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Terpyridine–Micelles for Inhibiting Bacterial Biofilm Development

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Abstract

Iron plays a critical role in bacterial infections and is especially critical for supporting biofilm formation. Until recently, Fe(III) was assumed to be the most relevant form of iron to chelate in therapeutic antimicrobial strategies due to its natural abundance under normal oxygen and physiologic conditions. Recent clinical data obtained from cystic fibrosis (CF) patients found that there is actually quite an abundance of Fe(II) present in sputum and that there exists a significant relationship between sputum Fe(II) concentration and severity of the disease. A biocompatible mixed micelle formed from the self-assembly of poly(lactic-*co*-glycolic acid)-*block*-methoxy poly-(ethylene glycol) (PLGA-*b*-mPEG) and poly(lactic-*co*-glycolic acid)-*block*-poly(terpyridine)₅ [PLGA-*b*-p(Tpy)₅] polymers was prepared to chelate Fe(II) (Tpy–micelle). Tpy–micelles showed high selectivity for Fe(II) over Fe(III), decreased biofilm mass more effectively under anaerobic conditions at >4 μ M Tpy–micelles, reduced bacteria growth in biofilms by >99.9% at 128 μ M Tpy–micelles, effectively penetrated throughout a 1-day old biofilm, and inhibited biofilm development in a concentration-dependent manner. This study reveals that Fe(II) chelating Tpy–micelles are a promising addition to Fe(III) chelating strategies to inhibit biofilm formation in CF lung infections.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfec-dis.8b00091.

Detailed syntheses, metal selectivity, competition studies, ¹H NMR spectra, and UV-vis spectra (PDF)

Keywords

Tpy-micelle; biofilm; cystic fibrosis

Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic Gram-negative bacterial pathogen that can cause diseases in plants, animals, and humans and is also one of the clinically relevant biofilm-forming bacteria. Patients with cystic fibrosis (CF) are highly susceptible to chronic *P. aeruginosa* infections due to abnormally high retention of mucus in the lungs.^{1–4} Consequently, pathogenic bacteria can easily colonize and form biofilms in the airways of CF patients, with *P. aeruginosa* displaying an enhanced capacity to form biofilms that are highly resistant to antibiotic penetration.^{5–7}

Iron is an essential nutrient for bacteria due to the significant role it plays in growth and biofilm development.^{8–11} Since most bacteria typically need ca. 10^{-8} M iron,¹² antimicrobial strategies focused on limiting the pool of Fe(III) available to bacteria have been promising areas of research. Encouraging studies have shown that sequestering Fe(III) with lactoferrin can indeed prevent *P. aeruginosa* biofilms from maturing from thin layers into large multicellular biofilm structures.¹³ Until recently, Fe(III) was assumed to be the most relevant form of iron to chelate in therapeutic antimicrobial strategies due to its natural abundance under normal oxygen and physiologic conditions. However, recent clinical data obtained from CF patients have found that there is quite an abundance of Fe(II) also present. The concentration of Fe(II) was reported to be $7 \pm 8 \mu\text{M}$ for normal to mild CF patients, and more elevated Fe(II) levels of $39 \pm 22 \mu\text{M}$ were found in severely infected patients.¹⁴ As such, a relationship between sputum Fe(II) concentration and severity of the disease appears to be relevant.^{14,15} This means that in poorly oxygenated micro-environments characteristic of late-stage CF,¹⁶ the sequestration of Fe(II) may complement Fe(III) chelation.

To the best of our knowledge, the antimicrobial effects of Fe(II) chelation have not been investigated as much and most antimicrobial chelation strategies have focused mainly on Fe(III). For example, the iron-chelating protein lactoferrin can bind up to two Fe(III) ions, each with a dissociation constant (K_d) of 10^{-20} M.¹⁷ Deferoxamine (DFO) and Deferasirox (DFX) are small molecule chelators currently FDA-approved for treating iron overload in humans and have also been investigated for their antimicrobial properties.¹⁸ Similarly, ethylenediaminetetraacetic acid (EDTA) and 2,2'-dipyridine (2DP) also have exhibited antimicrobial activity due to their sequestration of iron^{19,20} and, in the case of EDTA, also other divalent ions such as Mg(II) and Ca(II) that are critical to the integrity of the Gram-negative outer membrane.^{21–24} In order to investigate the antimicrobial effect of Fe(II)

chelation, desirable properties of the chelator include selectivity for Fe(II) ions and absence of interaction with natural iron-transport receptors. This is critical because, under iron-limited conditions, *P. aeruginosa* has been shown to express receptors capable of taking up Fe(III):DFO complexes for survival and growth.^{11,25} Although *P. aeruginosa* does not appear to possess lactoferrin receptors, other pathogens do express them in order to acquire Fe(III) from the host environment.^{26,27} To ensure sequestered Fe(II) does not end up being transported by bacteria as potential nutrient for growth, large macromolecular carriers such as micelles capable of chelating Fe(II) were developed in this study. The other advantage to using iron chelating macromolecules such as micelles is that they have already been shown to improve the solubility of some poorly water-soluble antibiotics through their encapsulation into the core of compatible micelles,^{28–30} and the combination of iron sequestering molecules with poorly soluble bactericidal antibiotics may serve as a promising addition for difficult-to-treat bacterial infections characterized by biofilm formations. To investigate Fe(II) chelating micelles, terpyridine (Tpy) was incorporated into the design to generate Tpy–micelles due to its well-reported selectivity for Fe(II) over other divalent and trivalent transition metals and has been reported to bind Fe(II) at a 2:1 ratio to form a complex $[\text{Fe}(\text{Tpy})_2]^{2+}$ that absorbs strongly at 570 nm with an equilibrium dissociation constant (K_d) of $10^{-20.9}$ M.³¹

Herein, we report on the synthesis, preparation, and characterization of a biocompatible Tpy–micelle formed from the self-assembly of poly(lactic-*co*-glycolic acid)-*block*-methoxy poly(ethylene glycol) (PLGA-*b*-mPEG) and poly-(lactic-*co*-glycolic acid)-*block*-poly(terpyridine)₅ [PLGA-*b*-p(Tpy)₅] polymers (Scheme 1). In this design, the FDA-approved PLGA block in both polymers is expected to form the hydrophobic core of the mixed micelle; the hydrophilic PEG block in PLGA-*b*-mPEG is present to provide stability and stealth properties, and the Tpy block in PLGA-*b*-p(Tpy)₅ is incorporated to ensure selective Fe(II) chelation. Since the Tpy block in PLGA-*b*-p(Tpy)₅ only averages about 5 repeating units in comparison to the PEG block (MW 5000 Da or ca. 114 repeating units) in PLGA-*b*-mPEG, iron chelation between Tpy and Fe(II) is expected to occur mostly within the micelle, rather than between micelles due to the steric effect of the PEG corona. Tpy–micelles investigated against *P. aeruginosa* reference strains PAO1 and ATCC 27853 demonstrated selectivity for Fe(II) over Fe(III) and encouraging antibiofilm activity under *in vitro* anaerobic conditions.

RESULTS AND DISCUSSION

Synthesis and Characterization of Tpy–Micelles

The detailed synthesis of PLGA-*b*-p(Tpy)₅ is described in the Supporting Information and was confirmed by ¹H NMR spectroscopy to have 70:70:5 GA:LA:Tpy repeating units (Figures S1–S5). On the basis of GPC, the final M_n of PLGA-*b*-p(Tpy)₅ averaged 13 000 Da with a PDI of 1.4. TEM images confirmed that both control PLGA-*b*-PEG micelles and Tpy–micelles possessed spherical morphological structures (Figure 1A,B). The diameter of control micelles averaged ca. 50 nm by TEM and ca. 64 nm by DLS. By TEM, Tpy–micelles averaged ca. 80 nm and ca. 100 nm by DLS with a PDI of 0.13; a PDI < 0.2 indicates that all micelles prepared were sufficiently monodisperse.

Tpy-micelles were characterized by an absorbance peak at 570 nm (A_{570}) only in the presence of Fe(II) in contrast to control PLGA-*b*-mPEG micelles (Figure 1C). On the basis of a linear regression equation correlating free Tpy to A_{570} (Figure S6A), we were able to determine that, after self-assembly of the polymer chains into a micelle, approximately 70% of the Tpy moieties were still available for Fe(II) chelation. The random self-assembly of polymer chains into a micelle means that not all Tpy may have been accessible for chelation. The final Tpy concentration was determined to be 100 μ M per 1 mg/mL polymer, but on the basis of A_{570} , only 70 μ M Tpy was available for chelation once the polymers self-assembled into micelles (Figure S6B). Next, the stability of Tpy-micelles in the presence of Fe(II) was monitored by dynamic light scattering (DLS). In general, the majority of Tpy-micelles averaged ca. 100 nm, with a small intensity peak appearing when Fe(II) was added due to possible cross-chelation effects between several micelles resulting from the presence of Tpy molecules on the surface of these micelles; however, the number average for these larger aggregates was much lower compared to the rest of the population of particles (Figure 1D). Overall, the general stability of Tpy-micelles can be attributed to the presence of a large population of PEG chains on the outer surface of the micelle, and the majority of the chelation process appeared to occur mainly within each particle.

Previous studies using spectral titrations have confirmed that terpyridine derivatives display higher selectivity for Fe(II) compared to other metal ions, and competitive binding studies based on absorbance spectra and ESI-MS have demonstrated that Tpy can indeed selectively bind to Fe(II) with high binding affinity.³² Since the selectivity of Tpy for Fe(II) has already been demonstrated, we were able to confirm the selectivity of Tpy-micelles for Fe(II) chelation through simple UV-vis absorption spectra. Tpy-micelles were independently incubated with 100 μ M Fe(II), Fe(III), Zn(II), Cu(II), Co(II), Ni(II), Ca(II), Mg(II), Mn(II), or Ce(IV) and monitored between 400 and 700 nm. As can be observed in Figure S7, only Fe(II) binding was characterized by a significant absorbance peak at 570 nm and the relative absorbance data for other metals evaluated was normalized with respect to Fe(II) absorption in Figure 2A. Note that our finding that other metals do not absorb strongly in the visible region agrees with reports showing that only iron and cobalt complexes exhibit intense absorption in the visible region ($\lambda > 400$ nm) due to metal-ligand charge-transfer effects.³³ This phenomenon allowed us to easily monitor Tpy to Fe(II) binding interactions by noting the consistent peak intensity of Fe(II):Tpy-micelles at 570 nm in the presence of other metal ions. Such metal competition studies for selectivity were performed by incubating Tpy-micelles with mixtures of 100 μ M Fe(II) and 100 μ M (1 $\times$) or 5 mM (50 $\times$) other metal ions simultaneously. The relative peak intensity of A_{570} corresponding to Fe(II):Tpy-micelles in the presence of mixtures of Fe(II) and competing metals added at 1 $\times$ or 50 $\times$ concentrations did not change (Figure 2B). This means that the high binding affinity between Fe(II) and Tpy-micelles is selective for Fe(II) and could not be disrupted by other metal ions in solution. The stability of Fe(II):Tpy-micelles in the presence of another Fe(II) chelator 2,2'-dipyridyl (2DP) was also investigated (Figure S8). When excess 2DP was added to Fe(II):Tpy-micelles, there was little change in the A_{570} up to 24 h incubation, which suggests that the Fe(II):Tpy-micelle complex is fairly stable and characterized by a binding constant greater than that of 2DP (17.5).³³

Selective Iron Chelation and Antibiofilm Properties of Tpy–Micelles

Since there is not enough residual iron present in M9 medium alone to satisfy *P. aeruginosa*'s biofilm growth, the influence of various Fe(II) and Fe(III) concentrations under aerobic and anaerobic conditions for PAO1 and ATCC 27853 bacteria was investigated using the crystal violet (CV) assay. The MBEC assay is normally the standard for evaluating biofilm eradication by antibiotics; however, since Tpy–micelles are not antimicrobials (they do not act on bacteria directly) but rather act indirectly on biofilm by sequestering iron from the extracellular medium, it means that the static CV assay can still be utilized to give relevant information about the ability of the Tpy–micelles to affect overall biofilm development. Under aerobic conditions, biofilm mass produced was similar between the two metal ions regardless of the level of Fe(II) or Fe(III) supplementation (8–128 μM) to the medium ($p = \text{ns}$ for all concentrations tested, Figure S9). In contrast, under anaerobic conditions, biofilm mass trends tended to increase with increasing iron supplementation for each metal ion, although it should be noted that Fe(II) addition resulted in more biofilm production overall compared to Fe(III) addition at 8–128 μM at $p < 0.001$ (Figure 3). This is likely because *P. aeruginosa* can take in Fe(II) ions directly through the dedicated Feo iron uptake system whereas extracellular Fe(III) uptake under anaerobic conditions may first require its reduction to the Fe(II) form by phenazine compounds released by the bacteria and would thus take more time to become available to the bacteria.^{34,35} Under anaerobic conditions, we found that *P. aeruginosa* biofilm formation is more dependent on iron concentrations than under aerobic conditions, which agrees with a previous report.²⁰

The colony counting assay was used to assess the effect of iron chelation by Tpy–micelles on bacterial cells in the biofilm (Figure 4). When 10 μM Fe(II) was supplied for bacteria growth under anaerobic conditions in the presence of Tpy–micelles, a significant downward trend ($p < 0.05$) in colony forming units (CFU) was observed for each concentration compared to untreated PAO1 bacteria (0 μM), but this decreasing CFU trend was not observed when 10 μM Fe(III) was supplied to the medium due to the inability of Tpy–micelles to selectively chelate Fe(III) ions (Figure 4A). A similar trend between Fe(II) and Fe(III) iron supplementation was observed for ATCC 27853 although this strain appeared less sensitive to the effect of chelating Fe(II) and there was no statistically significant difference in CFU compared to untreated bacteria until 128 μM Tpy–micelles ($p < 0.05$, Figure 4C). When 100 μM Fe(II) was supplemented, we saw a significant decrease in CFU for PAO1 and ATCC 27853 in the presence of 64 and 128 μM Tpy–micelles ($p < 0.05$ compared to untreated) but no statistical difference between untreated and treatment groups supplemented with 100 μM Fe(III) (Figure 4B,D). Note that, at 10 or 100 μM Fe(II) supplementation, the addition of 128 μM Tpy–micelles was sufficient to decrease the bacterial count by >99.9% compared to respective untreated cells (0 μM) for both bacterial strains.

The effect of chelating iron on biofilm mass was evaluated in M9 medium supplemented with 10 and 100 μM Fe(II). Under aerobic condition, >128 μM Tpy–micelles was needed to result in a decrease of biofilm formation in PAO1 and ATCC 27853 bacteria irrespective of Fe(II) supplementation levels (Figure S10). In contrast, biofilm mass significantly decreased under anaerobic condition with as little as >4 μM Tpy–micelles compared to untreated cells

for both PAO1 and ATCC 27853 strains ($p < 0.001$, Figure 5). Control studies with Fe(II) had demonstrated that biofilm production was highly dependent on iron concentrations under low-oxygen conditions (Figure 3); therefore, bacteria grown in the presence of Fe(II) and treated with Tpy-micelles would be expected to show impaired biofilm formation. This data is consistent with the general ability of small molecule Fe(II) chelators such as 2DP and EDTA to more effectively impair PAO1 biofilm formation under anaerobic conditions.²⁰ It should be noted that the effects of Tpy-micelles observed are due to the chelation of Fe(II) from the media and not due to the presence of the micelle carrier since similar studies conducted with control PLGA-b-mPEG micelles (not conjugated to Tpy) at equivalent mass concentrations demonstrated little effect on biofilm mass production in PAO1 or ATCC 27853 strains grown under anaerobic conditions (Figure S11).

Biofilm Penetration and Antibiofilm Properties of Tpy-Micelles

Biofilms largely consists of viscous polymeric substances that serve as a prominent diffusion barrier for many antibiotics that need to penetrate the film in order to exercise their antibiotic effect. To demonstrate the level of penetration and accumulation of Tpy-micelles into a PAO1 biofilm, Tpy-micelles were fluorescently labeled with RBITC (red channel). The penetration experiments were performed on a one-day old biofilm onto which Tpy-micelles were added and allowed to incubate for 2 h. After, the biofilm was washed with PBS and stained with the general cell permeable green fluorescent nucleic acid binding dye SYTO 13. As shown in Figure 6A–C, the CLSM images were obtained using two different fluorescence channels to simultaneously detect for the bacteria (green) and Tpy-micelles (red). On the basis of the overlay of the images, Tpy-micelles were able to diffuse into the matrix and dispersed throughout the biofilm, co-localizing with or near the bacteria. The ability of Tpy-micelles to effectively migrate throughout the one-day old PAO1 biofilm with limited hindrance may be due to the high density of PEG present on its surface³⁶ and appears to be essential for decreasing potential electrostatic interactions with charged polymeric substances within the biofilm matrix. Similarly, the incorporation of a dense PEG coating on nanoparticles in the range of 100–500 nm has been shown to improve their rapid diffusion through human mucus by at least 3 orders of magnitude³⁷ and implies that the ability of Tpy-micelles to easily disperse throughout the biofilm may also encouragingly extend to the mucus.

To directly observe the antibiofilm properties of Tpy-micelles with CLSM, Tpy-micelles were incubated at 0, 100, and 500 μM concentrations with PAO1 bacteria for 1 day under anaerobic condition in nutrient-rich MHB medium. Resulting biofilms were imaged after staining all bacteria in the biofilms with the nucleic acid binding dye SYTO 13 (green channel) (Figure 6D). In the absence of Tpy-micelles, images reveal that bacteria grew densely and was characterized by a thick biofilm (Figure 6D); however, bacteria growth decreased in the presence of 100 and 500 μM Tpy-micelles, and biofilm formation was also greatly inhibited (Figure 6E,F). This agrees with our studies which demonstrated that hypoxic biofilms are more sensitive to the depletion of Fe(II) (Figure 5).

Effect of Tpy–Micelles on Mammalian Cell Viability

To investigate the cytotoxicity of Tpy–micelles on mammalian cells, A549 lung cancer cells were treated with different concentrations of Tpy–micelles for 24 h under normal atmospheric oxygen levels. This study was not conducted under oxygen-deprived conditions due to the detrimental effect of low oxygen levels on mammalian cell growth. As shown in Figure 7, Tpy–micelles did not exhibit evidence of cytotoxicity against A549 cells, even at concentrations as high as 1024 μM equivalent Tpy in micelles, with cell viability remaining >80% for the highest concentration tested. Results of Tpy–micelle cytotoxicity are encouraging and suggest that they may be safe to mammalian cells at antimicrobial concentrations used.

CONCLUSIONS

Tpy–micelles displayed high selectivity for Fe(II) over other divalent and trivalent metals investigated. In general, chelation of Fe(II) by Tpy–micelles was more effective at decreasing biofilm mass formation under anaerobic conditions. CLSM images confirmed that Tpy–micelles can readily penetrate through a 1-day old biofilm and could indeed inhibit biofilm development in a concentration-dependent manner. Against lung epithelial A549 cells, Tpy–micelles did not exhibit cytotoxic properties at concentrations as high as 1024 μM equivalent Tpy, with cell viability remaining >80% under normal atmospheric oxygen levels. In combination with other Fe(III) chelating strategies and proper bacteriostatic antibiotics, Fe(II) chelating Tpy–micelles may serve as a clinically relevant antimicrobial addition to inhibit biofilm formation under anaerobic conditions.

EXPERIMENTAL SECTION

Synthesis of PLGA- β -p(Tpy)₅

PLGA-*b*-mPEG (MW 10 000:5000 Da, PDI 1.2) was purchased from PolySciTech and used as is. See the Supporting Information for detailed synthesis of PLGA-*b*-p(Tpy)₅ (Scheme S1). All the intermediate molecules and polymers synthesized were subjected to characterization by nuclear magnetic resonance (NMR) to confirm their molecular structures (Figures S1–S5). Polymers were also subjected to gel permeation chromatography (GPC) to confirm the degree of polymerization and polydispersity index (PDI).

Preparation of Tpy–Micelles

Control PLGA-*b*-mPEG micelles (nonchelating) and Fe(II) chelating mixed PLGA-*b*-mPEG/PLGA-*b*-p(Tpy)₅ micelles (Tpy–micelles) were prepared by nanoprecipitation using a DMSO–H₂O system. To make control micelles, 100 mg of PLGA-*b*-mPEG polymer was dissolved in 5 mL of DMSO and the mixture was added dropwise into 20 mL of deionized water under magnetic stirring. The resulting suspension was stirred for 4 h, dialyzed against deionized water using a dialysis membrane with a molecular weight cutoff (MWCO) of 3600 Da for 48 h to remove the organic solvent, and then lyophilized; Tpy–micelles were similarly prepared with PLGA-*b*-mPEG and PLGA-*b*-p(Tpy)₅ polymers at a 1:1 w/w. To fluorescently label Tpy–micelles for confocal microscopy studies, mixed micelles were formed by the self-assembly of PLGA-*b*-mPEG, PLGA-*b*-p(Tpy)₅, and PLGA-*b*-PEG-

RBITC. To prepare fluorescently labeled PLGA-*b*-PEG-RBITC, Rhodamine B isothiocyanate (RBITC) was conjugated to the terminal amine of PLGA-*b*-PEG-NH₂ (MW 12 000:5000 Da, PDI 1.8, PolySciTech) in DMSO and stirred overnight, followed by dialysis and lyophilization.

Characterization of Tpy–Micelles

The Fe(II) chelation capability of Tpy–micelles was probed via UV–vis absorption spectroscopy in the presence of a surplus concentration of Fe(II) by scanning between 400 and 700 nm (SpectraMax Plus, Molecular Devices). The resulting absorbance peak at 570 nm is characteristic of Fe(II):Tpy complexes ([Fe-(Tpy)₂]²⁺) and was therefore used to determine the amount of Tpy incorporated into the micelle system via an *A*₅₇₀ versus free Tpy standard curve (Figure S6A); approximately 70% of Tpy remained available for chelation after micelle formation compared to the free polymer before self-assembly (Figure S6B).

Average particle sizes and PDI of the micelles were determined by DLS with a Zetasizer Nano ZS (Malvern) and analyzed with Zetasizer software v7.10. Prior to measurements, micelle solutions were filtered through Millipore membranes with a 0.45 μm pore size. All the measurements were conducted on three batches of samples, and results are reported as mean ± standard deviation (SD). To investigate the morphology of micelles, transmission electron microscopy (TEM) images were taken on a Philips/FEI Tecnai20 instrument with an acceleration voltage of 120 kV. Samples were prepared by air-drying a drop of Tpy–micelle suspension on the copper grid prior to imaging whereas control PLGA-*b*-mPEG micelles were stained with sodium phosphotungstate solution (2% w/v, pH = 7) before imaging with TEM.

Metal Selectivity and Competition Studies

To confirm that Tpy–micelles can be applied in complex biological environments with sufficient selectivity for Fe(II) over other divalent and trivalent metal ions, *A*₅₇₀ (which correlates to the concentration of [Fe(Tpy)₂]²⁺ complexes) was monitored in the presence of other metal ions; briefly, a solution of Tpy–micelle (equivalent Tpy concentration of 100 μM) was added to 100 μM of each metal ion solution tested [Fe(II), Fe(III), Zn(II), Cu(II), Co(II), Ni(II), Mg(II), Mn(II), Ca(II), or Ce(IV)] and incubated at room temperature for 1 h prior to absorbance measurements. For the metal competition experiments, changes in the absorbance at 570 nm (corresponding to Fe(II):Tpy–micelles) were monitored in the presence of 100 μM Fe(II) and 100 μM (1× fold) or 5 mM (50× fold) of other metal ions [Fe(III), Zn(II), Cu(II), Co(II), Ni(II), Mg(II), Mn(II), Ca(II), or Ce(IV)]. Note that 1 mM ascorbic acid (in sodium salt form) was also added to the solutions to keep Fe(II) reduced during the selectivity and competition studies and that all the solutions were prepared in phosphate buffer at pH 7.2.

Stability of Fe(II):Tpy–Micelles in the Presence of a Competing Fe(II) Chelator

The stability of Fe(II):Tpy–micelle complex formation was investigated in the presence of another known Fe(II) chelator 2,2'-dipyridyl (2DP). For this study, 50 μM Fe(II) and 100 μM Tpy–micelles were incubated in solution to form the Fe(II):Tpy–micelle complexes; then,

100 μM (1 $\times$) or 1 mM (10 $\times$) 2DP was added to the solution. Changes in the A_{570} , characteristic of the Fe(II):Tpy–micelle complex formation, was monitored over 24 h to investigate the stability of Fe(II):Tpy–micelles in the presence of the competitor.

Bacterial Strains and Culture Conditions

P. aeruginosa strain PAO1 as the wild type (WT) and strain 27853 were obtained from American Type Culture Collection (ATCC). For antimicrobial and biofilm studies, strains were grown in M9 minimal medium (6.78 g of Na_2HPO_4 , 3 g of KH_2PO_4 , 0.5 g of NaCl, 1.0 g of NH_4Cl , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.011 g of CaCl_2 , and 4 g of glucose, per liter of water, pH 7.0) in the presence of different concentrations of $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ under aerobic and anaerobic conditions. The MGG Ananeropack System was used to grow bacteria under an anaerobic environment, and to permit anaerobic growth of strains, 1% KNO_3 was also added to the growth medium as a source of nitrate to provide a terminal electron acceptor for anaerobic respiration. All the bacterial strains were grown from frozen stocks and subcultured prior to their use in experiments. All growth media and Milli-Q water used for bacterial cultures were sterilized by an autoclave. Sterile polypropylene culture tubes and sterile polystyrene 96-well plates used for culturing were manufactured by VWR and Corning Incorporated, respectively.

Antibiofilm Activity of Tpy–Micelles

Antibiofilm studies with control PLGA-*b*-mPEG micelles and Tpy–micelles were conducted on PAO1 and ATCC 27853 strains. A single colony of *P. aeruginosa* was inoculated into 3 mL of MHB medium and grown to stationary phase at 37 °C in an incubator shaker until the optical density at 600 nm (OD_{600}) reached 0.4. The bacterial suspension was then transferred to an Eppendorf tube (2 mL), washed with saline twice, and then diluted to an OD_{600} value of 0.01 in M9 minimal medium. A 135 μL aliquot of the M9 medium containing different concentrations of micelles was then mixed with a 15 μL aliquot of bacterial solution in a 96-well plate; the plate was subsequently wrapped in parafilm and incubated at 37 °C for 18 h. Biofilm mass was analyzed by the crystal violet (CV) assay. Liquid in each of the 96-well plates was removed by inverting the plates with gentle shaking; wells were washed once with 200 μL of Milli-Q water and pipetted out slowly to avoid disrupting the biofilm. Next, 160 μL of CV (1%) in water was added to each well of the 96-well plates, incubated at room temperature for 20 min, and washed twice with water. Finally, 160 μL of ethanol was added to each well of the plates to solubilize the CV and allowed to incubate at room temperature for 10–15 min prior to measuring the absorbance at 595 nm in a plate reader.

Viability of Bacteria in the Biofilm

The colony counting assay was used to quantify the number of viable bacteria cells in the biofilm. Bacterial strains were treated with different concentrations of Tpy–micelles under anaerobic conditions as similarly described above for the biofilm mass assay using crystal violet. After 18 h of incubation, liquid in each of the 96-well plates was removed by inverting the plates with gentle shaking; wells were washed once with 200 μL of Milli-Q water and pipetted out slowly to avoid disrupting the biofilm. Next, 100 μL of saline was added to each well of the 96-well plates; the plates were then sonicated for 20 min, and the

sample was further dispersed with a pipetter. The solutions containing dispersed bacteria were then plated onto agar plates, and colony forming units were counted after incubation at 37 °C overnight.

Imaging Biofilms with Confocal Laser Scanning Microscopy (CLSM)

PAO1 strain was inoculated in 3 mL of MHB medium and grown to stationary phase at 37 °C. For the penetration study on a 1-day old established biofilm, seeding solution was made in MHB to an OD₆₀₀ of 0.01 and a 1 mL portion of the seeding solution was added to each well of a chamber slide. The slides were covered and incubated at 37 °C under static condition for 24 h. Next, planktonic bacteria were removed by washing with saline 3×, and 0.5 mg/mL Tpy-micelles-RBITC was added, and the slides were incubated for 2 h. Next, the biofilm was washed with phosphate buffer saline (PBS) 3×, and all bacteria were visualized by staining with SYTO 13 (green channel) prior to imaging with CLSM.

To visualize the effect of Tpy-micelles on biofilm development (antibiofilm studies), a seeding solution was made in MHB to an OD₆₀₀ of 0.01 and coincubated with 0, 100, or 500 μM Tpy-micelles under anaerobic conditions for 24 h. Planktonic bacteria was removed, and the remaining bacteria in the biofilm was stained with SYTO 13 as before and imaged with a Zeiss 710 confocal microscope. For all CLSM studies, the green fluorescent channel was collected between 496 and 554 nm and the red fluorescent channel was collected between 548 and 670 nm.

Mammalian Cell Viability Assay

The cytotoxicity of Tpy-micelles on mammalian cells was assessed by using the metabolism-based resazurin assay. A549 lung cancer cells were seeded in a 96-well plate at a density of 5000 cells per well. Cells were incubated in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, at 37 °C in 5% CO₂ for 24 h. Next, cells were incubated with growth medium containing Tpy-micelles at various concentrations. The micelle concentrations of each formulation were prepared by serial dilution with DMEM medium. After 24 h, the substrate resazurin was dissolved in cell culture medium at a concentration of 44 μM and 100 μL was added to each well for 4 h. Cell viability was determined by measuring fluorescence intensity at 590 nm (excitation at 560 nm) on a Spectra Max Gemini EM microplate reader. Readings from the wells without cells were used as the blank (I_{blank}), and the readings from control cells without treatment were used to represent 100% cell viability (I_{control}). All the measurements were conducted in triplicate, and cell viability was determined as follows using eq 1.

$$\text{cell viability (\%)} = 100 \times \frac{I_{\text{sample}} - I_{\text{blank}}}{I_{\text{control}} - I_{\text{blank}}} \quad (1)$$

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. (A) TEM images of Tpy-micelles and (B) control PLGA-*b*-mPEG micelles. (C) Fe(II) chelation by Tpy-micelles was confirmed by monitoring the absorbance of the complex at 570 nm, and (D) stability of Tpy-micelles before and after addition of Fe(II) was monitored by DLS.

Figure 2.

Metal selectivity and competition studies. (A) The selectivity of Tpy-micelles for binding Fe(II) was confirmed by scanning between 400–700 nm. Note that the relative A_{570} for other metals was plotted with respect to Fe(II). (B) For metal competition studies, Tpy-micelles were simultaneously incubated with 100 μM Fe(II) and competing metal ions at 100 μM (1 $\times$) or 5 mM (50-fold excess), and changes in the relative A_{570} absorbance peak were monitored. Note that 1 mM ascorbic acid (sodium salt) was also added to the solutions to keep Fe(II) reduced during the selectivity and competition studies. Error bars are SD ($n = 3$).

Figure 3.

Impact of Fe(II) and Fe(III) supplementation to M9 media on *P. aeruginosa* biofilm formation for strain (A) PAO1 and (B) ATCC 27853 under anaerobic conditions. Note that, for anaerobic conditions, 1% KNO₃ was also added to the medium. Error bars are SD ($n = 4$). *** $p < 0.001$; ** $p < 0.01$; ns means not statistically significant.

Figure 4.

Antibiofilm properties of Tpy-micelles against *P. aeruginosa* strains (A, B) PAO1 and (C, D) ATCC 27853 after 24 h incubation under anaerobic conditions. All assays were performed in M9 minimal medium supplemented with 10 or 100 μM concentrations of Fe(II) or Fe(III). Note that, for anaerobic conditions, 1% KNO₃ was also added to the medium. Error bars are SD ($n = 3$). * $p < 0.05$.

Figure 5.

Biofilm mass production in the presence of Tpy-micelles on *P. aeruginosa* reference strains PAO1 (A) and ATCC 27853 (B) after 24 h incubation under anaerobic condition. All assays were performed in M9 minimal medium supplemented with 10 or 100 μ M Fe(II); note that, for anaerobic conditions, 1% KNO₃ was also added to the medium. Error bars are SD ($n = 3$). * $p < 0.001$.

Figure 6.

(A–C) Confocal image stacks showing a one-day old PAO1 biofilm treated for 2 h with Tpy-micelles-RBITC at 0.5 mg/mL. Biofilm bacteria stained with SYTO 13 (green channel) and Tpy-micelles-RBITC (red channel) merged images reveal that micelles can diffuse through the biofilm. Scale bar: 20 μm . (D–F) Resulting PAO1 biofilms formed (stained with SYTO 13) after co-incubating with 0, 100, and 500 μM Tpy-micelles for 24 h under anaerobic conditions demonstrate inhibition of biofilm development; scale bar: 10 μm .

Figure 7.

Viability of A549 human lung cells treated with Tpy-micelles after incubating for 24 h. For reference, 100 μ M equivalent Tpy corresponds to 1 mg/mL of the polymer. All assays were performed in DMEM media supplemented with 10% fetal bovine serum and incubated at 37 °C, 5% CO₂. Error bars are SD ($n = 5$).

Scheme 1.

Schematic Illustration of Tpy-Micelles for Chelating Fe(II)^a

^aMixed micelles were formed from the self-assembly of PLGA-*b*-mPEG and PLGA-*b*-p(Tpy)₅ block copolymers. Note that free Tpy has been reported to chelate Fe(II) at a 2:1 stoichiometric ratio with a log $K = 20.9$, resulting in a strong absorbance at 570 nm.