Oxacillin coupled G-quadruplexes as a novel biofilm-specific antibiotic for *Staphylococcus aureus* biofilms

Renzo A. Fenati,[†] Katherine Locock[‡], Yue Qu,[‡] and Amanda V. Ellis^{*,†}

[†]School of Chemical and Biomedical Engineering, Melbourne School of Engineering, The University of Melbourne, Parkville, Victoria 3010, Australia

[‡]CSIRO Manufacturing, Clayton, Victoria 3168, Australia

[‡] Department of Infectious Diseases, The Alfred Hospital and Monash University, Clayton, Victoria 3800, Australia

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Corresponding Author

*Prof. Amanda V. Ellis

Email: Amanda.ellis@unimelb.edu.au

ABSTRACT. One of the most important traits of pathogenic microbial biofilms is their high tolerance to conventional antimicrobial agents, which is partially due to the presence of metabolically inactive and transiently resistant persister cells. Here, we use guanine-rich DNA structures known as G-quadruplexes (G4s) coupled with the β -lactam antibiotic, oxacillin (OX), and loaded with an iron-containing protoporphyrin IX (hemin), as an OXG4/hemin complex biofilm-specific antibiotic agent. By coupling the OX to the G4, to form an OXG4/hemin complex, the diffusion of the OX was facilitated into the biofilm. Further, by utilizing the known oxidizing behavior (peroxidase-mimicking) of the G4/hemin complex the entire system was found to be highly effectively against *Staphylococcus aureus* biofilms. By using G4 structures to penetrate biofilms, this work paves the way for an entirely new DNA-based therapy for biofilm eradication.

1. INTRODUCTION

The rise of chronic infections has been linked to biofilms formed by many pathogenic microorganisms.¹⁻⁵ Morphologically, biofilms are sessile cells (aggregated cells on a surface) embedded in a protective extracellular polymeric matrix that consist of polysaccharides, proteins and extracellular DNA (eDNA).⁶⁻¹¹ The presence of metabolically inactive and transiently resistant persister cells within a biofilm in combination with low drug diffusion and subsequent sub-lethal antimicrobial dose levels across biofilms contribute to the genotypical development of antimicrobial resistance.¹²⁻¹⁴ Strategies to combat biofilm forming bacteria, and reduce the prevalence of chronic infections, have included the use of high concentrations, or combinations of, conventional antimicrobial agents.^{5,15} New biofilm-effective agents that target persister cells, or disrupt the biofilm structure, have also been investigated such as, nanoparticles (e.g., silver,¹⁶ gold¹⁷ or palladium¹⁸) and enzymes (DNase¹⁹).

More recently, thiocyanate (SCN)-peroxidase-hydrogen peroxide (H_2O_2) treatments have been explored.²⁰ In this treatment the peroxidase enzyme reacts with H_2O_2 which then oxidizes the SCN. The oxidized thiocyanate then damages the essential structural and functional components of the bacteria cells, inhibiting their growth and eventually killing the cells. This approach has had success at eliminating planktonic bacteria but is yet to be tested on sessile bacteria within a biofilm. Further, SCN can be toxic and known to affect the thyroid and hormonal function, particularly in patients with reduced renal function. Thus, the SCN-peroxidase- H_2O_2 system has somewhat limited effectiveness as an antimicrobial system within the human body.^{20,21}

A potential method to replace the SCN-peroxidase- H_2O_2 system is to use the peroxidase-mimicking activity of catalytic DNA molecules (DNAzymes), in particular G-quadruplexes (G4s) which provide several advantages over enzymes; including cheaper synthesis and purification, wider operating conditions (pH and temperature) and the ability to react with a various substrates.^{22,23} G4s are assembled from guanine-rich (G-rich) DNA sequences that can stack two or more guanine-quartets (G-quartets) (see Figure 1(a)) in the presence of a metal cation.²⁴ Once assembled, the G4s can take on several conformations including parallel, anti-parallel and hybrid (see Figure 1(a)) and can be composed of a single DNA strand (intramolecular) or multiple DNA strands (intermolecular).^{25,26}

Of particular interest is that G4s are able to complex with hemin (Figure 1(b)), an iron containing protoporphyrin IX, which acquires peroxidase-mimicking activity in the presence of H_2O_2 .²⁷ The hemin (FeIII) first complexes with H_2O , which is then replaced with H_2O_2 (Figure 1(c)).²⁸ The peroxide is then broken down to form what is known as Compound I (a π radical cation protoporphyrin ($\text{FeIV}-\text{O}^+$)). The $\text{FeIV}-\text{O}^+$ is then able to react with a substrate to form $\text{FeIV}-\text{O}$

known as Compound II.²⁶ Compound II can then be recycled back into hemin (pre-Compound I) or Compound I, therefore acting as a DNAzyme that can continuously react and regenerate.²⁹

Importantly, from a diagnostic perspective, G4/hemin complexes in the presence of hydrogen peroxide are compatible with the well-established colorimetric peroxidase-based enzyme immunoassays (e.g., 3,3',5,5'-tetramethylbenzidine (TMB; $\lambda_{\text{ABS}} = 605 \text{ nm}$)³⁰ and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS; $\lambda_{\text{ABS}} = 410 \text{ nm}$ and 650 nm)³¹. This allows the concentration of G4/hemin complexes to be detected at sub- μM levels with the extent of the reaction being based on the H_2O_2 concentration.

Figure 1. (a) Schematic of general G-quadruplex (G4) conformations; G-quartet, anti-parallel, parallel and hybrid (formed from a single or multiple G-rich DNA sequence), (b) Schematic of the formation of a hemin (iron-containing protoporphyrin) with a G4 to form a G4/hemin complex and (c) Schematic of generalized reaction of hemin peroxidase-mimicking activity.

Given that G4/hemin complexes have been shown to have a wider range of reactants compared to peroxidase enzymes, but narrower than thiocyanate, in this work we use G4/hemin complexes as a possible method for eradication of biofilm bacteria.³² One of the important considerations is getting the complexes across the biofilm. Another route of interest is using these G4/hemin complexes in conjunction with antibiotics. Many drugs are able to easily penetrate biofilms (e.g., cefotaxime, amikacin), however the β -lactam antibiotic, oxacillin (OX) (Figure 2(a)), does not penetrate through *Staphylococcus aureus* biofilms at minimum inhibitory concentrations (MIC) of $1\ \mu\text{g mL}^{-1}$.³³ Therefore, by using OX coupled with the G4 complex may contribute to a synergistic killing effect.

Here, we detail a proof-of-concept peroxidase-mimicking system that utilizes G-rich DNA sequences chemically coupled with OX. These sequences are then folded into secondary three-dimensional G4 structures in the presence of hemin for use as an antimicrobial agent against *S. aureus* American Type Culture Collection (ATCC) 29213 biofilms. The OX was calculated at $1\ \mu\text{g mL}^{-1}$ in the resulting OXG4/hemin structure. The G4/hemin structure was shown to penetrate the biofilms (acting as a diffusion facilitator) leading to the coupled OX having an antimicrobial effect within the biofilm construct.

2. EXPERIMENTAL SECTION

2.1 Materials

All chemicals were purchased from Sigma-Aldrich (USA) unless stated otherwise. A stock Tris-EDTA buffer (10X, pH 7.5) was made up by dissolving tris(hydroxymethyl) aminomethane

(Tris) (15.8 g) and ethylenediamine tetraacetic acid (EDTA) (2.92 g, pH 8) in Milli-Q water (18.3 MΩ cm) (1 L) and adjusted to pH 7.5. Reaction buffer (10X) was made up by Tris (15.8 g) and KCl (37.3 g) in Milli-Q water (1 L) and adjusted to pH 7.5. 2-(*N*-morpholino)ethanesulfonic acid hemisodium salt (MES) buffer (1 M, pH 4.5) was made up by dissolving 2-(*N*-morpholino)ethanesulfonic acid (195.2 g) in Milli-Q water (1 L). The 3,3',5,5'-tetramethylbenzidine (TMB) Liquid Substrate System was used for the determination of peroxidase-mimicking activity. Mueller-Hinton broth (MHB) and Mueller-Hinton agar (MHA) was made according to the manufacturer's recommendations using reverse osmosis (RO) water, autoclaved at 121 °C for 10 min and stored at 4 °C. Bovine serum albumin (BSA) was used as purchased. *S. aureus* ATCC 25923 (non-biofilm forming³³) and *S. aureus* ATCC 29213 (biofilm forming³³) bacterial strains were purchased from ATCC, Inc., USA and stored at 4 °C on MHB agar plates. Table 1 shows the oligodeoxynucleotide (ODN) sequence (and melting temperature) used to form the G4 structures as well as the sequence used for coupling oxacillin.

Table 1. Sequences and melting temperatures of oligodeoxynucleotides (ODNs) used for fabricating G-quadruplexes (G4s) (the G4 ODN), including the sequence used for coupling with oxacillin (NH₂G4 ODN, tagged with the fluorophore Cy3.5). G4 structure forming sequences are labelled in bold.

Sequence ^a 5' ⇒ 3'	
G4 ODN	G₃A₃G₃CAC₂G₃C₂G₃
NH ₂ G4 ODN	NH ₂ - ATGA ₂ T ₃ C ₂ G₃A₃G₃CAC₂G₃C₂G₃CACAC₂A - Cy3.5

The G4 ODN was unlabeled while the NH₂G4 ODN was tagged at the 3' end with the fluorophore CY3.5 for concentration determination. All ODNs were purchased from IDT DNA (USA), made up to 100 μ M and stored at 4 °C.

2.2 Oxacillin coupling to the NH₂G4 ODN

The antibiotic, OX was coupled to the NH₂G4 ODN (see Table 1 for sequence). This was achieved by coupling the carboxylic acid functional group on OX to the amine functional group on the NH₂G4 ODN using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) coupling. Briefly, the NH₂G4 ODN (5 μ M, 5 μ L) was added to excess OX (15 μ M, 45 μ L) in 0.1 M MES buffer and vortexed for 60 s. EDC (20% (w/v), 50 μ L) in 0.1 M MES buffer was then added to the NH₂G4 ODN and OX mixture and vortexed for a further 60 s. The mixture was placed in a thermomixer at 25 °C for 20 min with alternating cycles of 30 s shaking (1400 rpm) and 30 s of standing. The resulting antibiotic attached ODN was denoted OXG4. DNA ethanol precipitation was performed to purify the OXG4.³⁶ The concentration of the precipitated OXG4 was determined using a standard curve carried out on a RotoGene Q 6-plex real-time polymerase chain reaction thermocycler (Qiagen, Germany).³⁷ By determining the concentration of the DNA, assuming 100% attachment of the OX to the NH₂G4 ODN, it was possible to determine the concentration of the OX present on the DNA.

2.3 Folding of G4 and G4/hemin structures

The concentrations of the as supplied ODNs (see Table 1 for sequences) were measured using a Nanodrop 2000 and then diluted down to 50 μ M in a Tris-EDTA buffer (1X). For the formation of the all G4 structures in this article the following procedure was followed: each G4 ODN and NH₂G4 ODN (50 μ M, 4 μ L) was diluted in Reaction buffer (32 μ L) containing K⁺ ions (50 mM).

Each solution was placed in a thermocycler and first heated to 95 °C then cooled to 30 °C over a 30 min time period. In order to prevent the OX from degrading at high temperatures the OXG4 was placed in Reaction buffer (final concentration of 10 µM and left at room temperature for 30 min.³⁸ The folded OXG4 structure was confirmed using the TMB assay described below.

2.4 TMB assay

In order to measure the peroxidase-mimicking activity of the folded G4, G4/hemin, OXG4, OXG4/hemin structures a TMB assay was performed. First, each solution (5 µM, 5 µL) was added to a 96-well plate. TMB Liquid Substrate solution (195 µL) was added to each well. The 96-well plate was then placed in a FLUOstar Omega microplate reader (BMG Labtech, Germany) and the absorbance measured at 605 nm every 30 s for 10 min. The results were then analyzed using an OriginPro 9.0 software linear fit function to determine the slope of each curve. Initial velocity (V_0) of the reaction was determined using a modified Beers Law Equation (Eq. 1):

$$V_0 = \frac{A}{\varepsilon} \quad (1)$$

Where A is the measured absorbance and ε is the molar extinction of TMB+ (39,000 L mol⁻¹ cm⁻¹).

2.5 Minimum inhibitory concentration (MIC)

The MIC was determined using the serial dilutions method set out in the Clinical and Laboratory Standards Institute (CLSI) and performed in aseptic conditions.²⁹ The bacteria used for this MIC determination was *S. aureus* ATCC 25923 (non-biofilm forming for MIC tests), which is susceptible to methicillin antibiotics such as OX. Both OX and the OXG4 were tested. OX was diluted from a stock solution (10240 µg mL⁻¹) to 8.0, 4.0, 2.0, 1.0, 0.5, 0.25 and 0.125 µg mL⁻¹.

The OXG4 was diluted to 10.000, 5.000, 2.500, 1.250 and 0.625 μM which is equivalent to 4.000, 2.000, 1.000, 0.500 and 0.250 $\mu\text{g mL}^{-1}$ of coupled antibiotic, based on a molecular weight of 401.436 g/mol of OX. This will result in a final concentration of 2.000, 1.000, 0.500, 0.250 and 0.125 $\mu\text{g mL}^{-1}$ of coupled antibiotic when determining the MIC. The OX and OXG4 serial dilutions (20 μL) were added to 96-well plates (ignoring the first column and row) and 20 μL of 5×10^5 *S. aureus* cells in MHB. Visual inspection was used to determine if any bacterial growth was successful. The only modification of the CLSI standard procedure was the use of flat bottomed 96-well plates as opposed to round bottom well plates. This has been previously published as an acceptable modification.³⁹

2.6 Biofilm growth in 96-well plates

Biofilms were grown by preparing a cell suspension of *S. aureus* ATCC 29213 (biofilm forming) in MHB (50 μL) that was then added into 3 x 96-well plates (ignoring the first column and row) and incubated at 37 °C for 90 min, shaking at 75 rpm. Afterwards the cell suspension was removed and MHB (1 mL) was replaced and the 96-well plates further incubated for 24 h at 37 °C, with shaking at 75 rpm. The 96-well plates were then washed with PBS solution to remove planktonic cells. One 96-well plate was used to determine the CFU of the biofilms. The two remaining 96-well plates were used for CV staining. These 96-well plates were dried upside down at 60 °C for 1 h prior to use.

The drop plate method was used to evaluate the CFU of the bacteria in the 96-well plates.⁸ Briefly, PBS solution (50 μL) was added to each well that contained a *S. aureus* ATCC 29213 biofilm. Then the biofilm was scratched off using a plastic pipette tip. This was performed 3 times for each well to ensure all the biofilm was removed. The PBS solution, with scratched off biofilm

from each well, was then placed in separate Eppendorf tubes and vortexed for 2 min with subsequent sonication for 10 min to break apart the biofilm. Each sample was diluted 10-fold eight times. Each dilution (10 μ L) was pipetted onto a horizontal MHA plate and the plate was lifted vertically to allow for the drop to run down the plate. The plates were incubated at 37 °C for 20 h. All the spots on each plate were counted, one spot was equivalent to 1 CFU, and multiplied by the dilution factor.

2.7 Biofilm growth on silicone coupons

In order to determine the successful growth of biofilm, sterilized silicone coupons (BioSurface Technologies Corporation, USA) that had been incubated in bovine serum albumin (BSA) overnight, were used. The coupons were placed into a 12-well plate (ignoring the first column and row) with a cell suspension (5×10^5 cells) of *S. aureus* ATCC 25293 in MHB (1 mL) and incubated at 37 °C for 90 min, with shaking at 75 rpm. Afterwards the cell suspension was removed and the MHB (1 mL) was replaced. The plate was further incubated for 24 h at 37 °C, with shaking at 75 rpm. The silicone coupons are then removed and placed into PBS solution and vortexed for 5 min to remove any planktonic cells and then stained using a LIVE/DEAD™ BacLight™ Bacterial Viability Kit for 40 min. The coupons were washed with PBS solution and analyzed using a Confocal TCSPC fluorescence scanning microscope (CFSM) with a 100x objective. Blue and yellow excitation with SYTO 9 stain and propidium emission filters were used to detect the staining.

3. RESULTS AND DISCUSSION

The ODN sequences used to prepare both a G4/hemin complex and an OX coupled G4 (OXG4) complex are shown in Table 1. The G4 ODN selected for coupling with the OX was a Cy3.5 fluorophore (Figure 2(b)) tagged NH₂G4 ODN sequence. This only differs from the G4 ODN by a spacer region of 6 nucleotides residues on either side of the G4 sequence to limit steric interaction of the fluorophore during folding. The coupling of the OX to the NH₂G4 ODN was carried out using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC).

Figure 2. Chemical structures (a) oxacillin (OX) and (b) the fluorophore Cy3.5.

Confirmation of OX coupling to the NH₂G4 ODN was performed using polyacrylamide electrophoresis (PAGE), shown in Figure 3. The NH₂G4 shows a mixture of single stranded ODNs and folded intramolecular G4 structures at approximately 27 nts.³⁴ The upper brighter band is a result of intermolecular (bimolecular) G4 structures at approximately 34 nts.³⁴ Once OX was coupled to the NH₂G4 an increase in the molecular weight was observed, indicating successful attachment. The intramolecular structures are still observed but now at a slightly higher molecular weights, at approximately 28 nts, and the intermolecular structures were likewise slightly increased at approximately 35 nts.

Figure 3. PAGE 12% (v/v) electrophoretogram of folded OXG4 and NH₂G4 ODNs, stained with 1X SYBR Green I and viewed using a Gel Doc. A DNA Ladder (20/100 Ladder Oligo length standards) was used for reference. The red lines indicate bands of interest with the approximate weights.

In order to determine if there were any negative effects caused by coupling the ODN with OX, such as loss of antimicrobial or peroxidase-mimicking activity, TMB assays and MIC assays were performed. First, a TMB assay was performed to determine the peroxidase-mimicking activity of the folded DNA structures with and without coupled OX. Figure 4 shows the plot of TMB⁺ concentration (μM) versus time (s) results of the TMB assay for hemin (control with no G4, green overlapping blue line), G4/hemin (black), NH₂G4/hemin (pink), OXG4/hemin (red) and OX/hemin (no G4, blue). All samples have hemin at 8 μM. The initial peroxidase-mimicking activity (V₀) calculated from the data is shown in Table 2.

Figure 4. TMB assay. Plot of TMB⁺ concentration (μM) versus time (s); hemin (control with no G4, green), G4/hemin (black), NH₂G₄/hemin (pink), OXG₄/hemin (red) and OX/hemin (no G4, blue). The data are an average of three UV/visible spectra calculated using the OriginPro 9.0 average curve function.

The G4/hemin complex had the highest V_0 at $0.204 \mu\text{M s}^{-1}$ compared to the NH₂G₄/hemin complex with a $V_0 = 0.130 \mu\text{M s}^{-1}$ (Table 2). This inhibition of the peroxidase mimicking activity by the NH₂G₄ ODN is likely due the additional functionalities (Cy3.5 fluorophore and spacer region (nucleotide residues)) in the structure compared to the G4 complex on its own.

After coupling of OX to the NH₂G₄ a slight increase in the peroxidase-mimicking activity of the OXG₄/hemin complex was observed ($V_0 = 0.170 \mu\text{M s}^{-1}$) compared to the NH₂G₄/hemin complex ($V_0 = 0.130 \mu\text{M s}^{-1}$). The difference in the peroxidase-mimicking activity between the NH₂G₄/hemin and OXG₄/hemin complex is proposed to be due to a difference in the structure of the G4 complex formed.

Table 2. Peroxidase-mimicking activity (V_0 , calculated using Eq. 1) of TMB assay for hemin (control, no G4), G4/hemin, NH₂G4/hemin, OXG4/hemin and OX/hemin (no G4). Data is calculated from Figure 4.

Sample	Peroxidase-mimicking activity, V_0 ,
with hemin at (8 μ M)	μ M s ⁻¹ , (Error = $\leq$ 1%)
hemin (control)	0.001
G4/hemin	0.204
NH ₂ G4/hemin	0.130
OXG4/hemin	0.170
OX/hemin (no G4)	0.001

Figure 5 shows the two possible conformations that the OXG4 with coupled OX (white ball) and Cy3.5 (yellow ball) can form in an intermolecular G4 structure. In the anti-parallel G4 conformation (Figure 5, top), the OX and Cy3.5 fluorophore are more likely to interact with each other and this can exert steric hinderance. This steric hindrance decreases the likelihood of the anti-parallel conformation forming. In the parallel G4 conformation (Figure 5, bottom), the OX and Cy3.5 are further spaced apart and therefore do not have the same steric hinderance as in the anti-parallel. Therefore, the parallel G4 conformation is favored and is well known to have higher peroxidase-mimicking activity than the anti-parallel G4 conformation (Cheng *et al.*²⁷). However, for the NH₂G4 (not coupled to OX) no such steric hindrance occurs which allows more of the anti-

parallel G4 conformation to form, resulting in the NH₂G4/hemin complex possessing a lower V_0 than the OXG4/hemin complex, as shown in Figure 4.

Figure 5. Schematic of general intermolecular (bimolecular) G-quadruplex (G4) conformations formed by two strands of OXG4 with coupled OX (white ball) and Cy3.5 (yellow ball), in an antiparallel and parallel conformation.

Minimum inhibitory concentration (MIC) of OX and OXG4 were then determined to further ascertain whether the OX was coupled to the NH₂G4 ODN. For this *S. aureus* ATCC 25923 (non-biofilm forming) cells were used. This was achieved by carrying out a MHB microdilution assay according to the CLSI guideline M7-A9.³⁵ In this manner, by comparing the MIC of the OXG4 structure with the NH₂G4 structure, clarification of whether the conjugation of OX to the G4 complex compromises its antibacterial activity. For the NH₂G4 structures there was no antimicrobial activity visually observed, as all the wells showed comparable cell growth to the control (cells grown in MHB with no OXG4 or NH₂G4) (data not shown).

It was found that both the OX and OXG4 structures had a MIC of $0.5 \mu\text{g mL}^{-1}$ (or $2.5 \mu\text{M}$ OXG4) (data not shown). This indicated that the coupling OX to the $\text{NH}_2\text{G4}$ ODN does not affect its antimicrobial activity profile. As a result, it showed promise to be used to investigate if the OXG4 structures could provide increased diffusion of the OX into the biofilm, without the need for added hydrogen peroxide. Singh *et al.*³³ have previously found that $1 \mu\text{g mL}^{-1}$ OX alone was unable to kill biofilm embedded cells. To this end, in order to perform biofilm diffusion studies, $1 \mu\text{g mL}^{-1}$ of OX and $2.5 \mu\text{M}$ OXG4 (equivalent to $1 \mu\text{g mL}^{-1}$ of free OX) was chosen. In this manner, any antimicrobial effects observed from the OXG4/hemin complex could be attributed to the peroxidase-mimicking activity, rather than the activity of the OX alone.

First, OX ($1 \mu\text{g mL}^{-1}$), hemin ($8 \mu\text{M}$) and various concentrations of G4/hemin complexes ($2 \mu\text{M}$, $3 \mu\text{M}$, $4 \mu\text{M}$ or $5 \mu\text{M}$, with hemin at $8 \mu\text{M}$) were tested on *S. aureus* ATCC 29213 (biofilm forming) biofilms to determine if they had an antimicrobial effect. Initially, *S. aureus* ATCC 29213 biofilms were established in 96-well plates and on silicone coupons using MHB with 20 h incubation at 37°C . Following this the MHB was removed and then either OX ($1 \mu\text{g mL}^{-1}$), hemin ($8 \mu\text{M}$) or G4/hemin complexes ($2 \mu\text{M}$, $3 \mu\text{M}$, $4 \mu\text{M}$ or $5 \mu\text{M}$, with hemin at $8 \mu\text{M}$) in MHB (containing 0.5% (v/v) H_2O_2) was added on top of each biofilm. The biofilm was then grown for a further 20 h to investigate antimicrobial activity and whether any persister cells remained after the initial growth and MHB removal (evidenced by further colony forming unit (CFU) described later). The wells and coupons were then washed with phosphate buffer saline (PBS) to remove any planktonic cells.

A LIVE/DEAD™ BacLight™ Bacterial Viability Kit and CFSEM were used to qualitatively confirm the presence of biofilms grown on silicone coupons (see Figure S1 for control). The validation of the biofilms by viable counts and CFSEM paved the foundation for further

experiments. The number of viable cells present in the biofilms was determined using CFU based viable count (see Figure S2 and S3). The control sample had a CFU of $\sim 10^7$ CFU mL⁻¹ (Figure S2 and S3).

Figure 6 and Table S1 shows the percentage $\Delta \log_{10}$ CFU mL⁻¹ compared to the control (cells only in MHB).

Figure 6. Bar graph showing the percentage $\Delta \log_{10}$ CFU mL⁻¹ present in the *S. aureus* biofilms after 20 h incubation with OX (1 μ g mL⁻¹), hemin (8 μ M) and G4/hemin complexes at 2 μ M, 3 μ M, 4 μ M and 5 μ M (with hemin at 8 μ M) in a MHB containing 0.5% H₂O₂ (v/v).

Figure 6 shows that the OX has only a slight effect on the cells within the biofilm, with a change in the number of cells of $-1.61 \pm 0.24\% \Delta\log_{10} \text{CFU mL}^{-1}$ compared to the control. This is expected as OX ($1 \mu\text{g mL}^{-1}$) is unable to enter or diffuse within biofilms.¹⁷ Another contributing factor is the presence of the 0.5% H_2O_2 , which may be degrading the antibiotic free in solution. In the presence of 0.5% (v/v) H_2O_2 , hemin ($8 \mu\text{M}$) showed an effective antimicrobial activity on the cells inside of the biofilm, with a reduction of $29.43 \pm 2.39\% \Delta\log_{10} \text{CFU mL}^{-1}$ of viable cells. This was not expected as hemin is thought to have low peroxidase-mimicking activity when by itself in solution.²⁷ The G4/hemin at $2 \mu\text{M}$ and $3 \mu\text{M}$ showed similar results to hemin alone, however, once the concentration of the G4/hemin reached $4 \mu\text{M}$ and $5 \mu\text{M}$ the effectiveness to reduce the number of viable *S. aureus* cells in the biofilm was doubled to $50.02 \pm 4.64\% \Delta\log_{10} \text{CFU mL}^{-1}$ and $52.58 \pm 4.36\% \Delta\log_{10} \text{CFU mL}^{-1}$, respectively.

Overall, these results are very positive, showing that hemin alone has an antimicrobial affect, but this is greatly increased by its complexation with G4 structures when H_2O_2 is present in the MHB. Further, these structures are able to enter and diffuse through the biofilm, unlike OX alone.³³ It is known that G4/hemin complexes within the body are able to react with peroxide species and subsequently cell components (e.g., cell walls) and damage cells in much the same manner as the SCN-peroxidase- H_2O_2 systems.²⁷ Therefore, it was next investigated if it was possible for the G4/hemin complex to have similar effects without the addition of the 0.5% H_2O_2 . Further investigation is required to determine how the G4/hemin complexes are able to penetrate and the exact mechanisms.

To improve the antimicrobial activity of the G4/hemin complexes, the impact of OX coupled G4/hemin complexes on the *S. aureus* biofilms was studied. In order to keep analysis consistent, the concentration of the OXG4 was calculated in terms of OX concentration assuming a near

~100% coupling of OX to G4. Therefore, a stock solution of 5 μM OXG4 was made up by diluting the purified OXG4 in MHB. 10 μL of the 5 μM OXG4 was added to a 96-well plate and 10 μL of MHB broth was added. To this end OX (1 $\mu\text{g mL}^{-1}$), hemin (8 μM), G4 (50 μM), G4/hemin complex (50 μM), OXG4 (2.5 μM) and OXG4/hemin complex (2.5 μM), were tested for antimicrobial activity against *S. aureus* ATCC 29213 biofilms in a similar manner to the previous biofilm studies (Figure 6). Since the peroxide was shown affect the OX in a negative way, based on the increase in the $\Delta\log_{10} \text{CFU mL}^{-1}$ ($-1.61 \pm 0.24\% \Delta\log_{10} \text{CFU mL}^{-1}$) the remaining experiments were performed without the aid of peroxide. It was hypothesized that the natural peroxide species produced within the biofilms would facilitate peroxidase-mimicking activity of the G4/hemin complexes.

Figure 7. Bar graph showing the percentage $\Delta\log_{10}$ CFU mL⁻¹ present in the *S. aureus* biofilms after 20 h incubation folded structures of OX (1 μ g mL⁻¹), hemin (8 μ M), G4 (50 μ M), G4/hemin complex (50 μ M), OXG4 (2.5 μ M) and OXG4/hemin complex (2.5 μ M).

Figure 7 and Table S2 show that the OXG4 and OXG4/hemin complex had the largest effect in regard to the reduction in the number of viable *S. aureus* cells, with a reduction of $28.76 \pm 0.51\%$ $\Delta\log_{10}$ CFU mL⁻¹ and $25.82 \pm 1.18\%$ $\Delta\log_{10}$ CFU mL⁻¹, respectively. This indicates that the OX attached to the G4 is having improved antibiotic activity compared to the G4 alone or G4/hemin complex. This is hypothesized to be due to the lack of endogenous peroxide species in the MHB and biofilm. As expected, OX had the lowest reduction of viable cells at $11.43 \pm 2.32\%$ $\Delta\log_{10}$ CFU mL⁻¹, again indicating that OX is not able to readily cross the biofilm. However, it should be noted that unlike Figure. 6, where there was an increase in $-1.61 \pm 0.24\%$ $\Delta\log_{10}$ CFU mL⁻¹, there was a decrease in the $\Delta\log_{10}$ CFU mL⁻¹.

Clearly, this work indicates that G4/hemin complexes alone can reduce *S. aureus* cell viability in biofilms in the presence of H₂O₂. Importantly by coupling these structures with the antibiotic OX, an increase in diffusion into the biofilm allows for further improved cell death compared to OX alone. This shows that there is a possibility to use DNA as a possible diffusion facilitator for other coupled drugs.

4. CONCLUSION

This work details a new antimicrobial system for combating *S. aureus* biofilm forming bacteria. Using a TMB assay and MIC analysis it was shown that OX could effectively be coupled to an amine-functionalized G-rich ODN. Preliminary studies of the unique G4/hemin complex systems, in the presence of 0.5% (v/v) H₂O₂, showed a reduction in biofilm cells of up to $52.02 \pm 4.64\%$ $\Delta \log \text{CFU mL}^{-1}$ at 5 μM compared to hemin alone, showing the G4 was able to penetrate and diffuse into the biofilms. The antimicrobial activity of the OXG4\hemin complex, without peroxide, was shown to be over twice as effective as OX alone, indicating that the G4 played an active role as a diffusion facilitator of OX into the biofilm matrix. This work shows promise for increasing the effectiveness of antibiotics using unique DNA nanostructuring assemblies, such as the G4 complexes used here. This system may provide a better candidate for peroxidase-like antimicrobial systems into the future.

SUPPORTING INFORMATION

The following files are available free of charge. Provides information on fluorescence confocal scanning microscope image of *S. aureus* ATCC 29213 biofilms and CFU data.

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ABBREVIATIONS

G4, G-Quadruplex; OX, oxacillin; OXG4, oxacillin coupled G-quadruplex; ODN, oligodeoxynucleotide; SCN, thiocyanate; H₂O₂, hydrogen peroxide; G-rich, guanine-rich; G-quartets, guanine-quartets; Fe, iron; TMB, 3,3,5,5-tetramethylbenzidine; MIC, minimum inhibitory concentrations; BSA, bovine serum albumin; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; nts, nucleotides; PAGE, polyacrylamide electrophoresis; MHB, Mueller-Hinton Broth; CFU, colony forming unit; PBS, phosphate buffer saline; CSFM, confocal fluorescence scanning microscopy.

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TABLE OF CONTENTS