP16/312/R). Personal information of patients who signed the consents, including age, name, treatments, and exacerbation records, was kept confidential. Samples were diluted 1:10 (w/v) in PBS, autoclaved, aliquoted in 50 mL tubes, and frozen at –80°C until use, as previously described.³⁸

Bronchoalveolar lavage

Male rats (250–300 g; rats were obtained from Animals' Experiment Care Unit, College of Pharmacy, King Saud University, Riyadh, Kingdom of Saudi Arabia) were maintained according to the animal welfare guidelines of KAIMRC and used for bronchoalveolar lavage (BAL) collection according to the protocol approved by KAIMRC's Institutional Review Board: SP16/312/R. Briefly, the rats were euthanized by using CO₂. The lungs and trachea were collected and then

laved with 3 mL cold PBS using a scalp vein (butterfly syringe). The lungs were gently filled with PBS and withdrawn by 10 mL syringe to a total volume of 30 mL, and stored in aliquots at -80°C until use, as previously described.³⁹

Stability of liposomal formulation in different environments

We tested the novel liposomal gentamicin formulations for their retention of gentamicin in different physiologic fluids for the time intervals 0, 2, 4, 6, 8, 12, 24, and 48 h. This test would give more information about the release of the content of nanoparticles in biologic milieus and with different routes of administration. Samples were washed as described previously³⁶ and suspended in 1 mL of PBS, BAL, plasma, or sputum. The mixtures were incubated with a mild agitation at 150 rpm at either 4°C for PBS or 37°C for the PBS, BAL, plasma, or sputum. Samples were harvested after the designated time intervals and centrifuged at $16,000\times g$ at 4°C for 15 min (centrifuge 5804R, Eppendorf). The supernatants were tested for gentamicin release using an agar diffusion assay as described previously.³⁷ Results were collected for the three separate experiments in duplicate. Retention of gentamicin in percentage was calculated as follows:

Retention of gentamicin (%)

$$= \left(\frac{\text{Conc. of encapsulated gentamicin} - \text{Conc. of released gentamicin}}{\text{Conc. of encapsulated gentamicin}} \right) \times 100$$

Antibacterial activity assay

The minimum inhibitory concentrations (MICs) of the free gentamicin and the liposomal gentamicin formulation were determined using a microbroth dilution method for *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *A. baumannii* BAA747, *K. oxytoca* 700324, *P. aeruginosa* BAA1744, *P. aeruginosa* 9721, *Pr. vulgaris* 6380, *S. aureus* BAA976, *S. aureus* BAA977, and *L. monocytogenes* BAA751, as reported previously.⁴⁰ Serial dilutions of gentamicin in free or liposomal formulations at 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, and 64 mg/L were prepared in the Mueller–Hinton broth. Empty liposomes were also suspended in Mueller–Hinton broth to be tested on bacteria. The overnight bacterial suspension was adjusted to follow the 0.5 McFarland standard. The bacteria were then diluted to achieve a final inoculum of 5×10^5 CFU/mL. The lowest concentration of the liposomal gentamicin formulations and the free gentamicin that inhibited

the visible bacterial growth (inhibition $\geq 80\%$) after 24 h at 37°C was defined as the MIC.⁴¹ Media with and without the same amount of inoculum were used as positive and negative controls, respectively. The minimum bactericidal concentration (MBC) was defined as the lowest concentration of either free or liposomal formulations that resulted in eliminating bacterial growth. Three separate experiments were performed in duplicate to confirm the results.

Time kill assays

We selected two of the bacterial strains, *P. aeruginosa* BAA1744 and *K. oxytoca* 700324, as model microbes to compare the optimal bactericidal activity time of the liposomal formulations and the free gentamicin in vitro. The killing time assay was performed for the liposomal gentamicin formulations and the free antibiotic using *P. aeruginosa* and *K. oxytoca* as described previously.⁴² Briefly, bacterial isolates at a final inoculum of 5×10^5 CFU/mL were suspended in Mueller–Hinton broth and incubated at 37°C for 2, 4, 6, 8, and 24 h in the presence of either free or liposomal formulation–encapsulated gentamicin at one, two, or four times the MICs. Samples were taken at each designated time interval and plated on Mueller–Hinton agar in triplicate for the bacterial count. Three separate experiments were performed and plates with CFUs between 30 and 300 colonies were included in this assay.

Biofilm assays

We evaluated the efficacy of our liposomal formulations to prevent and reduce bacterial biofilm formed by *P. aeruginosa* BAA1744 and *K. oxytoca* 700324. Biofilm assay was performed to test the ability of liposomal formulations at sub-MIC to prevent biofilm communities, as described previously.⁴³ Briefly, a standard static biofilm assay was performed by using an overnight bacterial culture adjusted to 0.5 McFarland standard and diluted to 1.5×10^7 CFU/mL. Samples were seeded in a 96-well polystyrene plate in the presence of $1/2\times$ MIC of NLG, NELG-1, NELG-2, or free gentamicin. The plate was then incubated for 18 h at 37°C . After the incubation period, unattached bacteria were removed and plates were washed three times with sterile PBS. The biofilm formed was stained with 0.1% crystal violet for 10 min and washed once to remove excess stain, and left to dry. Ethanol (95%) was added to the plates to solubilize the crystal violet for 15 min. Each plate was mixed, and solubilized biofilm was transferred to a new 96-well flat-bottomed plate. The OD was measured at 590 nm using a microplate reader PowerWav XS2 (BioTek Instruments

Inc., Winooski, VT, USA). The biofilm reduction assay was performed to determine whether the liposomal formulations at a concentration of $16\times$ MIC could inhibit the formation of biofilm, as described previously.⁴⁴ For biofilm reduction, bacteria were allowed to attach to the surface of a 96-well polystyrene plate and allowed to form a biofilm for 18 h. The next day, unadhered bacteria were washed and the biofilm was incubated again for 18 h with treatments at $16\times$ MIC. Biofilm formation was measured as above. All tests were performed three times in duplicate.

Statistical analysis

The data are presented as the mean and standard error of the mean of the three independent experiments. Comparisons of groups were made through one-way analysis of variance using InStat3 from GraphPad Software (version 5.0) followed by a Tukey-Kramer multiple comparisons test. *P*-values of $*P<0.05$, $**P<0.01$, and $***P<0.001$ were considered as statistically significant.

Results

Particle size and polydispersity index of liposomal formulations

We compared the physicochemical properties of our formulations in terms of their size, charge, polydispersity, and EE. The NLG yielded 625 ± 5.5 nm in size with relatively low EE, that is, $1.8\%\pm0.15\%$ (20 ± 1.5 mg/L). The formula net charge was neutral (-0.22 ± 0.17 mV). The sizes of negatively charged NELG-1 and NELG-2 were 691.3 ± 3.3 and 806.6 ± 3.4 nm with zeta potentials of -25.3 ± 2.5 and -31.7 ± 1.7 mV, respectively. The gentamicin loads in these formulations were $37.2\%\pm0.46\%$ (370 ± 4.6 mg/L) for NELG-1 and $43.6\%\pm0.65\%$ (440 ± 6.5 mg/L) for NELG-2, as shown in Table 1.

Stability of liposomal formulations

To test the stability of the liposomal formulations to hold gentamicin within the phospholipid vehicle, we exposed

the NLG, NELG-1, and NELG-2 formulations to different conditions and measured gentamicin release as an indicator of stability. The liposomal formulation NLG in PBS at 4°C retained $92.5\%\pm1.2\%$, NELG-1 retained $94.3\%\pm0.4\%$, and NELG-2 retained $95.7\%\pm0.7\%$ of gentamicin after 48 h. Liposomal formulations in PBS at 37°C retained gentamicin as follows: $75.0\%\pm2.8\%$ for NLG, $74.3\%\pm0.7\%$ for NELG-1, and $75.2\%\pm1.5\%$ for NELG-2 after 48 h. In the presence of BAL at 37°C , NLG retained $67.5\%\pm1.2\%$, NELG-1 retained $57.1\%\pm4.0\%$, and NELG-2 retained $45.2\%\pm2.6\%$ of gentamicin after 48 h. In the presence of plasma at 37°C , the NLG formulation retained $55.0\%\pm0.9\%$ of encapsulated gentamicin, whereas NELG-1 and NELG-2 formulations retained only $31.4\%\pm0.8\%$ and $22.7\%\pm3.2\%$ of gentamicin, respectively, after 48 h. In the presence of sputum at 37°C , NLG retained $61.7\%\pm2.4\%$ of the encapsulated gentamicin, whereas NELG-1 and NELG-2 retained $48.3\%\pm4.0\%$ and $40.2\%\pm1.1\%$, respectively, after 48 h. After 4 h of incubation from the initial period, the liposomal formulations' retention was decreased dramatically and then stabilized in all physiologic conditions (Figures 1–3).

Antimicrobial activity of liposomal gentamicin

We examined the MIC and MBC of encapsulated gentamicin in both neutral and negatively charged formulations and compared them to those of the free drug against several Gram-negative and Gram-positive bacterial strains. Empty liposomes consisting of DPPC:DMPG:Chol (2:3:1) and (2:4:1) and DPPC:Chol (2:1) had no effect on bacteria, as compared to the positive control. As shown in Table 2, the MICs and MBCs of the encapsulated gentamicin were significantly lower than those of free gentamicin in inhibiting the growth of bacterial strains tested. In particular, the MBCs of liposomal formulations were considerably lower than free gentamicin which exhibited a relatively high MBC of 8 mg/L against *K. oxytoca* 700324.

Table 1 Characterization of liposomal formulations

Liposomal formulations	Molar ratio	EE, %	Size, nm	Charge, mV
DPPC:Chol (NLG)	2:1	1.8 ± 0.15	625.0 ± 5.5	-0.22 ± 0.17
DPPC:DMPG:Chol (NELG-1)	2:3:1	37.2 ± 0.46	691.3 ± 3.3	-25.3 ± 2.5
DPPC:DMPG:Chol (NELG-2)	2:4:1	43.6 ± 0.65	806.6 ± 3.4	-31.7 ± 1.7

Notes: The EE, size, and charge of liposomal formulations are shown. Each value is represented as the mean \pm SEM.

Abbreviations: Chol, cholesterol; DMPG, 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol); DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; EE, encapsulation efficiency; NLG, dipalmitoyl-sn-glycero-3-phosphocholine and cholesterol; NELG-1, dipalmitoyl-sn-glycero-3-phosphocholine, 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol), and cholesterol (2:3:1); NELG-2, dipalmitoyl-sn-glycero-3-phosphocholine, 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol), and cholesterol (2:3:1); SEM, standard error of the mean.

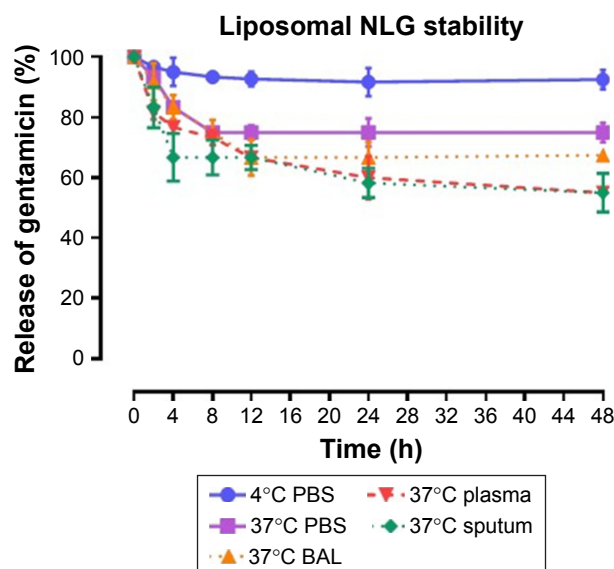


Figure 1 Stability of NLG formulation in PBS at 4°C (circle), in PBS (square) at 37°C, sputum (diamond) at 37°C, in BAL (triangle up) at 37°C and plasma (triangle down) 37°C.

Abbreviations: BAL, bronchoalveolar lavage; NLG, dipalmitoyl-*sn*-glycero-3-phosphocholine and cholesterol.

Time kill assays

Time kill of *P. aeruginosa*

The neutral liposomal gentamicin formulation at 1–4× MIC (0.25–1 mg/L) was more effective in killing planktonic *P. aeruginosa* in terms of time and dose. NLG (0.25 mg/L) and free gentamicin (1 mg/L) at 1× MIC had a comparable

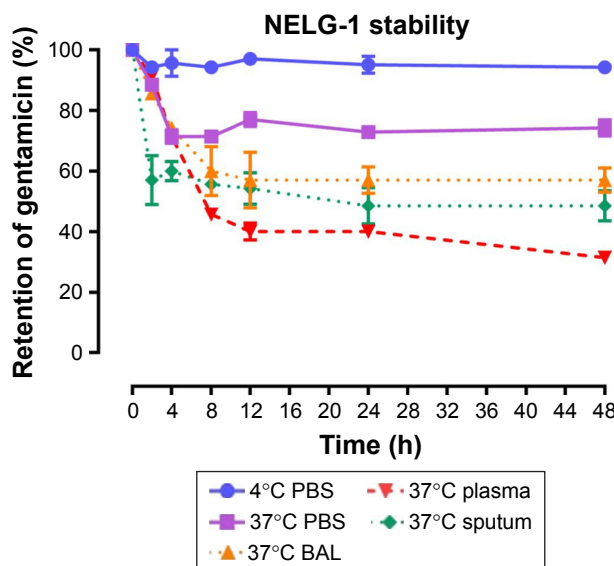


Figure 2 Stability of NELG-1 formulation in PBS at 4°C (circle), in PBS (square) at 37°C, sputum (diamond) at 37°C, in BAL (triangle up) at 37°C and plasma (triangle down) 37°C.

Abbreviations: BAL, bronchoalveolar lavage; NELG-1, dipalmitoyl-*sn*-glycero-3-phosphocholine, 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol), and cholesterol (2:3:1).

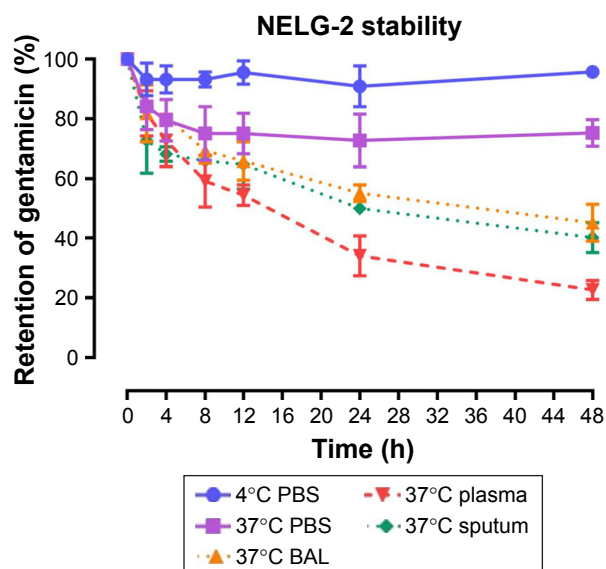


Figure 3 Stability of NELG-2 formulation in PBS at 4°C (circle), in PBS (square) at 37°C, sputum (diamond) at 37°C, in BAL (triangle up) at 37°C and plasma (triangle down) 37°C.

Abbreviations: BAL, bronchoalveolar lavage; NELG-2, dipalmitoyl-*sn*-glycero-3-phosphocholine, 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol), and cholesterol (2:3:1).

bacteriostatic effect at different time intervals between 0 and 24 h. However, the kill time at 2× MIC for NLG (0.5 mg/L) was slightly shorter than the free drug (2 mg/L), that is, 6 versus 8 h.

Considering the differences in MICs for NLG and free gentamicin (0.5 versus 2 mg/L), however, it was evident that the liposomal formulation exhibited a similar kill time for *P. aeruginosa* at a concentration lower than that of free gentamicin (Figure 4).

NELG-1 formulation and free gentamicin had comparable bacteriostatic and bactericidal effects on *P. aeruginosa*. NELG-1 and free gentamicin eliminated the bacteria at 2× MIC (2 versus 2 mg/L) in 8 h and inhibited their growth at 1× MIC (1 versus 1 mg/L) during a 24 h study period. Both forms of the drug had comparable MIC as well (Figure 5).

Finally, free gentamicin at concentrations 1, 2, 4 mg/L was equivalent to NELG-2 (1, 2, 4 mg/L) formulation in terms of preventing bacterial growth with the amount of antibiotic, but performed better in killing the organism in 8 h compared to 12 h time kill of liposomal gentamicin (Figure 6).

Time kill of *K. oxytoca*

This assay was also used to measure the killing time interval for NLG formulation against planktonic *K. oxytoca* 700324 strain in comparison to free gentamicin. The NLG formulation at 0.5 mg/L (1× MIC) exhibited suppression of bacterial

Table 2 Antibacterial activities of NLG, NELG-1, NELG-2, and free gentamicin against selected bacterial strains (in mg/L)

Bacterial strains	NLG		NELG-1		NELG-2		Free gentamicin	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
<i>Pseudomonas aeruginosa</i> ATCC 27853	0.5	1	1	2	1	2	1	2
<i>Acinetobacter baumannii</i> BAA747	0.5	1	2	2	1	4	2	4
<i>Klebsiella oxytoca</i> 700324	0.5	2	1	4	1	4	1	8
<i>P. aeruginosa</i> BAA1744	0.25	2	1	4	1	4	1	4
<i>P. aeruginosa</i> 9721	0.5	1	2	4	2	2	2	4
<i>Proteus vulgaris</i> 6380	0.5	1	2	4	2	4	2	4
<i>Staphylococcus aureus</i> BAA976	0.25	0.25	1	2	1	1	1	1
<i>S. aureus</i> BAA977	0.06	0.06	0.25	0.25	0.25	0.5	0.5	0.5
<i>Listeria monocytogenes</i> BAA751	0.25	0.5	1	1	1	1	1	1

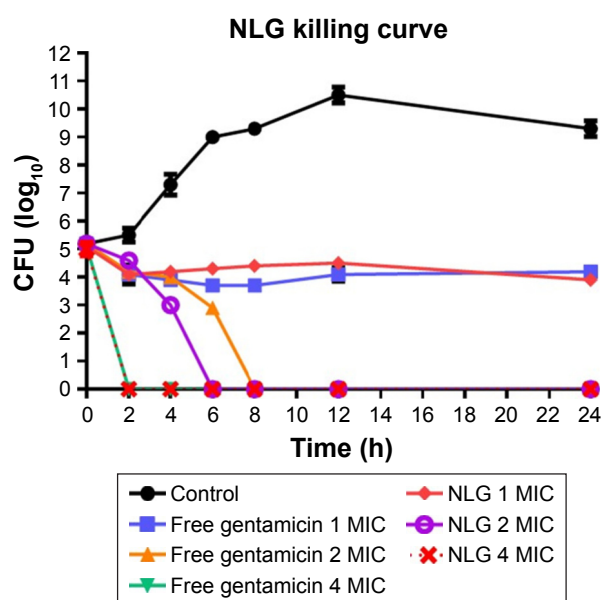
Abbreviations: MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; NLG, dipalmitoyl-*sn*-glycero-3-phosphocholine and cholesterol; NELG-1, dipalmitoyl-*sn*-glycero-3-phosphocholine, 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol), and cholesterol (2:3:1); NELG-2, dipalmitoyl-*sn*-glycero-3-phosphocholine, 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol), and cholesterol (2:3:1).

growth at \log_{10} 4.3 CFU/mL in 24 h. Free gentamicin at 1 mg/L (1× MIC) did not reach the suppression potency of NLG, since it was only able to suppress bacterial growth up to \log_{10} 6.6 CFU/mL in 24 h. Likewise, the NLG formulation at 1 mg/L (2× MIC) showed a decrease in bacterial colonies to \log_{10} 2.3 after 24 h, whereas free gentamicin at 2 mg/L (2× MIC) failed to reach this suppression potency of the NLG formulation over 24 h. Killing activity for NLG appeared at 2 mg/L (4× MIC), eradicating the bacteria at 2 h, whereas free gentamicin at 4 mg/L (4× MIC) exhibited killing activity against *K. oxytoca* 700324 after 4 h (Figure 7).

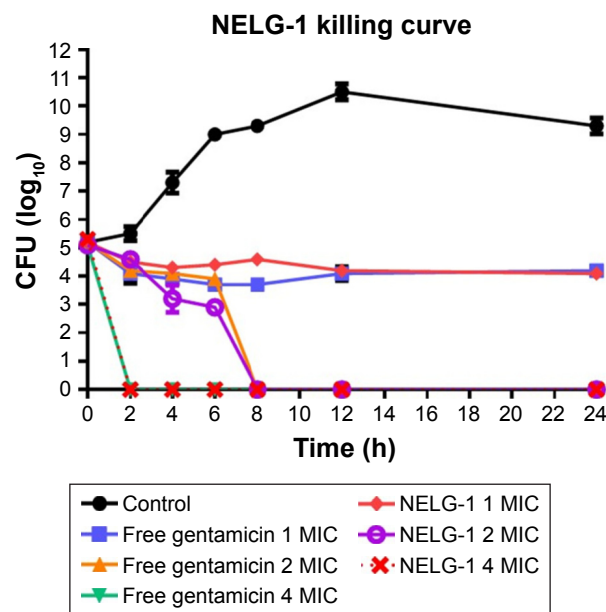
Bacterial growth was reduced to \log_{10} 4.3 CFU/mL by the NELG-1 formulation at 1 mg/L (1× MIC) in 24 h. However,

free gentamicin at 1 mg/L (1× MIC) only decreased bacterial growth to \log_{10} 6.6 CFU/mL in 24 h. Similarly, the NELG-1 formulation at 2 mg/L (2× MIC) reduced bacterial growth even more to \log_{10} 3.6 CFU/mL at 24 h. Free gentamicin at 2 mg/L (2× MIC) failed to reduce bacterial growth to the same level as the NELG-1 formulation; with the killing activity for both NELG-1 formulation and free gentamicin starting at 4 mg/L (4× MIC), the NELG-1 formulation was faster by one-time interval than the free gentamicin (2 versus 4 h), as shown in Figure 8.

The NELG-2 formulation at 1 mg/L (1× MIC) exhibited a bacteriostatic activity lower than that of free gentamicin

**Figure 4** Killing curve of bacterial strain *Pseudomonas aeruginosa* BAA1744 exposed to 0.25, 0.5, and 1 mg/L of NLG and 1, 2, and 4 mg/L of free gentamicin.

Abbreviations: CFU, colony forming unit; MIC, minimum inhibitory concentration; NLG, dipalmitoyl-*sn*-glycero-3-phosphocholine and cholesterol.

**Figure 5** Killing curve of bacterial strain *Pseudomonas aeruginosa* BAA1744 exposed to 1, 2, and 4 mg/L of NELG-1 and free gentamicin.

Abbreviations: CFU, colony forming unit; MIC, minimum inhibitory concentration; NELG-1, dipalmitoyl-*sn*-glycero-3-phosphocholine, 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol), and cholesterol (2:3:1).

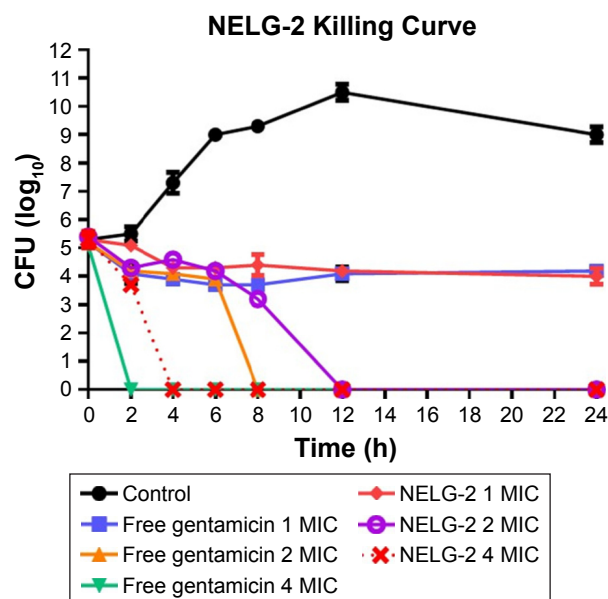


Figure 6 Killing curve of bacterial strain *Pseudomonas aeruginosa* BAA1744 exposed to 1, 2, and 4 mg/L of NELG-2 and free gentamicin.

Abbreviations: CFU, colony forming unit; MIC, minimum inhibitory concentration; NELG-2, dipalmitoyl-sn-glycero-3-phosphocholine, 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol), and cholesterol (2:3:1).

after 24 h. Similarly, the NELG-2 formulation at 2 mg/L (2× MIC) suppressed bacterial growth better than free gentamicin at the same concentration after 24 h. In contrast, the NELG-2 formulation and free gentamicin killed the bacterial strain at 4 mg/L (4× MIC). However, the killing time of free gentamicin was faster than that of NELG-2 (Figure 9).

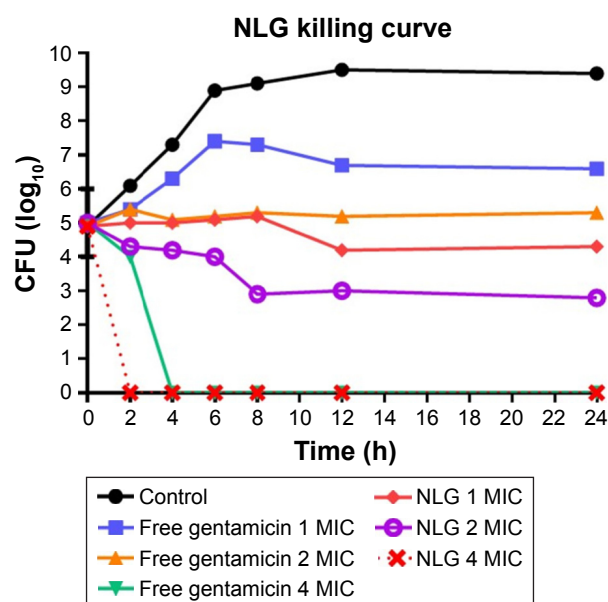


Figure 7 Killing curve of bacterial strain *Klebsiella oxytoca* 700324 exposed to 0.5, 1, and 2 mg/L of NLG and 1, 2, and 4 mg/L of free gentamicin.

Abbreviations: CFU, colony forming unit; MIC, minimum inhibitory concentration; NLG, dipalmitoyl-sn-glycero-3-phosphocholine and cholesterol.

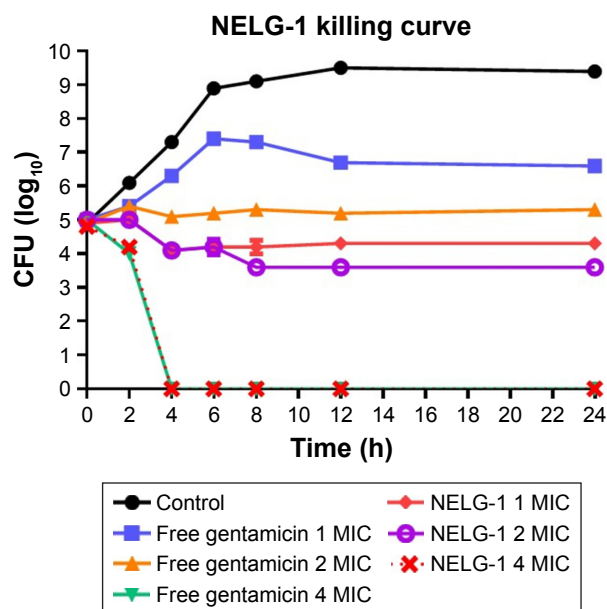


Figure 8 Killing curve of bacterial strain *Klebsiella oxytoca* 700324 exposed to 1, 2, and 4 mg/L of NELG-1 and free gentamicin.

Abbreviations: CFU, colony forming unit; MIC, minimum inhibitory concentration; NELG-1, dipalmitoyl-sn-glycero-3-phosphocholine, 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol), and cholesterol (2:3:1).

Biofilm assays

In the *P. aeruginosa* BAA1744 prevention assay, free gentamicin at 0.5 mg/L prevented biofilm formation less when compared to the control ($P<0.05$). However, NLG at

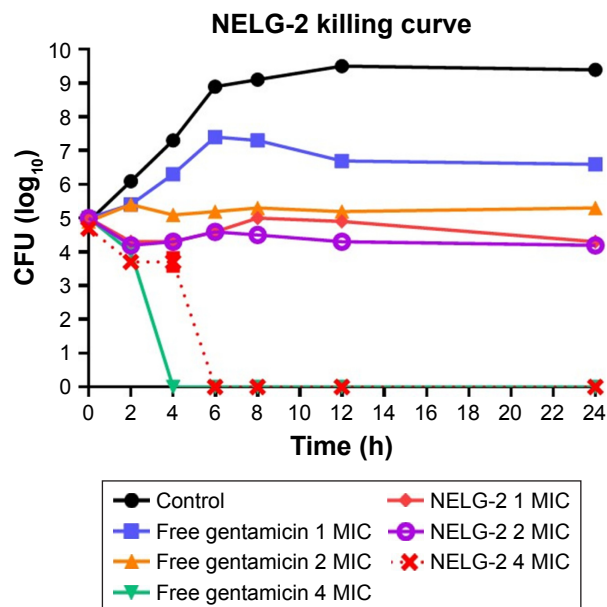


Figure 9 Killing curve of bacterial strain *Klebsiella oxytoca* 700324 exposed to 1, 2, and 4 mg/L of NELG-2 and free gentamicin.

Abbreviations: CFU, colony forming unit; MIC, minimum inhibitory concentration; NELG-2, dipalmitoyl-sn-glycero-3-phosphocholine, 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol), and cholesterol (2:3:1).

1/2× MICs (0.125 mg/L) was highly significant in preventing the formation of biofilm compared to the control ($P<0.001$), and NELG-1 at 0.5 mg/L also exhibited significantly higher biofilm prevention compared to the control ($P<0.01$). NELG-2 was able to prevent biofilm formation significantly as free gentamicin when compared to the control ($P<0.05$), as shown in Figure 10. However, when NLG (0.125 mg/L) and free gentamicin (0.5 mg/L) were compared, NLG (0.125 mg/L) was significantly better in preventing biofilm formation ($P<0.01$). In the *K. oxytoca* 700324 prevention assay, free gentamicin (0.5 mg/L) exhibited slight prevention of biofilm formation, but not significantly compared to the control. However, NLG (0.25 mg/L), NELG-1 (0.5 mg/L), and NELG-2 (0.5 mg/L) were able to prevent biofilm formation significantly better compared to the control ($P<0.001$). When comparing NLG to NELG-1 and NELG-2, NLG more significantly prevented biofilm formation by *K. oxytoca* ($P<0.01$), as shown in Figure 11.

The reduction effect of liposomal formulations and free gentamicin on structured biofilm formed by *P. aeruginosa* is shown in Figure 12. Free gentamicin at 16× MIC (16 mg/L) reduced biofilm, but the reduction was not significant when compared to the control, whereas NLG (4 mg/L) showed a significant reduction of biofilm compared to the control ($P<0.05$). NELG-1 (16 mg/L) also exhibited a significant reduction of biofilm, which was comparable to NLG ($P<0.05$). Furthermore, the increased reduction effect of NELG-2 (16 mg/L) on the biofilm was significant when compared to the control ($P<0.01$). In the *K. oxytoca*

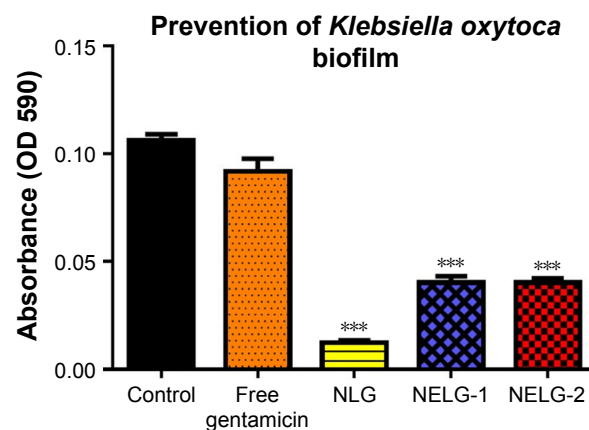


Figure 11 Prevention effect of 1/2× MIC of free gentamicin, NLG, NELG-1, and NELG-2 on *Klebsiella oxytoca* biofilm. *** $P<0.001$.

Abbreviations: MIC, minimum inhibitory concentration; NLG, dipalmitoyl-*sn*-glycero-3-phosphocholine and cholesterol; NELG-1, dipalmitoyl-*sn*-glycero-3-phosphocholine, 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol), and cholesterol (2:3:1); NELG-2, dipalmitoyl-*sn*-glycero-3-phosphocholine, 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol), and cholesterol (2:3:1).

700324 reduction assay, free gentamicin (16 mg/L) reduced the biofilm, but not significantly compared to the control. However, all liposomal formulations reduced structured biofilm formation compared to the control. For instance, NLG (8 mg/L) reduced biofilm significantly compared to the control ($P<0.05$). Notably, the negatively charged liposome formulations effectively reduced the biofilm. NELG-1 (16 mg/L) reduced the biofilm formation better than NLG and the difference was significant ($P<0.01$); NELG-2 (16 mg/L) reduced the biofilm formation better than NLG and NELG-1 when compared to the control ($P<0.001$), as shown in Figure 13.

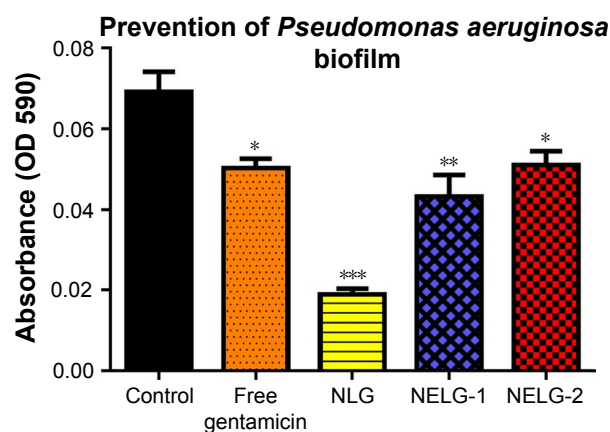


Figure 10 Prevention effect of 1/2× MIC of free gentamicin, NLG, NELG-1, and NELG-2 on *Pseudomonas aeruginosa* biofilm. * $P<0.05$, ** $P<0.01$, and *** $P<0.001$.

Abbreviations: MIC, minimum inhibitory concentration; NLG, dipalmitoyl-*sn*-glycero-3-phosphocholine and cholesterol; NELG-1, dipalmitoyl-*sn*-glycero-3-phosphocholine, 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol), and cholesterol (2:3:1); NELG-2, dipalmitoyl-*sn*-glycero-3-phosphocholine, 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol), and cholesterol (2:3:1).

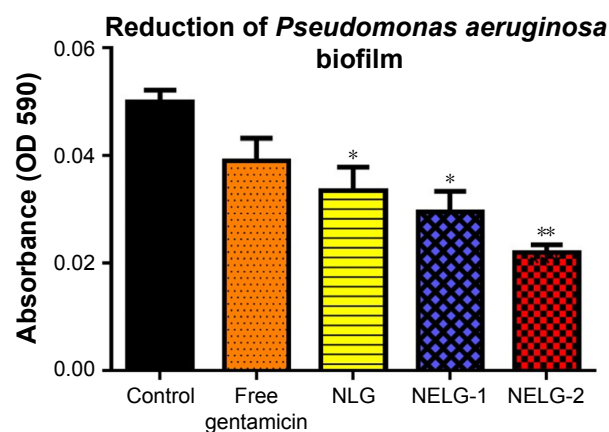


Figure 12 Reducing effect of 16× MIC of free gentamicin, NLG, NELG-1, and NELG-2 on *Pseudomonas aeruginosa* structured biofilm. * $P<0.05$ and ** $P<0.01$.

Abbreviations: MIC, minimum inhibitory concentration; NLG, dipalmitoyl-*sn*-glycero-3-phosphocholine and cholesterol; NELG-1, dipalmitoyl-*sn*-glycero-3-phosphocholine, 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol), and cholesterol (2:3:1); NELG-2, dipalmitoyl-*sn*-glycero-3-phosphocholine, 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol), and cholesterol (2:3:1).

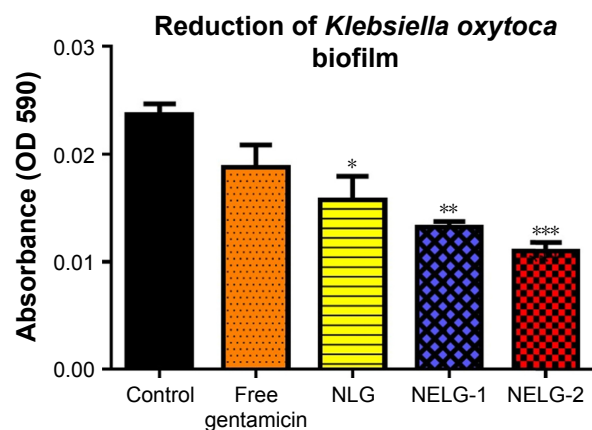


Figure 13 Reducing effect of 16× MIC of free gentamicin, NLG, NELG-1, and NELG-2 on *Klebsiella oxytoca* structured biofilm. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Abbreviations: MIC, minimum inhibitory concentration; NLG, dipalmitoyl-sn-glycero-3-phosphocholine and cholesterol; NELG-1, dipalmitoyl-sn-glycero-3-phosphocholine, 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol), and cholesterol (2:3:1); NELG-2, dipalmitoyl-sn-glycero-3-phosphocholine, 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol), and cholesterol (2:3:1).

Discussion

It is challenging to achieve a high percentage of encapsulation of aminoglycosides within liposomal nanoparticles. Electrostatic interaction between negatively and positively charged molecules is a particularly interesting approach to enhance the loading of drugs in nanoparticles.⁴⁵ In this study, we encapsulated gentamicin in different liposomal formulations including neutral and negatively charged liposomes. Increasing the negatively charged phospholipids ratio in different liposomal formulations (NELG-1 and NELG-2) played a role in increasing the EE of gentamicin compared to the neutral liposomes, due to an interaction between the positively charged gentamicin and the negatively charged liposomes. These results can be explained by the initiation of an electrostatic interaction force between the positively charged molecules such as gentamicin and the negatively charged phospholipids.⁴⁶ Our results are in agreement with a study that reported the enhancement of EE of the positively charged clarithromycin by negatively charged liposomes.³⁰

The size of the liposomes is an important characteristic parameter. The particle diameter of the negatively charged liposome gentamicin was larger than the diameter of the neutral liposomes. This might be due to the inclusion of the charge, which originates in an enlargement of space between the adjacent bilayers.⁴⁷ This aspect can be explained by the attraction of the positively charged gentamicin to the negatively charged liposomes and moving the phospholipid head group apart.⁴⁸

The stability of the liposome plays an important role in the development of a nanocarrier containing sufficient drug.

Therefore, we evaluated our formulations in different biologic environments to mimic physiologic conditions. The stability of liposomal formulations was found to be largely dependent on temperature. The neutral and negatively charged formulations retained >91% of encapsulated gentamicin in PBS at 4°C after 48 h. However, in PBS at 37°C, the stabilization of the loaded cargo was slightly reduced throughout the study period, compared to PBS at 4°C. The stability of the liposomal formulations can be explained by the extension in the acyl chain length of lipid compositions, and the presence of DPPC in our formulations contributed to a higher transition temperature. A previous study reported that saturated phospholipids with a higher transition temperature, such as DPPC, are more stable due to an increase of acyl chain.⁴⁹ Also, the presence of Chol in our formulation reduced the bilayer permeability of the liposomal membrane, resulting in high drug retention at a higher temperature.⁵⁰ In the presence of BAL, sputum, and human plasma, the release varied. NLG maintained a reasonable amount of gentamicin in these environments. NELG-1 and NELG-2 lost their gentamicin content whenever the negative lipid ratio was increased. This is explained by the avidity of negative formulations to interact with lipoproteins, albumin, immunoglobulins, and phospholipases present in BAL, sputum, and plasma, which have the ability to destabilize the liposomes depending on the lipid composition or the electrostatic charge.^{46,51,52} A study reported that liposomes containing DPPC bound less protein and were more stable than a negatively charged liposome.⁵³ Maximum release of gentamicin was noted after 4 h of incubation at 4°C and 37°C, and gentamicin was constant over the 48 h study period. This is due to the osmotic equilibrium of gentamicin concentration between the inside and outside of the liposomes.⁴⁹

In this in vitro study, we extensively examined the antimicrobial effect of liposomal gentamicin formulations on different bacterial strains. The results of MIC and MBC demonstrated that NLG enhanced the antimicrobial activity against tested bacteria (Table 2). Negatively charged liposomes exhibited MIC and MBC close to free gentamicin. These results are in agreement with a previous study which found that negatively charged liposome gentamicin had less activity on *Brucella abortus*.⁵⁴

To confirm our present MIC and MBC findings, time kill studies were performed (Figures 4–6). The NLG formulation at 2× MIC and 4× MIC improved the killing activity of gentamicin, eradicating the bacterial strains (*P. aeruginosa* BAA1744 and *K. oxytoca* 700324) at concentrations fourfold and twofold lower than free gentamicin,

respectively. Our results are in agreement with a previous study that confirmed the enhanced activity of gentamicin by encapsulating it into a neutral liposomal formulation against *P. aeruginosa*.⁴² Although NELG-1 at 2× MIC exhibited the same killing time compared to free gentamicin at 2× MIC, NELG-1 inhibited *P. aeruginosa* by \log_{10} 1 CFU at 6 h compared to free gentamicin (\log_{10} 3 CFU versus \log_{10} 4 CFU) as well as reduced *K. oxytoca* growth by \log_{10} 1 CFU throughout the study period. However, NELG-1 at 4× MIC eliminated *K. oxytoca* faster than free gentamicin (2 versus 4 h). A study demonstrated that negatively charged liposomes consisting of DPPC/DMPG and loaded with aminoglycoside exhibited an enhanced aminoglycoside antibacterial activity.⁵⁵ In our experiments, the negative formulations containing gentamicin showed a bactericidal efficacy equal to that of the free drug. Drulis-Kawa et al noted similar results and concluded that neutral and cationic liposomes interacted more with the Gram-negative negatively charged bacterial cell wall or the Gram-positive peptidoglycan than the negatively charged liposomes.⁵⁶ The interaction affinity between the negatively charged liposomes and the bacterial cell membrane could be delayed as a result of the repulsive force between the nanoparticles and bacteria.

We investigated the ability of our formulations to prevent bacterial biofilm formation. Free gentamicin at 1/2× MIC exhibited a preventive effect on *P. aeruginosa* biofilm formation; however, NLG and NELG-1 significantly prevented biofilm formation. Similarly, free gentamicin did not prevent *K. oxytoca* 700324 biofilm formation significantly compared to the control, whereas the liposomal formulations exhibited a potent prevention of biofilm formation. A previous study reported that the enhanced delivery of antibiotics to bacterial cells using liposomes is through a fusion mechanism,⁴⁰ thereby allowing the liposome-entrapped antibiotic to be injected directly into the bacterial cytoplasm. Our data showed an enhanced activity of liposomal formulations probably due to the increased delivery of gentamicin to the bacterial cytoplasm. We noticed a weak activity of NELG-2 with a strong negative charge, possibly because of limitation of liposomal fusion with a negatively charged bacterial cell wall, due to repulsive forces at close proximity.⁵⁶ Interestingly, the negatively charged liposomes exhibited a potent antibiofilm activity compared to NLG and free gentamicin against *P. aeruginosa* and *K. oxytoca*. Previous research reported an increase in activity of negatively charged liposome-encapsulated clarithromycin against *P. aeruginosa* biofilm.³⁰ Another study found that the encapsulated tobramycin in negatively charged liposomes becomes immobilized in close proximity

to a biofilm cluster which attracts the liposomes electrostatically leading to penetration and killing of bacteria.⁵⁷

Conclusion

The data presented here indicate that neutral liposomes enhance the antimicrobial activity of gentamicin against planktonic bacteria, while negatively charged liposomes increase encapsulation of gentamicin and significantly improve the activity of gentamicin against the biofilm community.

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Disclosure

The authors report no conflicts of interest in this work.

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