Biguanide-derived polymeric nanoparticles kill MRSA biofilm and suppress infection in vivo

Li, Jianghua; Zhong, Wenbin; Zhang, Kaixi; Wang, Dongwei; Hu, Jingbo; Chan-Park, Mary B.

2020

Li, J., Zhong, W., Zhang, K., Wang, D., Hu, J., & Chan-Park, M. B. (2020). Biguanide-derived polymeric nanoparticles kill MRSA biofilm and suppress infection in vivo. *ACS Applied Materials & Interfaces*, 12(19), 21231-21241. doi:10.1021/acsami.9b17747

<https://hdl.handle.net/10356/142365>

<https://doi.org/10.1021/acsami.9b17747>

This document is the Accepted Manuscript version of a Published Work that appeared in final form in *ACS Applied Materials & Interfaces*, copyright © American Chemical Society after peer review and technical editing by the publisher. To access the final edited and published work see <https://doi.org/10.1021/acsami.9b17747>

Downloaded on 26 Apr 2023 17:45:14 SGT

Biguanide-derived Polymeric Nanoparticles Kill MRSA Biofilm and Suppress Infection *In Vivo*

Jianghua Li,^a Wenbin Zhong,^a Kaixi Zhang,^a Dongwei Wang,^c Jingbo Hu^c and Mary B. Chan-Park^{a,b,*}

a. Centre for Antimicrobial Bioengineering, School of Chemical and Biomedical Engineering, Nanyang Technological University, 62 Nanyang Drive, 637459 Singapore

b. Lee Kong Chian School of Medicine, Nanyang Technological University, 59 Nanyang Drive, 636921 Singapore

c. Institute of Drug Discovery Technology, Ningbo University, Ningbo, 315000, China

***E-mail:** mbechan@ntu.edu.sg

Keywords: antibiofilm, multidrug resistance, nanoparticles, tannic acid, biocompatibility

Abstract: Methicillin-resistant *Staphylococcus aureus* (MRSA) is a significant cause of drug-resistant infections. Its propensity to develop biofilms makes it especially resistant to conventional antibiotics. We present a novel nanoparticle (NP) system made from biocompatible **F**-127 surfactant, **t**annic acid (TA) and biguanide-based **p**olymetformin (**PMET**) (termed **FTP** NPs), which can kill MRSA biofilm bacteria effectively *in vitro* and *in vivo* and which has excellent biocompatibility. FTP NPs exhibit biofilm bactericidal activity – ability to kill bacteria both inside and outside biofilm – significantly better than many antimicrobial peptides (AMPs) or polymers. At low concentrations (8-32 $\mu\text{g/mL}$) *in vitro*, FTP NPs outperformed PMET with around 100-fold ($\sim 2 \log_{10}$) greater reduction of MRSA USA300 biofilm bacterial cell counts, which we attribute to the antifouling property of the hydrophilic polyethylene glycol (PEG) contributed by F-127. Further, in an *in vivo* murine excisional wound model, FTP NPs achieved 1.8 $\log_{10}$ reduction of biofilm-associated MRSA USA300 bacteria, which significantly outperformed vancomycin (0.8 $\log_{10}$ reduction). Moreover, *in vitro* cytotoxicity tests showed that FTP NPs have less toxicity than PMET towards mammalian cells; and *in vivo* intravenous injection of FTP NPs at 10 mg/kg showed no acute toxicity to mice with negligible body weight loss and no significant perturbation of blood biomarkers. These biguanide-based FTP NPs are a promising approach to therapy of MRSA infections.

1. INTRODUCTION. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a leading cause of morbidity and mortality due to infection.¹ According to the US Centers for Disease Control and Prevention, in 2017, nearly 120,000 people suffered bloodstream infections caused by *Staphylococcus aureus* and about 1/6th of these infections were fatal.¹⁻² MRSA is resistant to many beta-lactam antibiotics such as methicillin and oxacillin, as well as other major classes of antibiotics including macrolides and some fluoroquinolones. MRSA is notorious for causing recalcitrant chronic wound infections and is the most common pathogen in skin and soft tissue infections.³ The ability of MRSA to develop robust biofilm on wound sites further complicates treatment, since biofilm bacteria are 10 to 1000 times more resistant than planktonic state bacteria to conventional antibiotics.⁴⁻⁵ Once a biofilm is formed, its three-dimensional matrix of extracellular polymeric substances (EPSs) can protect the biofilm cells from antibacterial agents and immune clearance.⁶ If untreated, MRSA can disseminate from the skin to blood to cause life-threatening sepsis.⁷ New drugs and technologies are urgently needed to eradicate difficult-to-treat biofilm infections, especially those developed by multidrug-resistant bacteria such as MRSA.

Cationic polymers have been studied as potential candidates to fight biofilm bacteria only in the past few years,⁸⁻¹⁰ although they have been widely used to combat planktonic bacteria, which are much easier to eradicate. Efforts have been made to develop novel antibiofilm agents, such as polysaccharide-based cationic polymer,¹¹⁻¹² polypeptides,⁸ cationic polyacrylates,¹³⁻¹⁵ and polycarbonate.⁹ Although these synthetic polymers show good antibacterial and/or antibiofilm efficacy, unselective toxicities and high raw materials cost have impeded their clinical application.¹⁶

In the effort to develop next-generation antibiofilm agents to meet both efficacy and biocompatibility requirements, nanotechnology has attracted much attention.¹⁷⁻¹⁸ The size, surface

morphology and charge of nanoparticles (NPs) can be tuned to enhance penetration into the biofilm matrix.^{4, 19-21} Moreover, polymeric nanocarriers can serve as drug delivery systems with controllable release, through pH-sensitive mechanisms, of antibacterial cargoes upon interaction with the biofilm matrix.²²⁻²³ Some other NPs act as antibiofilm agents in photodynamic therapy.^{4, 24} Nevertheless, there are still some limitations of current antibiofilm NPs. For example, many of these NPs are made from metals such as silver,²⁵⁻²⁷ gold²⁸ and iron,²⁹⁻³⁰ which has raised concerns about their toxicities²⁷ and resistance development.³¹ In our previous study, we reported a novel NP system made of dextran-*block*-poly(AMPTMA-*co*-BMA) (*i.e.* dextran-*block*-poly((3-acrylamidopropyl)trimethylammonium chloride)-*co*-(butyl methacrylate)) which has remarkably low toxicity and excellent biofilm dispersal ability via a mechanism we called “nanoscale bacterial debridement”.³² However, though this NP system effectively removes biofilm, it does not kill the dispersed bacteria – *i.e.* it is not intrinsically antibacterial. This is a drawback to potential clinical application of the material as the dispersed live bacteria may colonize other sites.

Biguanide-based compounds have been studied as small antibiofilm molecules. For instance, biguanide iridium (III) complex showed killing of mature biofilm bacteria of Gram-positive vancomycin-resistant *Enterococci* (VRE) with a mechanism of action possibly due to intracellular biguanide inhibition of some protein bio-synthesis.³³ Further, some guanidine and biguanide modified norspermidines also exhibited good biofilm inhibition and preformed biofilm disruption activity against Gram-positive *Staphylococcus aureus* by targeting the EPS of biofilm.³⁴ However, the *in vitro* and *in vivo* toxicities to mammalian cells of biguanide metal complexes and biguanidylated polyamines derived from norspermidine have yet to be determined so that the potential for development toward clinical applications of these compounds is unknown.

Herein, we report a new kind of NP made by polyphenol-assisted aggregation of biguanide-based polymetformin (PMET) which shows efficacy in both removal of biofilm and eradication of biofilm bacteria both inside and detached from biofilm, “intrinsic biofilm bacterial killing” whilst also having excellent biocompatibility. We have synthesized a novel biguanide-based PMET from linear polyethyleneimine (PEI) via a facile one-step reaction (**Scheme 1A**).³⁵ *In vitro* testing showed that PMET is active against various *Staphylococcus aureus* strains, including both community-associated and hospital-associated MRSA, with minimum inhibitory concentrations (MICs) of 8-16 µg/mL. However, in *in vitro* antibiofilm assay, PMET solution alone showed limited biofilm removal efficacy towards MRSA USA300 even at high concentrations (128-512 µg/mL), possibly because of its poor biofilm penetration ability.

To further improve the biofilm eradication efficacy, we developed a new polymeric NP system with three main components: PMET, tannic acid (TA), and Pluronic® F-127 (**Scheme 1B**). TA, a compound classed as “generally recognized as safe” (GRAS) by United States FDA, is a hydroxyl-rich polyphenol that can serve as building block in many supramolecular chemistries due to its ability to form hydrogen bonds and interact hydrophobically with other macromolecules.³⁶ We first examined the formation of **TA/PMET** NPs (thereafter called TP NPs) by transmission electron microscopy (TEM) and dynamic light scattering (DLS), and found that they would precipitate from aqueous solution due to their poor colloidal stability. We then further introduced another FDA approved compound, Pluronic® F-127 surfactant, which has been reported to interact with TA by hydrogen-bonding.³⁷ The **F-127/TA/PMET** combination forms stable NPs (hereinafter called FTP NPs). Compared with solution PMET, FTP NPs showed improved biocompatibility towards mammalian cells *in vitro*, and enhanced biofilm removal efficacy against MRSA USA300 biofilms in *in vitro* and *in vivo* testing. FTP NPs kill bacteria by membrane disruption. Our *in vivo* toxicity

studies found that a high systemic (intravenous) dose, 10 mg/kg, of FTP NPs induces no acute toxicity in mice, as evidenced by negligible loss of body weight and change of blood biomarkers.

2. RESULTS AND DISCUSSION.

2.1. Preparation and Characterization of TP and FTP NPs. PMET was first synthesized by reacting linear PEI with dicyandiamide under acidic conditions (**Scheme 1A**).³⁵ ¹H NMR, ¹³C NMR and GPC data (**Figure S1 and S2**) confirmed the successful synthesis of PMET. The degree of biguanide substitution (75 % - 78 %) of PMET was calculated from the ratio of the areas of the proton peaks of biguanide (“c” and “d”: 3.0 - 4.0 ppm, **Figure S1A**) to the total areas of unsubstituted PEI peaks (“a” and “b”: 2.5 - 2.7 ppm, **Figure S1A**) plus the biguanide peak areas (“c” and “d”: 3.0 - 4.0 ppm, **Figure S1A**). The measured molecular weight of PMET was 7.9 kDa which was close to the calculated value of 8.0 kDa for a starting PEI with molecular weight of 3.7 kDa (**Figure S1B**) with a grafting ratio of 75%-78%. Introducing biguanide group into PEI significantly improved the solubility of PMET in a neutral (physiological) pH aqueous environment, making it favorable for biological applications (**Figure S3**).

We then mixed PMET with TA to form TA/PMET (TP) NPs. The mixture formed a visibly turbid suspension (**Figure S4A**); TEM showed that these TP NPs have diameters of around 96 nm (**Figure S4B**). DLS data showed that the TP NPs have hydrodynamic diameter (D_h) of 96.9 ± 1.3 nm (**Table S1**), in excellent agreement with the TEM data. TP NPs are positively charged with a zeta potential of $+43.1 \pm 1.7$ mV. However, TP NPs exhibited poor stability in PBS, precipitating from suspension overnight at room temperature.

To improve the colloidal stability of the NP, we further PEGylated the TP complex by introducing the biocompatible surfactant Pluronic® F-127 (FTP NPs) (**Scheme 1B**). We prepared

stable FTP NPs by first mixing solutions of F-127 (1 mg/mL), TA (1 mg/mL) and PMET (10 mg/mL), followed by washing three times using a 50 kDa membrane ultrafiltration unit under centrifugation (5,000 r.p.m. for 30 min at 4 °C). The upper compartment of the centrifugal filter unit contained FTP NPs and the lower compartment contained filtrate. We then measured ^1H NMR and UV-Vis absorption spectra of the filtrate (**Figure S5**) to test for the presence of any components (F-127, TA or PMET) in it. No sign of these three components was detected in the ^1H NMR and UV-Vis absorption spectra, indicating that all three components were fully incorporated into the FTP NPs. DLS measurements of FTP NPs indicate $D_h = 121.6 \pm 3.5$ nm, larger than that of TP NPs at 96.9 ± 1.3 nm (**Figure 1A and Table S1**), which may be attributed to the hydrophilic PEG chains extending from the particle surface. FTP NPs are positively charged with a zeta potential of $+33.6 \pm 1.4$ mV (**Table S1**). The diameter of FTP NPs measured in TEM images was around 111 nm (**Figure 1A**), corroborating the D_h measured by DLS (**Figure 1A and Figure S6**). Prolonged standing study of the colloidal stability of FTP NPs showed that the D_h and polydispersity index (PDI) remained almost unchanged over two weeks (**Figure 1B**). These results indicate the successful fabrication of stable and uniform FTP NPs.

2.2. In vitro Antibacterial and Antibiofilm Activity. MICs of neat PMET were measured against a panel of *S. aureus* and MRSA strains (including community-associated MRSA USA300 and hospital-associated MRSA BAA40 and KKH5). PMET showed excellent anti-*Staphylococcal* and anti-MRSA activity with MICs of 8-16 $\mu\text{g/mL}$ (**Table 1**). However, for Gram-negative bacteria (**Table 1**), PMET showed moderate killing activity with MICs of 32-64 $\mu\text{g/mL}$ against various strains (*i.e.* *E. coli* 8739, *P. aeruginosa* PAO1, *K. pneumoniae* KPNR and *A. baumannii* AB-1). FTP NPs showed similar antibacterial activity to PMET against both Gram-positive and Gram-negative bacteria.

The ability of FTP NPs and PMET to disperse preformed biofilm of MRSA USA300 was evaluated using the MBECTM assay. Biofilm was established on MBECTM pegs and then the pegs were soaked in wells containing PMET or FTP NPs (**Figure S7**). Biofilms could be dispersed by PMET and FTP NPs solutions. The biofilm dispersal ability of FTP NPs is better than that of PMET (**Figure S8** and **Table 2**) : at concentrations of 8, 16, and 32 $\mu\text{g/mL}$, FTP NPs showed $\log_{10}$ reductions of 2.4, 2.6 and 3.5 respectively, while PMET had much lower (0.2, 0.4 and 1.5) $\log_{10}$ reductions at the same concentrations. The cell count reduction of PMET plateaued above 32 $\mu\text{g/mL}$ at about 3.4 $\log_{10}$. In contrast, FTP NPs showed a continual reduction of cell counts in a dose-dependent manner and better efficacy than PMET at all concentrations. At the highest tested concentration, 512 $\mu\text{g/mL}$, there was almost complete bacterial removal (5.2 $\log_{10}$ reduction) for FTP NPs compared to partial removal (3.2 $\log_{10}$ reduction) for PMET. To assess the agents' penetration into and action on biofilm matrix and cells, we grew MRSA USA300 biofilm on glass slides and incubated with rhodamine labelled FTP NPs and PMET at 16 $\mu\text{g/mL}$ for 30 mins. The penetration into and accumulation of FTP NPs and PMET in biofilm were examined by confocal laser scanning microscopy (CLSM). As shown in **Figure 2A**, FTP NPs showed more red fluorescence in the biofilm than did PMET after 30 mins treatment. Further, FTP NPs showed reduced green fluorescence (Syto 9: live bacteria) compared to PMET. Using ImageJ software to analyze the CLSM images, we quantified the penetration and accumulation of FTP NPs and PMET; the fluorescence intensity as a function of biofilm depth is shown in **Figure 2B**. The FTP NPs penetrated with high concentration significantly deeper into the biofilm than did PMET. In a separate experiment, CLSM images of MRSA USA300 biofilms treated with PMET (**Figure S9A**) and FTP NPs (**Figure S9B**) at the concentration of 16 $\mu\text{g/mL}$ for 2 h showed that FTP NPs have

better biofilm removal ability than PMET. There is a clear difference in the biofilm thickness: 12.8 μm after PMET treatment *versus* 3.2 μm after FTP NPs treatment.

Using field-emission scanning electron microscopy (FESEM), we visualized biofilm condition before and after treatment with FTP NPs. FESEM images (**Figure 3A**) showed a clear bacterial count reduction after a single dose (128 $\mu\text{g/mL}$) FTP NP treatment. We also determined the ability of FTP NPs to kill bacteria dispersed from biofilm. The bacteria were grown on MBECTM pegs to the biofilm state by submerging the pegs into a 96-well plate containing growth medium (for 24 h); the pegs were then placed into another 96-well plate containing the FTP NPs solution for 2 h. We then examined the viability of bacteria dispersed from the biofilm on the pegs; we transferred 20 μL of the dislodged bacteria suspension (containing the polymers and the dispersed-from-biofilm cells) into a fresh 96-well plate containing 180 μL growth medium and then incubated the bacteria with medium for 24 h before counting the colony forming unit (CFU) to determine the minimum bactericidal concentration (MBC) value of FTP NPs against bacteria dispersed from biofilm. We found that the FTP NPs have “intrinsic biofilm bactericidal activity” – the ability to kill bacteria *in situ* within the biofilm as well as after release from the biofilm through the agent’s biofilm dispersal effect -- and this is significantly better than many previously reported AMPs or polymers that do not do both well.^{32, 38-39} The FTP NPs have “dispersed from biofilm” bacterial MBC of 128 $\mu\text{g/mL}$ which is slightly better than PMET, which has MBC of 256 $\mu\text{g/mL}$ (**Figure 3B**).

Time-lapse CLSM confirmed the biofilm reduction effect of FTP NPs via removal of the biomass and killing of biofilm bacteria (**Figure 3C**). MRSA USA300 biofilms were pre-formed on the surface of glass slides and then FTP NPs (128 $\mu\text{g/mL}$) solution was dispensed on the biofilm before staining by LIVE/DEADTM dye. CLSM images (**Figure 3C**) showed that FTP NPs can

disperse the biofilm effectively in a very short period of time (30 min) with significant reduction of live bacteria (green fluorescence in **Figure 3C**) and dispersal of killed cells from the matrix (little red fluorescence from residual dead cells compared with the initial green fluorescence). After 120 min, few bacteria remained in the biofilm.

We also examined the ability of FTP NPs to inhibit biofilm formation at sub-MIC concentrations (1/2, 1/4 and 1/8 MIC). FTP NPs, which have PEG in their surfaces, are slightly better at biofilm formation inhibition than PMET or vancomycin at sub-MIC concentrations (1/2, 1/4 and 1/8 MIC), though none of the agents is highly effective (**Figure S10**).

2.3. *In vitro* Cytotoxicity. Both FTP NPs and PMET were non-hemolytic, with concentrations for 10% hemolysis of human red blood cells (HC₁₀) greater than 10,000 µg/mL (**Figure S11**). We also tested the cytotoxicity of FTP NPs and PMET against two mammalian cell lines, mouse embryonic fibroblast 3T3 cells and human dermal fibroblast (HDF) cells. The results (**Figure 4A**) show that FTP NPs have good biocompatibility with 70% inhibitory concentration (IC₇₀) >1024 µg/mL against 3T3 fibroblasts, a value much superior to the IC₇₀ = 239.8 µg/mL of PMET. For the human dermal fibroblasts, FTP NPs also showed much higher IC₇₀ (>1024 µg/mL) than PMET (125.6 µg/mL) (**Figure 4B**). The results demonstrate that FTP NPs have better *in vitro* biocompatibility than PMET toward the eukaryotic cells tested, which is possibly due to hydrophilic PEG chain of F-127.

2.4 Mechanism of Antibiofilm Action by FTP NPs. FTP NPs and PMET showed both biofilm removal and biofilm bacterial killing ability as mentioned above (**Table 2** and **Figure 3B**). To study the mechanism of action of FTP NPs and PMET against MRSA USA300, we used the cytoplasmic membrane depolarization assay with DiSC3(5) dye, FESEM and CLSM. Treatment with FTP NPs resulted in a rapid increase of the DiSC3(5) fluorescence intensity, indicating its

effect in depolarizing the cytoplasmic membrane (**Figure 5A**). Treatment with PMET also resulted in an increase of the DiSC3(5) fluorescence intensity (**Figure S12A**). FESEM images (**Figure 5B**) showed morphology changes in FTP NPs-treated ($1 \times \text{MIC}$: $16 \mu\text{g/mL}$) bacteria, which had wrinkled and deformed surfaces compared with the smooth and intact surfaces of untreated bacteria. PMET treated bacteria ($1 \times \text{MIC}$: $16 \mu\text{g/mL}$) also showed surface deformations in FESEM imagery (**Figure S12B**). We then utilized CLSM to observe MRSA USA300 bacteria stained with Syto 9 (green) and propidium iodide (red). For the untreated control (**Figure 5C**), most of cells were green and not stained by PI, indicating intact bacterial cell membrane. In contrast, both FTP NPs and PMET-treated ($1 \times \text{MIC}$: $16 \mu\text{g/mL}$) bacterial cells (**Figure 5C and S12C**) were stained by red-fluorescent PI dye, indicating dead bacteria with disrupted cell membrane. We also observed the agglomeration of bacteria and formation of clusters of MRSA treated by FTP NPs (**Figure 5C**) and PMET (**Figure S12C**). The clustering may due to a response to environment stress caused by antimicrobial agents such as FTP NPs and PMET, which is similar to some previous studies.⁴⁰⁻⁴¹

A liposome model of bacterial plasma membrane was employed in a calcein dye leakage assay to obtain a quantitative measure of membrane permeabilization caused by FTP NPs and PMET. We loaded calcein into phosphatidylglycerol (PG)/cardiolipin (CL) liposomes (PG:CL = 3:1), which were used to mimic Gram-positive bacterial membrane. FTP NPs induced ~50% dye leakage from PG/CL liposome at $16 \mu\text{g/mL}$ ($1 \times \text{MIC}$); at this wt/vol concentration, PMET induced around 30% leakage (**Figure S13**). These results corroborate the interpretation that both FTP NPs and PMET kill bacteria by a mechanism involving membrane permeabilizations.

The time-kill kinetics of FTP NPs and PMET against planktonic MRSA USA300 was investigated by CFU assay. As shown in **Figure 6**, at $1 \times \text{MIC}$ concentration, both FTP NPs and

PMET achieved 100% kill of MRSA USA300 in 4 h. Vancomycin showed much poorer killing efficacy ($\sim 1.6 \log_{10}$ reduction) after 4 h treatment at 1 x MIC. At 2 x MIC and 4 x MIC, FTP NPs and PMET both killed 100% of the bacteria within 60 mins at 2 x MIC and within 5 mins at 4 x MIC. Vancomycin required the full 4 h test period to eradicate 100% of the bacteria at both 2 x MIC and 4 x MIC. Against MRSA USA300, FTP NPs and PMET exhibit much faster kill kinetics than vancomycin.

2.5 Interaction Forces of the FTP NPs. We investigated the interaction between PMET and TA by investigating the mixture's optical property (turbidity) and isothermal titration calorimetry (ITC) behavior. Turbid suspension was formed at all tested PMET to TA mass ratios (**Figure S4A** and **Table S2**). The ITC curve (**Figure S14A**) showed a negative enthalpy change ($\Delta H = -57.3 \pm 3.78$ kcal/mol) and a positive entropy change ($-T\Delta S = 48.0$ kcal/mol), indicating the interaction between TA and PMET was enthalpically driven with unfavorable entropic change; this thermodynamic behavior and the known structures of the two components implies they bind primarily through hydrogen bonding. F-127 has previously been shown to interact with TA by hydrogen bonding.^{37,}
⁴² We utilized Fourier transform infrared spectrum (FTIR) to study the interaction between TA and F-127 (**Figure S14B**). The results showed that the C=O stretching vibration in TA shifted from 1713 cm^{-1} to 1731 cm^{-1} in the F-127/TA mixture. Also, compared to TA, the F-127/TA mixture showed a red shift in the -OH peak (from 3463 cm^{-1} in TA to 3605 cm^{-1} in the mixture), indicating the formation of hydrogen-bonding between TA and F-127. In summary, FTP NPs can be formed with TA as building block to interact with both F-127 and PMET (**Scheme 1B**).

2.6. *In vivo* Antibiofilm Efficacy and Toxicity. A murine excisional wound model was employed to examine the *in vivo* antibiofilm efficacy of FTP NPs. MRSA USA300 biofilms were first established by inoculation of 10^5 CFU/mL bacteria suspension onto excision wound sites (**Figure**

7A). Starting at 24 h post infection, a topical treatment with either 10 mg/kg vancomycin, FTP NPs, or PBS vehicle (control) was applied onto the infected wound site (three doses at 4-hour intervals: *i.e.* 4 mg/kg at 24 h post infection, followed by 3 mg/kg 4 h later, and another 3 mg/kg 4 h after the 2nd treatment). As shown in **Figure 7A**, FTP NPs significantly suppress MRSA USA300 biofilm bacteria with a log₁₀ reduction of 1.8, which is superior to vancomycin (with a log₁₀ reduction of around 0.8), indicating the superior antibiofilm efficacy of FTP NPs.

To determine the *in vivo* biocompatibility of FTP NPs, the material was intravenously injected into mice at 10 mg/kg followed by weight measurement and blood biochemistry analysis. The results (**Figure 7B**) showed no obvious weight loss and no change in behavior of mice receiving this substantial systemic dose of FTP NPs. Further, the blood biochemical analysis results demonstrated that no nephro- or hepato-toxicity was induced by intravenous injection of FTP NPs, as indicated by the negligible change of biomarkers (*i.e.* alanine transaminase (ALT), aspartate transferase (AST), blood urea nitrogen (BUN), and creatinine (CRE)) for treated groups 1-day and 7-days post injection. We also determined the LD₅₀ value (single lethal dose resulting in 50% mortality) via intraperitoneal injection into mice, and the results showed that FTP NPs have LD₅₀ at 40 mg/kg. Overall, these data support that our biguanide-based FTP NPs can effectively suppress MRSA USA 300 biofilm in a murine model with negligible acute toxicity.

2.7. Discussion.

We expected the biguanide group of metformin to provide multiple hydrogen-bonding donor sites, making it versatile as a building block for supramolecular structure and/or NP formation.³³ Further, we showed that PMET can form nanoparticles with the natural polyphenol TA which can serve as building block with strong affinity to multiple substances.⁴³ We improved the colloidal stability of TP NPs by adding the third component F-127, which introduces PEG into the NPs

(**Scheme 1B**), the resultant FTP NPs have good stability with almost unchanged particle size over two weeks (**Figure 1B**). We also found that these FTP NPs, compared with neat PMET alone, have better reduction (by $> 2 \log_{10}$) of biofilm cell counts at low concentrations (8-32 $\mu\text{g/mL}$) in MBECTM assay (**Table 2**). Further, 3D CLSM images (**Figure 2**) demonstrated that FTP NPs, with their antifouling PEG content, have better biofilm penetration and accumulation than PMET and better biofilm removal ability (**Figure S9**) than PMET alone at 16 $\mu\text{g/mL}$ (with residual biofilm thickness of 12.8 μm after PMET treatment as opposed to 3.2 μm thickness after FTP NPs treatment). FTP NPs also showed almost complete biofilm eradication ($5.2 \log_{10}$ reduction) at the concentration 512 $\mu\text{g/mL}$ (**Table 2**). Further, FESEM and time lapse CLSM images confirmed a clear reduction of bacterial counts within biofilm after a single dose (128 $\mu\text{g/mL}$) treatment of FTP NPs. All the results demonstrated that the FTP NPs have better antibiofilm efficacy than PMET alone. We then also determined the dispersed planktonic bacterial killing ability of FTP NPs and the results (**Figure 3B**) showed that FTP NPs can kill bacteria dispersed from biofilm with MBC of 128 $\mu\text{g/mL}$. PMET also kills bacteria dispersed from biofilm with MBC of 256 $\mu\text{g/mL}$.

FTP NPs can disperse biofilm bacteria and also kill the dispersed bacteria (**Table 2** and **Figure 3B**). We call this property “intrinsic biofilm bactericidal activity”. We utilized various assays, including DiSC3(5), CLSM, FESEM, calcein dye leakage and killing kinetics, to study the antibiofilm mechanism of FTP NPs and the results strongly suggest that the bacterial kill mechanism of FTP NPs involves membrane permeabilization. FTP NPs are superior to dextran-*block*-poly(AMPTMA-*co*-BMA) that only shows biofilm dispersal effect -- “nanoscale bacterial debridement” -- without bactericidal effects.³² Agents that disperse biofilm without killing the bacteria entail the risk that the dispersed bacteria can disseminate into and infect other parts of the body, unless an antibacterial agent is co-administered. We hypothesize that the improved efficacy

of FTP NPs may be attributed to the hydrophilic PEG chain of F-127 dangling at the surface of the NPs (**Scheme 1B**). This antifouling chain could facilitate the NPs penetration into the biofilm which was confirmed by confocal microscopy (**Figure 2**), so that the NPs can kill bacteria throughout the depth of the biofilm matrix. FTP NPs showed good ability to eradicate preformed biofilm by penetrating into the biofilm matrix and then killing the bacteria. The bacterial kill mechanism appears to involve membrane permeabilization; attachment of the NPs is presumably promoted by electrostatic interaction between the positively charged NPs and anionic bacterial envelope. FTP NPs are also able to kill live bacteria that detach from the biofilm matrix makes them a superior antibiofilm agent.

3. Conclusions

We have demonstrated for the first time that PMET has good anti-MRSA ability. TA, a polyphenol and F-127, a poloxamer, were introduced to assist the formation of FTP NPs. With good biofilm penetration and dispersion ability and intrinsic biofilm bacterial killing activity, FTP NPs can remove MRSA USA300 biofilm more effectively than neat PMET as shown by the *in vitro* MBECTM assay. In *In vivo* murine wound infection testing, FTP NPs exhibit significantly ($p \leq 0.01$) higher reduction ($1.8 \log_{10}$) in biofilm bacteria than the antibiotic vancomycin ($0.8 \log_{10}$ reduction). In *in vitro* biocompatibility tests, FTP NPs are less cytotoxic than PMET towards representative mammalian cells (3T3 and HDF). FTP NPs cause no acute *in vivo* toxicity to mice at 10 mg/kg systemic dose, with negligible loss of body weight and change of blood biomarkers. We expect that these FTP NPs can provide an alternative strategy to treat MRSA biofilm associated wound infections and other MRSA related infections.

4. Experimental Sections

4.1. Materials. Linear polyethyleneimine (PEI), dicyandiamide (99%), tannic acid, Pluronic® F-127, hydrochloric acid (37%), dimethyl sulfoxide (DMSO), crystal violet (CV), 3,3'-dipropylthiadicarbocyanine iodide (DiSC3(5)), Dulbecco's Modified Eagle's Medium (DMEM), trypsin-EDTA, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), glutaraldehyde, and Triton X-100 were purchased from Sigma-Aldrich and used as received.

4.2. Preparation of PMET. PMET was synthesized following a published protocol.³⁵ The characterizations of PMET are shown in supporting information **Figure S1** and **S2**.

4.3. Preparation and Characterization of TP and FTP NPs. To prepare TP NPs, 50 μ L TA (20 mg/mL) solution was dropped quickly into 1 mL PMET (10 mg/mL) aqueous solution under stirring at 1000 rpm and stirred for 10 mins. To prepare FTP NPs, a mixture solution (1:1, v/v) of 50 μ L TA (20 mg/mL) and 50 μ L F-127 (20 mg/mL) solution was first prepared. Subsequently, the mixture was quickly dropped into 1 mL PMET (10 mg/mL) aqueous solution under stirring at 1000 rpm and stirred for 10 mins. The final products were washed three times using a 50 kDa membrane ultrafiltration unit under centrifugation (5,000 r.p.m. for 30 min at 4 °C) and lyophilized. Particle sizes, polydispersity index (PDI) and zeta potential of FTP NPs were carried out by a Malvern Nano-ZS Particle Sizer (Malvern Instruments, Malvern, U.K.) at 25 °C. FTP NPs were dissolved in DI water with concentration of 100 μ g/mL for both DLS and zeta potential measurement. Transmission electron microscope (TEM) images of NPs were acquired with a JEOL JEM-2100Plus Electron Microscope (Tokyo, Japan).

4.4. Minimum Inhibitory Concentrations (MICs). Bacteria cells were grown in Mueller Hinton Broth (MHB) and diluted to 10^5 - 10^6 CFU/mL. A stock polymer solution (10.24 mg/mL) was

prepared and two-fold serially diluted in MHB in a 96-well microplate. After that, 50 μ L bacterial suspension in MHB (10^5 - 10^6 CFU/mL) was added into the polymer solution to achieve final volume of 100 μ L. After incubating at 37 °C for 16-18 h, the well ODs were measured at 600 nm wavelength using a microplate reader (TECAN, infinite F200). Positive control was bacteria in MHB alone, and negative control was MHB. MICs were determined as the lowest concentration that inhibited cell growth by more than 90%.

4.5. *In Vitro* Biocompatibility Studies. The biocompatibility study was carried out with two (3T3 and HDF) mammalian cell lines. Mammalian cells were seeded at 1×10^4 cells per well in a 96-well plate and cultured at 37 °C for 24 hours. A stock polymer solution (10.24 mg/mL) was prepared and two-fold serially diluted in DMEM complete medium. After that, 200 μ L of polymer solution at desired concentrations was added to the cells in the 96-well plate and incubated at 37 °C for 24 hours. After incubation, the NP and/or polymer-containing DMEM solution was removed and the cells were washed with PBS prior to the addition of MTT solution (100 μ L, 1 mg/mL in DMEM; initial MTT solution concentration was 5 mg/mL in PBS). After another 4 h incubation, the MTT medium was removed followed by addition of DMSO (100 μ L). The plate was shaken at 100 rpm for 15 min and the cell viability was determined by measuring OD at 570 nm wavelength.

4.6. Cytoplasmic Membrane Depolarization. MRSA USA300 were grown overnight in MHB and sub-cultured (1:100 diluted) in MHB and grown for 4 hours to mid-log phase. The bacteria suspension was centrifuged and washed twice with 5 mM HEPES buffer (pH 7.8) and diluted to a final concentration of 10^7 CFU/mL. The bacterial suspensions were incubated with 100 nM DiSC3(5) in a 1 cm cuvette at room temperature for around 30 minutes. After a stable fluorescence intensity was achieved, polymer solution was then added into the bacterial suspension to achieve a final concentration of 100 μ g/mL. Changes in fluorescence were recorded with a spectrometer

(Perkin Elmer LS-55 luminescence spectrometer) at an excitation wavelength of 622 nm and an emission wavelength of 670 nm. Sampling interval was set at 1.5 seconds. 1% TritonX-100 was used as positive control.

4.7. *In Vitro* Antibiofilm Assays. The MBECTM assay was used following ASTM E2799-17.⁴⁴ Briefly, a mid-log phase culture of MRSA USA300 was diluted into Tryptic soy broth to 10^5 CFU/mL. 150 μ L of the bacterial suspension was added into the 96-well growth plate covered by a lid containing pegs. After incubation at 37 °C for 24-48 hours, the lid was washed twice with PBS and transferred into the challenge plate containing 2-fold serial dilutions of polymer solution with total volume of 200 μ L in each well. After 2 hours treatment at room temperature, the peg lid was then washed in PBS before transfer to a recovery plate containing neutralizer. After that, the recovery plate was sonicated for 30 ± 5 min to dislodge the biofilm. The recovered bacteria were then 10-fold serial diluted in sterile PBS and spread on agar plates. After incubation at 37 °C for 24 h, the colonies were counted.

4.8. Minimum Bactericidal Concentrations (MBCs). To determine the MBC values of FTP NPs, PMET and vancomycin. 20 μ L of the solutions in the challenge plate wells described in *section 4.7* was transferred into a fresh 96-well plate containing 180 μ L MHB and incubated at 37 °C for 24 h. After incubation, bacteria were then 10-fold serial diluted in sterile PBS and spread on agar plates. The MBC was designated as the lowest concentration at which no bacterial colonies were formed on agar plates. The untreated bacteria suspension was employed as negative control, while vancomycin was used as positive control.

4.9. Confocal Laser Scanning Microscopy (CLSM) for Planktonic Bacteria and Biofilms.

LIVE/DEAD BacLight™ bacterial viability kit was used to investigate the membrane permeability before and after PMET treatment. Green-fluorescing Syto 9 can enter all cells, live or dead, whereas red fluorescing propidium iodide (PI) can only stain the DNA of dead/dying bacteria cell with damaged cytoplasmic membranes.⁴⁵ Briefly, MRSA USA300 were grown into mid-log phase in MHB, washed twice with PBS and diluted into PBS to $10^5 - 10^6$ CFU/mL. The polymer solution was added into the bacterial suspension at 1 X MIC for 30 mins. The cell images were captured using a Zeiss LSM780 confocal laser scanning microscope (CLSM; Carl Zeiss, Jena, Germany) with a 63× plan-apochromatic oil immersion objective lens (numerical aperture, 1.46).

Time-lapse images were captured to study the dynamics of biofilm dispersal effect of FTP NPs. MRSA USA300 biofilm was established on a sterile chambered coverglass slide by gently adding diluted culture ($10^5 - 10^6$ CFU/mL) and incubating statically at 37 °C for 24-48 hours. After washing the glass slide using PBS, bacteria were stained with Syto 9 and PI. FTP NPs at 128 µg/mL were then gently introduced into the cover slide and the cell images were captured at different time points by with a Zeiss LSM780 confocal laser scanning microscope (CLSM; Carl Zeiss, Jena, Germany) with a 63× plan-apochromatic oil immersion objective lens (numerical aperture, 1.46). Syto 9 and PI were excited at 488 nm and 561 nm, respectively. The captured images were further processed with Zen 2011 software (Carl Zeiss, Germany) and ImageJ software.

4.10. Field-Emission Scanning Electron Microscopy (FESEM) of Planktonic Bacteria and Biofilms.

The morphology change of MRSA USA300 was performed as described previously.⁴⁶ MRSA USA300 were grown into mid-log phase in MHB and washed twice with PBS and then diluted into PBS to $10^7 - 10^8$ CFU/mL. The polymer solution and NP suspension were then added into respective samples of bacterial suspension at 1 X MIC for 30 mins. After incubation, bacteria

were fixed by 2.5 % glutaraldehyde at 4 °C overnight followed by dehydration in a graded concentration ethanol series (25 %, 30 %, 50 %, 70%, 90%, 100%) and air dried for 24 hours. The dehydrated sample was coated with platinum and imaged by FESEM (JEOL JSM 6701F).

For the biofilm images, preformed biofilm was established on the pegs of an MBEC plate as previously described. After challenging at desired polymer and NP concentration, the peg lids were broken off using a pair of flame-sterilized pliers and rinsed in PBS. The pegs were then fixed in 2.5% glutaraldehyde at 4°C for 16 h, after which they were washed with PBS followed by dehydration in 70% ethanol for 20 minutes and air drying for 24 h before specimen mounting and examination by FESEM (JEOL JSM 6701F).

4.11. *In Vivo* Murine Wound Biofilm Model of MRSA USA300 Infection. Experiments were performed according to protocols approved by the institutional animal care and usage committee (IACUC) of Nanyang Technological University (protocol approval number IACUC A18051). Male C57BL6 mice (Invivos, Singapore) aged 7-8 weeks were used in the *in vivo* studies. Mice (n = 5 per group) were anesthetized using isoflurane and an excision wound was created on the dorsal area using a 5 mm diameter biopsy punch. 2.5 µL MRSA USA300 in PBS suspension (5×10^5 CFU/mL) was dispensed to wound sites using a 10 µL pipette and the wounds were subsequently covered by Tegaderm (3M™) to prevent contamination. After 24-hour inoculation, Tegaderm film was removed and first dose of vancomycin, FTP NPs or PBS (negative control) were added to infected wounds at 4 mg/kg. Afterwards, mice were returned to their cages and rested for 4 hours. 2nd (3 mg/kg) and 3rd (3 mg/kg) treatments were subsequently applied at 4-hour intervals. After the third treatment, mice were sacrificed using CO₂ and tissue samples were harvested using scalpel blade. Samples were further homogenized and plated on agar plates to determine CFU.

4.12. *In Vivo* Toxicology Evaluation. Experiments were performed according to protocols approved by animal ethics and welfare of Ningbo University (protocol approval No. AEWC-2018-07). 10 mg/kg of FTP NPs were injected intravenously (i.v.) into female balb/c mice (n = 5 mice per group). Mice without injection were used as control. Mice weight and condition were monitored daily. At 1 day and 7 days' post-injection, mice blood was collected from submandibular vein and blood biochemistry was analyzed using a Blood Chemistry Analyzer (Pointcare V2, MNCHIP).

4.13. Evaluation of LD₅₀ of FTP NPs. Experiments were performed according to protocols approved by animal ethics and welfare of Ningbo University (protocol approval No. AEWC-2018-07). For LD₅₀ determination, ICR mice (female, 5 weeks) were randomly divided into four groups (six mice per group). Each of the mice received single intraperitoneal injection of FTP NPs at varied concentrations (*i.e.* 20 mg/kg, 30 mg/kg, 40 mg/kg and 50 mg/kg). Mice health condition was monitored over 7 days and LD₅₀ was estimated from the survival rate of treated groups. The LD₅₀ determined for FTP NPs is 40mg/kg.

Statistical analysis was performed using either student's *t*-test or one-way analysis of ANOVA with Dunnett's correction where appropriate. *P*-values <0.05 were considered statistically significant.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publication website at DOI:

Details of experimental procedures of hemolytic activity, biofilm inhibition assay and calcein dye leakage assay. ^1H NMR and ^{13}C NMR of PEI and PMET (Figure S1-S2); Photo images of PEI and PMET (Figure S3); Photo and TEM image of TP NPs (Figure S4); ^1H NMR and UV-Vis absorption spectra of filtrate (Figure S5); DLS data of FTP NPs (Figure S6); Illustration of MBECTM assay (Figure S7); Bacterial CFU count of FTP NPs and PMET treated MRSA biofilm (Figure S8); Confocal microscopy of biofilm treated by FTP NPs and PMET at 16 $\mu\text{g/mL}$ for 2 h (Figure S9); Biofilm inhibition assay (Figure S10); hemolytic activity data (Figure S11); DiSC3(5), FESEM and confocal microscopy of PMET treated cells (Figure S12); calcein dye leakage data (Figure S13); ITC and FTIR data (Figure S14); DLS and zeta potential data (Table S1); solution properties of mixture of TA and PMET at different mass ratio (Table S2).

AUTHOR INFORMATION

Corresponding Authors

***E-mail:** mbechan@ntu.edu.sg

Notes

The authors declare no competing financial interest.

Acknowledgements

We thank the funding support from a Singapore Ministry of Education Tier 3 grants (MOE2013-T3-1-002, MOE2018-T3-1-003), and a Singapore Ministry of Health Industry Alignment Fund grant (NMRC/ MOHIAFCAT2/003/2014). This research was also supported by an ASTAR RIE2020 Advanced Manufacturing and Engineering (AME) IAP-PP Specialty Chemicals Programme grant (No. A1786a0032). Li Jianghua acknowledges the support of NTU through a PhD scholarship.

References:

1. Deadly Staph Infections Still Threaten the U.S. <https://www.cdc.gov/media/releases/2019/p0305-deadly-staph-infections.html>.
2. Mataraci, E.; Dosler, S., In Vitro Activities of Antibiotics and Antimicrobial Cationic Peptides Alone and in Combination against Methicillin-Resistant *Staphylococcus aureus* Biofilms. *Antimicrob. Agents Chemother.* **2012**, *56*, 6366-6371.
3. Drugs for MRSA Skin and Soft-Tissue Infections. *JAMA* **2014**, *312*, 1583-1584.
4. Hu, D.; Li, H.; Wang, B.; Ye, Z.; Lei, W.; Jia, F.; Jin, Q.; Ren, K. F.; Ji, J., Surface-Adaptive Gold Nanoparticles with Effective Adherence and Enhanced Photothermal Ablation of Methicillin-Resistant *Staphylococcus aureus* Biofilm. *ACS Nano* **2017**, *11*, 9330-9339.
5. Goswami, S.; Thiagarajan, D.; Das, G.; Ramesh, A., Biocompatible Nanocarrier Fortified with a Dipyrindinium-Based Amphiphile for Eradication of Biofilm. *ACS Appl. Mater. Interfaces* **2014**, *6*, 16384-16394.
6. Flemming, H.-C.; Wingender, J., The Biofilm Matrix. *Nat. Rev. Microbiol.* **2010**, *8*, 623-633.
7. Surewaard, B. G. J.; Thanabalasuriar, A.; Zeng, Z.; Tkaczyk, C.; Cohen, T. S.; Bardoel, B. W.; Jorch, S. K.; Deppermann, C.; Bubeck-Wardenburg, J.; Davis, R. P.; Jenne, C. N.; Stover, K. C.; Sellman, B. R.; Kubes, P., α -Toxin Induces Platelet Aggregation and Liver Injury during *Staphylococcus aureus* Sepsis. *Cell Host Microbe*. **2018**, *24*, 271-284 e3.
8. Liu, R.; Chen, X.; Falk, S. P.; Masters, K. S.; Weisblum, B.; Gellman, S. H., Nylon-3 Polymers Active against Drug-Resistant *Candida albicans* Biofilms. *J. Am. Chem. Soc.* **2015**, *137*, 2183-2186.
9. Tan, J. P. K.; Coady, D. J.; Sardon, H.; Yuen, A.; Gao, S.; Lim, S. W.; Liang, Z. C.; Tan, E. W.; Venkataraman, S.; Engler, A. C.; Fevre, M.; Ono, R.; Yang, Y. Y.; Hedrick, J. L., Broad Spectrum Macromolecular Antimicrobials with Biofilm Disruption Capability and In Vivo Efficacy. *Adv. Healthc. Mater.* **2017**, *6*, 1601420.
10. Zhang, K.; Du, Y.; Si, Z.; Liu, Y.; Turvey, M. E.; Raju, C.; Keogh, D.; Ruan, L.; Jothy, S. L.; Reghu, S.; Marimuthu, K.; De, P. P.; Ng, O. T.; Mediavilla, J. R.; Kreiswirth, B. N.; Chi, Y. R.; Ren, J.; Tam, K. C.; Liu, X. W.; Duan, H.; Zhu, Y.; Mu, Y.; Hammond, P. T.; Bazan, G. C.; Pethe, K.; Chan-Park, M. B., Enantiomeric Glycosylated Cationic Block co-Beta-Peptides Eradicate *Staphylococcus aureus* Biofilms and Antibiotic-Tolerant Persisters. *Nat. Commun.* **2019**, *10*, 4792.
11. Sahariah, P.; Masson, M.; Meyer, R. L., Quaternary Ammoniumyl Chitosan Derivatives for Eradication of *Staphylococcus aureus* Biofilms. *Biomacromolecules* **2018**, *19*, 3649-3658.
12. Junter, G. A.; Thebault, P.; Lebrun, L., Polysaccharide-Based Antibiofilm Surfaces. *Acta. Biomater.* **2016**, *30*, 13-25.
13. Jennings, M. C.; Minbiole, K. P.; Wuest, W. M., Quaternary Ammonium Compounds: An Antimicrobial Mainstay and Platform for Innovation to Address Bacterial Resistance. *ACS Infect. Dis.* **2015**, *1*, 288-303.
14. Yavvari, P. S.; Gupta, S.; Arora, D.; Nandicoori, V. K.; Srivastava, A.; Bajaj, A., Clathrin-Independent Killing of Intracellular Mycobacteria and Biofilm Disruptions Using Synthetic Antimicrobial Polymers. *Biomacromolecules* **2017**, *18*, 2024-2033.
15. Uppu, D. S.; Samaddar, S.; Ghosh, C.; Paramanandham, K.; Shome, B. R.; Haldar, J., Amide Side Chain Amphiphilic Polymers Disrupt Surface Established Bacterial Bio-films and Protect Mice from Chronic *Acinetobacter baumannii* Infection. *Biomaterials* **2016**, *74*, 131-143.
16. Wolfmeier, H.; Pletzer, D.; Mansour, S. C.; Hancock, R. E. W., New Perspectives in Biofilm Eradication. *ACS Infect Dis* **2018**, *4*, 93-106.

17. Lam, S. J.; Wong, E. H. H.; Boyer, C.; Qiao, G. G., Antimicrobial Polymeric Nanoparticles. *Prog. Polym. Sci.* **2018**, *76*, 40-64.
18. Liu, Y.; Shi, L.; Su, L.; van der Mei, H. C.; Jutte, P. C.; Ren, Y.; Busscher, H. J., Nanotechnology-Based Antimicrobials and Delivery Systems for Biofilm-Infection Control. *Chem. Soc. Rev.* **2019**, *48*, 428-446.
19. Duncan, B.; Li, X.; Landis, R. F.; Kim, S. T.; Gupta, A.; Wang, L. S.; Ramanathan, R.; Tang, R.; Boerth, J. A.; Rotello, V. M., Nanoparticle-Stabilized Capsules for the Treatment of Bacterial Biofilms. *ACS Nano* **2015**, *9*, 7775-7782.
20. Qiao, J.; Purro, M.; Liu, Z.; Xiong, M. P., Terpyridine-Micelles for Inhibiting Bacterial Biofilm Development. *ACS Infect. Dis.* **2018**, *4*, 1346-1354.
21. Li, X.; Yeh, Y.-C.; Giri, K.; Mout, R.; Landis, R. F.; Prakash, Y.; Rotello, V. M., Control of Nanoparticle Penetration into Biofilms Through Surface Design. *Chem. Commun.* **2015**, *51*, 282-285.
22. Horev, B.; Klein, M. I.; Hwang, G.; Li, Y.; Kim, D.; Koo, H.; Benoit, D. S., pH-Activated Nanoparticles for Controlled Topical Delivery of Farnesol to Disrupt Oral Biofilm Virulence. *ACS Nano* **2015**, *9*, 2390-404.
23. Cao, J.; Zhao, Y.; Liu, Y.; Tian, S.; Zheng, C.; Liu, C.; Zhai, Y.; An, Y.; Busscher, H. J.; Shi, L., Phosphorylcholine-Based Polymer Encapsulated Chitosan Nanoparticles Enhance the Penetration of Antimicrobials in a Staphylococcal Biofilm. *ACS Macro Let.* **2019**, *8*, 651-657.
24. Liu, Y.; van der Mei, H. C.; Zhao, B.; Zhai, Y.; Cheng, T.; Li, Y.; Zhang, Z.; Busscher, H. J.; Ren, Y.; Shi, L., Eradication of Multidrug-Resistant Staphylococcal Infections by Light-Activatable Micellar Nanocarriers in a Murine Model. *Adv. Funct. Mater.* **2017**, *27*, 1701974.
25. Guo, Q.; Zhao, Y.; Dai, X.; Zhang, T.; Yu, Y.; Zhang, X.; Li, C., Functional Silver Nanocomposites as Broad-Spectrum Antimicrobial and Biofilm-Disrupting Agents. *ACS Appl. Mater. Interfaces* **2017**, *9*, 16834-16847.
26. Fei, X.; Jia, M.; Du, X.; Yang, Y.; Zhang, R.; Shao, Z.; Zhao, X.; Chen, X., Green Synthesis of Silk Fibroin-Silver Nanoparticle Composites with Effective Antibacterial and Biofilm-Disrupting Properties. *Biomacromolecules* **2013**, *14*, 4483-4488.
27. Loo, C. Y.; Rohanizadeh, R.; Young, P. M.; Traini, D.; Cavaliere, R.; Whitchurch, C. B.; Lee, W. H., Combination of Silver Nanoparticles and Curcumin Nanoparticles for Enhanced Antibiofilm Activities. *J. Agric. Food Chem.* **2016**, *64*, 2513-2522.
28. Gupta, A.; Mumtaz, S.; Li, C.-H.; Hussain, I.; Rotello, V. M., Combatting Antibiotic-Resistant Bacteria Using Nanomaterials. *Chem. Soc. Rev.* **2019**, *48*, 415-427.
29. Durmus, N. G.; Taylor, E. N.; Kummer, K. M.; Webster, T. J., Enhanced Efficacy of Superparamagnetic Iron Oxide Nanoparticles Against Antibiotic-Resistant Biofilms in the Presence of Metabolites. *Adv. Mater.* **2013**, *25*, 5706-5713.
30. Naha, P. C.; Liu, Y.; Hwang, G.; Huang, Y.; Gubara, S.; Jonnakuti, V.; Simon-Soro, A.; Kim, D.; Gao, L.; Koo, H.; Cormode, D. P., Dextran-Coated Iron Oxide Nanoparticles as Biomimetic Catalysts for Localized and pH-Activated Biofilm Disruption. *ACS Nano* **2019**, *13*, 4960-4971.
31. Panáček, A.; Kvítek, L.; Smékalová, M.; Večeřová, R.; Kolář, M.; Röderová, M.; Dyčka, F.; Šebela, M.; Pucek, R.; Tomanec, O., Bacterial Resistance to Silver Nanoparticles and How to Overcome It. *Nat. Nanotechnol.* **2018**, *13*, 65-71.
32. Li, J.; Zhang, K.; Ruan, L.; Chin, S. F.; Wickramasinghe, N.; Liu, H.; Ravikumar, V.; Ren, J.; Duan, H.; Yang, L.; Chan-Park, M. B., Block Copolymer Nanoparticles Remove Biofilms of

Drug-Resistant Gram-Positive Bacteria by Nanoscale Bacterial Debridement. *Nano Lett.* **2018**, *18*, 4180-4187.

33. Chen, F.; Moat, J.; McFeely, D.; Clarkson, G.; Hands-Portman, I. J.; Furner-Pardoe, J. P.; Harrison, F.; Dowson, C. G.; Sadler, P. J., Biguanide Iridium (III) Complexes with Potent Antimicrobial Activity. *J. Med. Chem.* **2018**, *61*, 7330-7344.

34. Böttcher, T.; Kolodkin-Gal, I.; Kolter, R.; Losick, R.; Clardy, J., Synthesis and Activity of Biomimetic Biofilm Disruptors. *J. Am. Chem. Soc.* **2013**, *135*, 2927-2930.

35. Zhao, Y.; Wang, W.; Guo, S.; Wang, Y.; Miao, L.; Xiong, Y.; Huang, L., PolyMetformin Combines Carrier and Anticancer Activities for *In Vivo* siRNA Delivery. *Nat. Commun.* **2016**, *7*, 11822.

36. Le, Z.; Chen, Y.; Han, H.; Tian, H.; Zhao, P.; Yang, C.; He, Z.; Liu, L.; Leong, K. W.; Mao, H.-Q., Hydrogen-Bonded Tannic Acid-Based Anticancer Nanoparticle for Enhancement of Oral Chemotherapy. *ACS Appl. Mater. Interfaces* **2018**, *10*, 42186-42197.

37. Wang, X.; Yan, J.; Pan, D.; Yang, R.; Wang, L.; Xu, Y.; Sheng, J.; Yue, Y.; Huang, Q.; Wang, Y., Polyphenol-Poloxamer Self-Assembled Supramolecular Nanoparticles for Tumor NIRF/PET Imaging. *Adv. Healthc. Mater.* **2018**, *7*, 1701505.

38. de la Fuente-Núñez, C.; Korolik, V.; Bains, M.; Nguyen, U.; Breidenstein, E. B.; Horsman, S.; Lewenza, S.; Burrows, L.; Hancock, R. E., Inhibition of Bacterial Biofilm Formation and Swarming Motility by a Small Synthetic Cationic Peptide. *Antimicrob. Agents Chemother.* **2012**, *56*, 2696-2704.

39. de la Fuente-Núñez, C.; Reffuveille, F.; Haney, E. F.; Straus, S. K.; Hancock, R. E., Broad-Spectrum Anti-Biofilm Peptide that Targets a Cellular Stress Response. *PLOS Pathog.* **2014**, *10*, e1004152.

40. Hill, E. H.; Pappas, H. C.; Evans, D. G.; Whitten, D. G., Cationic Oligo-*p*-Phenylene Ethynylenes form Complexes with Surfactants for Long-Term Light-Activated Biocidal Applications. *Photochem. Photobiol. Sci.* **2014**, *13*, 247-253.

41. Hall-Stoodley, L.; Costerton, J. W.; Stoodley, P., Bacterial Biofilms: from the Natural Environment to Infectious Diseases. *Nat. Rev. Microbiol.* **2004**, *2*, 95-108.

42. Zhang, P.; Wang, L.; Yang, S.; Schott, J. A.; Liu, X.; Mahurin, S. M.; Huang, C.; Zhang, Y.; Fulvio, P. F.; Chisholm, M. F., Solid-State Synthesis of Ordered Mesoporous Carbon Catalysts Via a Mechanochemical Assembly Through Coordination Cross-Linking. *Nat. Commun.* **2017**, *8*, 15020.

43. Shin, M.; Lee, H. A.; Lee, M.; Shin, Y.; Song, J. J.; Kang, S. W.; Nam, D. H.; Jeon, E. J.; Cho, M.; Do, M.; Park, S.; Lee, M. S.; Jang, J. H.; Cho, S. W.; Kim, K. S.; Lee, H., Targeting Protein and Peptide Therapeutics to the Heart Via Tannic Acid Modification. *Nat. Biomed. Eng.* **2018**, *2*, 304-317.

44. Ceri, H.; Olson, M.; Stremick, C.; Read, R.; Morck, D.; Buret, A., The Calgary Biofilm Device: New Technology for Rapid Determination of Antibiotic Susceptibilities of Bacterial Biofilms. *J. Clin. Microbiol.* **1999**, *37*, 1771-1776.

45. Rahman, M. A.; Bam, M.; Luat, E.; Jui, M. S.; Ganewatta, M. S.; Shokfai, T.; Nagarkatti, M.; Decho, A. W.; Tang, C., Macromolecular-Clustered Facial Amphiphilic Antimicrobials. *Nat. Commun.* **2018**, *9*, 5231.

46. Li, P.; Zhou, C.; Rayatpisheh, S.; Ye, K.; Poon, Y. F.; Hammond, P. T.; Duan, H.; Chan-Park, M. B., Cationic Peptidopolysaccharides Show Excellent Broad-Spectrum Antimicrobial Activities and High Selectivity. *Adv. Mater.* **2012**, *24*, 4130-4137.

Scheme 1. (A) Synthesis of PMET by reacting linear PEI with dicyandiamide. (B) Preparation of FTP NPs.

Figure 1. Characterizations of FTP NPs. (A) Hydrodynamic diameter (D_h) of FTP NPs using DLS. Inset is the TEM image of FTP NPs with scale bar = 200 nm. (B) Stability of FTP NPs no significant change of D_h and PDI with passage of time. Data are presented as mean $\pm$ standard deviation and represent three independent experiments.

Figure 2. (A) Penetration and accumulation of rhodamine-labeled (red) FTP NPs (upper panel) and PMET (lower panel) into MRSA USA300 biofilm (Syto 9: green) at 16 $\mu\text{g/mL}$ for 30 min. (B) Fluorescence intensities of rhodamine-labeled (red) FTP NPs and PMET as a function of depth in the biofilm (dashed vertical line represents the bottommost layer of biofilm). Scale bar is 20 μm .

Figure 3. (A) FESEM images of MRSA USA300 biofilm before and after FTP NPs treatment (128 μg/mL). Scale bars are 1 μm. (B) Minimum bactericidal concentration (MBC) values of FTP NPs and PMET against dispersed planktonic bacteria in the MBECTM challenge wells, measured by CFU count (circles represent zero count). Untreated bacteria suspension was employed as negative control, while vancomycin was used as positive control. Data are presented as mean ± standard deviation and represent three independent experiments. (C) 3D confocal images of MRSA USA300 biofilms treated by FTP NPs at 128 μg/mL with incubation time: 0 min, 30 min, and 120 min, showing the removal of MRSA USA300 biofilm.

Figure 4. *In vitro* biocompatibility of PMET and FTP NPs towards mammalian cells. (A) mouse embryonic fibroblast 3T3 cells. (B) human dermal fibroblast (HDF) cells. Data are presented as mean ± standard deviation and represent three independent experiments.

Figure 5. (A) MRSA USA300 membrane potential depolarization assessed by DiSC3(5) fluorescence after FTP NPs treatment. (B) FESEM images of MRSA USA300 (i) before and (ii) after FTP NPs treatment at MIC (16 $\mu\text{g/mL}$). Scale bar is 1 μm . (C) CLSM images of MRSA USA300 bacteria of (upper panel) untreated control and (lower panel) FTP NPs treatment at MIC (16 $\mu\text{g/mL}$). Live and dead bacterial cells were stained by Syto 9 (green) while only dead cells were stained by propidium iodide (red). Scale bar is 10 μm .

Figure 6. Killing kinetics of planktonic MRSA USA300 at different concentrations: (A) 1 x, (B) 2 x and (C) 4 x MIC of FTP NPs and PMET. Untreated bacteria was employed as negative control, while vancomycin was used as positive control. Data are presented as mean $\pm$ standard deviation and represent three independent experiments.

Figure 7. (A) Illustration of murine wound model and *in vivo* antibiofilm activity. Log_{10} CFU per wound from PBS alone (control), vancomycin (10 mg/kg), and FTP NPs (10 mg/kg). ** $p \leq 0.01$ and **** $p \leq 0.0001$ by two-tailed Student's *t*-test. (B) Mice weight monitoring for 7 days post intravenous injection of FTP NPs at 10 mg/kg. The average weight was plotted *versus* time, with error bars representing the sample standard deviation within the experimental group at each day. Blood biochemistry analysis at 1 day and 7 days post intravenous injection of FTP NPs at 10 mg/kg. Blood biochemical parameters from each mouse are plotted as individual points and error bars represent the sample standard deviation within an experimental group. *P* values were calculated using one-way ANOVA analysis.

Table 1. Antimicrobial of PMET, FTP NPs and reference antibiotics against planktonic Gram-positive and Gram-negative bacteria.

Samples	MICs: $\mu\text{g/mL}$							
	Gram-positive				Gram-negative			
	Methicillin							
	<i>S. aureus</i>	<i>S. aureus</i>	<i>S. aureus</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>K. pneumoniae</i>	<i>A. baumannii</i>
	(SA 29123)	(MRSA BAA40)	(MRSA USA300)	(MRSA KKH5 ^a)	(EC8739)	(PAO1)	(KPNR)	(AB-1)
PMET	16	8	16	8	32	32	64	64
FTP NPs	16	8	16	8	16/32	32	64	64
Vancomycin	1	1	0.5	0.5	128	>128	>128	>128
Colistin	128	>128	128	128	2	1	2	2

^aMRSA KKH5 provided by KK Women's and Children's Hospital, Singapore

Table 2. Log₁₀ reduction of MRSA USA300 biofilm cell counts treated by FTP NPs, PMET and vancomycin compared to untreated control.

Concentration ($\mu\text{g/mL}$)	Samples		
	FTP NPs	PMET	Vancomycin
4	1.1	-0.1	0.1
8	2.4	0.2	0.9
16	2.6	0.4	0.7
32	3.5	1.5	0.9
64	3.6	3.4	0.8
128	4.0	3.5	1.1
256	4.2	3.2	1.1
512	5.2	3.2	1.4

TOC GRAPHICS

Supporting information

Biguanide-derived Polymeric Nanoparticles Kill MRSA Biofilm and Suppress Infection *In Vivo*

Jianghua Li,^a Wenbin Zhong,^a Kaixi Zhang,^a Dongwei Wang,^c Jingbo Hu^c and Mary B. Chan-Park^{a,b,*}

a. Centre for Antimicrobial Bioengineering, School of Chemical and Biomedical Engineering, Nanyang Technological University, 62 Nanyang Drive, 637459 Singapore

b. Lee Kong Chian School of Medicine, Nanyang Technological University, 59 Nanyang Drive, 636921 Singapore

c. Institute of Drug Discovery Technology, Ningbo University, Ningbo, 315000, China

***E-mail:** mbechan@ntu.edu.sg

Keywords: antibiofilm, multidrug resistance, nanoparticles, tannic acid, biocompatibility

Hemolysis assay. Human red blood cells were collected and prepared in Tris buffer (pH 7.2) to a concentration of 5 % in volume ratio. Polymer solution in Tris buffer with two-fold dilutions was prepared and added into 50 μ L blood cells solution to reach a total volume of 100 μ L. After incubation at 37 °C for 1 h, the 96-well microplate was centrifuged for 10 min at 1,000 $\times$ g. 80 μ L samples of the supernatant were removed and added into 80 μ L of Tris buffer to achieve total volume of 160 μ L. Hemolytic activity was determined by measuring OD at 540 nm using a TECAN microplate reader. The positive control was 0.1 % Triton X-100 and the negative control was Tris buffer. The hemolysis percentage was calculated based on following equation:

$$\% \text{ Hemolysis} = \left(\frac{O_p - O_b}{O_t - O_b} \right) \times 100$$

where O_p is the OD value of polymer treated solution, O_b is OD value of negative control, and O_t is the OD value of positive control.

Analysis of biofilm inhibition. Briefly, MRSA USA300 was cultured in MHB and diluted to 10^6 - 10^7 CFU/mL. The NP or polymer solutions were prepared in the same medium with two-fold serial dilution and 75 μ L of the FTP NPs and PMET solution (at 64, 32, 16, 8, 4, 2 μ g/mL) were added into a 96-well microplate. After that, 75 μ L of bacterial suspension was added into each well to achieve a final volume of 150 μ L. The microtiter plate was incubated for 24 hours at 37°C. After incubation, the bacterial suspension was discarded and the microplate was rinsed with DI water twice followed by adding 125 μ L of 0.1 % crystal violet (CV). After that, the microplate was kept at room temperature for 10 min followed by washing twice with DI water. 125 μ L of 30 % acetic acid solution was added into each well to dissolve the CV. After an interval of 15 min to dissolve the CV, a TECAN microplate reader was used to measure OD₅₅₀ value to determine the biomass. Vancomycin was used as positive control and MHB was used as negative control.

Calcein dye leakage assay. To prepare 3:1 PG/CL liposomes, 450 μL of PG (25 mg/mL in CHCl_3) and 150 μL of CL (25 mg/mL in CHCl_3) were added to 2 mL CHCl_3 . The solvent was evaporated by vacuum to obtain a thin lipid film, which was then hydrated with 1 mL of calcein solution (40 mM calcein, 10 mM Na_2HPO_4 in H_2O at pH = 7.0, buffer A). The mixture was subjected to at least 5 freeze–thaw cycles using liquid nitrogen and warm water. The suspension was extruded 20 times through a polycarbonate membrane with 200 nm pore diameter. The excess dye was removed using Sephadex G-50 column and buffer B, consisting of 10 mM Na_2HPO_4 and 90 mM NaCl, as the eluent. The dye-filled vesicle fractions were diluted 1000 times with buffer B (final lipid concentration: ~ 5.0 mM). This suspension (90 μL) was subsequently mixed with polymer stock solutions (10 μL) on a 96-well black microplate. Buffer B (10 μL) and Triton X-100 (0.1% v/v, 10 μL) were employed as the negative and positive controls, respectively. The time evolution of fluorescence intensity in each well was recorded using a microplate reader (TECAN, Switzerland) with excitation and emission wavelengths of 490 and 515 nm, respectively. The percentage of leaked calcein dye in each well was determined as follows: leakage (%) = $[(F - F_0)/(F_T - F_0)] \times 100\%$ where F is the fluorescence intensity recorded in the well, F_0 is the intensity in the negative control well, and F_T is the intensity in the positive control well.

Figure S1. (A) ^1H NMR of Linear PEI in DMSO- d_6 and PMET in D_2O . (B) GPC of linear PEI and PMET.

Figure S3. Visual assessment of solubility of linear PEI and PMET in PBS.

Figure S4. (A) Optical images of turbidity of TA, PMET and TP NPs. (B) TEM image of TP NPs. Scale bar=100 nm.

Figure S5. (A) ^1H NMR and (B) UV-Vis absorption spectra of filtrate in lower compartment of centrifugal filter units. (C) and (D) UV-Vis absorption of free PMET and TA as standard with different concentrations.

Figure S6. Particle size distribution curves (intensity vs. particle diameter) of FTP NPs using DLS analysis.

Figure S7. Illustration of MBEC™ assay.

Figure S8. Bacterial cell counts after treatment with FTP NPs or PMET. Untreated biofilm was employed as negative control, while vancomycin was used as positive control. Data are presented as mean $\pm$ standard deviation and represent three independent experiments.

Figure S9. 3D confocal microscopy images of MRSA USA300 biofilms treated with (A) PMET (upper panel) and (B) FTP NPs (lower panel) at 16 µg/mL for 2 h. Live and dead bacterial cells were stained by Syto 9 (green) while only dead cells were stained by propidium iodide (red).

Figure S10. Biofilm inhibition of FTP NPs and PMET at sub-MIC concentrations by crystal violet assay. Untreated biofilm was employed as negative control, while vancomycin was used as positive control. Data are presented as mean $\pm$ standard deviation and represent three independent experiments.

Figure S11. Hemolytic activity of PEMT And FTP NPs against human red blood cells.

Figure S12. (A) MRSA USA300 membrane potential depolarization assessed by DiSC3(5) fluorescence after FTP NPs treatment. (B) FESEM images of MRSA USA300 (i) before and (ii) after PMET treatment at MIC (16 μ g/mL). Scale bar is 1 μ m. (C) CLSM images of MRSA USA300 bacteria with (upper panel) no treatment (control) and (lower panel) PMET treatment at MIC (16 μ g/mL). Live and dead bacterial cells were stained by Syto 9 (green) while only dead cells were stained by propidium iodide (red). Scale bar is 10 μ m.

Figure S13. Calcein dye leakage caused by addition of FTP NPs and PMET at 16 $\mu\text{g/mL}$ (1 x MIC). Liposome composition: PG/CL (3:1, w/w; membrane mimic of Gram-positive MRSA USA300) vesicles. Triton X-100 was employed as positive control to cause 100% dye leakage.

Figure S14. (A) ITC data: titration of TA (1.47 mM) into PMET (147 μ M), indicating the interaction between TA and PMET was enthalpically driven with unfavorable entropic change. (B) FTIR spectra of TA, F-127 and F-127/TA mixture (1:1, w/w).

Table S1. DLS and zeta potential of PMET, TA, TP NPs and FTP NPs.

	Size (nm)	ζ (mV)
PMET	1.0 ± 0.2	14.5 ± 0.7
TA	2.2 ± 0.7	-1.3 ± 0.5
TP NPs	96.9 ± 1.3	43.1 ± 1.7
FTP NPs	121.6 ± 3.5	33.6 ± 1.4

Table S2. Solution appearance of mixtures of PMET and TA at different mass ratios.

Samples	Solution turbidity
PMET	clean
TA	clean
PMET:TA =1:2	turbid
PMET:TA =1:1	turbid
PMET:TA =2:1	turbid
PMET:TA=10:1	turbid