

**Research Article**

Design and Evaluation Novel Gene-Specific, Cell-Permeable Antisense Peptide Nucleic Acids to Prevent *Staphylococcus Aureus* Biofilm Formation

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Abstract

Staphylococcus aureus, a Gram-positive bacterium, is a leading cause of various biofilm-associated infections in humans and animals, posing significant economic and healthcare challenges. Biofilms exhibit heightened resistance to antimicrobial agents as well as to immune-mediated clearance, thus persisting for long periods of time. Hence, novel therapeutic approaches are needed to eradicate *S. aureus* biofilms. Peptide nucleic acids (PNAs), synthetic DNA analogs with a peptide backbone instead of sugar backbone, offer a promising approach. In this study, we designed, synthesized and tested the efficacy of several synthetic antisense PNAs coupled with cell-penetrating peptides (CPPs), targeting essential and biofilm related *S. aureus* genes to inhibit staphylococcal biofilm growth using standard microtiter plate and tygon catheter biofilm assays. P-PNAs targeting the genes for intercellular adhesion locus, *ica*, cell wall/membrane/envelope biogenesis, *fmhb*, accessory regulator, *sarA*, sensor histidine kinase, *saeS*, repressor of toxins, *rot*, response regulator, *yyCF* and histidine kinase, *yyCG* genes were tested. Two scrambled PNAs and CPP alone were used as controls. Only one P-PNA, targeting *sarA*, showed the strongest biofilm inhibitory activity (up to 40 %) at a concentration of 50 μ M or higher. This novel P-PNA could be a useful adjunct for the treatment *S. aureus* biofilm infections.

Keywords: Peptide nucleic acids (PNAs); *Staphylococcus aureus*; Biofilm; *sarA*; Antisense technology

Introduction

The Gram-positive opportunistic pathogen, *Staphylococcus aureus*, is the major causative agent of diverse biofilm-associated infections in humans and animals. Being a part of the normal microbiota of the skin and upper airways, *S. aureus* can readily establish various kinds of biofilm infections of the living tissue (biotic surfaces) such as the skin, soft tissue, bones and cystic fibrosis airways among others [1-6]. *Staphylococcus aureus* can also easily reach implanted devices such as catheters and prosthetic joints to establish biofilm infections on these abiotic surfaces [7]. In most cases, the *S. aureus* infection remains localized on these infected surfaces. However, the infection can spread to other parts of the body via the bloodstream causing serious complications [8].

The unique structural organization of the biofilms, with complex extracellular matrix, prevent the deeper penetration of antimicrobial agents into the biofilms and reaching the organisms at sufficient concentration to mediate antibacterial effects. This confers bacterial antibiotic resistance without the presence of resistance genes [9-11]. It has been demonstrated that the staphylococcal biofilms are also resistant to immune-mediated clearance [12, 13]. Overall, the biofilms can persist on biotic and abiotic surfaces for prolonged periods of time by multiple mechanisms [14-19]. Unfortunately, there are currently no antimicrobial therapies available that specifically target bacteria in biofilms, resulting in significant health as well as economic impact [20, 21]. For example, in patients with persistent biofilm infection of prosthetic joints, either replacement of the infected joint or even amputation of the limb may be the only viable options [22, 23]. Hence, novel anti-biofilm therapies are urgently needed.

Antisense technology has been widely applied in the medical field because of their ability to limit, and in some cases eliminate, the expression of targeted genes through sequence-specific recognition without much off-target effects. While antisense technology has been used to block the expression of human genes in the biomedical field, in microbiology they are used as an antimicrobial agent for targeting the expression of specific gene(s) in pathogens. Peptide nucleic acids (PNAs) are synthetic DNA mimics that have a peptide backbone that replaces the sugar-phosphate backbone of DNA, which confers excellent stability *in vivo*. They also bind to

target DNA or RNA sequences with a higher affinity than natural nucleic acids and have been used in a variety of applications, including inhibition of bacterial growth [24, 25]. Several studies have tested the ability of PNAs to block the expression of specific genes in *S. aureus* [26-30]. However, the effectiveness of PNA to inhibit biofilm formation in *S. aureus* has not been studied until recently. In this study, we have identified a novel PNA that effectively inhibited *S. aureus* biofilm formation *in vitro*.

Materials and Methods

Strain and growth conditions

Throughout this study, a pathogenic *S. aureus* isolate that expresses the luciferase gene and can also form biofilms (*S. aureus* P231) and a non-biofilm forming *S. epidermidis* isolate were used. Expression of the lux operon in *S. aureus* facilitates rapid screening of the effect of PNAs on bacterial growth/viability using *in vivo* imaging system “IVIS” [31, 32]. All bacterial strains were stored as 15–25 % (v/v) glycerol stocks at -70 °C as cryogenic stocks and plated on tryptic soy agar (TSA) when needed. Cultures were revived by streaking the stock onto TSA agar followed by overnight incubation at 37 °C. Single colonies were picked to inoculate pre-cultures in tryptic soy broth (TSB) with 1% glucose and incubated at 37 °C and 220 rpm for 16–18 h in a shaking incubator.

Design and Synthesis of P-PNAs

The genome was searched for genes that are known or theorized to be necessary in biofilm formation. The candidate bacterial genes were selected by scanning among those relevant to biofilm formation of *S. aureus*. Commonly targeted seven bacterial gene functions and pathways are those essential for biofilm formation such as adherence of cells to a surface and several global regulators. Suitable antisense PNAs for target genes were obtained from NCBI Database and designed to bind their translation initiation regions within the coding strands of each mRNA, which covered the AUG start codon [33, 34] (Table 1). Another two sequences (scrambled PNAs) mismatching the base pair with the target genes were designed as controls. All PNAs linked to a cell penetrating peptide (CPP) were designed and submitted to a commercial company (Table 1). All the P-PNAs were synthesized, purified, and conjugated with either (RXR)4XB, or (KFF)3K bacterial penetration peptide by PNA Bio (Newberry Park, CA).

Genbank Gene Reference Number and/or Name	Function	Target DNA Sequence (5'-3')	PNA Sequence (5'-3') with Attached CPP
AAD52055.1 The intercellular adhesion (<i>ica</i>) locus	It is involved in the synthesis of poly N-acetylglucosamine for intercellular adhesion in <i>S. aureus</i> .	TGTATTATGTCT	(RXR)4XB-TGACATATTTCT
NBR005743 Cell wall/membrane/envelope biogenesis (<i>fmb</i>)	The essential gene that encodes the factor that catalyzes the first step in the synthesis of the characteristic pentaglycine interpeptide in the <i>S. aureus</i> peptidoglycan.	ATAAATCATGGA	(RXR)4XB-TCCTTGATTAT
AF515775.1 <i>Staphylococcus aureus</i> variant staphylococcal accessory regulator region (<i>SarA</i>)	An important transcriptional regulator that activates the <i>agr</i> operon and modulates the expression of many virulence genes and is a positive regulator of <i>S. aureus</i> PNAG-dependent biofilm formation.	TTTAAACATGGCA	(RXR)4XB-TGCCATGTTATA
AJ556795.1 <i>Staphylococcus aureus</i> Sensor histidine kinase (<i>SaeS</i>)	Coordinate environmental signals with the internal regulatory circuitry governing virulence and other adaptive processes	GAGCCGATAATG	(RXR)4XB-CATTTGGGCTC
AF189239.2 <i>Staphylococcus aureus</i> repressor of toxins Rot (<i>rot</i>)	Plays a key role in regulating <i>S. aureus</i> virulence through activation or repression of promoters that control expression of many critical virulence factors.	AGAATAATGCA	(KFF)3K-O-TGCATTATTCT
AF136709.1 Two-component system YycFG <i>Staphylococcus aureus</i> response regulator YycF (<i>yycF</i>)	YycF binds to the upstream promoter regions of the target genes to positively and negatively regulate their expression.	AAGAGGTTAACG	(KFF)3K-O-CATTAACCTCTT
AF136709.1 Two-component system YycFG <i>Staphylococcus aureus</i> histidine kinase YycG (<i>yycG</i>)	YycG is a sensor protein with histidine kinase activity	GAAACGAATGA	(KFF)3K-O-TCATTCGTTTC
Scramble PNA	Random (non-specific)	NONE	(RXR)4XB- TTTTGCAT
Scramble PNA	Random (non-specific)	NONE	(RXR)4XB- CCCTGATATA

Cell Penetrating Peptide	Short peptides that can deliver PNAs across cellular membranes	N/A	(RXR)4XB
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Table 1: Sequences of designed P-PNAs and CPP for *Staphylococcus aureus*.

Inhibitory activities of synthesized P-PNAs against *Staphylococcus aureus* biofilm formation

Briefly, frozen stock of *S. aureus* P231 was streaked on TSA (Tryptic Soy Agar) plates to obtain single, well-isolated colonies. Plates were incubated overnight at 37 °C with 5 % CO₂. One colony was inoculated to 10 ml of TSB (trypticase soya broth) supplemented with 1 % glucose and incubated for 20-24 h at 37 °C in shaker at 200 rpm. The overnight cultures grown in TSB-1 % glucose was diluted 180 folds (approximately 10⁵ CFU/ml) with 2x TSB-1 % glucose. Fifty microliters of the diluted cultures were incubated with indicated concentrations of PNA in a volume of 50 µl, in duplicates, in a 96-well, low adhesion, flat-bottomed microtiter plates (cat. # 3474; Corning Inc., Corning, NY), making the total volume per well to 100 µl. The plates were incubated in a humidified incubator at 37 °C with 5 % CO₂. To reduce edge effects and evaporation, 200 µl TSB was added to circumferential wells on the plate. For determining biofilm formation by crystal violet (CV) staining, the plates were incubated for 48 hours, and the liquid culture medium was removed by gently tapping the plates on paper towel to remove planktonic bacteria. Subsequently, 200 µl 0.1 % aqueous CV was added to each well and incubated for 10-15 minutes at room temperature. The plates were washed by submerging the plates in a tray of water, filling the wells and tapping the plates gently upside down on a stack of paper towels. After 2 washes, 200 µl of 30 % glacial Acetic acid was added to each well, incubated at room temperature for 10-15 mins to dissolve the biofilm-bound CV and the absorbance was determined using a microtiter plate reader at 570 nm. The average and standard deviation of the OD₅₇₀ values obtained for each strain were calculated [35]. For experiments with the bioluminescent feature of *S. aureus* isolate, the plates were incubated for 24 h before determining bioluminescence using IVIS (R) imaging system.

Inhibitory activities of anti-*sarA* P-PNA against *S. aureus* biofilm formation on tygon tubing

The tygon catheter tubes mimics with an inner diameter of 1/16 in. and a wall thickness of 1/16 in, giving a total diameter of 3/16 in (Part No.5894K31, McMaster Carr, Santa Fe Springs, CA, USA) [36, 37] were cut into 3.5 cm to 4 cm long segments and sterilized by soaking in 10 % bleach for 15 minutes. Silicon stoppers were also sterilized similarly. After sterilization, catheter fragments and stoppers were washed with sterile distilled water (diH₂O) several times in a laminar flow hood and allowed to dry

prior to inoculation with *S. aureus* P231. For tygon biofilm assay, 50 µl of the bacteria (10⁵ CFU/ml) was transferred into a low-binding 1.5 ml microfuge tube and centrifuged at 13,300 rpm for 3 minutes. After aspirating the supernatant, the bacterial pellets were resuspended in 50 µl anti-*sarA* P-PNA (50 or 100 µM) in TSB-1 % glucose and the mixes were applied into the lumen of the catheter fragments. After incubation for 48 hours at 37 °C with 5 % CO₂, the liquid culture medium was removed from the catheter lumen by gentle aspiration to remove planktonic bacteria. Then, 50 µl of 0.1 % aqueous CV was added to each lumen of the catheter and incubated for 10-15 minutes at room temperature. The CV-stained bacterial biofilm on the catheter pieces was washed several times by pipetting with water. After washing, they were carefully dried with a tissue and the catheter pieces were transferred to 96 well flat bottom microtiter plates. To dissolve the biofilm bound CV, 50 µl of 30 % glacial acetic acid was added to the lumen of each the catheter piece, incubated at room temperature for 10-15 minutes and the absorbance was determined at 570 nm using a microtiter plate reader.

Statistics

All statistical analyses were performed with Student's two-tailed t-test using Microsoft Excel (Microsoft Corp., Redmond, WA) or using GraphPad Prism software (Version 10.4). P-values of ≤ 0.05 were considered significant.

Results and Discussion

Biofilm formation by *S. aureus* is a complex process which is not fully understood. However, several genes and pathways have been shown to be critical in this process. Therefore, we hypothesized that targeting important genes using cell-penetrating antisense PNAs will be a viable approach to treat *S. aureus* biofilm infections. For this, we first systematically reviewed the literature and identified several candidate bacterial genes that have been shown to regulate staphylococcal biofilm formation or critical for staphylococcal growth and virulence. The *icaA* gene, which plays a role in the synthesis of poly-N-acetylglucosamine for intercellular adhesion, has been reported to play an important role in biofilm formation [38, 39]. The *fmhB* gene, encodes for the factor that catalyzes the first step in the synthesis of the characteristic pentaglycine interpeptide in the *S. aureus* peptidoglycan, which is involved in wall biosynthesis [40]. *SarA* has been shown to be an important transcriptional regulator that activates the *agr* operon and modulates the expression of many virulence genes and is a positive

regulator of PNAG-dependent biofilm formation of *S. aureus* [41-44]. Another global autoinducer regulator, *sae* regulon, includes both biofilm formation factors (i.e., *Coa*, *Emp*, *Eap*, *FnBPA*, *FnBPB*, *Hla*, *Hlb*) and biofilm degradation factors [45-50].

The DNA-binding protein Rot, which belongs to the *sarA* family of *S. aureus* regulators, controls *S. aureus* virulence by activating or repressing promoters that regulate the expression of several important virulence factors [51-52]. Several studies have demonstrated a relationship between the presence of the *Rot* gene and the formation of *S. aureus* biofilms [53]. One of the major properties of *S. aureus* is that it can adapt to the environment by coordinating gene expression thereby allowing it to withstand a variety of environmental stresses. The two-component systems (TCS), plays a central role in this process [54]. The *YycFG* TCS regulates cellular physiology, structure and biofilm organization [55]. The *YycG* TCS controls genes related to cell wall metabolism, virulence regulation, biofilm formation, oxidation stress resistance and antibiotic resistance through a direct or indirect regulation of autolysins. It acts as a sensor protein kinase that automatically phosphorylates a histidine residue in the dimerization domain and then shifts the phosphate group to the aspartic acid residue conserved in the regulatory domain of *YycF*. The upstream promoter regions of the target genes can control the expression of these genes either positively or negatively [56, 57]. Anti-sense PNAs targeting these genes were synthesized and tested in in vitro assays.

First, we tested whether the PNAs had direct bactericidal activity in 96-well microtiter plate bioluminescence assay. The extent of bioluminescence was comparable in all wells, when treated with test PNAs, scrambled PNAs, cell-permeable peptide or medium alone at concentrations ranging from 15 to 50 μ M (Fig. 1). This indicated that our PNAs are not bactericidal. Next, we tested the ability of the compounds to specifically inhibit biofilm formation in CV assay. In this assay, only anti-*sarA* P-PNA exhibited the strongest biofilm inhibitory effect at concentration of 50 μ M or higher. Biofilm formation was reduced by 95 % after 48 h of treatment with anti-*sarA* P-PNA at concentration 50 μ M (Fig 2). As expected, neither of the scrambled PNAs nor cell-permeable peptide alone had any inhibitory effect. Interestingly, certain PNAs, such as *icaA*, *fnhb* and lower concentrations of *sarA* appeared to promote biofilm formation. While we do not understand the mechanisms, it is possible that the PNAs may interact with the extracellular DNA that is known to be present in the biofilm matrix and promote biofilm formation.

Since only anti-*sarA* P-PNA showed strong inhibitory activity in microtiter plate assay, we next tested the ability of anti-*sarA* P-PNA to inhibit *S. aureus* biofilm formation on tygon tubes. In this assay, anti-*sarA* P-PNA caused approximately 50 % inhibition of biofilm formation of *S. aureus* strain P231 on the surface of tygon catheter tubes at a concentration of 50 μ M and over 95 % inhibition at a concentration of 100 μ M compared to the control (Fig 3). The CPP alone had no inhibitory effect on biofilm formation (Fig 3). In conclusion, we have designed a novel PNA that significantly inhibited biofilm formation by pathogenic *S. aureus*. However, there are some limitations associated with our study. While anti-*sarA* PNA clearly inhibited biofilm formation, the mechanisms need to be worked out. Fully understanding the molecular pathways regulated by *sarA* that are important for biofilm formation may lead to novel therapies to inhibit biofilm formation. Once the PNA is tested in in vivo models, this could be as an alternative and probably as a complementary therapeutic strategy with broad spectrum antimicrobials.

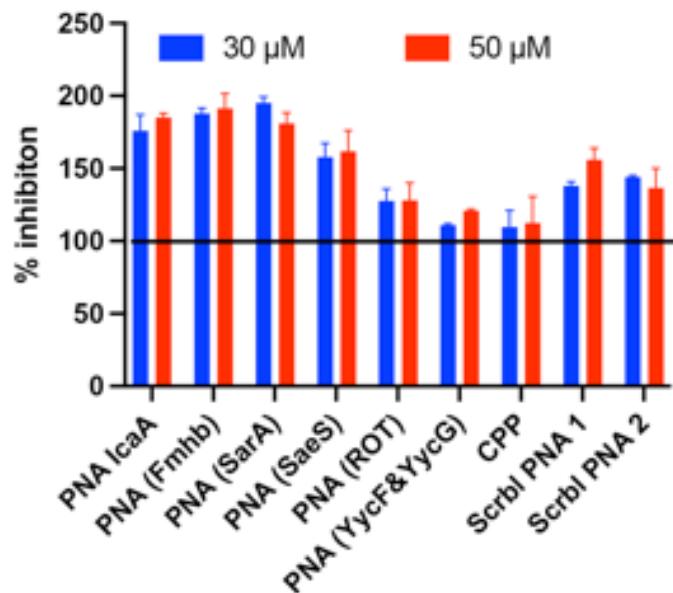


Figure 1: Determination of direct antibacterial activity of PNAs. To investigate whether the PNAs have direct antibacterial activity, the *S. aureus* P231 bioluminescent strain was grown in microtiter plates in the presence of various gene-specific PNAs, scrambled PNAs or CPP ((RXR)4XB) alone for 24 hours. The luminescence intensities were calculated using IVIS system as described in methods. The percent of inhibition of bioluminescence by the test compounds was calculated by comparing the luminescence with medium alone. The bars represent Mean \pm SE of two to three independent experiments.

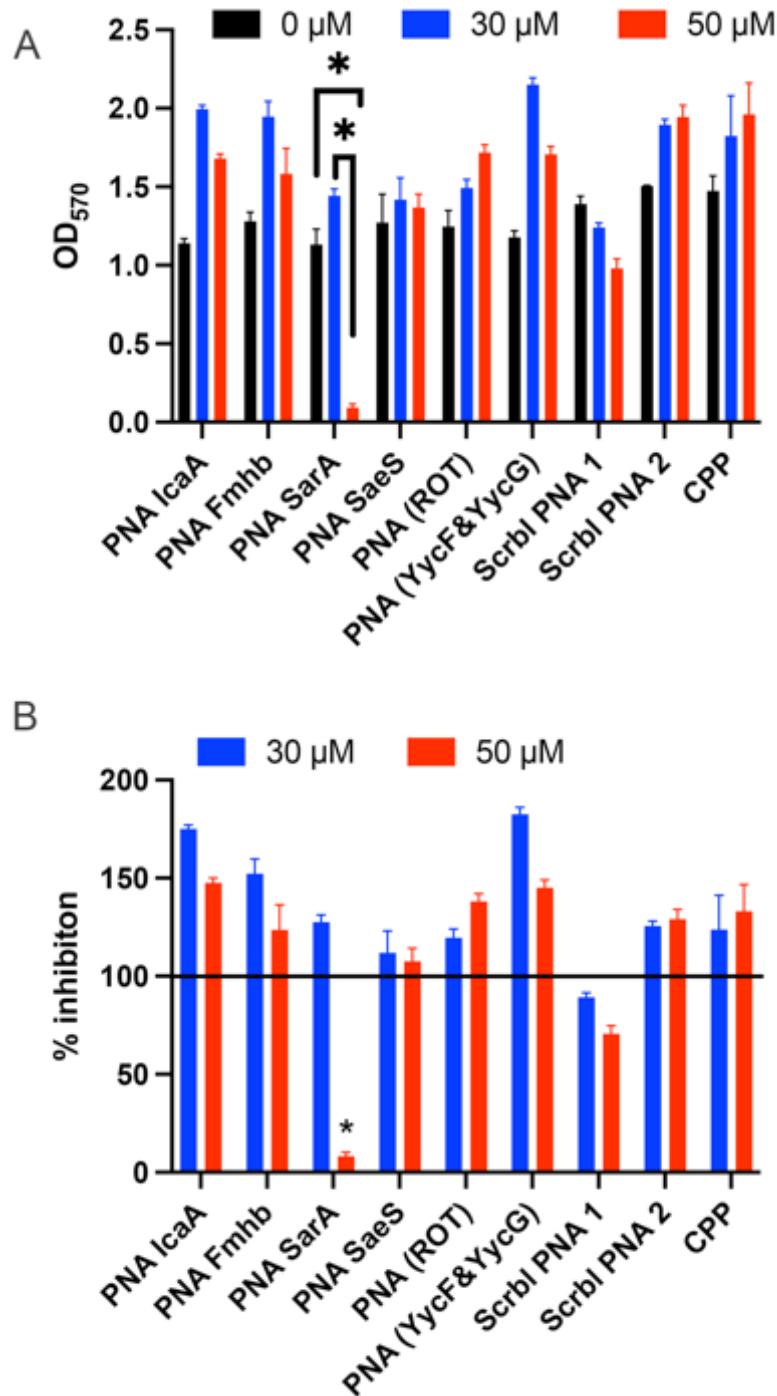


Figure 2: Inhibition of *S. aureus* biofilm formation by PNAs. *S. aureus* P231 strain biofilms were grown in 96-well low binding microtiter plate. Indicated concentrations of PNAs were added in duplicates and the extent of biofilm formation was determined by crystal violet staining assay at 48 hours. (A) OD₅₇₀ values by crystal violet staining assay. (B) Percentage inhibition by PNAs compared to medium alone. The bars represent Mean ± SE of two to three independent experiments. * p<0.05

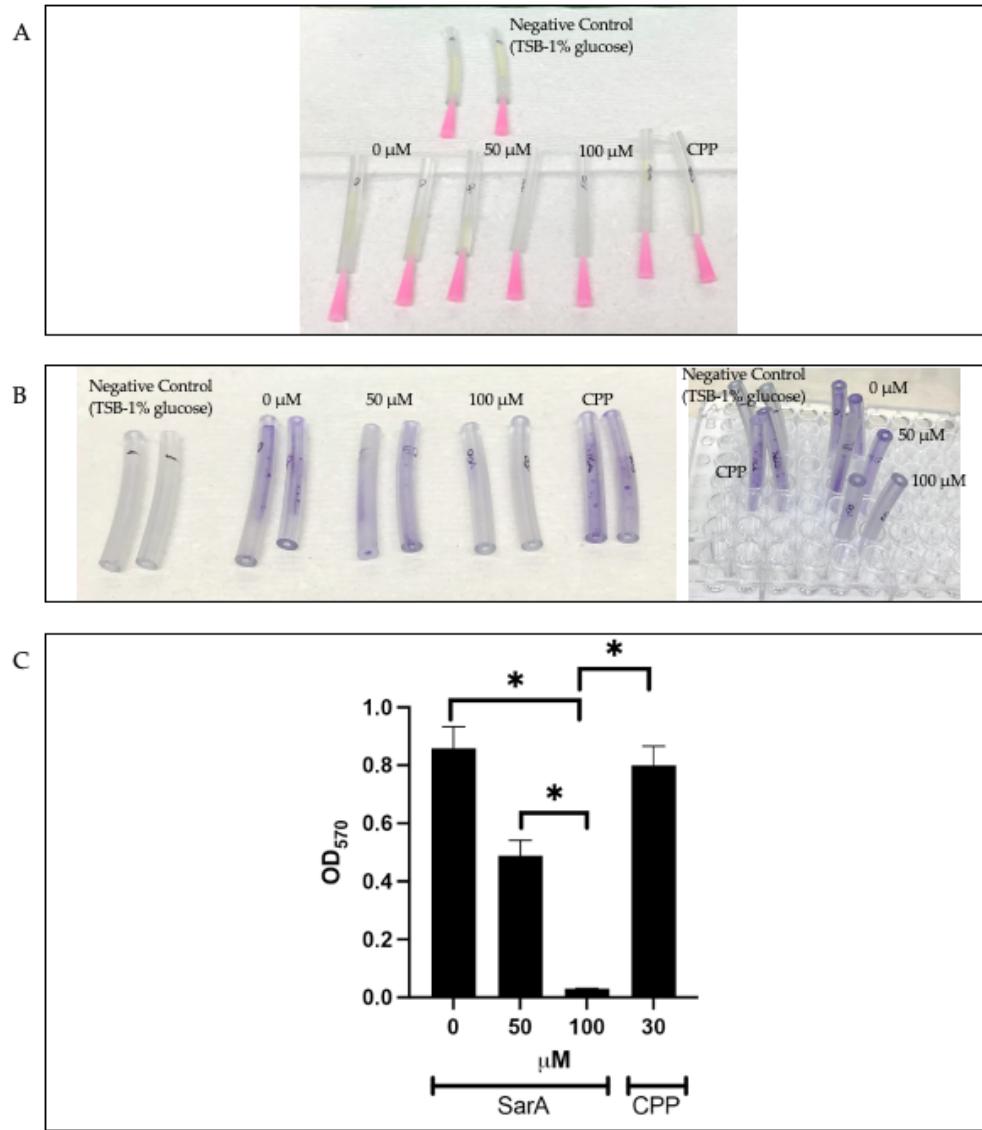


Figure 3: Inhibition of biofilm formation by *S. aureus* P231 strain on tygon catheters by (RXR)4XB-linked anti-*sarA* P-PNA. (A) Biofilms of *S. aureus* P231 strain were grown for 48 h on the tygon catheter segments. (B) Crystal violet-stained biofilms of *S. aureus* P231 on tygon catheter tube segments. (C) OD₅₇₀ values by Crystal violet staining to determine the extent of biofilm inhibition at 48 hours. Anti-*sarA* P-PNA was tested at 50 and 100 μM and the control CPP was used at 30 μM. * p<0.05.

Studies with antisense peptide-PNAs targeting essential genes have shown growth inhibitory effects in *S. aureus* [27]. PNAs' potential as antibacterial agents has been thoroughly evaluated, and it has been found that design parameters like the base sequence, the location of the membrane penetrating peptide, and the presence of a linker can affect the activity of P-PNAs. As a result, the most effective PNAs for Gram-positive bacteria had a base pare length of 10 nucleotides, the most effective PNAs for mRNA regions that bind to the ribosomal binding sequence of the mRNA (RBS region), and the PNA activity of different carrier agents produced distinct antibacterial effects on both Gram-positive and Gram-negative bacteria [57].

Conclusion

In conclusion, we have designed a novel PNA that significantly inhibited biofilm formation by pathogenic *S. aureus*. Once the PNA is tested in *in vivo* models, this could be as an alternative and probably as a complementary therapeutic strategy with broad spectrum antimicrobials.

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Conflicts of Interest: The authors declare no conflict of interest.

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