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Diclofenac Sodium and Gentamicin Co-Encapsulated PLGA Nanoparticles: Targeting Extracellular Matrix Components to Combat Biofilm Formation in *Pseudomonas aeruginosa* PAO1

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) have received attention to be used in combination with antibiotics in antibacterial chemotherapy. However, this study is the first to explore the impact of dual encapsulation of diclofenac sodium and gentamicin within PLGA nanoparticles (DS-GEN-PLGA NPs) on inhibiting extracellular matrix components and biofilm eradication of *Pseudomonas aeruginosa* PAO1. DS-GEN-PLGA NPs were prepared using the double emulsion solvent evaporation (DESE) technique and characterized by various characterization techniques. Subsequently, the inhibition and eradication potential of DS-GEN-PLGA NPs against *P. aeruginosa* biofilm was explored. The DS-GEN-PLGA NPs are spherical and oval and 80–200 nm in diameter. DS-GEN-PLGA NPs significantly reduced biofilm formation by 76.28%, biofilm metabolic level by 69.8%, biofilm exopolysaccharide by 75.3%, alginate production by 32.56%, and eDNA release by 60.2%. The expression level of the *lasI* and *rhlI* decreased by 0.29 and 0.44 folds compared with untreated cells. This study indicates that DS-GEN-PLGA NPs have promising antibiofilm activity against *P. aeruginosa*, highlighting its potential as a novel therapeutic formulation to combat biofilm-related infections.

Keywords Antibiofilm · Extracellular DNA · Alginate · Diclofenac sodium · Quorum sensing

Introduction

Pseudomonas aeruginosa is a dominant bacterial pathogen that contributes to a variety of chronic infections by expressing various virulence traits, such as toxins, pigments, and biofilm formation. Biofilm is a three-dimensional and structured bacterial community embedded in a self-synthesized matrix of extracellular polymeric substances (EPSs) consisting of polysaccharides, nucleic acids, proteins, and lipids, which collectively establish a distinctive microenvironment within the biofilm community (Olivares et al. 2020). Biofilm formation is a crucial resistance mechanism in *P. aeruginosa*, serving as a major obstacle to drug penetration.

Three vital polysaccharides, including alginate, pellicle loci (Pel), and polysaccharide synthesis loci (Psl), are crucial for initial surface adhesion and preserving the structural stability of biofilms. They serve as a scaffold for biofilm architecture and shield cells from antibiotics and host defenses (Harimawan and Ting, 2016). Alginate is a negatively charged exopolysaccharide, consisting of β-D-mannuronic acid and α-L-guluronic acid and is associated with chronic *P. aeruginosa* infections by providing protection against phagocytosis and opsonization and facilitating biofilm maturation. Pel is a glucose-rich and cationic polymer comprised of D-mannose, D-glucose, and L-rhamnose and secreted during planktonic growth which plays a vital role in the initial phases of biofilm development through mediating attachment to surfaces and microcolony formation. Besides exopolysaccharides, extracellular DNA (eDNA) is another essential component of the biofilm

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matrix, that serves as a nutrient reservoir and contributes to improved biofilm stability. Moreover, it promotes antibiotic resistance by altering the acidity of the biofilm environment (Thi et al., 2020).

In addition to biofilm formation, numerous virulence traits of *P. aeruginosa* are regulated by the quorum sensing (QS) regulatory system. QS is a communication mechanism among bacterial cells, controlling various aspects of bacterial behavior based on cell density. The Las, Rhl, PQS, and IQS are the identified QS systems in *P. aeruginosa*, with Las and Rhl being the most significant QS systems (Flores-Percino et al., 2023). N-(3-oxododecanoyl)-l-homoserine lactone (C_{12}-HSL) and N-butanoyl-l-homoserine lactone (C_4-HSL) are signaling molecules of the Las and Rhl QS systems, which can modulate bacterial virulence through binding to their transcriptional regulators, LasR and RhlR (Feathers et al. 2022).

The rapid and widespread dissemination of antibiotic-resistant bacteria underscores the critical requirement for novel treatments. Non-steroidal anti-inflammatory drugs (NSAIDs) are pharmaceutical agents that are extensively prescribed painkiller and anti-inflammatory drugs. Furthermore, the antimicrobial activity of various NSAIDs and their inhibitory impact on biofilms and the QS system has been demonstrated in previous studies (Paes Leme and da Silva, 2021). Additionally, the enhanced antimicrobial efficacy of conventional antibiotics in combination with different NSAIDs against pathogenic bacteria, including *P. aeruginosa*, has been established (Tabatabaeifar et al., 2022). Therefore, combining NSAIDs with antibiotics may represent a novel approach to treating microbial infections. Diclofenac sodium (DS) is an NSAID, primarily used for treating painful conditions. Similar to other NSAIDs, diclofenac is linked to some gastrointestinal and renal side effects (Aycan et al., 2018). Drug encapsulation in biocompatible polymers such as poly (lactic-co-glycolic acid) (PLGA) has been introduced to reduce the adverse effects of medicines and improve their stability and efficiency (Yeh et al. 2020). Furthermore, drug encapsulation provides sustained drug release and the possibility of encapsulating drugs with different antibacterial mechanisms. Öztürk et al. (2020) reported that encapsulation efficiency of DS in PLGA NPs was in a range of 41.4–77.8% and showed up to 24 h of DS release. In our previous study, we showed an intense release of DS from PLGA NPs during the first four h followed by gradual release up to 16 h (Rostamnejad et al. 2024). Additionally, we illustrated the anti-QS and antibacterial properties of diclofenac sodium encapsulated in PLGA nanoparticles (DS-PLGA NPs) against *P. aeruginosa*. However, the combined effect of diclofenac sodium and gentamicin (GEN) on biofilm formation and extracellular matrix components in *P. aeruginosa* has not been investigated to date. Considering the role of bacterial biofilm in antibiotic resistance, the current research aims to dual encapsulation of DS and GEN in PLGA nanoparticles (DS-GEN-PLGA NPs) and characterize their effects on the characteristics of *P. aeruginosa* biofilm.

Materials and Methods

Bacterial Strain and Chemicals

P. aeruginosa PAO1 was kindly provided by Dr. A. Elikaei (Alzahra Univeriority, Iran). For long-term storage, the bacterial suspension was prepared in tryptic soy broth (TSB) medium supplemented with 20% glycerol and stored at −80 °C. Initially, the bacterial stock was inoculated into TSB and subsequently subcultured onto Mueller-Hinton agar (MHA). PLGA (50:50, P2191) and polyvinyl alcohol (PVA, 341584) were purchased from Sigma-Aldrich. Also, DS, GEN, 2,3,5-triphenyltetrazolium chloride (TTC, 108380), and carbazole were obtained from Merck (Germany). All chemicals and reagents of analytical grade were commercially available.

Synthesis of NPs

The synthesis of DS-GEN-PLGA NPS was carried out using the double emulsion solvent evaporation (DESE) technique. Initially, DS (10.24 mg/mL) and GEN (0.256 mg/mL) were dissolved in distilled water to prepare the water phase 1 (W_1). Next, PLGA (2% w/v) was dissolved in 5 mL of pure acetone to produce the organic phase (O). Then, the W_1 phase was added dropwise to the O phase, and the mixture was sonicated for 5 min using an ultrasonic water bath to produce a primary (W_1/O) emulsion. Water phase 2 (W_2) was then created by dissolving PVA (1% w/v) in 10 mL of distilled water, and the primary emulsion was added drop by drop into the W_2 phase under sonication to form a double emulsion (W_1/O/W_2). The excess organic solvents were evaporated by stirring the mixture for 18 h. The DS-GEN-PLGA NPs were collected by centrifugation at 10,000 rpm for 20 min and freeze-dried (Paswan and Saini, 2017).

Characterization of NPs

The size and morphology of the NPs were assessed through Scanning Electron Microscopy (SEM) imaging. Initially, the NPs were affixed to carbon tape and then, sputter-coated with a thin layer of gold. Subsequently, imaging was done using a Tescan Mira 2 microscope (Czech Republic) at a voltage of 15 kV. To identify functional groups, the samples were combined with potassium bromide (KBr) and

subjected to a vacuum to eliminate the moisture. The dehydrated samples were compacted into a pellet and analyzed by Fourier transform infrared (FTIR) spectroscopy (Thermo Nicolet Avatar 360, USA). To assess the hydrodynamic size of the NPs in an aqueous medium, the NPs were dispersed in water, and the hydrodynamic radius was measured using a Dynamic Light Scattering (DLS) analyzer at a scattering angle of 90°. Furthermore, the surface charge of the NPs was evaluated using a Horiba SZ-100Z Nanoparticle analyzer (Horiba Jobin Yovin, Japan).

Minimum Inhibitory Concentration (MIC)

The antimicrobial activity of DS-PLGA NPs, GEN-PLGA NPs, and DS-GEN-PLGA NPs against *P. aeruginosa* PAO1 was assessed by determining their minimal inhibitory concentration (MIC) using the broth micro-dilution technique. In brief, bacterial cells (1.5×10^6 CFU/mL) were treated with various concentrations of the NPs in a 96-well microtiter plate. Mueller-Hinton broth (MHB) served as the negative control, while the bacterial suspension was used as the positive control. The plates were incubated at 37 °C for 24 h. Finally, 20 µL of TTC dye (0.5% w/v) was added to the wells, and the MIC values were determined as the lowest concentration of the NPs, where no color change occurred after a 1-hour incubation (CLSI, 2021).

Cytocompatibility Assay

To assess the cytocompatibility of DS-GEN-PLGA NPs, a hemolysis assay was conducted according to the method applied by Kahbasi et al. with some modifications (Kahbasi et al. 2019). Sheep red blood cells (RBCs) (Darvash Co., Iran) were washed with normal saline, and the RBCs were diluted in normal saline to prepare a 2% RBC solution. Subsequently, 1 mL of varying concentrations (1∕2, 1∕4, 1∕8, 1∕16 MIC) of DS-GEN-PLGA NPs were added to 1 mL of the RBC solution and incubated at 37 °C with agitation for 2 h. The samples were centrifuged at 3000 rpm for 15 min, and the optical density of the resulting supernatant was measured at 430 nm (Unico 2100 spectrophotometer, USA). Normal saline and distilled water were used as the negative and positive controls, respectively. The hemolysis ratio (%) was calculated using the following formula: HR (%) = (A sample − A negative)/(A positive − A negative) × 100%.

(A referred to the absorbance)

Qualitative Biofilm Formation

Qualitative analysis of biofilm formation by *P. aeruginosa* was performed following the prior description (Deka, 2014). Briefly, a loopful of colonies from an overnight culture was inoculated into test tubes containing Luria-Bertani (LB) broth with or without NPs at ½ MIC. The tubes were then incubated at 37 °C for 48 h without agitation. After incubation, the contents of the tubes were carefully aspirated and slightly rinsed three times with distilled water. Subsequently, the tubes were left to air dry, and the attached cells were stained with crystal violet at room temperature for 5 min. After staining, the excess crystal violet was decanted, and the tubes were washed with distilled water. Finally, the tubes were inverted and allowed to dry, and the presence of a violet ring was examined by the naked eye.

Biofilm Inhibition Assay

The biofilm inhibitory effect of the NPs on *P. aeruginosa* PAO1 was quantified by the crystal violet staining method (Haney et al., 2021). Initially, gradient concentrations (10X of the 1/2, 1/4, 1/8, 1/16, 1/32 MIC) of DS-PLGA NPs, GEN-PLGA NPs, and DS-GEN-PLGA NPs were prepared in 96-well plates with 10 µL per well. Then, 90 µL of bacterial cells ($OD_{600} = 0.01$) were added to each well. Untreated bacteria were used as controls. After 48 h of incubation under stagnant conditions at 37 °C, the medium was discarded, and the wells were gently rinsed three times with distilled water. Subsequently, the adhered biomass in each well was stained with 105 µL of 0.1% crystal violet and incubated for 30 min at room temperature with gentle agitation. After incubation, the dye was discarded, and the plate was washed three times with distilled water to eliminate any unbound stain. Finally, the bound dye was solubilized with 110 µL of 30% acetic acid for 30 min, and the optical density was measured at 595 nm.

Quantification of Biofilm Metabolic Activity

Metabolic activity measurement was performed in 96-well plates using TTC dye. Initially, 100 µL of bacterial suspension in LB broth ($OD_{600} = 0.01$) was added to each well, followed by incubation at 37 °C for 48 h. Afterward, the wells containing non-adherent cells were aspirated, and the plate was gently rinsed with 150 µL of sterile medium. The adhered biofilm was then treated with different concentrations (1/2, 1/4, 1/8, 1/16, 1/32 MIC) of DS-PLGA NPs, GEN-PLGA NPs, and DS-GEN-PLGA NPs. Then, 2 µL of a 5% (wt/vol) sterile TTC solution was added to each well, and the plate was incubated at 37 °C, overnight. After discarding the growth medium by gently inverting the plate, the wells were washed three times with distilled water. Finally, 205 µL of DMSO was added to each well, and the absorbance was measured at 500 nm (Haney et al. 2021).

Biofilm Eradication Level

The eradication efficiency of DS-PLGA NPs, GEN-PLGA NPs, and DS-GEN-PLGA NPs on *P. aeruginosa* PAO1 biofilms was investigated using colony counting. Initially, *P. aeruginosa* PAO1 biofilm was formed in a 96-well microtiter plate by inoculating 100 µL of bacterial cells (OD_{600} nm = 0.01) for 48 h. Next, free-floating planktonic cells were aspirated, and the wells were filled with ½ MIC of DS-PLGA NPs, GEN-PLGA NPs, and DS-GEN-PLGA NPs and incubated at 37 °C overnight. Then, the wells were aspirated, and adherent cells were washed with 200 µL of fresh LB broth. The adhered biofilm was scraped with a sterile cotton swab and resuspended in 1 mL of LB broth. Cell suspensions were diluted up to 10^{-4} dilutions, and 10 µL of diluted suspensions were inoculated on nutrient agar plates. The plates were incubated at 37 °C overnight, and the number of colonies was counted to calculate CFUs (Haney et al. 2021).

Quantification of Total Exopolysaccharide

The effect of synthesized NPs on exopolysaccharide production was assessed using total polysaccharide quantification. In summary, *P. aeruginosa* was inoculated in LB broth with and without ½ MIC of DS-PLGA NPs, GEN-PLGA NPs, and DS-GEN PLGA NPs. After overnight incubation at 37 °C, bacterial cultures were centrifuged, and pellets were suspended using PBS. Then, 1 mL of the exopolysaccharide solution was mixed with 1 mL of chilled 5% phenol and five volumes of concentrated sulfuric acid. Following a one-hour incubation in the dark, the optical density of the resulting red solution was assessed at 490 nm (Rashmi et al., 2018).

Alginate Assay

The effect of synthesized NPs on alginate production by *P. aeruginosa* PAO1 was investigated using the carbazole-sulfuric acid method. Initially, *P. aeruginosa* was cultured in LB broth supplemented with or without ½ MIC of DS-PLGA NPs, GEN-PLGA NPs, and DS-GEN PLGA NPs, then incubated for 24 h at 37 °C. Following this, 70 µL of treated and untreated cultures were mixed with 600 µL of boric acid (2.5 M) and concentrated sulfuric acid solution (1:40) in an ice bath. The mixture was vortexed for 10 s, and 20 µL of 0.2% carbazole solution in ethanol was added to the mixture and vortexed for another 10 s. The mixture was then incubated in a water bath at 55ºC for 30 min, after which the optical density was measured at 530 nm (Knutson and Jeanes, 1968).

eDNA Assay

eDNA plays an important role in the composition of *P. aeruginosa* biofilm. The impact of DS-PLGA NPs, GEN-PLGA NPs, and DS-GEN PLGA NPs on the eDNA level was assessed through agarose gel electrophoresis and spectrophotometry assays. Initially, *P. aeruginosa* biofilm was allowed to form with or without the presence of the NPs. Afterward, the biofilm was harvested from the well, and eDNA extraction was carried out using the Cinnagen DNA extraction kit. Subsequently, the obtained DNA was loaded onto a 1% agarose gel and visualized using a gel documentation system. Also, DNA concentration was quantified by nanodrop spectrophotometry.

Pellicle Formation Assay

The pellicle formation assay was performed following the method outlined by Friedman and Kolter (2004). In brief, *P. aeruginosa* was cultured in LB broth supplemented with ½ MIC NPs at 37 °C for 48 h under stagnant conditions. Subsequently, the pellicles formed at the air-liquid interface in both NP-treated and control groups were observed visually. For quantitative analysis, 1 mL of ethyl alcohol was added beneath each pellicle layer. The floating pellicles were resuspended in PBS, and their absorbance was measured at OD_{600} nm.

Expression of *rhlI* and *rhlR* Genes

Real-Time PCR was used to investigate the effect of the NPs on the relative expression of the *rhlI* and *lasI* genes in *P. aeruginosa*. Initially, *P. aeruginosa* was cultured in LB broth containing ½ MIC of NPs in a 37 °C shaker incubator for 16 h. Bacterial cells of the control group were grown in the absence of NPs. Subsequently, bacterial cultures were centrifuged at 4000 g for 5 min, and resulting cell pellets underwent total RNA extraction using the TriZol reagent (Thermo Fisher Scientific, USA), according to the manual. The quality and purity of the extracted RNA were assessed using gel electrophoresis and a NanoDrop spectrophotometer. To eliminate DNA impurities, the extracted RNA was treated with DNase I, and cDNA synthesis was performed using the Sinnaclon First Strand cDNA Synthesis Kit. RT-PCR was conducted using the LightCycler 96 real-time PCR system (Roche, Switzerland). The characteristics of the gene-specific primers are described in Table 1. The *rpsL* gene was employed as an internal reference gene for data normalization, and the relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 1 The list of the primers that were used in this work

Primer	Sequence (5′-3′)
rhlI-forward	CAAACCCGCTACATCGTCG
rhlI-reverse	GTTTCGCTGCACAGGTAGG
lasI-forward	GGCTGGGACGTTAGTGTCAT
lasI-reverse	AAAACCTGGGCTTCAGGAGT
rpsL-forward	AAGCGCATGGTCGACAAGA
rpsL-reverse	TGTGCTCTTGCAGGTTGTGA

Statistical Analysis

Differences between the control and treatment groups were assessed by one-way analysis of variance (ANOVA) using GraphPad Prism 9 software. p values < 0.05 were considered significant.

Results

Characterization of NPs

SEM was employed to examine the morphology of the produced DS-GEN-PLGA NPs. As illustrated in Fig. 1a, the DS-GEN-PLGA NPs are spherical and oval and have a size range of 80 to 200 nm. The hydrodynamic size and surface charge of the NPs were determined through DLS and zeta potential analyses, respectively. The average hydrodynamic diameter of the NPs was 426.6 nm (Fig. 1b), while the zeta potential value of the NPs was −4.2 mV(Fig. 1c). Since DLS analysis measures particle dimensions in an aqueous medium, the particles appear larger than in the microscopic analysis, due to water absorption. As shown in the FT-IR
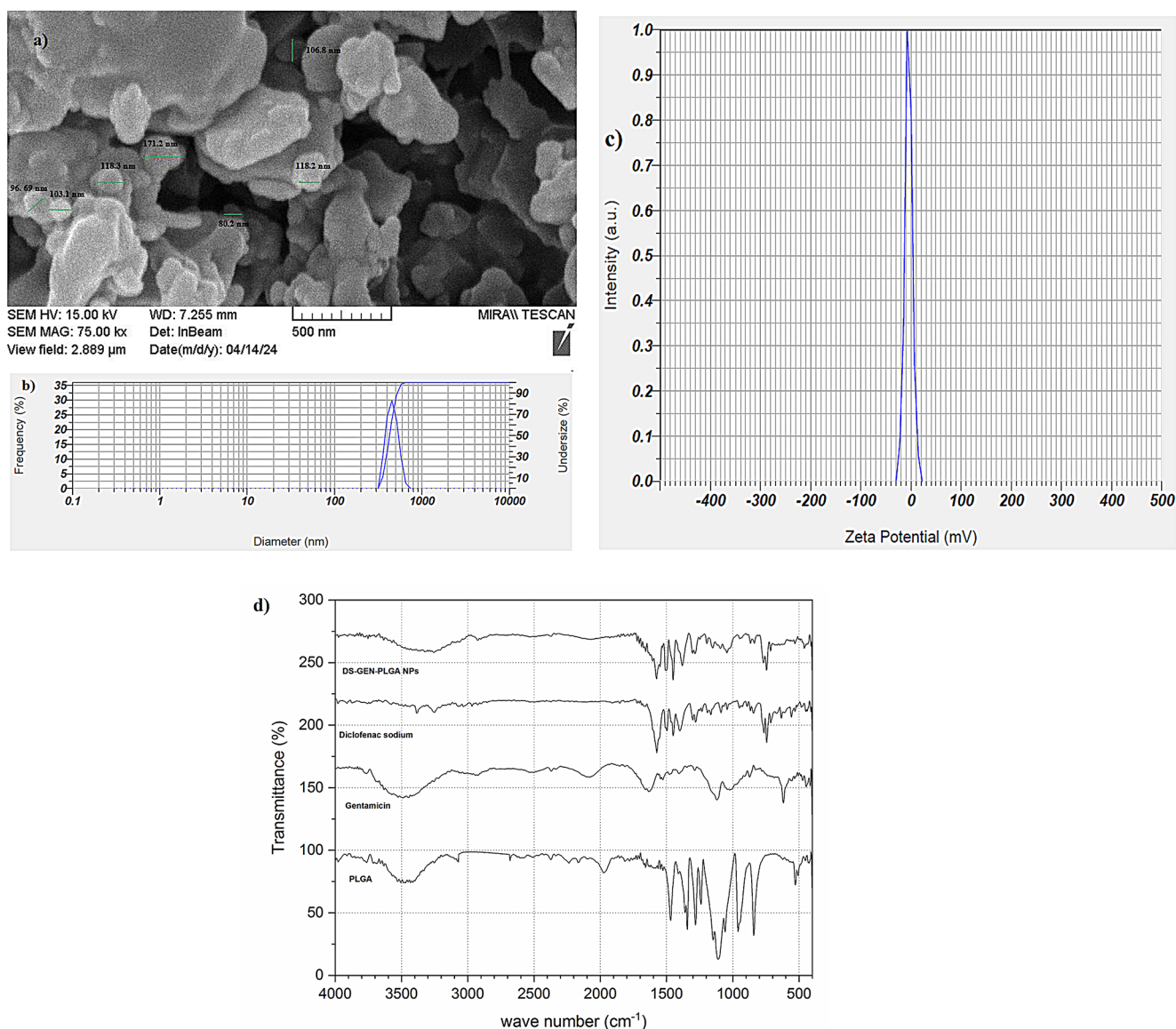

Fig. 1 Characteristics **of** DS-GEN-PLGA (**a**) SEM, (**b**) DLS, (**c**) Zeta, (**d**) FT-IR analyses

Fig. 2 The hemolysis images of different concentrations of DS-GEN-PLGA (1–4: equivalent to 1⁄2, 1⁄4, 1⁄8, and 1⁄16 MIC for *P. aeruginosa* PAO1, NS: normal saline)
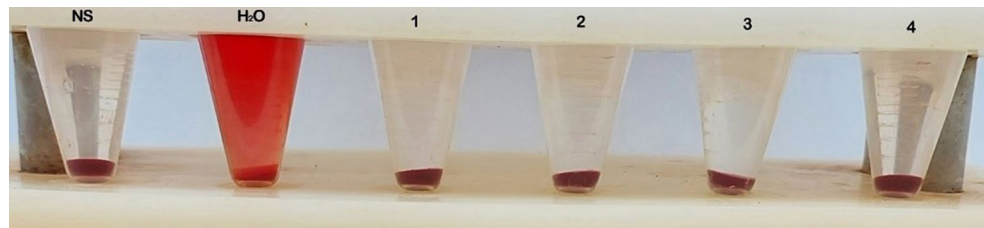
Fig. 3 Minimum inhibitory concentration determination of DS-PLGA NPs, GEN-PLGA NPs, and DS-GEN-PLGA NPs against *P. aeruginosa* PAO1. −ve Ctrl: negative control (Mueller-Hinton broth); +ve Ctrl: positive control (bacterial suspension)
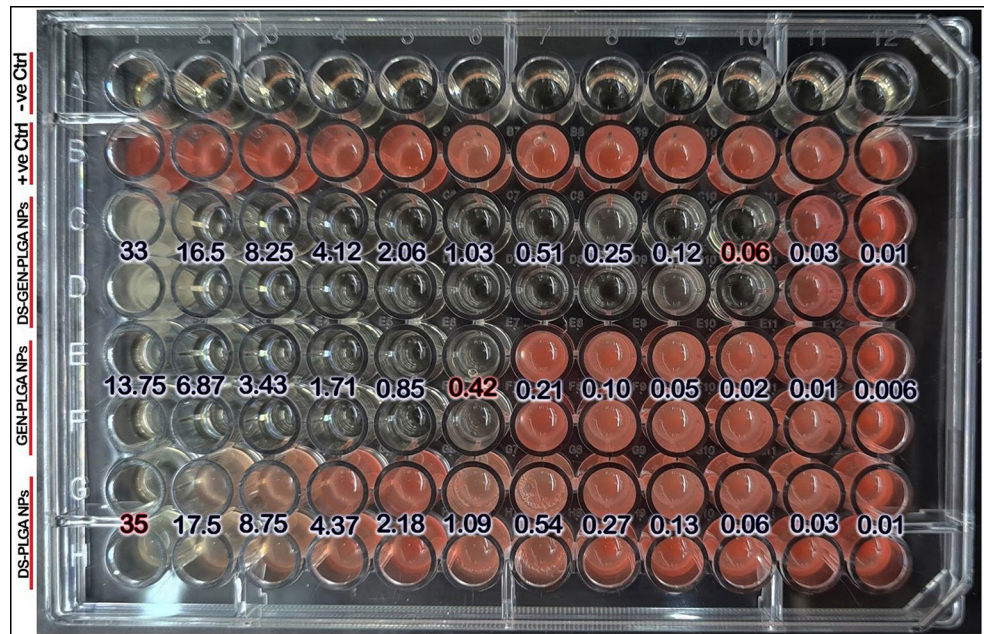
spectrum of DS (Fig. 1d), the band at 3382.91 cm^{-1} represents N–H stretching of the secondary amine groups, and the band at 1573.81 cm^{-1} represents the presence of the –C=O stretching of the carboxyl ion. Also, the C=C ring stretching and C–Cl stretching were observed at 1556.45 cm^{-1} and 744.47 cm^{-1}, respectively. In the FT-IR spectra of GEN, the bands at 1627.81 cm^{-1} and 1527.52 cm^{-1} are assigned to the N-H bending vibrations of aromatic amine. The band at 1120.56 cm^{-1} is attributed to the HSO$_4$$^{-1}$ vibrational. The bands at 1037.63 cm^{-1} and 619.11 cm^{-1} correspond to S-O bending and S–O stretch, respectively. The FT-IR spectrum of PLGA reveals some peaks at 3300 to 3500 cm^{-1} that contribute to –OH stretching vibrations. The C-O stretching bands are observed at about 1100, 1290, and 1320 cm^{-1}. The band observed at about 1480 cm^{-1} contributes to the O-H bending. Considering the FT-IR spectrum of DS-GEN-PLGA NPs, the bands related to GEN, DS, and PLGA can be observed.

Cytocompatibility of DS-GEN-PLGA NPs

Figure 2 presents the hemolysis level caused by DS-GEN-PLGA NPs. As illustrated, sub-MICs of DS-GEN-PLGA NPs did not induce remarkable hemolysis compared to the

positive control, with a hemolysis rate of less than 3%. Consequently, the synthesized NPs demonstrate favorable biocompatibility and are non-toxic.

Growth Inhibitory Effect of Synythesized NPs

The MIC of the synthesized NPs against *P. aeruginosa* PAO1 was quantified using the broth microdilution method (Fig. 3). Based on the results, the growth of *P. aeruginosa* was inhibited at 35 mg/mL and 0.42 mg/mL of DS-PLGA NPs and GEN-PLGA NPs, respectively, while the MIC value of DS-GEN-PLGA NPs against *P. aeruginosa* was determined to be 0.06 mg/mL. DS may have facilitated the entry of GEN into the cell by increasing membrane permeability, leading to the lowest MIC value in the dual-encapsulated NPs.

Qualitative Assessment of Biofilm Formation

The tube method was used for the qualitative evaluation of biofilm formation by *P. aeruginosa*. As depicted in Fig. 4, DS-PLGA NPs, GEN-PLGA NPs, and DS-GEN-PLGA NPs reduced the visible film on the tube walls compared to the control. However, the ring formed in the DS-GEN-PLGA

Fig. 4 Qualitative evaluation of biofilm formation in *P. aeruginosa* PAO1 using tube method (1: Untreated, 2: DS-PLGA NPs, 3: GEN-PLGA NPs, and 4: DS-GEN-PLGA NPs treated groups)
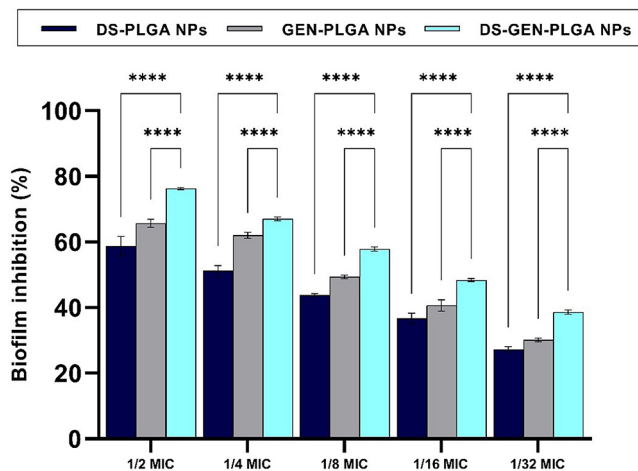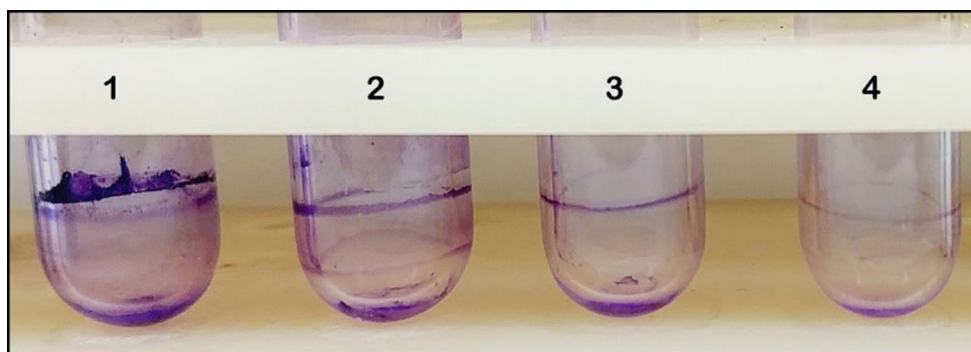
Fig. 5 Quantitative assesment of biofilm-inhibition by DS-PLGA NPs, GEN-PLGA NPs, and DS-GEN-PLGA NPs against *P. aeruginosa*

NPs treated group showed a significant reduction compared to the untreated group.

Biofilm Inhibition

The inhibitory effect of DS-PLGA NPs, GEN-PLGA NPs, and DS-GEN-PLGA NPs at sub-MICs on *P. aeruginosa* biofilm formation was examined using crystal violet staining. The results revealed a dose-dependent pattern in the inhibitory potential of the NPs. The highest biofilm inhibition of 76.28% was achieved by DS-GEN-PLGA NPs at a ½ MIC concentration. At this concentration, GEN-PLGA NPs and DS-PLGA NPs demonstrated biofilm inhibition of 65.69% and 58.79%, respectively. Our findings indicate that GEN amplifies the anti-biofilm activity of DS. The percentage of biofilm inhibition by varying concentrations of NPs is depicted in Fig. 5.

Biofilm Eradication and Metabolic Level

Eliminating mature biofilms is crucial in managing bacterial infections. Hence, the effect of synthesized NPs on biofilm metabolism and eradication level of *P. aeruginosa*

PAO1 biofilm was assessed using TTC staining and colony counting methods. Based on the TTC assay, the minimum metabolic activity was documented to be 69.23%, 40.74%, and 30.2% at ½ MIC of DS-PLGA NPs, GEN-PLGA NPs, and DS-GEN-PLGA NPs, respectively (Fig. 6a). Additionally, colony counting assay revealed that at ½ MIC of DS-PLGA NPs, GEN-PLGA NPs, and DS-GEN-PLGA NPs, viable biofilm cells reduced to 6.79, 5.76, and 5.38 log CFU, respectively (Fig. 6b).

Quantification of Exopolysaccharides

Exopolysaccharides, an essential component of biofilms, play a crucial role in cell adhesion and biofilm formation. Our findings demonstrated that exposure of bacterial cells to synthesized NPs reduced exopolysaccharide release by *P. aeruginosa* (Fig. 7a). Exopolysaccharide production was inhibited by 12.68% and 39.86% in the DS-PLGA NPs and GEN-PLGA NPs treated groups, respectively, compared to the untreated group. Additionally, the highest reduction in exopolysaccharide production was observed with DS-GEN-PLGA NPs, with an inhibition percentage of 75.30%.

Alginate Assay

Based on the carbazole assay, DS-PLGA NPs caused a slight decrease in alginate production (8.71%), while GEN-PLGA NPs and DS-GEN-PLGA NPs decreased alginate production by 23.17% and 32.56%, respectively (Fig. 7b).

eDNA Assay

Considering the crucial function of eDNA in the formation of biofilms, we examined the eDNA levels in formed biofilms in the presence or absence of synthesized NPs. Analysis via agarose gel electrophoresis revealed a decrease in band intensity in samples treated with NPs compared to the untreated group. Additionally, quantitative measurement of eDNA using a nanodrop revealed that the concentration of eDNA in the biofilms of the NP-treated groups is

Fig. 6 Biofilm-eradication effect of DS-PLGA NPs, GEN-PLGA NPs, and DS-GEN-PLGA NPs on *P. aeruginosa*. (**a**) Metabolic activity, (**b**) Log CFU graph (left), colony counting plates (right). $*p \leq 0.05$, $***p \leq 0.001$, and $****p \leq 0.0001$ were considered as statistically significant
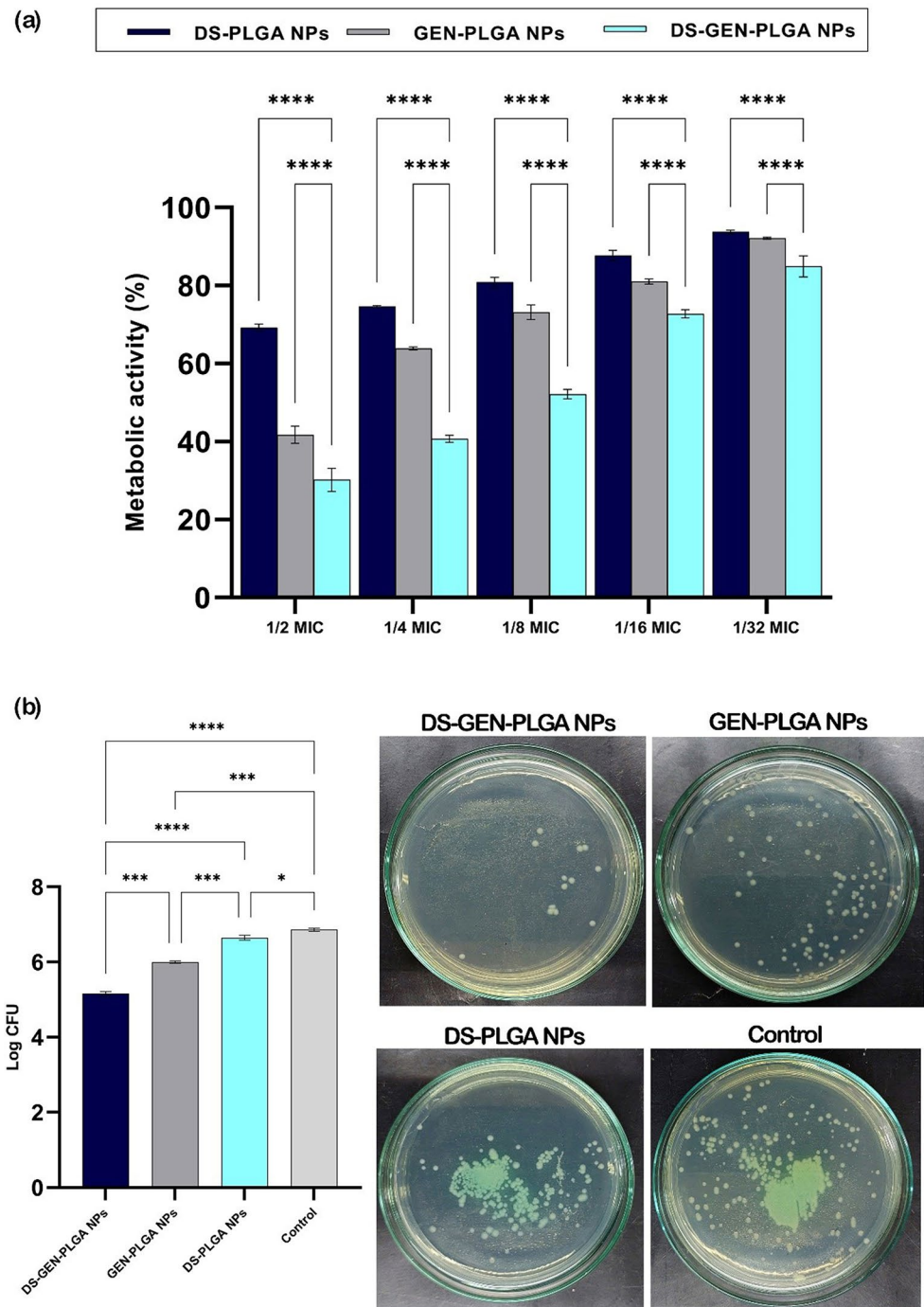
significantly lower than that of the control group (Fig. 7c and supplementary Fig. 1).

Pellicle Formation Assay

Pellicles are biofilms that are formed at the interface between air and liquid. As depicted in Fig. 8, DS-PLGA caused a negligible reduction in the pellicle formation of *P. aeruginosa*. Conversely, GEN-PLGA NPs and DS-GEN-PLGA NPs displayed a robust inhibitory effect on pellicle formation. Based on quantitative measurement, the amount of pellicle formation decreased by 19.74% in the DS-PLGA treated group compared to the control group. In comparison, GEN-PLGA NPs and DS-GEN-PLGA NPs inhibited pellicle formation by 68.44% and 86.52%, respectively.

Fig. 7 Inhibition of *P. aeruginosa* EPSs by DS-PLGA NPs, GEN-PLGA NPs, and DS-GEN-PLGA NPs at ½ MIC. Quantitative analysis (left) and qualitative observation (right) of exopolysaccharide (**a**), alginate (**b**), and eDNA (**c**). (M: Ladder, 1: Untreated, 2: DS-PLGA NPs, 3: GEN-PLGA NPs, and 4: DS-GEN-PLGA NPs treated groups). $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$ were considered as statistically significant, whereas ns is non-significance
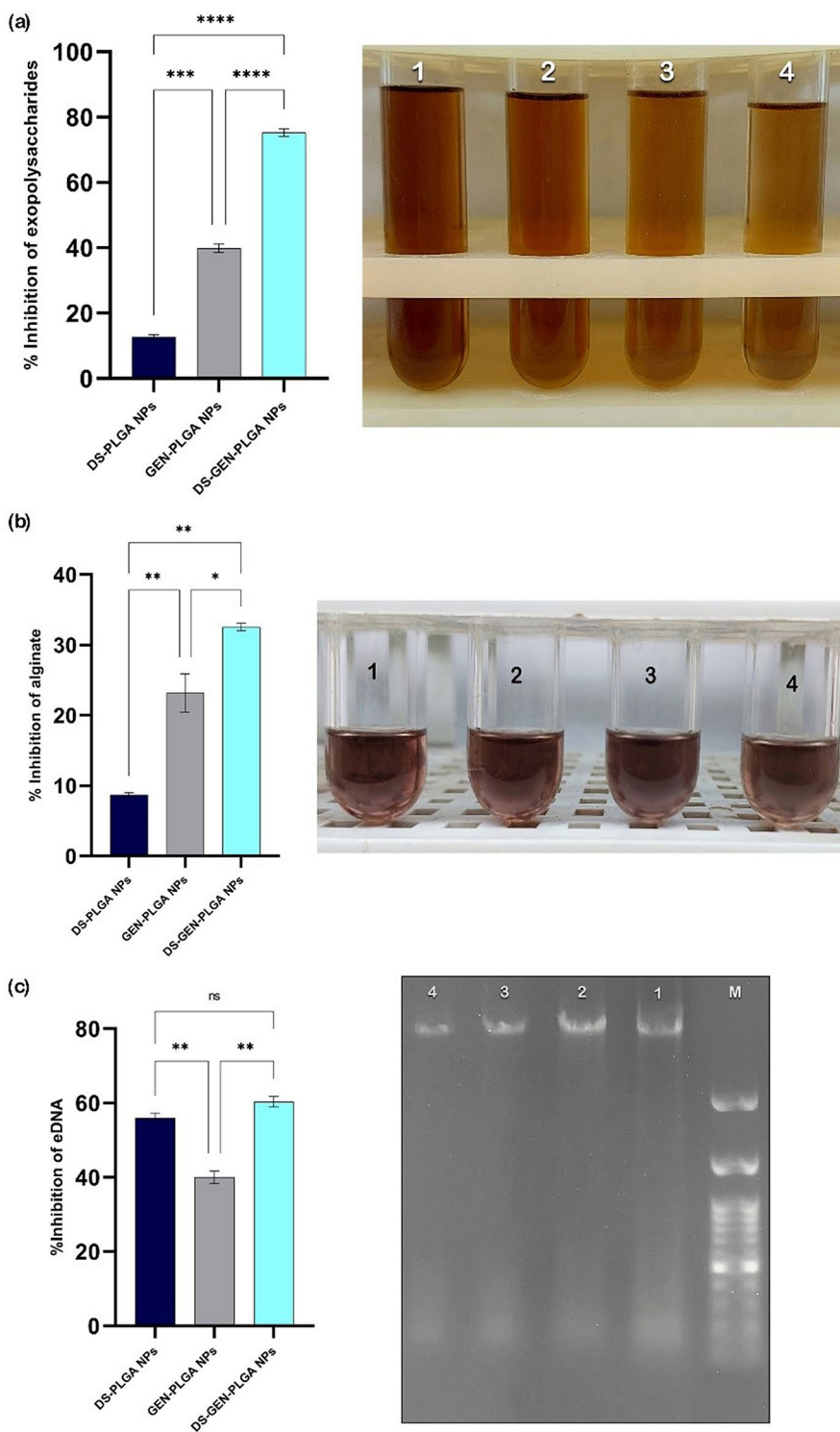

Gene Expression Assay

Real-time PCR was employed to examine the impact of NPs on the mRNA level of the *rhlI* and *lasI* genes, the genes associated with the QS autoinducer synthesis. The expression of the *rhlI* in *P. aeruginosa* reduced by 0.33, 0.03, and 0.29 folds after treatment with DS-PLGA NPs, GEN-PLGA NPs, and DS-GEN-PLGA NPs, respectively. According to the results, DS-PLGA NPs, and DS-GEN-PLGA NPs caused a significant reduction of the *rhlI* gene compared with untreated cells. Treatment of *P. aeruginosa* with DS-PLGA NPs, GEN-PLGA NPs, and DS-GEN-PLGA NPs resulted in a reduction of the *lasI* gene by 0.34, 0.19, and

Fig. 8 Inhibition of *P. aeruginosa* PAO1 pellicle formation by DS-PLGA NPs, GEN-PLGA NPs, and DS-GEN-PLGA NPs at ½ MIC. Bar graphs represent the percentage of pellicle inhibition (left), Tubes illustrate pellicle formation (right). (1: Untreated, 2: DS-PLGA NPs, 3: GEN-PLGA NPs, and 4: DS-GEN-PLGA NPs treated groups). $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$ were considered as statistically significant
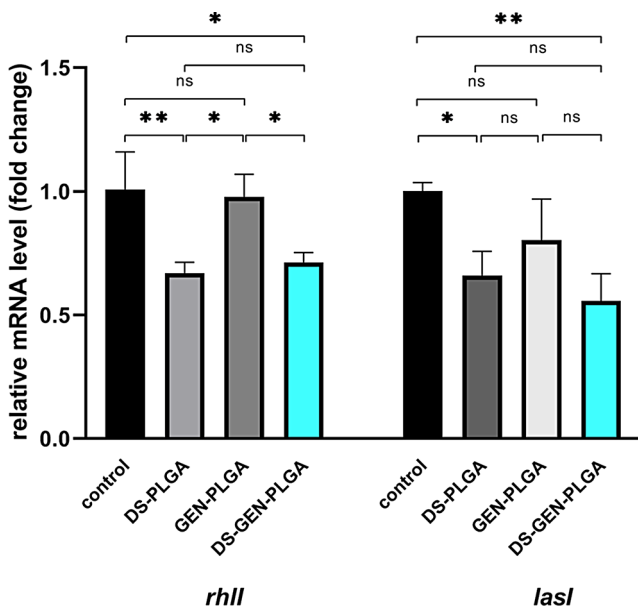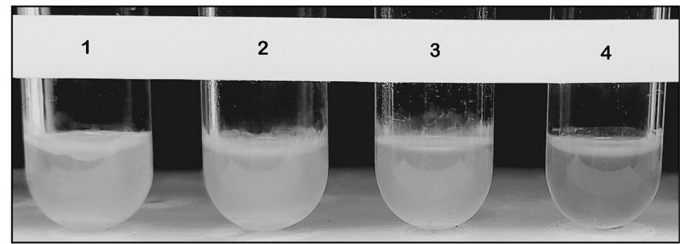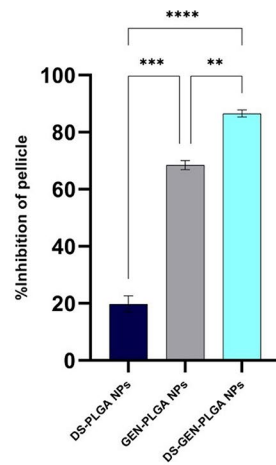
Fig. 9 Relative mRNA level of the *rhlI* and *lasI* genes in *P. aeruginosa* PAO1 treated with DS-PLGA NPs, GEN-PLGA NPs, and DS-GEN-PLGA NPs at ½ MIC. $*p \leq 0.05$, and $**p \leq 0.01$ were considered as statistically significant. ns: non-significant

0.44 folds, respectively. Similarly, DS-PLGA NPs and DS-GEN-PLGA NPs caused a significant attenuation of the *lasI* compared with the control group (Fig. 9). Since the expression of *rhlI* and *lasI* genes in the GEN-PLGA NPs-treated group was not significantly different from the control group, it can be concluded that the DS, that is present in the DS-GEN-PLGA NPs, decreased the expression levels of these genes.

Discussion

P. aeruginosa is a frequent cause of nosocomial infections in patients with immunocompromised immune system (Haque et al. 2018). Biofilm formation by microbial pathogens like

P. aeruginosa contributes to the failure of antibiotic treatments. EPSs are major barriers to drug penetration, leading to insufficient drug concentrations for antimicrobial effects. Biofilm formation relies on a sophisticated intracellular communication network called QS (Maurice et al., 2018). In *P. aeruginosa*, LasI-LasR, RhlI-RhlR, and PQS-MvfR are the main QS systems that secrete signaling molecules termed autoinducers in response to increasing cell density. The interaction between autoinducers and their receptor proteins leads to the regulation of various virulence traits, including biofilm formation (Liao et al., 2022). Hence, biofilm and QS systems are promising targets for drug development to combat bacterial infections.

Evaluation of the cytocompatibility of DS-GEN-PLGA NPs showed no considerable hemolytic activity, compared with positive control. This suggests favorable compatibility of the DS-GEN-PLGA NPs as a hemolysis rate of less than 5% is considered non-toxic and safe (Malehmir et al., 2023).

The anti-biofilm and anti-QS effects of PLGA NPs loaded with non-steroidal anti-inflammatory drugs have been established in prior research. Accordingly, in this study, we conducted the simultaneous encapsulation of DS and GEN in PLGA NPs to combat biofilm formation and interfere with the QS system of *P. aeruginosa*. The antimicrobial activity of the synthesized NPs against *P. aeruginosa* was assessed using the MIC assay. Based on the results, the MIC value of DS-GEN-PLGA NPs was several folds lower than those of DS-PLGA NPs and GEN-PLGA NPs, indicating an increase in the antimicrobial activity of the dual-encapsulated NPs. The enhanced anti-bacterial effect of the DS-GEN-PLGA NPs can be attributed to the simultaneous antibacterial effects of DS and GEN. Previous research has indicated that NSAIDs can increase bacterial membrane permeability (Luchnikova et al., 2022). According to this finding, it could be hypothesized that in our current investigation, DS within DS-GEN-PLGA NPs may have heightened membrane permeability, consequently facilitating increased penetration of

GEN into bacterial cells, resulting in amplified antimicrobial effects.

The synthesized NPs demonstrated a concentration-dependent inhibitory potential against biofilm formation, with the highest anti-biofilm effect observed at ½ MIC concentration. At this concentration, DS-PLGA NPs and GEN-PLGA NPs inhibited biofilm formation by 58.79% and 65.69%, respectively, while DS-GEN-PLGA NPs exhibited the highest inhibition (76.28%). It has been reported that DS could interacts with QS system components in *P. aeruginosa*, resulting in the attenuation of bacterial virulence traits, such as bacterial colonization and biofilm formation, that are in agreement with this study (Ulusoy and Bosgelmez-Tinaz, 2013; Soheili et al., 2015). Moreover, encapsulation of DS and GEN increases their surface-to-volume ratio, which leads to greater interaction with bacterial surfaces compared to the non-encapsulated form. This increased interaction is associated with cell damage, cytoplasmic leakage, and biofilm inhibition.

Additionally, to elucidate the anti-biofilm mechanism of the synthesized NPs, we examined the production level of crucial biofilm components like extracellular polysaccharides and eDNA. Pel is an extracellular matrix polysaccharide that assists in the formation of pellicle biofilms. The structure formed at the air-liquid interface in a static bacterial culture is called a pellicle (Friedman and Kolter 2004). In this study, the synthesized NPs considerably reduced pellicle production. The decrease in the pellicle formation by *P. aeruginosa* following treatment with DS-GEN-PLGA NPs might be due to the down-regulation of *pel* genes by GEN. This hypothesis was supported by a previous study that showed that sub-MICs of GEN decrease the expression of the *pelA* gene, the gene involved in the synthesis of the Pel polysaccharide (Davarzani et al. 2021). In addition, in the study conducted by Rastgar et al., ibuprofen, an NSAID, notably reduced the expression of pivotal genes associated with the synthesis and development of Pel polysaccharide (*pelD* and *pelF*) in clinical strains of *P. aeruginosa* (Rastgar et al. 2022). Given the role of the pellicle in the early steps of biofilm formation, the reduction in this polysaccharide could be a contributing factor to biofilm inhibition in the present study. Alginate is another matrix polysaccharide that serves as an adhesin by adhering to mucin in the respiratory tract. Its acetyl groups elevate viscosity, trapping water and nutrients within biofilms. Furthermore, alginate protects *P. aeruginosa* from phagocytosis and oxidative stress, bolstering its endurance. Moreover, it binds to aminoglycoside antibiotics, impeding their penetration into biofilms, thus exacerbating antibiotic resistance (Mann and Wozniak 2012; Strateva and Mitov 2011). In our study, DS-GEN-PLGA NPs showed a significant reduction in the production of alginate by *P. aeruginosa* PAO1. This result is in line with the findings of Davarzani et al., where GEN was found to decrease alginate production in *P. aeruginosa*. Additionally, their research indicated a notable decrease in the expression of genes responsible for alginate production (*algD* and *algU*) (Davarzani et al., 2021). Similarly, Khodaparast et al. demonstrated that ibuprofen alone and in combination with ciprofloxacin caused a significant decrease in the expression of the *alg44* gene in *P. aeruginosa* (Khodaparast et al. 2022). Based on these findings, the reduction in alginate levels, observed in our study, may be attributed to the inhibitory effect of DS-GEN-PLGA NPs on the expression of genes within the alg operon. Restricting the production of essential matrix components hampers biofilm formation and consequently lowers bacterial resistance to antimicrobial agents.

A large number of biofilm-related genes are regulated by the LasR/LasI and RhlR/RhlI systems. Pel and Psl polysaccharides are crucial components of the matrix that contribute to the formation and maintenance of biofilm structure (Colvin et al. 2012). Transcription of the *pel* gene cluster is necessary to synthesize extracellular exopolysaccharides in *P. aeruginosa* (Sakuragi and Kolter, 2007). The LasR/LasI system positively regulates the expression of *pel* and *psl*, playing an important role in biofilm formation. In contrast, the RhlR/RhlI system is involved in the maturation stage of biofilm development (Wang et al. 2023). In addition, eDNA is an essential component of EPSs that plays a crucial role in bacterial accumulation, biofilm initiation, and development through its interaction with extracellular calcium. The PQS system is involved in biofilm lysis and the release of eDNA (Allesen-Holm et al., 2006). According to the literature, the *las*, *rhl*, and *pqs* mutants form unstable and thin biofilms with less eDNA compared to wild-type *P. aeruginosa* (Wang et al. 2023). Hence, the biofilm inhibition caused by DS-GEN-PLGA NPs might stem from the attenuation of the Las and Rhl QS systems, leading to decreased production of essential biofilm components like exopolysaccharides and eDNA.

In this study, the sub-inhibitory level of DS-GEN-PLGA NPs notably decreased the expression of the *rhlI* and *lasI* genes in *P. aeruginosa*. Previous works have demonstrated the anti-QS potential of NSAIDs, including DS (Tajani et al., 2021; Rostamnejad et al., 2024). One possible mechanism by which NSAIDs inhibit the QS system is their interaction with the active sites of QS molecules, resulting in the disruption of bacterial QS. Supporting this hypothesis, molecular docking studies have demonstrated that some NSAIDs, such as ibuprofen (Dai et al., 2019), naproxen (Esnaashari et al., 2023), and ketoprofen (Mirpour and Zahmatkesh, 2024), effectively bind to the active sites of Las and Rhl proteins. Given the mentioned instances and the fact that GEN-PLGA NPs did not significantly alter the expression of *lasI* and *rhlI*

genes, it can be concluded that the DS in the synthesized NPs is responsible for attenuating the QS system.

The alteration in the activity of efflux pumps may be another mechanism by which DS-GEN-PLGA NPs inhibit the QS system. Besides expelling antimicrobial agents from bacterial cytoplasm, efflux pumps also participate in transporting autoinducer molecules and facilitating cell communication. The preceding studies have indicated that numerous NSAIDs act as substrates for the Mex efflux pumps in *P. aeruginosa* (Laudy et al. 2016). Based on this, DS, present in DS-GEN-PLGA NPs, may inhibit the extrusion of QS signaling molecules, thereby disrupting cell-to-cell signaling (Evans et al. 1998).

Attenuating the *rhlI* and *lasI* genes, which are responsible for the synthesis of QS autoinducers, reveals the anti-QS effects of DS-GEN-PLGA NPs. Various virulence traits are regulated by the Las and Rhl QS systems in *P. aeruginosa*, such as exotoxin production, biofilm formation, motility, pyocyanin level, and proteolytic activity (Strateva and Mitov, 2011). Hence, in addition to biofilm inhibition, the disruption of the Las and Rhl QS systems by DS-GEN-PLGA NPs may result in the attenuation of bacterial virulence.

The dense matrix and persistent cells in biofilms render them more resistant than regular cells, forming a barrier against antimicrobial agents. Therefore, the eradication of biofilms is crucial for treating biofilm-associated infections. To assess the effectiveness of the NPs in eradicating biofilms, the 48-hour biofilm was exposed to the same sub-MIC concentrations used in the biofilm inhibition assay. According to the findings, the eradication of biofilm by GEN-PLGA NPS and DS-GEN-PLGA NPs was more significant than DS-PLGA NPs.

Since in crystal violet staining, both live and dead cells are stained, we used TTC to assess biofilm metabolic activity. TTC is a tetrazolium-based dye that is converted into colored formazan due to reduction by cellular NADH (Sabaeifard et al., 2014). In this study, treatment of mature biofilms with DS-GEN-PLGA NPs significantly decreased the metabolic activity of biofilm cells, indicating a reduction in the number of living cells within the biofilm. We also demonstrated a decrease in viable cells within a biofilm following treatment with DS-GEN-PLGA NPs. The reduction in metabolic activity, consequent to the decrease in the number of viable cells within the mature biofilm post-exposure to DS-GEN-PLGA NPs, represents a noteworthy discovery, leading to a decline in bacterial virulence (Richardson, 2019).

Conclusion

In this study, DS-GEN-PLGA NPs were synthesized using the DESE method, resulting in particles sized 80–200 nm with a zeta potential of -4.2 mV. The DS-GEN-PLGA NPs demonstrated a considerable biofilm inhibition rate of 76.28%. Additionally, the dual-encapsulated NPs resulted in a reduction of 69.23% in biofilm metabolic activity and a decrease of 5.38 log CFU in viable cells of the *P. aeruginosa* biofilm. Moreover, DS-GEN-PLGA NPs significantly attenuated the expression of QS system autoinducer synthesis genes, which could reduce bacterial virulence and improve treatment efficacy. Given the critical role of *P. aeruginosa* biofilm in drug resistance and bacterial virulence, this study highlights the potential of co-encapsulating DS and GEN in PLGA NPs as a combined therapeutic approach to synergically combat biofilm-associated infections. However, the lack of study on multiple clinical strains and in-vivo validation using animal models are the limitations of the present study, which should be considered in future works to explore the potential of DS-GEN-PLGA NPs for inhibiting and eradicating *P. aeruginosa* biofilm in the clinical setting.

Declarations

Ethical Approval Not applicable.

Competing Interests The authors declare no competing interests.

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