Original Research

The Effect Of Vancomycin-Loaded Niosomes On MRSA Strains Of Staphylococcus Aureus And Expression of MECA, HLA, and HLB Genes

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Abstract:

Background:

Niosomes have received a lot of attention today due to their better penetration and controlled release. This study aimed to evaluate the antimicrobial effect of vancomycin-loaded niosomes on the microbial species of staphylococcus aureus.

Materials and methods:

In this study, 250 clinical samples of different patients' specimens including blood, wounds, skin, and urine were collected from different medical centers (Imam Hossein, Atieh, and Sarem) in 2020. To synthesize nanodrugs, vancomycin was encapsulated in nosocomial nanocarriers by thin-film hydration, and optimization was performed based on the three main characteristics of nanocarriers. The process of drug release from nanocarriers and stability were investigated. Then, the antimicrobial properties against microbial species of staphylococcus aureus were investigated, and finally, the expression of mecA, hla, and hlb genes was determined using the real-time PCR technique.

Results:

According to the designed tests, a Span60 to Tween60 ratio of 50:50 and lipid content of 300 µmol were selected as the optimal form. The optimal nanoliposomes showed a size of 190.7 nm, a particle dispersion index (PDI) of 0.177, and retention efficiency of 71.22%. The process of drug release from the nanocarrier showed about 50% drug release from the niosome during 24 hours and about 60% after 72 hours. Stability studies over 3 months at two temperatures of 25 °C and 4 °C on the optimal sample showed that the samples stored in the refrigerator were more stable. The antimicrobial properties of the vancomycin-loaded niosomes against the mentioned microbial species showed better results compared to the free form of the drug. The nanoparticle was also able to further reduce the expression of virulence genes including mecA, hla, and hlb compared to the free form of the drug.

Conclusion:

The favorable physical properties, efficient antimicrobial effects, and low toxicity make vancomycin-loaded niosome nanocarriers a suitable candidate for the treatment of some common bacterial wound infections.

Keywords: Niosome, Vancomycin, Bacterial Infection, Thin-film Hydration, Virulence Gene.

Submitted: 14 November 2022, Revised: 23 November 2022 , Accepted: 2 Decenber 2022

Introduction

Staphylococcus aureus can be actively involved in human and animal diseases due to its pathogen encoding genes (1). This bacterium is resistant to many antibiotics, especially the penicillin family (2). Vancomycin has been introduced today as a potential drug for bacterial infections (3, 4). However, in the last decade, resistance to this antibiotic has been observed in clinical strains of Staphylococcus aureus, indicating the need for introducing a new method to deal with this problem. To effectively deliver drugs to the scar tissue, control microbial colonization, and prevent the progression of invasive infections, the use of new drug delivery systems including liposomes, autosomes, and niosomes has received much attention (5). Niosomes are a special type of vesicular drug delivery system made from nonionic surfactants and cholesterol that produce microscopic lamellar structures (6). Like other vesicular systems, niosomes possess many advantages, such as prolonged residence time of the drug in the circulatory system, targeted drug delivery to specific organs and tissues, controlled release of the drug, and biodegradable and non-immunogenic features (7).

As one of the most prominent vesicles in drug delivery systems, niosomes have attracted a lot of attention for drug delivery (6-10). Parthasarthi et al. prepared vincristine sulfate-loaded niosomes with lower toxicity and improved anticancer activity (11). Nikoonahad et al. (12) showed that lipofectamine, as a cationic liposome, can effectively transfect miR-101 into the cell and exert antitumor effects by increasing HECTH9 expression. Noisome preparation methods include thin-film hydration, freeze-drying, reverse phase evaporation, ether injection, sonication and microfluidization, bubble (13-15). Studies have shown that niosome formulation is the most important parameter that can affect niosome properties (9). The results of a study by Abdelaziz et al. (16) showed that optimal niosomes are more efficient than the drug solution. The researchers suggested that niosomes are promising in increasing antibacterial activity and reducing antibiotic resistance. Recently, the results of a study showed that ciprofloxacin-loaded niosomes could restore ciprofloxacin activity against resistant S.aureus isolates (17). This study aimed to determine the antimicrobial effect of vancomycin-loaded **n**iosomes against methicillin-resistant strains of Staphylococcus aureus and the expression of hla, hlb and mecA genes.

Materials and methods

Preparation and optimization of niosomes by thin-film hydration technique

To prepare the niosomes, the required amounts of cholesterol and surfactant were weighed using a digital scale and dissolved in chloroform to reach the desired lipid concentration. A certain number of glass spheres was added to the round-bottom flask containing the lipid solution. Evaporation of organic solvent was performed using a rotary apparatus at 60 °C in vacuum condition. The evaporation time of the solvent was half an hour. At the end of this step, the lipid was placed as a thin film on the wall of the round-bottom flask and the glass spheres. In the hydration step of the prepared lipid film, 15 mg of vancomycin in 10 cc of phosphate buffer pH=7.3 was added to the thin film to acquire the desired drug concentration and rotated at 120 rpm for half an hour at 60 °C. After completion of the hydration steps, sonication was performed to reduce the particle size.

In this study, lipid content and spin60:tween60 ratio (molar ratio) were considered as experimental variables, and nanoparticle size, dispersion index (PDI), particle size, and encapsulation efficiency (% EE) were considered as experimental responses.

Characterization of vanesomycin-loaded niosomes

A zetasizer device equipped with a green laser with a wavelength of 633 nm was used to measure the dynamic diameter of nanoparticles. Dynamic light scattering method was used to investigate the particle size at a temperature of 25 °C. The particle dispersion index (PDI) was calculated using the Malvern nanosizer device ($PDI=Mw^2/Mn^3$). To separate the free drug from the drug-loaded niosume, the formulation was centrifuged at 14000 g for 30 minutes at 4 °C. Drug-loaded nanoparticles precipitated and free drug remains as the supernatant. The amount of supernatant absorbance at 760 nm was determined using the calorimetric technique. Using a calibration curve, the amount of free drug was calculated and subtracted from the initial amount of drug. Finally, the percentage of encapsulation efficiency was calculated from the following formula:

Encapsulation efficiency = 100 x (the amount of initial drug – the amount of free drug)/ the amount of initial drug

$$\text{Encapsulation efficiency(EE\%)} = \frac{\text{the amount of initial drug} - \text{the amount of free drug}}{\text{the amount of initial drug}} \times 100$$

To evaluate the stability, the optimal samples were stored both at ambient temperature and in the refrigerator for 2 months and were evaluated for the EE% and particle size at specified time intervals.

Collecting clinical samples

The strains were collected from the hospitals of Tehran, Iran. In this study, 250 clinical samples of different patients' specimens including blood, wounds, skin, and urine were collected from different medical centers (Imam Hossein, Atieh, and Sarem) in 2020.

Genomic DNA extraction

First, clinical samples were cultured on Lb broth medium for 24 hours at 37 ° C. After the samples were cultured, they were centrifuged at 13000 rpm. After removing the aqueous phase, the remaining precipitate was added by 600 µl of lysis buffer (Tris-Hcl, pH 7.4; EDTA 50mM), 13 µl of sodium dodecyl sulfate (25% SDS), and 3 µl of proteinase K (20mg / ml) and stored at 60 °C for one hour. Using gloves at this stage, 600 µl of phenol: chloroform: isoamyl alcohol solution (prepared with a ratio of 25:24: 1) was added under the hood until a uniform milky phase was formed. The Eppendorfs were then centrifuged at 1300 rpm for 5 minutes and three different phases were formed (with a bottom-up order: organic phase containing non-polar material, thin protein phase, and an aqueous phase containing DNA). The upper phase (aqueous phase) was transferred to the new Eppendorf and steps 4 and 5 were repeated twice to completely remove the proteins from the aqueous phase. At this stage, to completely remove the phenol from the aqueous phase, 600 µl of chloroform was added to each Eppendorf and the aqueous phase was removed again and transferred to the new Eppendorf. To precipitate DNA, an equal volume of cold and pure ethanol to the aqueous phase along with 0.1 µl of sodium acetate (1M) was added and stored in the refrigerator for 10 minutes. Then, the supernatant aqueous phase was removed and rinsed again with cold ethanol 70% and centrifuged (at 13000 rpm for 5 minutes) and the aqueous phase was extracted. The extracted DNA is now collected at the bottom of the Eppendorf. The Eppendorfs were dried using a vacuum apparatus, and finally, a TE buffer (10 mM Tris, pH: 7.4; 0.1 mM EDTA) containing RNase was added to each and stored in a freezer at -20 °C.

Performing PCR for mecA, hla and hlb genes

The primers were lyophilized and a certain amount of distilled water was added to each forward and reverse primer according to the datasheet of the manufacturer (Macrogen, South Korea). In this case, the primer concentration was 100 pmol/µl. For PCR, a concentration of 10 pmol/µl was used. Replication of mecA, hlb, and hla genes was performed to investigate the efflux pump genes. To this end, a PCR mixture was prepared and gene replication was performed using the thermal cycle of an Eppendorf thermal cycler.

RNA extraction from Staphylococcus aureus cells treated with niosomal subMIC concentrations

Containers and tubes used for RNA were treated with 0.1% DE PC8 solution for one hour at 37 °C or overnight at room temperature and then autoclaved at 15 psi for 15 minutes.

Producing complementary DNA (cDNA)

To produce cDNA from the extracted RNA, NanoDrop kit (Revert AidTM First Strand cDNA Synthesis Kit (Fermentas)) was used to determine the amount and concentration of RNA from Staphylococcus aureus MRSA cells adjacent to the MIC concentrations of drug-loaded noisomes, according to the fabrication kit.

Evaluation of mecA, hla, and hlb genes by Real-Time PCR

In this study, SYBER green method and 16S rRNA housekeeping gene (reference gene) were used to study the expression of mecA, hla and hlb genes in bacteria treated with drug-loaded niosomes. It should be noted that before the PCR reaction, the same concentrations of cDNAs from the bacteria treated with the extract were measured using a NanoDrop. In this study, Mater mix (Bioneer) and primers of 16S rRNA, hla, hlb, and mecA genes were used in duplicate. The data were then analyzed using the software on the device to evaluate gene expression and the results were evaluated using agarose gel electrophoresis.

Statistical analysis

The obtained data were statistically analyzed using SPSS 16 software. The results were analyzed using one-way ANOVA, and the differences in the expression of target genes between the control and treatment samples were calculated using Tukey's HSD post-hoc test. Data were presented as mean ± standard deviation (SD) and $P<0.05$ was considered as significant. Real-time PCR data was analyzed based on cycle threshold comparison. In this study, the difference in cycle thresholds obtained from the experimental samples (drug-treated cells) and the control (untreated cells) was calculated. The ratio of the target gene to the reference gene (16 S rRNA) was calculated by $2^{-\Delta\Delta Ct}$ formula. The calculation formula was as follows:

ΔCt= Ct target-Ct reference

$\Delta\Delta Ct=\Delta Ct$ test sample - ΔCt control sample

Relative expression: $2^{-\Delta\Delta Ct}$

After obtaining the Ct, $\Delta\Delta Ct$ of each sample was calculated using Rest 2009 software. Then, the expression change of each sample was calculated using the One-way ANOVA method in SPSS 16 software.

Results

Validation method for quantification of vancomycin

Table 1 shows the studied parameters, including light absorption, Beer-Lambert index, accuracy, and precision. Drawing the vancomycin calibration curve (standard) by the calorimetric method resulted in an R^2 value of 0.9997, indicating a linear relationship between absorption and drug concentration.

Formulation and properties of vancomycin-loaded niosomes

In this study, different formulations of vancomycin-loaded niosomes were investigated based on surfactant type, Span60 to Tween60 ratio, lipid content, vancomycin, sonication time, and surfactant to cholesterol ratio. Of these indices, only lipid content and the Span60 to Tween60 ratio were changed in this study. Table 2 shows the synthesis results of vancomycin-loaded niosomal formulations using the thin-film hydration method based on three variables of particle size, PDI, and EE%. The results of this study showed that the average particle size (Z-average) for niosomes was 157.9 nm and for vancomycin-loaded niosomes were 190.7 nm. Moreover, the PDI reported for niosomes and vancomycin-loaded niosomes was 0.164 and 0.177, respectively.

Investigation of the drug release process

Figure 1 shows the cumulative release process of the drug solution form of the release medium and drug-loaded nanocarriers in the PBS release medium over 72 hours.

In this study, drug release from the nanocarriers (61.33%) was less than that from drug solution (98.02%) during 72 hours of release. In drug solution release, 51% of the drug was released within the first 6 hours, while for drug-loaded nanocarriers, 50% of the drug was released from the nanoniosomes within 24 hours. After analyzing the release rate of vancomycin from the optimal formulation, a linear form of different kinetic models was drawn to define the release kinetics. The model with the closest linear regression coefficient to 1 was selected as the optimal model. The best model for describing the release kinetics of vancomycin was Korsmeyer Peppas (R^2=0.9611 and n=0.5148), which represented Fickian diffusion.

Isolation and differentiation of strains from clinical specimens

Table 3 shows the frequency of clinical samples by type of sample from which clinical strains were isolated and their distribution by sex.

To investigate the presence of mecA, hlb, and hla genes in isolated methicillin-resistant strains of Staphylococcus aureus, specific primers of this gene were used. The mecA gene was found in all methicillin-resistant strains (15 samples). The hlb gene was observed in 10 samples and the hla gene in 9 samples. In addition, three genes of mecA, hla, and hlb were observed simultaneously in 9 strains.

Antimicrobial effects of drug-loaded niosome and free drug

Methicillin-resistant Staphylococcus aureus (MRSA) strains were exposed to 100-125.3 μg/ml drug-loaded niosome and free drug for 24 hours. The results showed that the antibacterial effect of drug-loaded niosomes was more significant than that of free drugs so that MIC concentration was 2 to 4 times lower compared with that in free drugs (Table 4).

Evaluation of mecA, hla and hlb gene expression in MRSA strains after treatment with drug-loaded niosomes

The graph of fluorescence irradiation of different PCR reaction products is shown in Figure 2.

The relative expression of mecA, hla, and hlb genes in MRSA isolates was studied by Real-Time PCR. The results showed that the expression of mecA, hla, and hlb genes in MRSA strains significantly decreased in sub-MIC concentrations of drug-loaded niosomes and was statistically significantly different from the expression of 16S rRNA gene.

Today, overusing antibiotics in the treatment of human and animal diseases has led to multidrug antibiotic resistance in bacteria (18). This research aimed to produce a new form of

antibiotics with superior performance characteristics over the free form by preparing vancomycin-loaded niosomes. In this project, based on three main characteristics of nanocarriers, i.e., size, particle size dispersion, and drug encapsulation efficiency, vancomycin-loaded niosomal nanocarriers were prepared and optimized based on two parameters of lipid content and span60 to Tween60 ratio using thin-film hydration method (19). According to the results of screening studies, the parameters of surfactant type (Span 60), sonication time (7 minutes), surfactant to cholesterol ratio (1: 1), and amount of drug (1 mg/ml) were considered as constants and two parameters of lipid content and Span to Tween ratio were considered as the main variables. Vancomycin-loaded niosomal formulations were produced to achieve the optimal formulation. The optimal formulation was obtained with a size of 190.7, particle dispersion index of 0.177, and encapsulation efficiency of 71.22. Changings in the optimal loading efficiency are greater in the thin-film hydration method than in the handshaking method and the ether injection method (20). Examination of the parameters affecting the particle size also revealed that the appropriate ultrasound exposure time (5-7 minutes) is required to obtain a medium and appropriate particle size with a narrower size distribution. Spans and tweens are non-ionic surfactants with a variety of advantages such as improved stability, wide compatibility, and formulation flexibility. The rigidity of Tween 60 as the niosome membrane is poor due to its high hydrophilicity. In addition, span 60 with high lipophilicity and proper encapsulation properties and of cholesterol and surfactant (Span60:Tween60) in a 1:1 molar ratio can lead to the density of niosome films (21).

The results of the present study showed that all different ratios of lipid content (cholesterol and surfactant) are involved in the optimal formation of niosomes. Different Span60:Tween60 ratios for the preparation of niosomal formulations caused nanoparticles with different sizes. Moreover, the morphology of the niosomes prepared by FESEM microscopy showed that the optimal formulation prepared by the thin-layer hydration method is usually multilayer vesicles with a size of about 50 nm and suitable particle distribution that contain a large amount of drug and have a slower drug release (22). In vitro studies under physiological conditions help to implement and complete the in vivo phase for a proposed drug delivery system (23). In vitro studies are performed under physiological conditions with a temperature of 37 °C and pH=7.4. The results of in vitro drug-release experiments showed that drug encapsulation inside the niosomes has the potential for optimizing the drug-release control over a long time. The results also showed that the drug release from nosomal formulation is a two-step process. In the first step, drug release is relatively rapid while in the second step, the drug is released at a slower rate. The rapid release of the drug in the first stage is due to drug excretion from the outer surface of the niosome, and the slower release of the drug in the second stage is due to the penetration of the drug through the niosome (24).

The results for antimicrobial effects of S. aureus suggested that much lower concentrations of vancomycin-loaded nanoparticles are needed to inhibit bacterial growth than its free form. Accordingly, it can be concluded that the antibacterial effectiveness of drug-loaded nanoparticles is higher than that of the free form of the drug. The results of real-time PCR also showed that vancomycin-loaded niosomes significantly down-regulated the expression of bacterial virulence genes (hla, hlb, and mecA) compared to both the reference gene and the free drug-treated group. Akbarzadeh et al. (25) optimized the cephalexin-loading neosomal formulation using Spen60 and Tween60. They investigated

the antimicrobial properties of the prepared formulations against the microbial species of S. aureus and E. coli. The results showed that loading the drug into nosomal formulation improved the antimicrobial properties of the drug against these microbial species. Haeri et al. (26) also investigated the effect of moxifloxacin niosomes on the microbial species P.aeruginosa and S. aureus and observed that the formulations prepared were more effective against these microbes compared to the free drug. The results of another study showed that the improvement of cephalexin bioavailability and prolonged drug release profile could be achieved by niosomal formulation as a desirable antibiotic drug delivery system (27). Recently, another study reported that the optimized formulation exhibited enhanced antibacterial effects than the free drug solution (27).

Mirzaie et al. (29) conducted a study aimed at optimizing noisome-encapsulated ciprofloxacin and evaluating their antibacterial and biofilm inhibition effects against ciprofloxacin-resistant methicillin-resistance Staphylococcus aureus (CRMRSA). In this study, the formulation of noisome-encapsulated ciprofloxacin was optimized by changing the Tween60/Span 60/cholesterol ratio. The optimized ciprofloxacin encapsulated formulations based on Span 60 and Tween 60 were prepared and characterized using scanning electron microscopy (SEM), transmission electron microscopy (TEM), and dynamic light scattering (DLS). The results of SEM and TEM showed that noisome-encapsulated ciprofloxacin was spherical with a size between 50 and 150 nm. The prepared neosomal formulations showed great storage stability for up to 30 days through a slight change in size and drug entrapment during storage, making them good candidates for drug delivery systems. Optimized niosome-encapsulated ciprofloxacin enhanced antibacterial activity against CR-MRSA strains by reducing the minimum inhibitory concentration (MIC) and significantly inhibited biofilm formation. Niosome-encapsulated ciprofloxacin also significantly downregulated the expression of icaB biofilm formation gene. The authors reported that ciprofloxacin encapsulation in niosomes is a promising approach to improve antibacterial activity, biofilm inhibition, and decrease antibiotic resistance in CR-MRSA strains.

Conclusion

Vancomycin-loaded niosome with its favorable physicochemical properties and antimicrobial effects on Staphylococcus aureus was able to significantly down-regulate the expression of virulence factors of this bacterium. Therefore, vancomycin-loaded niosome is a good candidate against Staphylococcus aureus.

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Tables

Table 1. Validation of vancomycin parameters in PBS (pH 7.4)

Parameter	**Results**
λ_{max}(nm)	280
Beer's law range(µg/ml)	20-200
Accuracy	97.1652
Precision(%RSD)	4.5543

Table 2. Synthesis results of vancomycin-loaded niosomal formulations using thin-film hydration method

Formulation	Size (nm)	PDI	EE (%)
F1	221.9	0.264	53.65
F2	183.5	0.168	66.36
F3	240.5	0.315	51.24
F4	238.6	0.231	72.55
F5	190.7	0.177	71.22
F6	274.3	0.359	65.90
F5-B	157.9	0.164	-

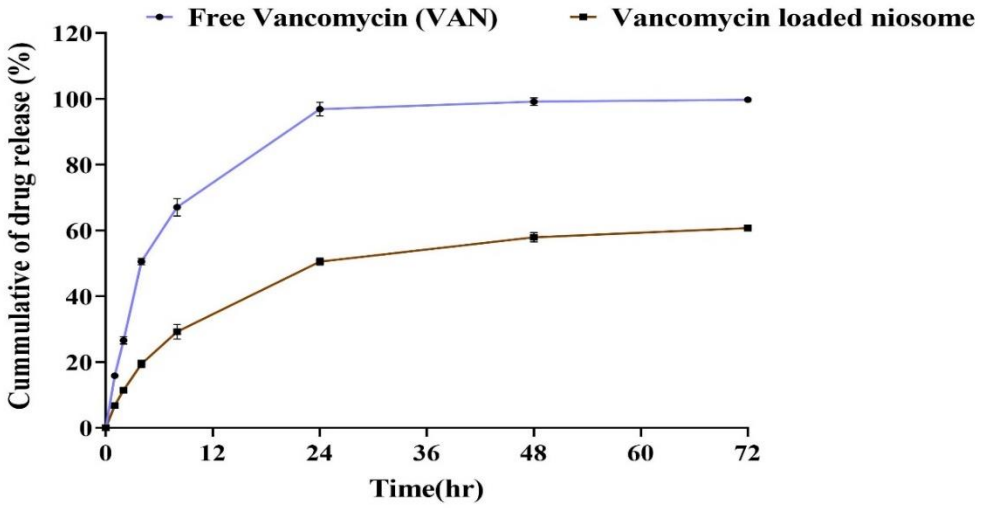

Figure 1. The release process of drug solution and drug-loaded nanocarriers in PBS receptor phase

Table 3. Absolute and relative frequency of clinical samples by type of sample from which clinical strains were isolated and their distribution by sex.

Gender	Female		Male		Total	
Sample type	No.	%	No.	%	No.	%
Blood	15	6	20	8	35	14
Wound	32	12.8	10	4	42	16.8
Skin	28	11.2	15	6	43	17.2
Urine	60	50	70	28	130	52

Table 4. MIC and sub-MIC concentrations calculated for drug-loaded niosomes and free drugs against MRSA isolates

Strain No.	MIC of free drug (µg/ml)	MIC of drug-loaded niosome (µg/ml)	Sub-MIC of drug-loaded niosome (µg/ml)
4	50	6.25	3.125
8	100	25	12.5
15	25	3.125	<3.125
19	50	6.25	3.125
26	50	6.25	3.125
29	100	25	12.5
32	25	3.125	<3.125
34	25	3.125	<3.125
38	50	12.5	6.25
40	50	3.125	<3.125
42	50	6.25	3.125
45	100	12.5	6.25
46	50	3.125	<3.125
48	100	12.5	6.25
49	50	3.125	<3.125

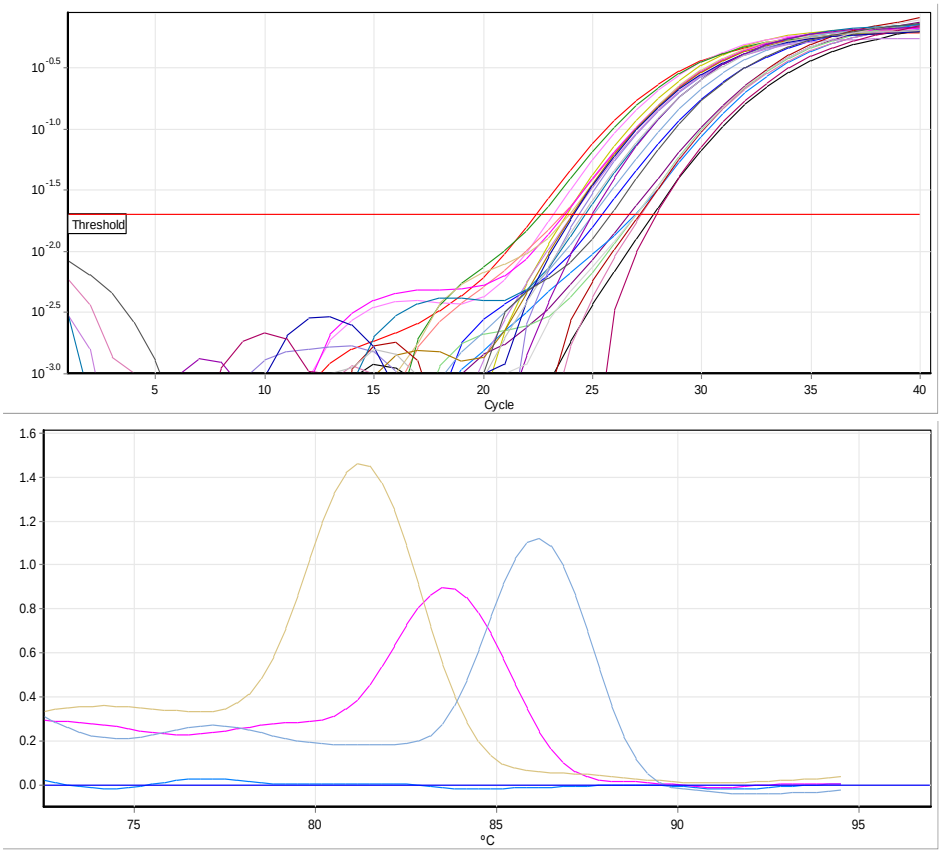

Figure 2. Real-time replication and melting curve analysis for three genes of hla, hlb, and mecA

Table 5. Changes in the expression of mecA, hla, and hlb genes after treatment with drug-loaded niosomes

Strain No.	***hla***	***Hlb***	***mecA***	***16S rRNA***
8	0.29±0.07	0.36±0.05	0.61±0.07	0.97±0.04
15	0.46±0.08	0.66±0.06	0.52±0.03	0.97±0.04
19	0.53±0.09	0.58±0.05	0.63±0.04	0.97±0.04
26	0.31±0.08	0.42±0.07	0.34±0.08	0.97±0.04
29	0.68±0.08	0.49±0.06	0.63±0.04	0.97±0.04
34	0.58±0.06	0.62±0.07	0.52±0.06	0.97±0.04
38	0.61±0.08	0.37±0.06	0.49±0.07	0.97±0.04
40	0.39±0.07	0.28±0.04	0.39±0.04	0.97±0.04
45	0.22±0.07	0.62±0.08	0.62±0.07	0.97±0.04

Table 6. Changes in the expression of mecA, hla, and hlb genes after treatment with the drug

Strain No.	***Hla***	***Hlb***	***mecA***	***16S rRNA***
8	0.63±0.09	0.56±0.05	0.73±0.07	0.97±0.04
15	0.52±0.08	0.76±0.06	0.81±0.03	0.97±0.04
19	0.69±0.07	0.68±0.05	0.91±0.04	0.97±0.04
26	0.79±0.08	0.72±0.07	0.69±0.08	0.97±0.04
29	0.88±0.08	0.89±0.06	0.72±0.04	0.97±0.04
34	0.78±0.06	0.72±0.07	0.76±0.06	0.97±0.04
38	0.61±0.08	0.67±0.06	0.68±0.07	0.97±0.04
40	0.79±0.07	0.78±0.04	0.83±0.04	0.97±0.04
45	0.82±0.07	0.82±0.08	0.79±0.07	0.97±0.04