

RESEARCH PAPER

Effect of the nanoliposomal formulations of rifampin and N-acetyl cysteine on staphylococcus epidermidis biofilm

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ABSTRACT

Objective(s): Staphylococcus epidermidis is a common cause of medical device-associated infections due to biofilm formation, and its elimination is extremely challenging. Although rifampin efficacy against S. epidermidis biofilms has been confirmed, its use as a single agent may lead to resistance. As such, it is assumed that the combination of rifampin and N-acetylcysteine (NAC) could exert additive effects as a mucolytic agent. The present study aimed to use a liposomal system for the delivery of these compounds to bacterial biofilm.

Materials and Methods: Liposomal formulations were prepared using the dehydration-rehydration method and characterized in terms of the size, zeta potential, and encapsulation efficacy. In addition, the ability of various formulations in the eradication of bacterial biofilm and inhibition of biofilm formation was assessed based on the optical density ratio.

Results: The zeta potential of the liposomes was positive, and the mean size of these liposomal formulations was less than 200 nanometers. Liposomal rifampin was the most effective formulation against S. epidermidis, and the anti-biofilm activity of most of the formulations was concentration-dependent and time-dependent.

Conclusion: According to the results, the rifampin-loaded liposomes were effective against S. epidermidis biofilm formation.

Keywords: Biofilm, Nanoliposomes, N-acetyl cysteine, Rifampin

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INTRODUCTION

Staphylococcus epidermidis and other coagulase-negative staphylococci have become the leading cause of the infections induced by implanted medical devices. These infections are directly caused by the formation of a multi-layer structure and biofilms on artificial surfaces. The microorganisms in these structures produce an extracellular matrix, which is a crucial virulence factor. The matrix advocates bacterial adhesion, thereby making the elimination of the bacteria difficult [1, 2].

Detection of biofilms is challenging in conventional diagnostics as they are naturally

tolerant to the human immune system and typical antibiotic therapies [3]. Additionally, the development of antibiotic resistance and slow improvement in finding new classes of antibiotics have urged researchers to seek novel therapeutic methods [4]. N-acetylcysteine (NAC) is a non-antibiotic agent with antibacterial effects. It is a mucolytic drug that disrupts the disulfide bonds in the mucus [5]. NAC is generally used in the medical treatment of chronic bronchitis, cancer, and acetaminophen intoxication [6]. Furthermore, NAC affects several processes that are vital for bacterial biofilm formation, reducing the extracellular polysaccharide production and demonstrating anti-biofilm properties [7]. Combinations therapy represents a therapeutic strategy against *S. epidermidis* biofilm [7].

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Previous studies have indicated that rifampin is the most potent antibiotic against the biofilms of *S. epidermidis* [8-10]. Therefore, we used the combination of rifampin and NAC in order to eradicate *S. epidermidis* biofilm.

Today, novel drug delivery systems are commonly used these to benefit from more effective therapeutic methods. These carriers could improve the effects of antibacterial drugs and reduce drug side-effects, while effectively decrease antibacterial resistance [11, 12]. Among these, liposomal formulations have been investigated as lipid-based carriers [13]. In addition to the simple manufacturing process, liposomes have potential applications for the delivery of oil-soluble or water-soluble antibacterial agents against various bacterial infections [14-16].

Several mechanisms have been described for the enhancement of the anti-biofilm efficacy of liposomal formulations in terms of free forms. The better penetration and adhesion of liposomal antibiotics to bacterial biofilms are important mechanisms in this regard, which have been studied extensively. According to the literature, the optimal size for penetration is within the range of 100-130 nanometers, and the unilamellar vesicle exhibited better penetration [17]. Better penetration and adhesion to bacterial biofilm structure has also been reported in cationic liposomes [18].

The present study aimed to examine the *in-vitro* antimicrobial activities of nanoliposomal formulations loaded with NAC or/and rifampin against *S. epidermidis* biofilm.

MATERIALS AND METHODS

Experimental materials

Hydrogenated soybean phosphatidylcholine (HSPC) was purchased from the Lipoid Company (USA), stearylamine (SA) and cholesterol (Chol) were purchased from Merck (Germany), rifampin was provided by Hakim Pharmaceutical Company (Tehran, Iran), NAC was obtained from Avicenna Pharmaceutical Company (Tehran, Iran), and chloroform, methanol, trimethyl tetrazolium chloride, and trypticase soy broth (TSB) were purchased from Merck (Germany).

Liposome preparation and characterization

Liposomes encapsulated with rifampin were prepared using the solvent evaporation method. To this end, lipids and rifampin were dissolved in

chloroform and methanol (2:1). Following that, the lipid solution was dried, and a thin film was formed in a round-bottom flask using a rotary evaporator (Heidolph, Germany). Following that, the lipid film was hydrated with the addition of phosphate buffered saline (PBS) and vortexing of the dispersion. The lipid phase was composed of HSPC, SA, and Chol with the molar ratio of 1:0.1:1 [19]. The amount of rifampin was twice as high as the minimum bactericidal concentration (MBC), which was used for biofilm eradication.

The dehydration and rehydration (DRV) method was used for the preparation of the liposomes containing NAC. The liposomes were composed of HSPC, Chol and SA using the solvent evaporation method. At the next stage, NAC was added to the prepared liposomes. After the freezing of the formulation, the frozen liposomes were vacuum-dried for 12 hours, and the dried particles were rehydrated using distilled water and vortexed to form the liposomal suspension. In addition, free NAC was separated from the liposome-loaded drug through dialysis, and the dialysis membrane was used with an appropriate cutoff (12 kDa).

In order to prepare the liposomes containing the combination of rifampin and NAC, the same method was applied, while during the process, the liposomes loaded with rifampin were combined with the NAC solution instead of empty liposomes [8].

To reduce particle size and form the unilamellar vesicle and the liposomes were extruded through 1,000-, 800, 600, 400, and 100- nanometer polycarbonate filters using the thermobarrel extruder (Northern Lipids, Burna Boy, Canada). The mean particle size and surface charge of the provided liposomes were also assessed using dynamic light scattering (DLS) (model: Zeta Sizer Nano-ZS; Malvern Instruments Ltd., United Kingdom) after proper dilution [10].

The encapsulation efficacy of the liposomal formulation was determined using validated high-performance liquid chromatography (HPLC) method. Moreover, the chromatographic analysis of rifampin was performed by injecting the sample into a C18 column (4.6×250 mm), and isocratic elution was performed by the solvent system using 0.05 M phosphate buffer, including acetonitrile (55:45 v/v) at the flow rate of 1 ml/min (8). The chromatographic analysis for NAC was also carried out using a C18 chromatography column

(4.6×150 mm, 5µm) and a mobile phase consisting of a mixture of 0.05 M potassium phosphate monobasic (pH=3) acidified by phosphoric acid and acetonitrile 88:12 at the flow rate of 0.8 ml/min [20]. After injection to the HPLC system, EE% was determined using the following equation:

$$\text{Encapsulation Efficiency (\%)} = \frac{W_{\text{entrapped drug}}}{W_{\text{initial drug}}} \times 100$$

where $W_{\text{initial drug}}$ and $W_{\text{entrapped drug}}$ show the drug amount initially used and entrapped drug into the solid lipid nanoparticles, respectively.

Determination of planktonic minimum inhibitory concentration

The minimum inhibitory concentration (MIC) was determined using the broth microdilution method in accordance with the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS) [21]. *S. epidermidis* strain DSMZ3270 (DSMZ Cloning, Braunschweig, Germany) was used as the microbial strain, along with the stock culture of the frozen bacteria (-75°C) on the brain-heart infusion broth medium (BHI; Biomieux, France) containing 25% glycerol. The overnight (37°C) subculture of *S. epidermidis* was prepared on the BHI broth medium. In the next step, *S. epidermidis* was cultivated on the MHB medium supplemented with 0.25% glucose in order to reach and match 0.5 MacFarland standard, and the stock suspension was approximately 10⁸ CFU/ml.

Rifampin and NAC were prepared from the serial two-fold dilutions (0.003-30 µg/ml for rifampin, 40-4 mg/ml for NAC). In total, 250 microliters of the drugs were added to each well of a microtiter plate, followed by the addition of 25 microliters of 1:20 dilution of the bacterial stock suspension. Afterwards, the inoculated microplate was incubated for 24 hours at the temperature of 37°C. By definition, the MIC is the lowest antibiotic concentration that yields no visible growth. The wells containing the test medium (MHB) and inoculated medium (510⁵ CFU/ml) were considered as controls in the present study. The MIC was determined by adding trimethyl tetrazolium chloride (TTC) to each well and incubation at the temperature of 37°C for 30 minutes.

Promotion of biofilm formation

Rifampin, NAC, and their combination were diluted onto a nutrient medium containing the

S. epidermidis cell suspension (10⁶ cells/ml). Afterwards, 200 microliters of each suspension was added to each well of the 96-well microtiter plates, and the plates were incubated at the temperature of 37°C for 24 hours. Finally, each well was washed twice with 200 microliters of saline solution, and crystal violet staining was used as an indicator of total biofilm biomass. They were stained with 0.3% crystal violet for five minutes. In order to solubilize the bounded crystal violet, 200 microliters of 96% ethanol were added to each well, and the optical density (OD) at 540 nanometers was determined using a microplate reader.

Crystal violet assay

This assay was aimed at assessing the efficacy of each formulation for biofilm biomass reduction. The ability of *S. epidermidis* to form biofilms on non-living surfaces was determined as described previously [22]. The bacterial suspension with the approximate concentration of 10⁸ CFU/ml was prepared in TSB (enriched with 0.25% glucose) from the overnight culture of *S. epidermidis*. Following that, the bacterial cultures were diluted 1:40 in the same diluent, and 200 microliters was added to each well of the 96-well microtiter plates; the plates were incubated at the temperature of 37°C for 24 hours. After eight hours, the supernatant was replaced with fresh enrichment culture medium. At the end of the incubation period, the bacterial biofilms were attached to the bottom of a 96-well microtiter plate. For the extracting of the unattached bacteria, each well was rinsed trice with PBS (200 µl).

The concentration of each drug was adjusted at 1,000-fold of the MIC values. In order to examine the anti-biofilm activity of the drugs, the serial dilution of each formulation was added to each well, and the microplates were incubated for various periods (24, 48, and 72 hours) to evaluate the effect of time exposure. After incubation, the biofilms were rinsed trice with 200 microliters of PBS and stained with 0.3% crystal violet for five minutes. To solubilize the bounded crystal violet, 200 microliters of 96% ethanol was added to each well, and OD at 540 nanometers was determined using a microplate reader (Awareness, Palm City, FL). Each experiment was performed in triplicate.

To assess the efficacy of the liposomal formulations, bacterial biofilm formation was performed as described earlier. Afterwards, 200

microliters of each formulation was added to an individual well of the microplate and incubated at the temperature of 37°C for 24, 48, and 72 hours. To examine the effect of the liposomal concentration, seven-fold serial dilutions of various formulations were prepared in PBS, and each dilution series was tested as described earlier. In addition, the initial concentration of the free form of antibiotics and liposomal antibiotics was adjusted at the same level. The quantitative measurement of the OD ratio (ODr) was also calculated by dividing the OD of each antimicrobial agent to the OD of the positive control (native biofilm).

Statistical analysis

All the tests were performed in triplicate. Data analysis was performed using one-way analysis of variance (ANOVA) to determine the difference in the OD, which were considered statistically significant at the P-value of less than 0.05.

RESULTS

Table 1 shows liposome characterization in terms of the mean size, polydispersity index, and zeta potential of different formulations. Accordingly, the mean sizes in all the formulations were less than 200 nanometers. Moreover, the zeta potential of all the formulations was positive, and the MIC of rifampin and NAC against *S. epidermidis* was estimated at 0.03 and 4,000 µg/ml, respectively. According to the obtained results, the free form of the antibiotics was ineffective in the eradication of the biofilm at 1,000-fold MIC. It is also notable that the combination of rifampin and NAC did not change the required antimicrobial concentration for the effective eradication. As is depicted in Fig 1, the liposomal formulations significantly contributed to the eradication of the formed biofilms. In the assessment of the effect of various formulations on the inhibition of biofilm formation, the free form of rifampin was observed to effectively inhibit biofilm formation.

Table 1. Z-average, Polydispersity Index (PDI), and Zeta Potential of Each Liposomal Formulation (Mean±SD; n=3)

Formulations	Size (nm)	PDI	Zeta Potential (mV)	EE%
Empty Liposome Formulation	164.66±3.53	0.339±0.01	45.2±2.51	-----
Rifampin Liposomal Formulation	125.23±6.40	0.124±0.001	30.76±1.31	90±5%
NAC Liposomal Formulation	153±0.43	0.112±0.01	48.03±1.46	20±3%
Dual Liposomal Rifampin and NAC	180±2.32	0.200±0.02	54.33±2.96	80±3% (rifampin) 18±2% (NAC)

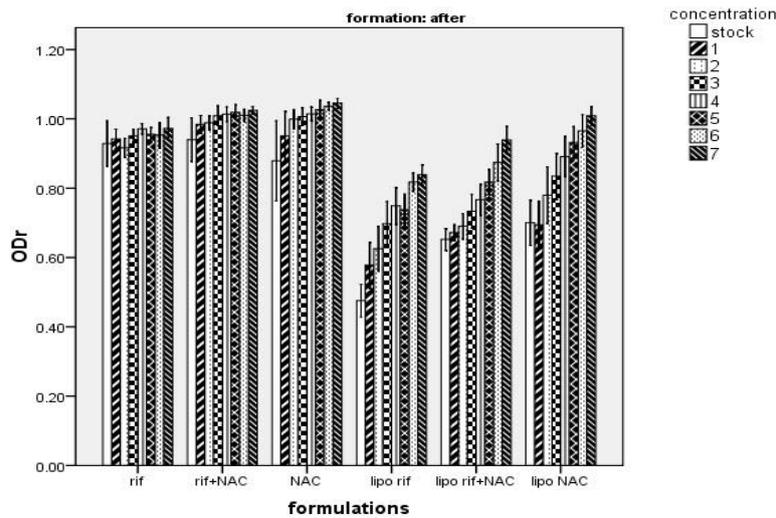


Fig 1. Optical Density Ratio (ODr) of Rifampin, NAC, and Their Combination in Liposomal and Free Forms Used to Eradicate *Staphylococcus epidermidis* Biofilm (Mean±SD; n=3)

However, this function in NAC was completely dose-dependent.

According to our findings, combination therapy had no significant effects compared to rifampin alone (Fig 2). Furthermore, the results regarding the liposomal formulations indicated that both liposomal NAC and liposomal combination had insufficient anti-biofilm activity compared to liposomal rifampin. It is also notable that rifampin had no significant difference with the liposomal form ($P>0.05$).

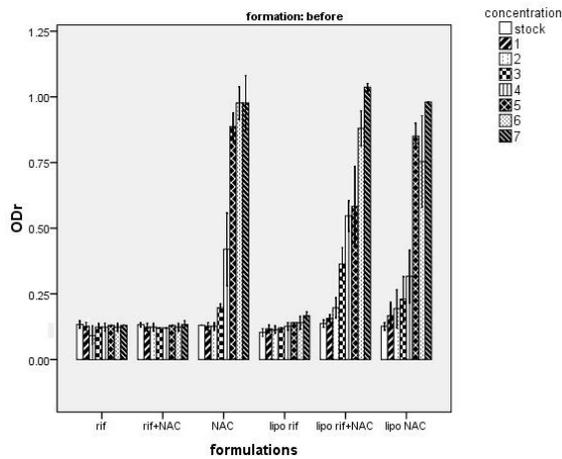


Fig 2. ODr of Rifampin, NAC, and Their Combination in Liposomal and Free Forms Used to Inhibit Staphylococcus epidermidis Biofilm Formation (Mean±SD; n=3)

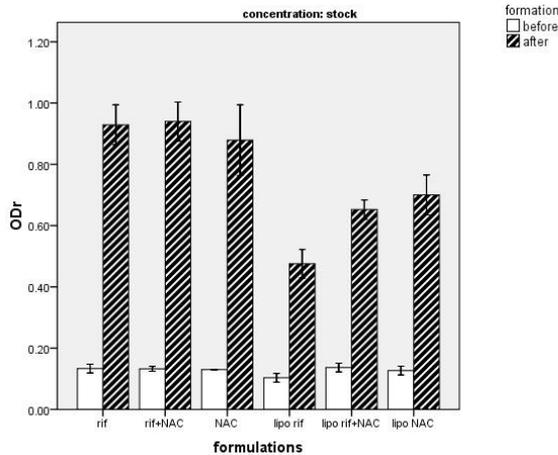


Fig 3. ODr of the Rifampin, NAC, and Their Combination in Liposomal and Free Forms Used to Inhibit Staphylococcus epidermidis Biofilm Formation Compared to Biofilm Eradication (Mean±SD; n=3)

Fig 3 shows the effectiveness of the formulations before and after biofilm formation. As can be seen, after biofilm formation, even rifampin with significant antibiofilm effects

could not destroy the formed biofilm. We also investigated the effect of the exposure time, and the findings indicated that the ability of the formulations to eradicate the biofilm improved with the increased incubation time (Fig 4).

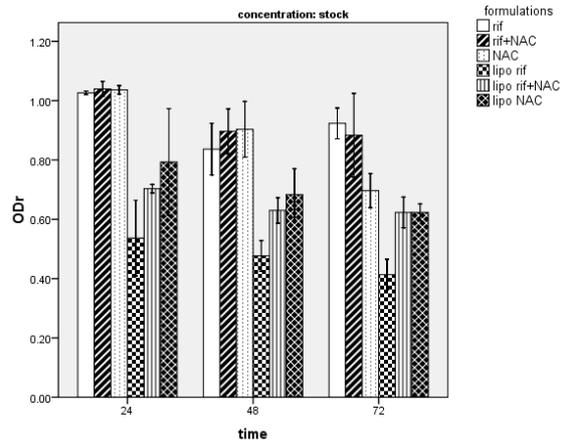


Fig 4. ODr of the Rifampin, NAC, and Their Combination in Liposomal and Free Forms Used to Eradicate Staphylococcus epidermidis Biofilm at 24, 48, and 72 Hours of Incubation (Mean±SD; n=3)

DISCUSSION

In the past decades, several strategies have been developed for the prevention and/or eradication of biofilm formation. Previous studies in this regard have strongly suggested that particular combinations of antimicrobial agents have considerable differences with monotherapies [23, 24]. For instance, Zheng and Stewart detected rifampin-resistant mutants when biofilms were exposed to rifampin for more than 48 hours [25]. Furthermore, lipid nanoparticles have been considered as delivery systems, showing a promising approach to combating microbial biofilm. In the present study, the theoretical foundation was the reported results regarding the effect of NAC on the disruption of mature biofilms and higher efficacy of rifampin against the bacteria adhering to biomaterials compared to other common antibiotics [26]. Therefore, it was assumed that the combination of these agents could have a synergistic effect due to their different mechanism of action.

According to the results of the present study (Tables 1), the zeta potential of all the formulations was higher than +30 mV, and EE% was estimated at 20% and 80% for NAC and rifampin, respectively. This is in line with the previously published data, suggesting that hydrophilic drugs have lower

EE% compared to hydrophobic drugs [8, 15]. Additionally, the positive charge of particles could contribute to the better interaction of the particulate system with bacterial biofilm [2, 8].

In the current research, rifampin alone showed high efficacy in the prevention of biofilm formation. However, the liposomal formulation of rifampin had no significant difference with the free form (Fig 2). This is consistent with the previous studies in this regard. Rifampin solely has high antibiofilm activity, which could not increase by its encapsulated form [27, 28].

The findings of the current research confirmed the efficacy of NAC in the inhibition of biofilm formation in a completely dose-dependent manner (Fig 2). Accordingly, the NAC concentrations of 2.5-40 mg/ml could effectively inhibit biofilm formation, while no such function was observed at lower concentrations; however, the difference was not considered significant compared to the controls. In the study by Leite et al., the effectiveness of NAC against all the planktonic cells of Gram-positive and Gram-negative strains was confirmed. However, NAC could not reduce the growth of all the tested strains [29].

According to the results of the present study, NAC in the liposomal formulations could effectively destroy the formed biofilm, which could be attributed to the better interaction between the liposomal formulations and biofilm (Fig 1). Biofilm has a negative charge due to the presence of carboxylate groups in the extra polysaccharides matrix. We used stearylamin in our liposomal formulations to induce a positive charge, which may be the reason for the better interaction of the liposomal forms. Another reason for this result might be cholesterol as it increases the release of hydrophilic drugs. Therefore, the hydrophilicity characteristic of NAC made the use of cholesterol effective in the present study. In addition, the small size of the liposomes facilitated their entry to the target biofilm. In contrast to our hypothesis, the combination of rifampin and NAC had no synergistic or additive effects on the control of *S. epidermidis* biofilm formation (Fig 2). In a study in this regard [30], the synergistic effects of NAC in combination with tigecycline were observed on *S. epidermidis* biofilms using NAC at 20 MIC (80 mg/ml) and tigecycline at 1000MIC (1 mg/ml). Therefore, it could be assumed that rifampin is absolutely potent in the prevention of biofilm formation, and its combination with other anti-

biofilm agents leads to no significant difference in this regard. Moreover, a hydrophilic drug such as NAC may affect the rifampin profile of release, so that rifampin could not reach its maximum effect.

As is depicted in Fig 1, the liposomal formulations had a significant effect on the eradication of *S. epidermidis* biofilm, which could be due to the fact that the selected formulation had better interactions with the drug delivery system and biofilm. In addition, the concentration of the formulations played an important role in the anti-biofilm activity. In this regard, a serial dilutions of the formulations was prepared, and their anti-biofilm activity was investigated. Particularly in case of NAC, the antibiofilm effects extremely decreased at lower concentrations, while in case of rifampin, even at lower concentrations than the MIC, the agent was completely effective in the prevention of biofilm formation (Fig 2). These findings are consistent with the previous studies in this regard [9, 29]. According to our findings, incubation time significantly affected the efficacy of the liposome formulations (Fig 4). In other words, increased time exposure resulted in the improved biofilm eradication, which is in line with the previous findings in this regard [31]. As mentioned earlier, all the applied formulations in the present study (liposomal formulations and free form of drugs) were highly effective in the inhibition of biofilm formation. After biofilm formation, the power of the formulations in biofilm eradication significantly decreased, indicating that the stage of biofilm formation plays a key role in the response to anti-biofilm agents.

CONCLUSION

According to the results, liposomal rifampin with the desired physicochemical properties was the most effective formulation in the inhibition of biofilm formation, while no synergistic effect was observed between rifampin and NAC in biofilm eradication. Therefore, it could be concluded that liposome formulations are novel drug delivery systems for biofilm eradication.

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