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Identification of New Nitric Oxide-donating Peptides with Dual Biofilms Eradication and Antibacterial Activities for Intervention of Device-related Infections

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ABSTRACT: Implantable medical device-related infections with biofilms have become a significant challenge in clinic. Based on the potential bacteria biofilm dispersing effect of nitric oxide (NO) and the unique antibacterial activity of antimicrobial peptides (AMP), we synthesized five peptides and selected the most potent one to conjugate its *N*-terminal with a furoxan moiety to offer a hitherto-unknown NO-donating antimicrobial peptide (**FOTyr-AMP**), which exhibited *S. aureus* and *E. coli* biofilms dispersing and eradication, and potent antibacterial activities in vitro. In an implanted biofilms infection mice model, topical subcutaneous injection of **FOTyr-AMP** allowed synergetic eradication of bacterial biofilms and potent antibacterial activity, superior to antibiotic Cephalosporin C. Given low hemolysis effect, little influence on the blood pressure and potent in vivo efficacy of **FOTyr-AMP**, it is clear that subcutaneous administration of **FOTyr-AMP** could be a

promising approach for the intervention of medical device-related biofilm infections with desirable safety.

INTRODUTION

Implantable medical devices, such as intravascular catheters, heart valves, pace-makers, prosthetic joints, dialysis catheters, dental implants, orthopaedic and trauma devices, have benefited healthcare, treatment, and recovery of patients. However, high risk of the medical device-related infections has remarkably threated the health of patients, causing undue distress[1]. Especially, the biofilm formation on the surfaces of the implants could protect bacterial species against antimicrobial agents and immune defenses, resulting in obstinate infection and increasing morbidity and mortality[2-3]. Biofilms are aggregates of microorganisms in which cells are frequently embedded in a self-produced matrix of extracellular polymeric substances[4]. Once the biofilm is formed, bacterial cells being protected inside the biofilm could perform high resistance to antimicrobial agents over 10^3-folds compared to their planktonic counterparts[2, 5]. In this regard, most clinical drugs are assessed failed for treatment of such situations. Given the key roles of biofilms in device-related infections and limited treatment options, solutions towards opening the ‘black box’ of biofilm infections are urgently needed.

As a well-known gas messenger molecule, nitric oxide (NO) is necessary in body's innate immune response to foreign pathogens, especially in macrophage defenses against intracellular bacteria. NO as such, or together with reactive nitrogen species (RNS) which derived from the reaction of NO with reactive oxygen species (ROS), could cause oxidative and nitrosative damage to microbial proteins, DNA, metabolic enzymes, or exterior membrane structure. Furthermore, it was reported that NO could effectively induce biofilm dispersal in *Pseudomonas aeruginosa*[6], *Staphylococcus aureus*[7-8] and other bacterial species[9-10]. The possible mechanism is that NO could reduce the levels of cyclic diguanylate (c-di-GMP), which promotes bacteria-surface attachment and then formation of biofilms[11-13]. Therefore, NO-based antimicrobials, including enzyme-triggered NO-donating agents, NO-releasing macromolecules and NO donor coated nanoparticles, have attracted many attentions, representing a promising alternative for the treatment of chronic biofilm infections[14-16]. However, in vivo therapeutic efficacy against biofilms infection for these NO donating agents were not reported yet.

Furoxans, a kind of NO donor, containing 1,2,5-oxadiazole 2-oxide heterocyclic scaffold, can efficiently release NO under the attack of thiol-containing molecules (Scheme S1), especially glutathione (GSH), at positions 3 and 4[17-18]. Compared to other NO donors, furoxans have a characteristic of prolonged duration of action[19-21]. Notably, it was reported that GSH played an important role in *P. aeruginosa* biofilm formation and infection[22]. Therefore, topical administration of furoxans could specifically release NO under attack of GSH at the infection site to achieve biofilm dispersing effect. Importantly, besides the biofilm eradication, bacteria killing is indispensable for biofilm infections. Antimicrobial peptides (AMPs), possess unique membrane-permeabilizing activity, representing a promising therapeutic option against bacterial infections[23]. Cationic peptides[24], a group of AMPs playing an important role in immune system, possesses broad spectrum of antibacterial activity, low resistance and hemolysis. In these regards, we hypothesized that whether hybridization of furoxan moiety with AMPs could produce a hitherto-unknown NO-donating AMPs with dual biofilms eradication and antimicrobial activities that could be more effective for the intervention of medical device-related biofilm infections than monotherapy in vivo. To test this hypothesis, we

selected and synthesized five cationic peptides **1-5** with straight chain which were reported to have potentially broad antibacterial activity[25-27] and tested the antibacterial activity against Gram-positive *S. aureus* and Gram-negative *E. coli*. As shown in table S1, peptide **5** exhibited the most potent antibacterial activity. Therefore, we conjugated the *N*-terminal of peptide **5** with a furoxan moiety via a tyrosine-linkage and a click reaction to produce a NO-donating AMP, **FOTyr-AMP** (Figure 1A). It was proposed that **FOTyr-AMP**, containing a furoxan head and an AMP tail, could be endowed enhanced antimicrobial efficacy to biofilm-exist infection owing to biofilms dispersing assisting with antimicrobial activity (Figure 1B). We investigated the antimicrobial activities, biofilms eradication effect of **FOTyr-AMP** against *S. aureus* and *E. coli* in vitro. Furthermore, we evaluated the potential of **FOTyr-AMP** for the intervention of medical device-related infections via topical administration strategy by using a biofilms infection model in mice.

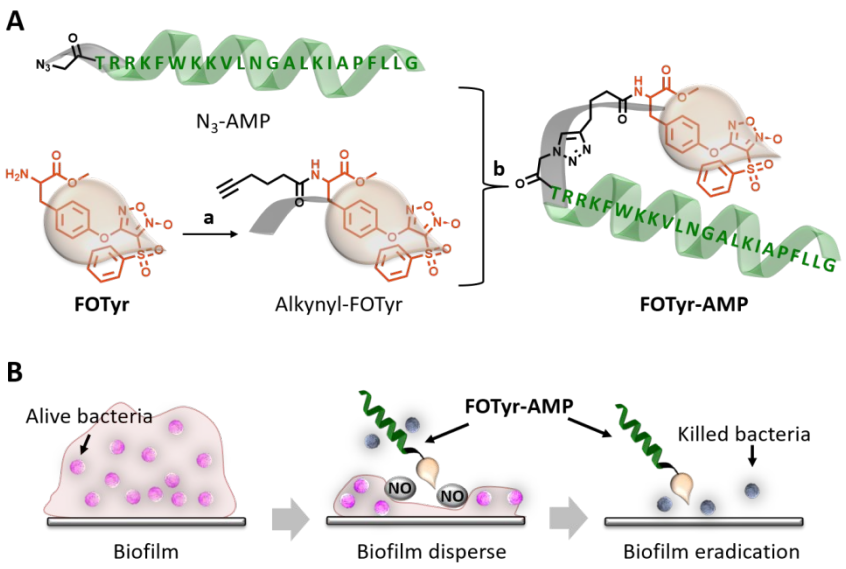

Figure 1. A) The synthetic route of **FOTyr-AMP**. N_3-AMP (green) was synthesized based on solid-phase peptide synthesis (SPPS) approach. Furoxan moiety FOTyr was synthesized and characterized. Reagents and conditions: a) 5-hexynoic acid, HBTU, N-methylmorpholine, DMF, overnight; b) $CuSO_4$, sodium ascorbate, THF, H_2O. B) Schematic illustration of biofilms eradi-cation and enhanced antibacterial activity of **FOTyr-AMP** against biofilm protected bacterial infection.

RESULTS AND DISCUSSION

The five peptides (RLARIVVIRVAR-NH_2 **1**; ILAWKWAWWAWRR-NH_2 **2**; KSRIVPAIPVSLL-NH_2 **3**; ILPWKWPWWPWRR **4**; TRRKFWKKVLNGALKIAPFLLG **5**)

were synthesized by using a solid phase method and the procedure are described in Scheme S2-6. The synthetic route of **FOTyr-AMP** is depicted in Figure 1A (detailed in Scheme S7). The reaction of the *N*-terminus of peptide **5**-resin with azide acetate, and the subsequent deprotection by 5% triisopropylsilane (TIPS) in trifluoroacetic acid (TFA) solution produced azide derivative N_3-AMP. Meanwhile, tyrosine methyl ester substituted furoxan derivative FOTyr (**11**) was synthesized according to the previously reported method[28] (Scheme S7). The condensation reaction of FOTyr (**11**) with 5-hexynoic acid furnished alkynyl-FOTyr (**12**). Finally, the click reaction of N_3-AMP and alkynyl-FOTyr (**12**) produced target molecule **FOTyr-AMP**.

The NO release from FOTyr and **FOTyr-AMP** in Gram-positive bacteria *S. aureus* and Gram-negative *E. coli* cells were firstly investigated by measuring the concentrations of nitrite (an oxidative metabolite of NO) in cell lysis after co-incubation for 4 h by Griess assay. As shown in Table S2 and S3, **FOTyr-AMP** released significantly more amount of NO than FOTyr in both *S. aureus* and *E. coli* strains, while the pretreatment of 1-chloro-2,4-nitrobenzene [29](CDNB, a well-known GSH-depleted agent) significantly decreased the NO release from **FOTyr-AMP** in *S. aureus* strains, suggesting that **FOTyr-AMP** could

effectively penetrate into bacteria cells and release NO under attack of thiol-containing molecules. Additionally, the dose dependent hemolysis assay provided that **FOTyr-AMP** possessed less hemolysis than AMP (Figure S1), and the half hemolytic concentration (HC_{50}) of **FOTyr-AMP** was greater than 380 μM.

The antibacterial activity of FOTyr, peptide **5** (AMP) and **FOTyr-AMP** against *S. aureus* and *E. coli* was evaluated by microdilution assay, and the corresponding minimum inhibitory concentration (MIC) values were calculated and summarized in Table S4. It was found that furoxan moiety FOTyr showed weak bacterial growth inhibitory activity (MIC values were greater than 50 μM), and **FOTyr-AMP** exhibited slightly enhanced bacterial growth inhibitory activity than AMP against *S. aureus* (2.2 vs 3.1 μM) and *E coli* (1.3 vs 2.0 μM), suggesting the introduction of furoxan moiety at the *N*-terminus of AMP may not affect the membrane-permeabilizing activity of AMP, but somehow improve the antibacterial activity due to the release of NO. Furthermore, by using scanning electron microscopy (SEM), the morphologies of *S. aureus* and *E. coli* cells treated by **FOTyr-AMP** (200 μM, 60 mins) confirmed that the membrane of bacteria

was ruptured and the contents were partially spilled (Figure 2), indicating potent bacteria killing effect of **FOTyr-AMP**.

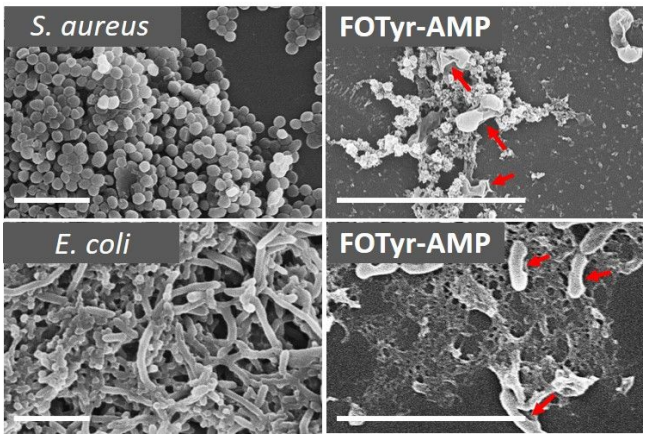

Figure 2. SEM images of **FOTyr-AMP** treated *S. aureus* and *E. coli* strains. Left: Controls; Right: **FOTyr-AMP** (200 μM) treated bacteria (Up: *S. aureus*; Down: *E. coli*) for 60 mins. Red arrows: bacteria cells with broken cell wall/membrane and leakage of contents. Bar: 5 μm.

Biofilm dispersal effect of FOTyr, AMP and **FOTyr-AMP** against *S. aureus* and *E. coli* were quantitatively investigated by crystal violet staining assay. As shown in Figure 3, the biofilm biomass of both *S. aureus* and *E. coli* in AMP treated groups (100 μM for 12 h) were similar to those in the vehicle control group, indicating little biofilm dispersing

effect of AMP (Figure 3A). In sharp contrast, treatment of FOTyr and **FOTyr-AMP** (100 μM for 12 h) significantly reduced the biofilm biomass of *S. aureus* by 44.4% and 64.1%, and reduced the biofilm biomass of *E. coli* by 81.2% and 80.9%, respectively (Figure 3A). Interestingly, the addition of a NO scavenger PTIO (100 μM) totally abrogated the biofilm eradication effect of **FOTyr-AMP** (Figure S2). Collectively, these data suggest that the NO released from furoxan moiety under the attack of thiol-containing molecules mainly contributed the biofilm dispersing effect of **FOTyr-AMP**. In order to visualize the morphologies of the biofilms after treatment, the SEM images of all groups were recorded. Briefly, *S. aureus* and *E. coli* were cultured on the silicon wafers in Trypticase Soy Broth (TSB) medium for 24 h to allow the formation of biofilms, followed by the addition of blank TBS, or AMP, FOTyr and **FOTyr-AMP** in TBS, respectively, at a dose of 100 μM for further incubation of 12 h. As shown in Figure 3B, in the blank TSB treated group (control), it can be seen a densified and thick biofilm, while the treatment of AMP displayed weak biofilm dispersing effect. In sharp contrast, the treatment of **FOTyr-AMP** dramatically dispersed the formed biofilms superior to FOTyr.

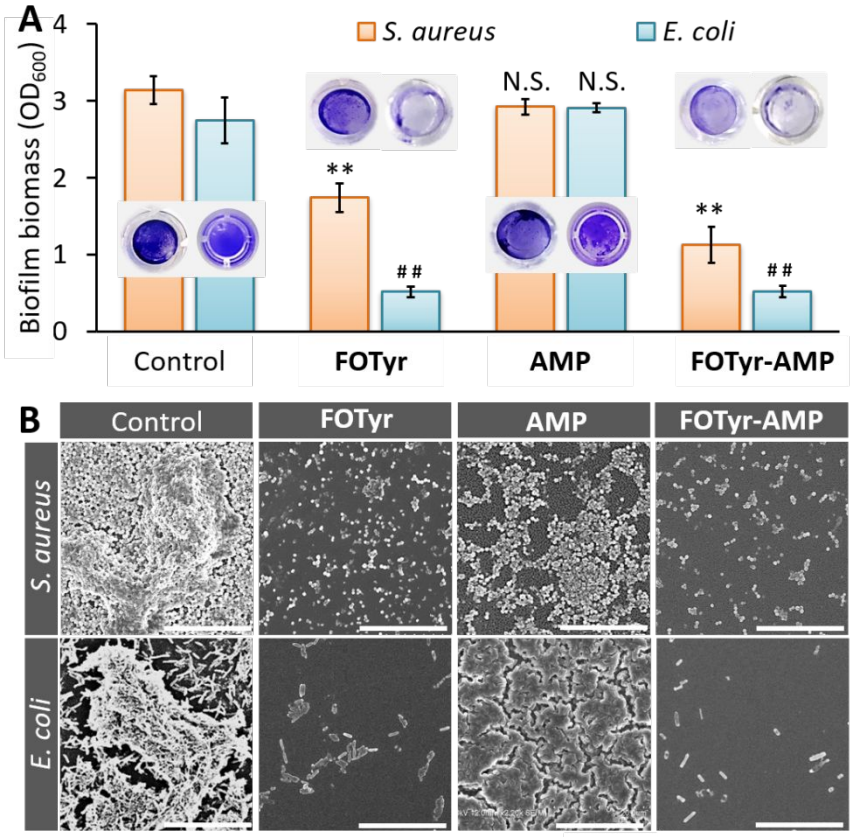

Figure 3. A) Quantitative calculation of biofilm biomass (*S. aureus* and *E. coli*) by crystal violet staining assay. The statistical analysis of FOTyr, AMP and **FOTyr-AMP** (100 μM for 12 h) were all compared with the control. Statistical significance; **/# #, $p < 0.01$; two-sample t-Test analysis. Data: mean ± S.D. (n = 3). B) SEM images of biofilms after treatment of blank TSB, 100 μM of FOTyr, AMP or **FOTyr-AMP** in TSB for 12 h. Bar: 20 μm.

To fully understand the biofilm eradication dynamics and planktonic bacteria killing effect of **FOTyr-AMP**, we designed a mixed assay (Figure 4A and 4C). Briefly, *S. aureus* was added into a 16-cell plate and incubated for 24 h to allow biofilm grow on the bottom, which were timely recorded by using a real-time cell counter assay[30]. After discarding the supernatant and washing with saline, blank TSB, AMP, **FOTyr-AMP** and a known antibiotic Cephalosporin C (CPC) in TSB were respectively added with a final concentration of 100 μM and incubated for further 9 h. During this period, the biofilms on the bottom of each groups were continually monitored every 15 mins to obtain a biofilm quantity-time profile (Figure 4A). It was found that during the initial 24 h before the administration of the agents, the biofilms of all groups in the 16-cell plate showed an exponential growth between 0~7 h, then a stable maintenance lasted up to 24 h (Figure S3). Compared with the naturally growth of the biofilm (TSB control), the group of AMP and CPC had a significant inhibition of the continuous biofilm growth after treatment for 1-2 h, while the **FOTyr-AMP** treated group showed downward trend at the beginning of the treatment (Figure 4B). These results indicated that AMP and CPC could efficiently kill the freed bacteria outside of biofilm leading to the inhibition of the biofilm growth,

whereas **FOTyr-AMP** actively disperse the biofilm, freed the protected bacteria and kill planktonic bacteria resulting a dispersing and eradication of the biofilm. The same assay was conducted for FOTyr and FOTyr plus AMP**.** As shown in Figure S4-S5, FOTyr plus AMP had a sustaining and stable inhibitory effect on the biofilm growth during the whole period after treatment, while FOTyr alone was not able to suppress the biofilm growth during the last 2h, indicating the addition of AMP indeed synergistically inhibited the biofilm growth due its antibacterial activity. To further confirm this conclusion, we carried out another assay to evaluate both biofilm eradication (biofilm on the bottom) and antibacterial activity (in supernatant) of the test compounds (Figure 4C). *S. aureus* strains was added into a confocal dish and incubated for 24 h. After discarding the supernatant and washing with saline, blank TSB, FOTyr, AMP, **FOTyr-AMP** and CPC in TBS were respectively added with a final concentration of 100 μM and incubated for further 12 h. After incubation finished, the biofilms on the bottom of the confocal dish and the supernatant were separated. The former ones were conducted 3D confocal imaging to visualize the live bacteria cells by using live cells fluorescence staining assay (Figure 4D), while the latter ones were measured the OD values to calculate the living

planktonic bacterial cells in the supernatant (Figure 4E). 3D confocal imaging exhibited that the treatments of CPC were not able to effectively decrease the living bacterial cells in the biofilm on the bottom of the confocal dish compared with the solvent control (Figure 4D). Dramatically, the treatment of **FOTyr-AMP** almost completely killed the bacterial cells in the biofilm on the bottom, superior to AMP and FOTyr (Figure 4D). Accordingly, **FOTyr-AMP** also significantly killed the living planktonic bacterial cells in the supernatant, superior to CPC (Figure 4E). Interestingly, although FOTyr significantly decreased the living bacterial cells in the biofilm on the bottom of the confocal dish (Figure 4D), it displayed weak bacterial cells killing activity in the supernatant (Figure 4E). Collectively, these data demonstrated that **FOTyr-AMP**, containing furoxan head and AMP tail, not only effectively dispersed biofilm, but also exhibited potent antibacterial activity superior to AMP and CPC, suggesting a promising agent for the intervention of biofilm infection.

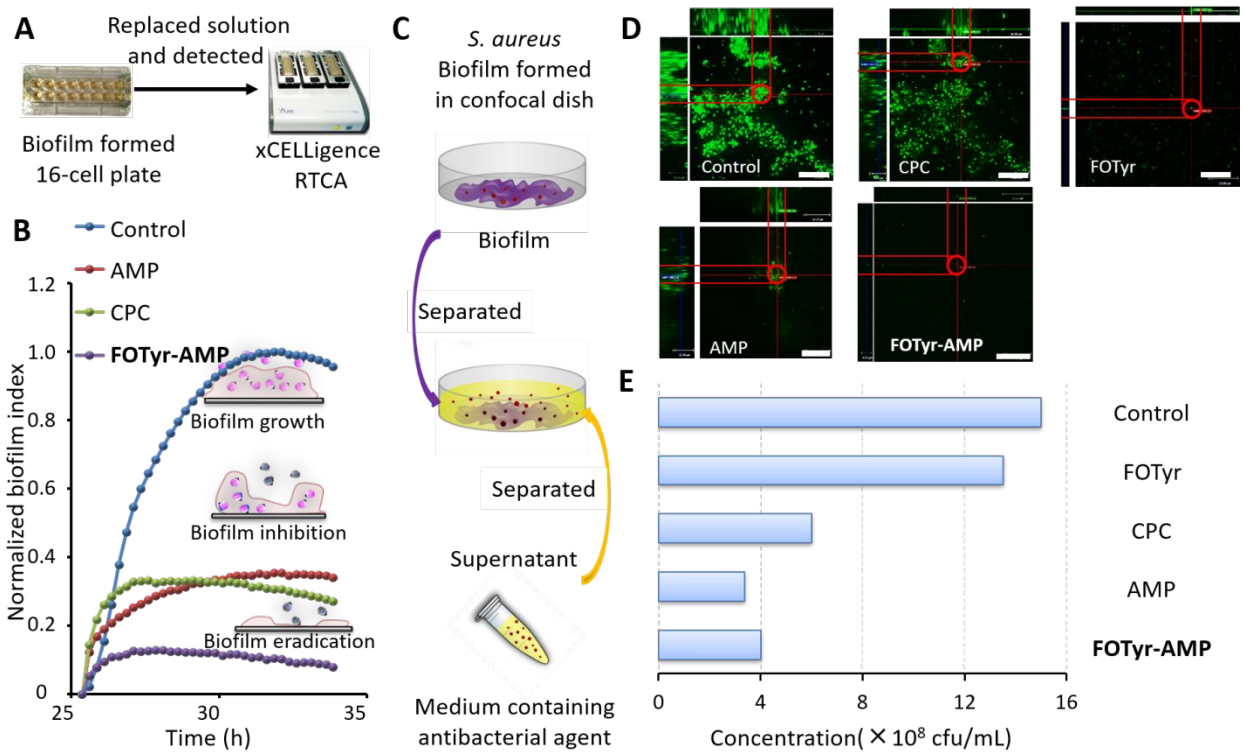

Figure 4. A) Schematic illustration of dynamic detection of biofilm. B) Biofilm index of S. aureus by the treatment of the test compounds. After one day of biofilm formation (for 24 h, at 37 ℃ under 5% CO_2), the medium was changed to TSB, AMP (100 μM in TSB), CPC (100 μM in TSB), **FOTyr-AMP** (100 μM in TSB), respectively, and the biofilm indexes were continuously monitored every 15 mins for 9 h by xCELLigence RTCA DP Instrument. C) Schematic illustration of the mixed assay to evaluate the biofilm eradication (biofilm on the bottom) and antibacterial activity (in supernatant) of the test compounds. D) 3D confocal images of the biofilm on the bottom of the confocal dishes

for each group after 12 h of incubation. Bar 20 μm. E) Calculation of the living bacterial cells in the supernatant separated from the confocal dishes.

To verify the potential of biofilms eradication and antibacterial activity of **FOTyr-AMP** in vivo, we modified a previously reported biofilms infection model to mice[31-32]. As shown in Figure 5A, we cultured biofilms of *S. aureus* on the surface of polydimethylsiloxane (PDMS) slices (0.3 × 0.3 cm) overnight, and then the PDMS slices were subcutaneously implanted into the back of the mice. Twelve hours after implantation, blank solvent, FOTyr, AMP, **FOTyr-AMP**, **FOTyr-AMP** plus NO scavenger PTIO, and CPC with the same dose of 0.25 μmol/kg in 5% DMSO in saline, were respectively subcutaneously administrated to mice every 12 h for 3 days. Then, the PDMS slices were removed from mice for ultrasound, and the bacterial cells dropped from the PDMS slices were quantitatively counted. It was found that in the control group the skin near the implanted site had obvious ulceration, indicating a serious bacterial infection, while in the **FOTyr-AMP** treated group no visible ulceration was observed (Figure 5B). Plate colony counting assay[33] demonstrated that **FOTyr-AMP** (Log_{10}CFU

value 4.54 ± 0.30) significantly decreased colony counts than solvent control (Log_{10}CFU value 6.40 ± 0.28), superior to AMP (5.40 ± 0.14), CPC (4.97 ± 0.21) and FOTyr (4.91 ± 0.49). Notably, the co-treatment of NO scavenger PTIO (0.25 μmol/kg) significantly abrogated the activity of **FOTyr-AMP**, decreasing Log_{10}CFU value to 5.92 ± 0.13 (Figure 5C), indicating the contribution of NO. In addition, the vicinity tissues of the implanted site were taken to perform H&E staining to evaluate bacterial infection. We found that **FOTyr-AMP** significantly decreased the bacterial inflammation in the vicinity tissues of the implanted site, superior to the antibiotic CPC and AMP groups (Figure 5D). Notably, we found that the subcutaneous administration of **FOTyr-AMP** (0.172 μmol/kg) in rats had little influence on the blood pressure within 6 h after injection (Table S5). Given the low hemolysis effect, little influence on the blood pressure and potent in vivo efficacy of **FOTyr-AMP**, it is clear that subcutaneous administration of **FOTyr-AMP** could be a promising approach for the intervention of medical device-related infections with desirable safety.

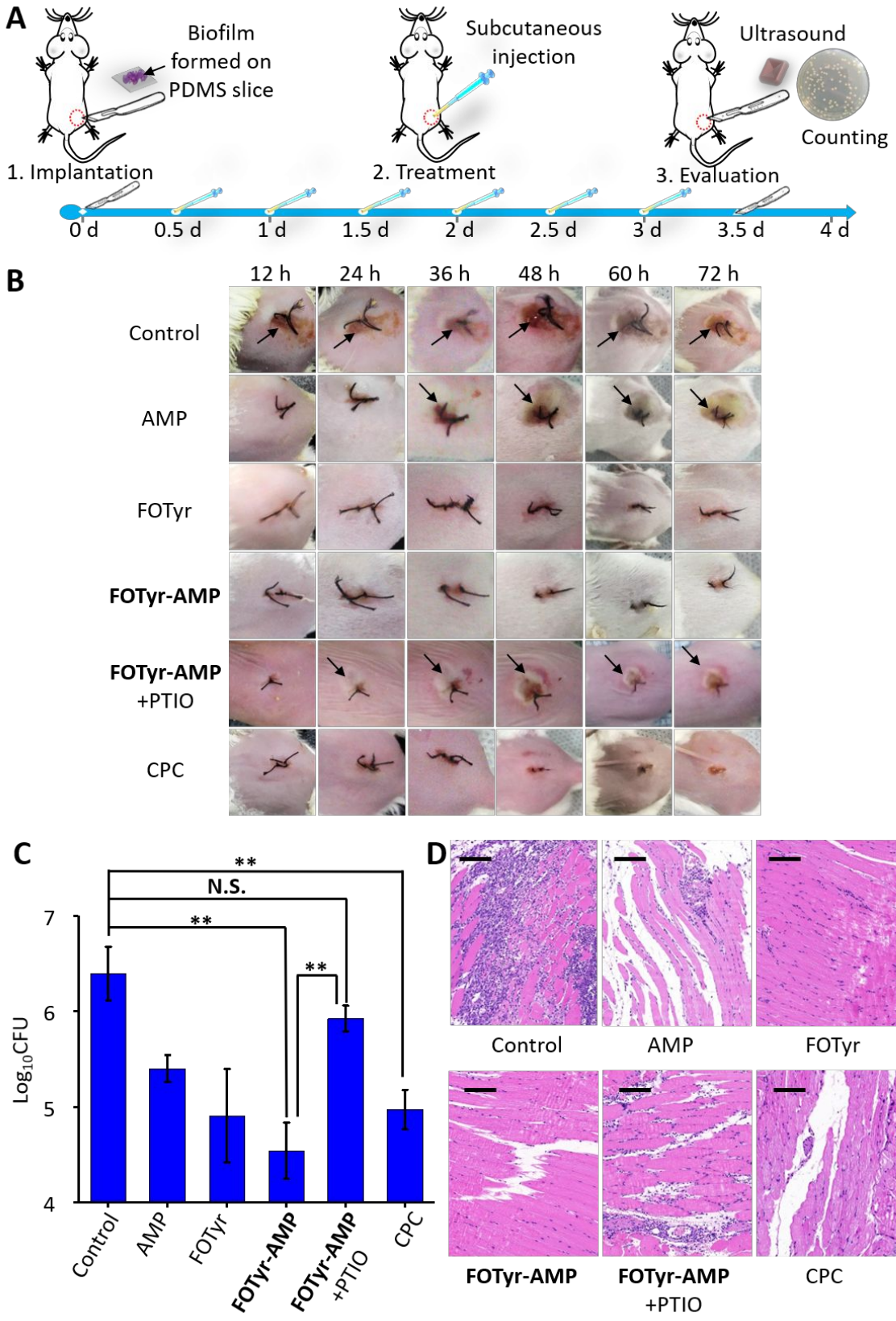
A
Biofilm formed on PDMS slice
1. Implantation
Subcutaneous injection
2. Treatment
Ultrasound
Counting
3. Evaluation
0 d
0.5 d
1 d
1.5 d
2 d
2.5 d
3 d
3.5 d
4 d
B
12 h
24 h
36 h
48 h
60 h
72 h
Control
AMP
FOTyr
FOTyr-AMP
FOTyr-AMP +PTIO
CPC
C
**
N.S.
**
**
Log10CFU
7
6
5
4
Control
AMP
FOTyr
FOTyr-AMP
FOTyr-AMP +PTIO
CPC
D
Control
AMP
FOTyr
FOTyr-AMP
FOTyr-AMP +PTIO
CPC

Figure 5. A) Schematic diagram of biofilm infection mice model. B) State diagram of the implanted site every 12 h after PDMS slices were surgically implanted. Black arrows: obvious ulceration sites. C) Bacterial culture counting on the implanted PDMS after ultra-sound. The data were expressed as mean ± SD (n = 3), one-way ANOVA with a Turkey comparisons post hoc test. ** $p<0.05$. D) H&E staining of the vicinity tissues of the implanted site (Bar 50 μm).

CONCLUSION

Herein, we have synthesized five antibacterial peptides and selected the most potent one to conjugate its *N*-terminal with a furoxan moiety to offer a hitherto-unknown NO-donating AMP **FOTyr-AMP**. Notably, the conjugation of AMP with the NO donor provided dual biofunctions of biofilm eradiation and antibacterial activity. Dynamics studies of biofilm dispersing showed that **FOTyr-AMP** released NO from the furoxan head to sustainably eradicate biofilm, subsequently free the bacterial cells, which could be more sensitive to the AMP tail. Furthermore, we established an implanted biofilms infection mice model, to the best of our knowledge for the first time, to evaluate the in

vivo therapeutic efficacy of the NO donating AMP for implantable medical device-related biofilms infections. It was found that **FOTyr-AMP** significantly decreased bacterial cells on the PDMS slices, relieved the skin ulceration and decreased the bacterial inflammation in the vicinity tissues of the implanted site, superior to AMP and antibiotic CPC. Importantly, subcutaneous administration, GSH specific NO-release and bacterial targeting of AMP all contributed to minimize NO-mediated off-target effects. In conclusion, **FOTyr-AMP** exhibited dual biofilms eradication and antibacterial activities, warranting further investigation for the intervention of implantable medical device-related biofilm infections. Our strategy may also expand the NO-based therapeutics for clearing biofilm-based infections.

EXPERIMENTAL SECTION

Chemistry. General information. All commercially available compounds were used without further purification, unless otherwise noted. Analytical and preparative TLC was performed on silica gel (200 - 300 mesh) GF/UV 254 plates, and the chromatograms were visualized under UV light at 254 and 365 nm. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker AVANCE III HD 400 (^{1}H, 400 MHz; ^{13}C, 100 MHz) spectrometer at 303 K, using TMS as an internal standard. MS spectra were recorded with a Mariner mass spectrometer (ESI) and high-resolution

mass spectrometry (HRMS) spectra on an Agilent Technologies LC/MSD TOF instrument. Solutions after reactions and extractions were concentrated using a rotary evaporator operating at a reduced pressure of ~20 Torr. HPLC was performed on a Shimadzu Series (LC-20AT) using an InertSustain 5 μm C18 column (4.6 × 250 mm) to determine the purity of the test compounds. Individual compounds with a purity of > 95% were used for biological experiments.

General synthesis protocol for peptides. The resin was soaked in DMF for 2 h and then soaked in DMF containing 20% piperidine for 15 min at the room temperature to remove the Fmoc group. Then the resin was washed for 3 times with dichloromethane and DMF alternately, after that 3-5 particles were taken out into 1.5 mL centrifuge tube containing Kaiser test solution, heated to boiling about 1min (if particles turned black, the protective group has been removed). The obtained resin was filtered and reacted with the Fmoc-AA-OH (10 equiv.) in the presence of hexafluorophosphate benzotriazole tetramethyl uronium (HBTU, 10 equiv.) and *N*-methylmorpholine (5%) in DMF. One hour later, the resin was washed for 3 times with dichloromethane and DMF alternately, 3-5 particles were taken out into 1.5 mL centrifuge tube containing Kaiser test solution, heated to boiling about 1min (if particles did not turn black, the coupling has been completed). After that, the resin was filtered and then soaked in DMF containing 20%

piperidine to remove the Fmoc group. The similar condensation and deprotection reactions were conducted in the order of the AA sequence to eventually furnish peptide resin. Finally, 5% triisopropylsilane (TIPS) in trifluoroacetic acid (TFA) solution was used to slice peptide from resin, as well as the Boc groups from Lysine and Tryptophan, Pbf group form Arginine, tBu group from Threonine. The Kaiser test solution was composed of one drop Ninhydrin solution (0.5 g Ninhydrin in 10 mL Ethanol), one drop phenol solution (20 g phenol in 5 mL Ethanol) and one drop ascorbic acid solution (0.1 g ascorbic acid in 5 mL Ethanol).

Peptide **1**, RLARIVVIRVAR-NH_2, a total yield of 17%, R: Arginine; V: Valine; L: Leucine; A: Alanine; I: Isoleucine. ESI MS (m/z): $[M + 2H]^{2+}$ calcd for $C_{63}H_{123}N_{25}O_{12}$, 711.0; found, 711.7. Peptide **1** combines two protons, therefore, the molecular weight of the peptide **1** is 711.7*2-2=1421.4, M calcd for $C_{63}H_{123}N_{25}O_{12}$, 1420.8.

Peptide **2**, ILAWKWAWWAWRR-NH_2, a total yield of 15%, R: Arginine; K: Lysine; W: Tryptophan; L: Leucine; A: Alanine; I: Isoleucine. ESI MS (m/z): $[M + 2H]^{2+}$ calcd for $C_{94}H_{128}N_{26}O_{13}$, 914.5; found, 914.7. Peptide **2** combines two protons, therefore, the molecular weight of the peptide **2** is 914.7*2-2=1827.4, M calcd for $C_{94}H_{128}N_{26}O_{13}$, 1827.0.

Peptide **3**, KSRIVPAIPVSLL-NH_2, a total yield of 16%, K: Lysine; S: serine; R: Arginine; I: Isoleucine; V: Valine; P: Proline; A: Alanine; L: Leucine. ESI MS (m/z): $[M + 3H]^{3+}$ calcd for

$C_{65}H_{121}N_{18}O_{15}$, 464.9; found, 464.4. Peptide **3** combines three protons, therefore, the molecular weight of the peptide **3** is 464.4*3-3=1390.2, M calcd for $C_{65}H_{121}N_{18}O_{15}$, 1391.0.

Peptide **4**, ILPWKWPWWPWRR, a total yield of 30%, I: Isoleucine; L: Leucine; P: Proline; W: Tryptophan; R: Arginine; K: Lysine; V: Valine. ESI MS (m/z): $[M + 3H]^{3+}$ calcd for $C_{100}H_{134}N_{25}O_{14}$, 636.8; found, 636.4. Peptide **4** combines three protons, therefore, the molecular weight of the peptide **4** is 636.4*3-3=1906.2, M calcd for $C_{100}H_{134}N_{25}O_{14}$, 1906.0.

Peptide **5**, TRRKFWKKVLNGALKIAPFLLG, a total yield of 27%, T: Threonine; R: Arginine; K: Lysine; F: Phenylalanine; W: Tryptophan; V: Valine; L: Leucine; N: Asparagine; G: Glycine; A: Alanine; I: Isoleucine; P: Proline. MALDI-TOF MS (m/z): $[M - H]^-$ calcd for $C_{123}H_{201}N_{34}O_{25}$, 2554.5; found, 2554.4.

Synthesis of N_3-AMP (N_3-TRRKFWKKVLNGALKIAPFLLG): Fmoc-Gly-OH Wang-resin was soaked in DMF for 2 h and then soaked in DMF containing 20% piperidine for 15 min at the room temperature to remove the Fmoc group. The obtained Gly-OH Wang-resin was filtered and reacted with the second Fmoc-AA-OH (10 equiv.) in the presence of HBTU (10 equiv.) and N-methylmorpholine (5%) in DMF. After the condensation reaction was completed, the resin was filtered and then soaked in DMF containing 20% piperidine to remove the Fmoc group. The similar condensation and deprotection reactions were conducted in the order of the AA sequence to eventually

furnish peptide **5**-resin. Subsequently, N_3CH_2COOH (10 equiv.) was reacted with the free amino group of peptide **5**-resin in the presence of HBTU (10 equiv.) and DIPEA (10 equiv.) in DMF. Finally, 5% TIPS in TFA solution was used to slice N_3-AMP from resin, as well as the Boc groups from lysine and tryptophan, Pbf group from arginine, tBu group from threonine. N_3-AMP, N_3-TRRKFWKKVLNGALKIAPFLLG, a total yield of 39%, T: Threonine; R: Arginine; K: Lysine; F: Phenylalanine; W: Tryptophan; V: Valine; L: Leucine; N: Asparagine; G: Glycine; A: Alanine; I: Isoleucine; P: Proline. MALDI-TOF MS (m/z): $[M - H]^-$ calcd for $C_{125}H_{206}N_{37}O_{26}$, 2637.6; found, 2636.7.

Synthesis of 4-(4-((N-Tert-butoxycarbonyl) L-alanine methyl ester-3-yl) phenoxy)-3-(phenylsulfonyl)-1,2,5-oxadiazole 2-oxide (compound 10). Compound **9** was prepared starting from thiophenol (**6**), via an addition reaction with chloroacetic acid, an oxidation reaction with hydrogen peroxide, and the subsequent reaction with fuming nitric acid in the presence of acetic acid, by using a previously reported method[28]. ^{1}H NMR (300 MHz, $CDCl_3$) for compound **9**: δ 8.24 – 8.11 (m, 2H), 7.81 (dd, J = 12.5, 7.3 Hz, 1H), 7.66 (q, J = 7.8 Hz, 2H). Compound **9** (0.36 g, 1.00 mmol) was reacted with Boc-Tyr methyl ester (0.44 g, 1.50 mmol) in CH_2Cl_2 (15 mL) in the presence of DBU (0.46 g, 3.00 mmol) for 2 h

under vigorous stirring. After the reaction was completed monitored by TLC, the mixture was washed with water (50 mL), HCl aqueous solution (2 M, 50 mL) and brine (50 mL), dried over Na_2SO_4 and then evaporated under vacuum. The crude product was purified by flash chromatography on silica gel using petroleum ether and ethyl acetate (100:1 to 1:10) for eluting to give target compound **10** (0.33 g, 62.8%) as a white solid. MS (m/z): $[M + Na]^+$ calcd for $C_{23}H_{25}N_3NaO_9S$, 542.1; found, 542.3. ^1H NMR (300 MHz, $CDCl_3$) δ 8.13 – 8.05 (m, 2H), 7.79 (t, *J* = 7.5 Hz, 1H), 7.65 (t, *J* = 7.8 Hz, 2H), 7.26 – 7.20 (m, 4H), 5.04 (d, *J* = 6.9 Hz, 1H), 4.59 (d, *J* = 6.2 Hz, 1H), 3.72 (s, 3H), 3.11 (ddd, *J* = 32.8, 13.9, 6.1 Hz, 2H), 1.42 (s, 9H) .

Synthesis of 4-(4-(L-Alanine methyl ester-3-yl) phenoxy)-3-(phenylsulfonyl)-1,2,5-oxadiazole 2-oxide (compound 11, FOTyr). Compound **10** (0.20 g, 0.39 mmol) was dissolved in 20% trifluoroacetic acid in CH_2Cl_2 (5 mL), and the obtained mixture was stirred at the room temperature for 3 h until compound **10** was totally deprotected. The solvent was removed under vacuum to give the crude product, which was purified by flash chromatography on silica gel using CH_2Cl_2 and methanol (100:1 to 1:5) for eluting to give target compound **11** (0.14 g, 87.0%) as a white solid. MS (m/z): $[M + H]^+$ calcd

for $C_{18}H_{18}N_3O_7S$, 420.1; found, 420.2. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.09 (d, *J* = 7.6 Hz, 2H), 7.80 (dd, *J* = 15.1, 7.5 Hz, 1H), 7.65 (t, *J* = 7.8 Hz, 2H), 7.28 (s, 4H), 4.29 (s, 1H), 3.85 – 3.72 (m, 3H), 3.31 (s, 2H).

Synthesis of 4-(4-(*N*-5'-Hexynoyl-*L*-alanine methyl ester-3-yl) phenoxy)-3-(phenylsulfonyl)-1,2,5-oxadiazole 2-oxide (compound 12, alkynyl-FOTyr). 5-Hexynoic acid (0.08 g, 0.79 mmol) and HBTU (0.26 g, 0.68 mmol) were dissolved in DMF (3 mL), to which was added FOTyr (**11**) (0.10 g, 0.24 mmol) and *N*-methylmorpholine (0.070 g, 0.70 mmol). The obtained mixture was stirred at the room temperature overnight. The mixture was poured into cold water (30 mL), and was extracted with CH_2Cl_2 (5 mL × 5). The combined organic layers were washed with brine (5 mL), dried over Na_2SO_4 and then evaporated under vacuum. The crude product was purified by flash chromatography on silica gel using petroleum ether and ethyl acetate (40:1 to 1:1) for eluting to give target compound **12** (0.12 g, 97%) as a white solid. MS (m/z): $[M + Na]^+$ calcd for $C_{24}H_{23}N_3NaO_8S$, 536.1; found, 536.0. HRMS (m/z): $[M + H]^+$ calcd for $C_{24}H_{24}N_3O_8S$, 514.1284; found, 514.1278. Error[ppm]=0.2. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.13 (d, *J* = 8.0 Hz, 2H), 7.82 (t, *J* = 8.0 Hz, 1H), 7.68 (t, *J* = 8.0 Hz, 2H), 7.22 – 7.20 (m,

4H), 5.98 (d, *J* = 8.0 Hz, 1H), 4.96-4.92 (m, 1H), 3.77 (s, 3H), 3.24-3.20 (m, 1H), 3.16-3.14 (m, 1H), 2.37 (t, *J* = 8.0 Hz, 2H), 2.25 (d, *J* = 8.0 Hz, 2H), 2.00 (s, 1H), 1.89-1.82 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.92, 170.88, 157.41, 150.74, 137.01, 134.92, 133.80, 129.89, 128.88, 127.75, 119.08, 109.81, 82.40, 68.48, 52.01, 51.60, 36.50, 33.82, 22.98, 16.80.

Synthesis of FOTyr-AMP: Compound **12** (0.01 g, 0.019 mmol) and N_3-AMP (0.05 g, 0.019 mmol) were dissolved in a solution of water (2 mL) and tetrahydrofuran (2 mL) containing copper sulfate (1.28 mg, 0.008 mmol) and vitamin C sodium salt (3.17 mg, 0.016mmol). The obtained mixture was stirred at the room temperature overnight. The reaction solution was filtered and the filtrate was collected for dialysis (the molecular weight cut off is 2000 D) to remove small molecules. Further lyophilization offered **FOTyr-AMP** as a brown solid with a purity of 98.4% determined by HPLC. MALDI-TOF MS (m/z): $[M - H]^-$ calcd for $C_{149}H_{227}N_{40}O_{34}S$, 3152.7; found, 3153.6.

Biology. General information. Bacterial strains of Staphylococcus aureus (ATCC 6538) and Escherichia coli (ATCC 8099) were obtained from China General

Microbiological Culture Collection Center. All experiments involved the mice and rats were performed in accordance with the guidelines of Animal Management and Ethics Committee of National Center for Nanoscience and Technology (NCNST).

MIC experiments. Bacterial TSB medium (100 μL, 2×10^5 cfu/mL) and TSB medium solution (100 μL) containing test compound with different concentrations (the concentrations of peptide 1-4 were 100 μM, 50 μM, 25 μM, 12.5 μM, 6.25 μM, the concentrations of peptide 5 and **FOTyr-AMP** were 10 mg/mL, 9 mg/mL, 8 mg/mL, 7 mg/mL, 6 mg/mL, 5 mg/mL, 4 mg/mL, 3 mg/mL, 2 mg/mL, 1 mg/mL) was added into the 96-well plate. After 20 h of incubation (37 °C ± 0.5 °C under 5% CO_2), the absorbance was measured by a microplate reader. When the positive control group (bacterial TSB medium) showed obvious growth, the negative control group (TSB medium) had no bacterial growth, indicating the experimental results were credible. If the growth of bacteria was completely inhibited by the test compound (OD value did not increase significantly compared to the negative control group), the minimum concentration of the compound is the minimum inhibitory concentration (MIC).

NO release experiments. Physiological saline, FOTyr, and **FOTyr-AMP** were added to the physiological saline solution of bacteria (10^8 cfu/mL), and the concentrations of the test compounds were 10 μM. After incubation for 4 h (37 °C ± 0.5 °C under 5% CO_2), the bacteria were collected by centrifugation, and an appropriate amount of lysate was added. After lysis, lysate was centrifuged at 10,000 g for 5 min. The supernatant (50 μL) for each group were added to a 96-well plate, and 50 μL of Griess Reagent I and 50 μL of Griess Reagent II were subsequently added into each well. The absorbance was measured with a microplate reader at

540 nm and the concentrations of NO_2^- (an oxidative metabolite of NO) for each group were calculated by the standard curve of absorbance to the concentrations of $NaNO_2$. When measuring the NO release in bacteria without GSH, CDNB was added to the physiological saline solution of bacteria (10^8 cfu/mL), and the concentrations of CDNB was 100 μM. After incubation for 0.5 h (37 °C ± 0.5 °C under 5% CO_2), the bacteria were collected by centrifugation, then 10 μM physiological saline solution (FOTyr or **FOTyr-AMP**) was added to the bacteria and incubated for 4 h (37 °C ± 0.5 °C under 5% CO_2). The following procedures are the same as described above.

Hemolysis experiment. 2% red blood cell suspension was obtained from the blood of mice. The clean centrifuge tubes were taken and marked them (negative control tube, positive control tube, and sample tubes). After that, 1.25 mL of 2% red blood cell suspension and 1.25 mL of 0.9% sodium chloride solution was added to the negative control tube. Besides, 1.25 mL of 2% red blood cell suspension and 1.25 mL of water was added to the positive control tube. The sample tube contained 1.25 mL of 2% red blood cell suspension, 1.1 mL 0.9% sodium chloride solution and 0.15 mL sample solution. Then, all samples were cultured in incubator (37 °C ± 0.5 °C under 5% CO_2) for 3 hours. Finally, the standard curve method was used to calculate the degree of hemolysis.

Sample preparation method of scanning electron microscope (SEM). SEM of biofilm (Figure 3B): The silicon slice was placed in bacterial culture medium (10^8 cfu/mL) for 24 hours, and then the medium was removed. After washed by physiological saline for 3 times, TSB solution containing test compounds was added into the silicon slice and incubated for 12 hours. Next, the silicon slice was washed by water for 3 times, added 4% paraformaldehyde solution,

and soaked for 2 hours. After that, the samples were immersed in 30%, 50%, 70%, 90% ethanol for 10 minutes. After immersing in 100% ethanol for 30 minutes, the solution was changed to 85% ethanol solution for 15 minutes, the silicon slice was aspirated and naturally dried. The silicon slice was adhered to the sample stage with a conductive adhesive to spray gold. Finally, the sample was detected by a cold field emission scanning electron microscope (Hitachi S4800＋EDS). SEM of dead bacteria (Figure 2): *S. aureus* and *E. coli* bacterial cells were incubated in TSB only, or in TSB containing **FOTyr-AMP** (200 μM) for 60 mins. Then, the solution was centrifuged, and the supernatant was discarded. The precipitated sample was added 4% paraformaldehyde solution, dropped on silicon slice and prepared for SEM detection as described above.

Crystal violet staining. Bacterial culture solution (10^8 cfu/mL) was added to each well (100 μL) for 24 hours in a 96-well plate. Then, the culture solution was aspirated and each well was washed by 200 μL of physiological saline for 3 times. After that, 100 μL TSB medium containing the test compound was added to each well and incubated for12 h. Then, the culture solution was aspirated and 100 μL of methanol was added to each well for 15 min, then the methanol was aspirated and dry. Subsequently, 100 μL of 1% crystal violet solution was added to each well and stained for 5 min at the room temperature. After aspirating the crystal violet staining solution in the culture well, the excess dye was rinsed off by water. Then, the 96-well plate was desiccated at room temperature, and 100 μL of ethanol was added to each well to dissolve crystal violet for 30 minutes in incubator (37 °C ± 0.5 °C under 5% CO_2). Lastly, The OD value of the solution in the culture well is measured by a microplate reader at 600 nm.

Biofilm dynamic detection experiment. TSB and *S. aureus* TSB solution (10^8 cfu/mL) were added to the well plate, then xCELLigence RTCA DP Instrument was started and placed in incubator (37°C ± 0.5°C under 5% CO_2) for one day to form biofilms. During this period, the biofilm indexes were continuously monitored every 15 mins for 9 h by xCELLigence RTCA DP Instrument. Subsequently, the well plate was taken out, and the supernatant was aspirated. After washed by physiological saline for three times, the medium solutions containing each compound (100 μM) was added. The well was placed in an incubator (37°C ± 0.5°C under 5% CO_2) and the data was collected every 15 mins for 9 h by using an xCELLigence RTCA DP Instrument. The blank data was deducted and normalized it (Set the lowest point to 0 and the highest value to 1, deducted the data fluctuation after adding samples).

Simultaneous evaluation of antibacterial and dispelling biofilm. 2 mL of *S. aureus* (10^8 cfu/mL) medium was added to the confocal dish, cultured for one day (37°C ± 0.5°C under 5% CO_2). After that, the supernatant was aspirated and washed three times with physiological saline. Next, each group of TSB solution containing test compound (100 μM) was added to the confocal dish. After incubation for 12 h, 1 mL of the supernatant was taken and added TSB to adjust its OD_{600} to 0.1 (10^8 cfu/mL), then the original bacterial concentration was calculated from the added volume ($C_{0.1}$*(V_{sample}+ V_{add}) =C_{sample}*V_{sample}). The confocal dish was washed three times with physiological saline, and 1 mL of stain was added. After staining for 15 min, the formation of biofilm was observed by confocal microscopy (UltraVIEW VoX).

Biofilms infection in vivo. Polydimethylsiloxane (PDMS) slices (0.3×0.3 cm) were placed in bacterial medium (10^8 cfu/mL), cultured for 24 hours (37°C ± 0.5°C under 5% CO_2) to form biofilms on the surface of the PDMS slices. The PDMS slices were then surgically implanted

into the back of the mice. The PDMS slices were sandwiched between the muscle and the epidermis, separated from the muscle by the peritoneum. Twelve hours after implantation, FOTyr, AMP, **FOTyr-AMP, FOTyr-AMP** plus PTIO, and CPC with the same dose of 0.25 μmol/kg, or blank solvent, were respectively subcutaneously administrated to mice every 12 h for 3 days. After 6 administrations (3 days), PDMS slices were taken out from mice for ultrasonic culture counting. After the PDMS was rinsed with physiological saline, it was ultrasonicated for 15 mins in a centrifuge tube containing 1 mL of physiological saline, and the solution was diluted at 10, 10^2, 10^3, 10^4, 10^5 times and cultured on a solid medium. The total amount of bacteria was calculated in a petri dish with a colony number of 30 to 300 (The number of bacteria was the number of colonies multiplied by the dilution factor). The muscle tissues near the implanted site were taken, and the spread of the bacteria was observed by H&E stain. The tissues were immersed in tissue fixative for 24 h, trimmed and placed in an embedding cassette, rinsed with water for 30 min, and dehydrated with different concentrations of alcohol. After the dehydration was completed, the tissue was placed in xylene to replace the alcohol in the tissue. The tissue was embedded in the dissolved paraffin, solidified and sliced. Finally, using hematoxylin stained, then rinsed and stained with eosin.

Rat blood pressure influence experiment. An aqueous solution of saline and **FOTyr-AMP** (0.172 μmol/kg) was subcutaneously injected into the healthy rats, and the blood pressure in the tail artery was measured at 0, 1, 3, and 6 h by Non-invasive blood pressure monitor (BP-2010A).

ASSOCIATED CONTENT

The Supporting Information is available free of charge on the ACS Publications website. The mechanism of GSH-mediated NO release from furoxan and synthetic schemes for peptides **1-5** and FOTyr-AMP, and additional data, including Scheme S1–S7, Tables S1–S5, and Figures S1−S5. Molecular Formula Strings was saved in CSV file.

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Notes

The authors declare no competing financial interests.

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ABBREVIATIONS

NO, nitric oxide; AMPs, antimicrobial peptides; RNS, reactive nitrogen species; ROS, reactive oxygen species; c-di-GMP, cyclic diguanylate; GSH, glutathione; CATH, Cathelicidin peptides; MIC, minimum inhibitory concentration; PDMS, polydimethylsiloxane; PTIO, 2-phenyl-4, 4, 5, 5-tetramethylimidazoline-1-oxyl 3-oxide; Boc, t-Butyloxy carbonyl; Pbf, 2, 2, 4, 6, 7-pentamethyldihydrobenzofuran-5-sulfonyl; tBu, tert-butyl, CDNB, 1-chloro-2,4-nitrobenzene; H&E staining, hematoxylin-eosin staining.

REFERENCES

(1) Hall-Stoodley, L.; Costerton, J. W.; Stoodley, P. Bacterial biofilms: from the Natural environment to infectious diseases. *Nat. Rev. Microbiol.* **2004**, *2*, 95-108.

(2) Davies, D. Understanding biofilm resistance to antibacterial agents. *Nat. Rev. Drug Discov.* **2003**, *2*, 114-122.

(3) Wu, H.; Moser, C.; Wang, H.-Z.; Høiby, N.; Song, Z.-J. Strategies for combating bacterial biofilm infections. *Int. J. Oral. Sci.* **2015**, *7*, 1-7.

(4) Flemming, H.-C.; Wingender, J.; Szewzyk, U.; Steinberg, P.; Rice, S. A.; Kjelleberg, S. Biofilms: an emergent form of bacterial life. *Nat. Rev. Microbiol.* **2016**, *14*, 563-575.

(5) Hu, J.; Quan, Y.; Lai, Y.; Zheng, Z.; Hu, Z.; Wang, X.; Dai, T.; Zhang, Q.; Cheng, Y. A smart aminoglycoside hydrogel with tunable gel degradation, on-demand drug release, and high antibacterial activity. *J. Control Release*. **2017**, *247*, 145-152.

(6) Reighard, K. P.; Hill, D. B.; Dixon, G. A.; Worley, B. V.; Schoenfisch, M. H. Disruption and eradication of P. aeruginosa biofilms using nitric oxide-releasing chitosan oligosaccharides. *Biofouling.* **2015**, *31*, 775-787.

(7) Jardeleza, C.; Foreman, A.; Baker, L.; Paramasivan, S.; Field, J.; Tan, L. W.; Wormald, P. J. The effects of nitric oxide on Staphylococcus aureus biofilm growth and its implications in chronic rhinosinusitis. *Int. Forum Allergy Rh*. **2011**, *1*, 438-444.

(8) Jardeleza, C.; Rao, S.; Thierry, B.; Gajjar, P.; Vreugde, S.; Prestidge, C. A.; Wormald, P. J. Liposome-encapsulated ISMN: a novel nitric oxide-based therapeutic agent against Staphylococcus aureus biofilms. *Plos One*. **2014**, *9*, e92117.

(9) Tang, R.; Jiang, F.; Wen, J.; Deng, Y.; Sun, Y. Managing bacterial biofilms with chitosan-based polymeric nitric oxides: Inactivation of biofilm bacteria and synergistic effects with antibiotics. *J. Bioact. Compat. Pol*. **2016**, *31*, 393-410.

(10) Wonoputri, V.; Gunawan, C.; Liu, S.; Barraud, N.; Yee, L. H.; Lim, M.; Amal, R. Copper Complex in Poly (vinyl chloride) as a Nitric Oxide-Generating Catalyst for the Control of Nitrifying Bacterial Biofilms. *ACS Appl. Mater. Inter*. **2015**, *7*, 22148-22156.

(11) Jenal, U.; Reinders, A.; Lori, C. Cyclic di-GMP: second messenger extraordinaire. *Nat. Rev. Microbiol*. **2017**, *15*, 271-284.

(12) Barraud, N.; Hassett, D. J.; Hwang, S.-H.; Rice, S. A.; Kjelleberg, S.; Webb, J. S. Involvement of nitric oxide in biofilm dispersal of Pseudomonas aeruginosa. *J. Bacteriol*. **2006**, *188*, 7344-7353.

(13) Koo, H.; Allan, R. N.; Howlin, R. P.; Stoodley, P.; Hall-Stoodley, L. Targeting microbial biofilms: current and prospective therapeutic strategies. *Nat. Rev. Microbiol*. **2017**, *15*, 740-755.

(14) Yang, L.; Feura, E. S.; Ahonen, M.; Schoenfisch, M. H. Nitric Oxide-Releasing Macromolecular Scaffolds for Antibacterial Applications. *Adv. Healthc. Mater*. **2018**, *7*, 1800155.

(15) Mihu, M. R.; Cabral, V.; Pattabhi, R.; Tar, M. T.; Davies, K. P.; Fried-man, A. J.; Martinez, L. R.; Nosanchuk, J. D. Sustained Nitric Oxide-Releasing Nanoparticles Interfere with Methicillin-Resistant Staphylococcus aureus Adhesion and Biofilm Formation in a Rat Central Venous Catheter Model. *Antimicrob. Agents Ch*. **2017**, *61*, e02020-16.

(16) Howlin, R. P.; Cathie, K.; Hallstoodley, L.; Cornelius, V.; Duignan, C.; Allan, R. N.; Fernandez, B. O.; Barraud, N.; Bruce, K. D.; Jefferies, J. Low-Dose Nitric Oxide as Targeted Anti-biofilm Adjunctive Therapy to Treat Chronic Pseudomonas aeruginosa Infection in Cystic Fibrosis. *Mol. Ther*. **2017**, *25*, 2104-2116.

(17) Medana, C.; Ermondi, G.; Fruttero, R.; Di Stilo, A.; Ferretti, C.; Gasco, A. Furoxans as Nitric Oxide Donors. 4-Phenyl-3-furoxancarbonitrile: Thiol-Mediated Nitric Oxide Release and Biological Evaluation. *J. Med. Chem*. **1994**, *37*, 4412-4416.

(18) Feelisch, M.; Schonafinger, K.; Noack, E., Thiol-mediated generation of nitric-oxide accounts for the vasodilator action of furoxans. *Biochem. Pharmacol.* **1992**, *44*, 1149-1157.

(19) Sadrearhami, Z.; Yeow, J.; Nguyen, T.-K.; Ho, K. K.; Kumar, N.; Boyer, C. Biofilm dispersal using nitric oxide loaded nanoparticles fabricated by photo-PISA: influence of morphology. *Chem. Commun.* **2017**, *53*, 12894-12897.

(20) Barraud, N.; Kardak, B. G.; Yepuri, N. R.; Howlin, R. P.; Webb, J. S.; Faust, S. N.; Kjelleberg, S.; Rice, S. A.; Kelso, M. J. Cephalosporin-3'-diazeniumdiolates: Targeted NO-Donor Prodrugs for Dispersing Bacterial Biofilms. *Angew. Chem. Int. Ed*. **2012**, *51*, 9057-9060.

(21) Poh, W. H.; Barraud, N.; Guglielmo, S.; Lazzarato, L.; Rolando, B.; Fruttero, R.; Rice, S. A. Furoxan Nitric Oxide Donors Disperse Pseudomonas aeruginosa Biofilms, Accelerate Growth, and Repress Pyoverdine Production. *ACS Chem. Biol*. **2017**, *12*, 2097-2106.

(22) Laar, T. A. V.; Esani, S.; Birges, T. J.; Hazen, B.; Thomas, J. M.; Rawat, M. Pseudomonas aeruginosa gshA Mutant Is Defective in Biofilm Formation, Swarming, and Pyocyanin Production. *Msphere* **2018**, *3*, e00155-18.

(23) Hussain, S.; Joo, J.; Kang, J.; Kim, B.; Braun, G. B.; She, Z. G.; Kim, D.; Mann, A. P.; Mölder, T.; Teesalu, T. Antibiotic-loaded nanoparticles targeted to the site of infection enhance antibacterial efficacy. *Nat. Biomed. Eng*. **2018**, *2*, 95-103.

(24) Chen, Y.; Cai, S.; Qiao, X.; Wu, M.; Guo, Z.; Wang, R.; Kuang, Y.-Q.; Yu, H.; Wang, Y. As-CATH1-6, novel cathelicidins with potent antimicrobial and immunomodulatory properties from Alligator sinensis, play pivotal roles in host antimicrobial immune responses. *Biochem. J*. **2017**, *474*, 2861-2885.

(25) Jenssen, H.; Hamill, P.; Hancock, R. E. W., Peptide antimicrobial agents. *Clin. Microbiol. Rev*. **2006**, *19*, 491-511.

(26) Hilchie, A. L.; Wuerth, K.; Hancock, R. E. W., Immune modulation by multifaceted cationic host defense (antimicrobial) peptides. *Nat. Chem. Biol*. **2013**, *9*, 761-768.

(27) Hancock, R. E. W.; Lehrer, R., Cationic peptides: a new source of antibiotics. *Trends Biotechnol*. **1998**, *16*, 82-88.

(28) Nortcliffe, A.; Botting, N. P.; O'Hagan, D. Novel amino acids: synthesis of furoxan and sydnonimine containing amino acids and peptides as potential nitric oxide releasing motifs. *Org. Biomol. Chem*. **2013**, *11*, 4657-4671.

(29) Schon, M. R.; Kauffman, F. C.; Thurman, R. G., Selective depletion and measurement of glutathione in periportal and pericentral regions in the perfused rat liver. *Toxicology letters.* **1988**, *42*, 265-272.

(30) Diana, G.; Claudio, H. C.; Ana, R.; Pilar, G.; Patricia, R. M. Monitoring in Real Time the Formation and Removal of Biofilms from Clinical Related Pathogens Using an Impedance-Based Technology. *Plos One*. **2016**, *11*, e0163966.

(31) Nair, P. N. R.; Malin, B.; Goran, S.; Ulf, S. G. Building biofilms in vital host tissues: a survival strategy of Actinomyces radicidentis. *Oral. Surg. Oral. Med. O*. **2008**, *106*, 595-603.

(32) Antonoplis, A.; Zang, X.; Huttner, M. A.; Chong, K. K. L.; Lee, Y. B.; Co, J. Y.; Amieva, M. R.; Kline, K. A.; Wender, P. A.; Cegelski, L. A Dual-Function Antibiotic-Transporter Conjugate Exhibits Superior Activity in Sterilizing MRSA Biofilms and Killing Persister Cells. *J. Am. Chem. Soc*. **2018**, *140*, 16140-16151.

(33) Carmen, J. C.; Roeder, B. L.; Nelson, J. L.; Beckstead, B. L.; Runyan, C. M.; Schaalje, G. B.; Robison, R. A.; Pitt, W. G. Ultrasonically enhanced vancomycin activity against Staphylococcus epidermidis biofilms in vivo. *J. Biomater. Appl*. **2004**, *18*, 237-245.

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