Electronic Supplementary Information

Desferrioxamine:Gallium-Pluronic Micelles Increase Outer Membrane Permeability and Potentiate Antibiotic Activity Against *Pseudomonas aeruginosa*

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Materials

Pluronic® F127 (F127), sodium hypochlorite solution (NaClO solution), sodium bromide (NaBr), 2,2,6,6-tetramethyl-1-piperidinyloxy free radical (TEMPO), N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), N,N-diisopropylethylamine (DIPEA), gallium(III) nitrate hydrate ($Ga(NO_3)_3 \cdot xH_2O$), ferric chloride ($FeCl_3$), erythromycin (ERY), rifampicin (RIF), vancomycin hydrochloride (VAN), Mueller-Hinton Broth II (MHB), and Mueller-Hinton Agar II (MHA) were purchased from Sigma-Aldrich (St. Louis, MO). Tetraphenylethylene (TPE) was purchased from TCI America (Portland, OR). 1-hydroxybenzotriazole hydrate (HOBt) was purchased from Chem-Impex International (Wood Dale, IL). Desferrioxamine mesylate (DFO) was purchased from Hospira, Inc. (Lake Forest, IL) via the University of Wisconsin Hospital Pharmacy Services. Hydrochloric acid 12N (HCl), dichloromethane (DCM), and diethyl ether were purchased from Fisher Scientific (Fair Lawn, NJ). Dimethylformamide (DMF) was purchased from Acros Organics (Morris Plains, NJ). Ethanol (EtOH) was purchased from Decon Labs (King of Prussia, PA).

Pseudomonas aeruginosa reference strains used were ATCC 27853 and ATCC 15692 (PAO1). Clinically-isolated MDR *Pseudomonas aeruginosa* strains used (2638, 3072, and 24530) were generously provided by Dr. David Andes at the University of Wisconsin School of Medicine and Public Health. The MDR strains selected for this study were chosen based on their resistance phenotypes, with MDR2638 representing a pan-resistant organism, while MDR3072 and MDR24530 representing carbapenem-resistant organisms that are still susceptible to at least one clinically-relevant antipseudomonal agent. The *Escherichia coli* reference strain used was ATCC 25922. Antibiotic resistance profiles of each strain are given in **Table S1**.[1]

Methods

Synthesis of F127-$(COOH)_2$: Oxidation of the terminal hydroxy groups in F127 was performed as described in Liu *et al*.[2] 3.78g F127 (0.6 mmol -OH) was oxidized in DI water (100 mL) with 100mg NaBr (0.972 mmol), 100mg TEMPO (0.640 mmol), and 10mL NaClO solution (10-15% available chlorine) at pH 10 for 15 minutes (r.t.). The reaction was quenched by the addition of EtOH (10 mL) and acidified to pH 2 with HCl. The oxidized polymer was extracted with 3x100 mL of DCM followed by drying via rotary evaporation. The dried polymer was then recrystallized twice from EtOH to obtain F127-$(COOH)_2$. Conversion to carboxylic acid groups quantified by titration with 1 N NaOH. The chemical structure of F127-$(COOH)_2$ was confirmed by ^{1}H NMR (**Figure S1**) and MALDI MS (**Figure S2**).

Synthesis of F127-$(DFO)_2$: Amide coupling of F127-$(COOH)_2$ to DFO was performed as described in Liu *et al*[2] with a few modifications. 1.575g F127-$(COOH)_2$ (0.25 mmol -COOH) was reacted with 57.4mg HOBt (0.375 mmol), 71.9mg EDC (0.375 mmol), 197mg DFO (0.3 mmol), and 109μL DIPEA (0.625 mmol) in 75mL DMF for 24 hours (r.t.). The reaction mixture was poured into 375mL EtOH and cooled to -20°C to precipitate the F127-$(DFO)_2$ conjugate. The polymer was then recrystallized twice from EtOH to obtain F127-$(DFO)_2$. The chemical structure of F127-$(DFO)_2$ was confirmed by ^{1}H NMR (**Figure S3**) and MALDI MS (**Figure S4**). Complexation efficiency was also confirmed by UV-Vis experiments. DFO chelates Fe^{III} in a 1:1 ratio and the DFO:Fe complex has a characteristic absorbance peak at 430 nm. A DFO standard curve was generated by mixing DFO solutions of known concentrations with excess aqueous $FeCl_3$ and measuring the A430 by UV-Vis spectroscopy (SpectraMax® Plus, Molecular Devices). F127-

$(DFO)_2$ was dissolved in DI water with excess $FeCl_3$ and the DFO concentration was back calculated from the standard curve (93% of -COOH groups capped with DFO). F127-$(DFO)_2$ was dissolved in DI water, titrated with aqueous $FeCl_3$, and the absorbance of the F127-$(DFO:Fe)_2$ complex was monitored at 430 nm. The maximum absorbance was observed after adding 1.9 eq. $FeCl_3$ per 1 eq. F127-$(DFO)_2$, which corresponds to ca. 95% of COOH groups capped with DFO (**Figure S5**).

Complexation of Ga(III) to F127-$(DFO)_2$: Complexation of F127-$(DFO)_2$ with Ga^{III} was performed in aqueous solution. 1.25g F127-$(DFO)_2$ (0.15 mmol DFO) was dissolved in water (50mL) with 69mg $Ga(NO_3)_3 \cdot xH_2O$ (0.165 mmol Ga) and stirred for one hour (r.t.). The solution was dialyzed against a 10,000 MWCO membrane for 24 hours to remove uncomplexed Ga(III) and lyophilized. The lyophilized product was recrystallized from EtOH to obtain purified F127-DG_2. Complexation to Ga(III)) was confirmed by monitoring of the disappearance of hydroxamic acid protons of DFO (**Figure S6**) and by MALDI-MS (**Figure S7**).

Complexation efficiency of Ga(III) in F127-DG_2: The amount of Ga complexed to F127-$(DFO)_2$ was quantified by atomic absorption spectroscopy (AAS) on a 932 AA, GBC Scientific using a gallium hollow cathode lamp with Ga detection at 294.4 nm. A gallium standard curve from 10 ppm to 100 ppm was generated by diluting a standardized solution of Ga^{III}, 5% HNO_3 in DI water. F127-DG_2 was dissolved in DI water and the Ga concentration was back calculated from the standard curve (1 DFO : 0.96 Ga). The final conjugation efficiency of F127-DG_2 was 90% of the initial end groups being converted to DG.

Preparation and physical characterization of micelles: F127 and F127-DG_2 micelles loaded with TPE were prepared by the thin-film rehydration method as in Liu *et al*.[2] 300mg of either F127 or F127-DG_2 and 10mg TPE were dissolved in 20mL DCM and the solvent removed by rotary evaporation followed by drying in a vacuum oven overnight. The resultant thin film was rehydrated in 10mL PBS (10 mM, pH 7.4) for 30 minutes at 37°C to generate micelles and filtered through a 0.22 μm filter to remove unencapsulated TPE. Micelles were characterized by dynamic light scattering (DLS) and morphology was confirmed by transmission electron microscopy (TEM) (**Figure S8**).

CLSM studies into OM accumulation on bacterial surfaces: OM binding assays of TPE loaded micelles were performed as follows. *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 were grown in cation-adjusted MHB until the turbidity of the culture matched a 0.5 McFarland Standard, after which aliquots were centrifuged and resuspended in 10 mM PBS (pH 7.4) containing either 512 μM F127-DG_2-TPE or 512 μM F127-TPE. Samples were incubated at 37°C for 20 minutes and washed with PBS. Control staining was performed with FM 4-64FX, fixed with 4% paraformaldehyde, and directly imaged with a Zeiss LSM 710 Confocal Microscope (**Figure S9**).

CLSM studies on OM permeabilization: OM permeabilization of *P. aeruginosa* was evaluated using nitrocefin (NCF). *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 were grown to the stationary phase in cation-adjusted MHB and then suspended in 10 mM PBS (pH 7.4) containing either 128 μM F127-DG_2 or 128 μM F127 plus 256 μM DG. NCF was added to each sample for a

final concentration of 250 μg/mL and hydrolysis was monitored by measuring the absorbance of the hydrolyzed product at 485 nm over two hours at 37°C. OM permeabilization was visualized by confocal laser scanning microscopy (CLSM) using hexidium iodide (HI), a fluorescent stain for Gram-positive organisms which does not cross the OM on its own. *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 were grown in cation-adjusted MHB until the turbidity of the culture matched a 0.5 McFarland Standard, after which aliquots were centrifuged and resuspended in 10 mM PBS (pH 7.4) containing either 128 μM F127-DG_2 or 128 μM F127 plus 256 μM DG. Samples were incubated at 37°C for two hours, resuspended in 10 mM Tris (pH 7.4), and stained with red fluorescent HI for one hour and green fluorescent SYTO13 for five minutes.[4] Stained bacterial cells were washed several times with 10 mM Tris (pH 7.4) to remove any excess dye and were directly imaged with a Zeiss LSM 710 Confocal Microscope (**Figure S10**).

Antimicrobial efficacy dose response curves: Antimicrobial efficacy was assessed by the checkerboard broth microdilution assay in accordance with CLSI guidelines.[5] Organisms were grown overnight on cation-adjusted MHA plates to obtain single colonies. Three morphologically distinct colonies were transferred to a culture tube containing cation-adjusted MHB, and the broth culture was incubated at 37°C until its turbidity matched a 0.5 McFarland Standard. The inoculum was then diluted 1:20 in sterile water, followed by a 1:10 dilution when inoculating wells of a 96-well plate. Plates were then incubated at 37°C for 18 hours, and the MIC was read as the lowest concentration of antimicrobial agent that had no visible growth and resulted in an OD600 < 0.04 after normalization to the untreated growth control. The extent of bacterial growth for combinations of F127-DG_2 or F127 plus DG with each antibiotic shows a dose-dependent relationship (**Figure S11-S16**). Colony counting assays were performed on cation-adjusted MHA

plates using the single-plate serial dilution spotting method.[6] Synergistic activity was calculated using the following equation:

$$FICI = \frac{C_{ABX,combination}}{MIC_{ABX,alone}} + \frac{C_{F127-(DFO-Ga)_2,combination}}{MIC_{F127-(DFO-Ga)_2,alone}}$$

Cytocompatibility studies: The cytotoxicity of F127-DG_2 was evaluated against HeLa cells in Dulbecco's Modified Eagle Medium (DMEM). Cells were seeded at 3000 cells/well in 96-well plates and cultured at 37°C for 24 hours before treatment. Following a 48 hour incubation, cell viability was assessed using a metabolism-based resazurin assay, in which the resazurin substrate was added to each well and incubated for 4 hours before measuring the fluorescence with 560 nm excitation and 590 nm emission (SpectraMax® Gemini, Molecular Devices). Cell viability was calculated using the following equation:

$$Cell\ Viability\ (\%) = 100 \times \frac{E_{sample} - E_{blank}}{E_{control} - E_{blank}}$$

Hemolytic activity was assessed by measuring hemoglobin release from sheep RBCs. RBCs were washed with 10 mM PBS (pH 7.4) and diluted to a final concentration of 2 x 10^6 cells per well and incubated with F127-DG_2, F127, DG, or F127 plus DG in a humidified, 5% CO_2 atmosphere at 37°C for two hours. Samples were then centrifuged to pellet intact RBCs and hemoglobin released into the supernatant was quantified by measuring the A_{405}. Data was normalized with PBS representing 0% hemolysis and 1% Triton X-100 in PBS as a 100% hemolysis positive control using the following equation:

$$Hemolysis\ (\%) = 100 \times \frac{A_{sample} - A_{NC}}{A_{PC} - A_{NC}}$$

Table S1. Intrinsic resistance profiles for all *P. aeruginosa* organisms used.

	E. coli	*P. aeruginosa*				
	ATCC 25922	ATCC 27853	PAO1	MDR 2638	MDR 3072	MDR 24530
Doripenem	S	S	S	**R**	**R**	**R**
Imipenem	S	S	S	**R**	**R**	**R**
Meropenem	S	S	S	**R**	**R**	**R**
Ceftazidime	S	S	S	**R**	S	**R**
Cefepime	S	S	S	**R**	S	**R**
Ciprofloxacin	S	S	S	**R**	**R**	S
Tobramycin	S	S	S	**R**	**R**	S
Erythromycin	**R**	**R**	**R**	**R**	**R**	**R**
Rifampicin	**R**	**R**	**R**	**R**	**R**	**R**
Vancomycin	**R**	**R**	**R**	**R**	**R**	**R**

Key: S = Susceptible, **R** = Resistant

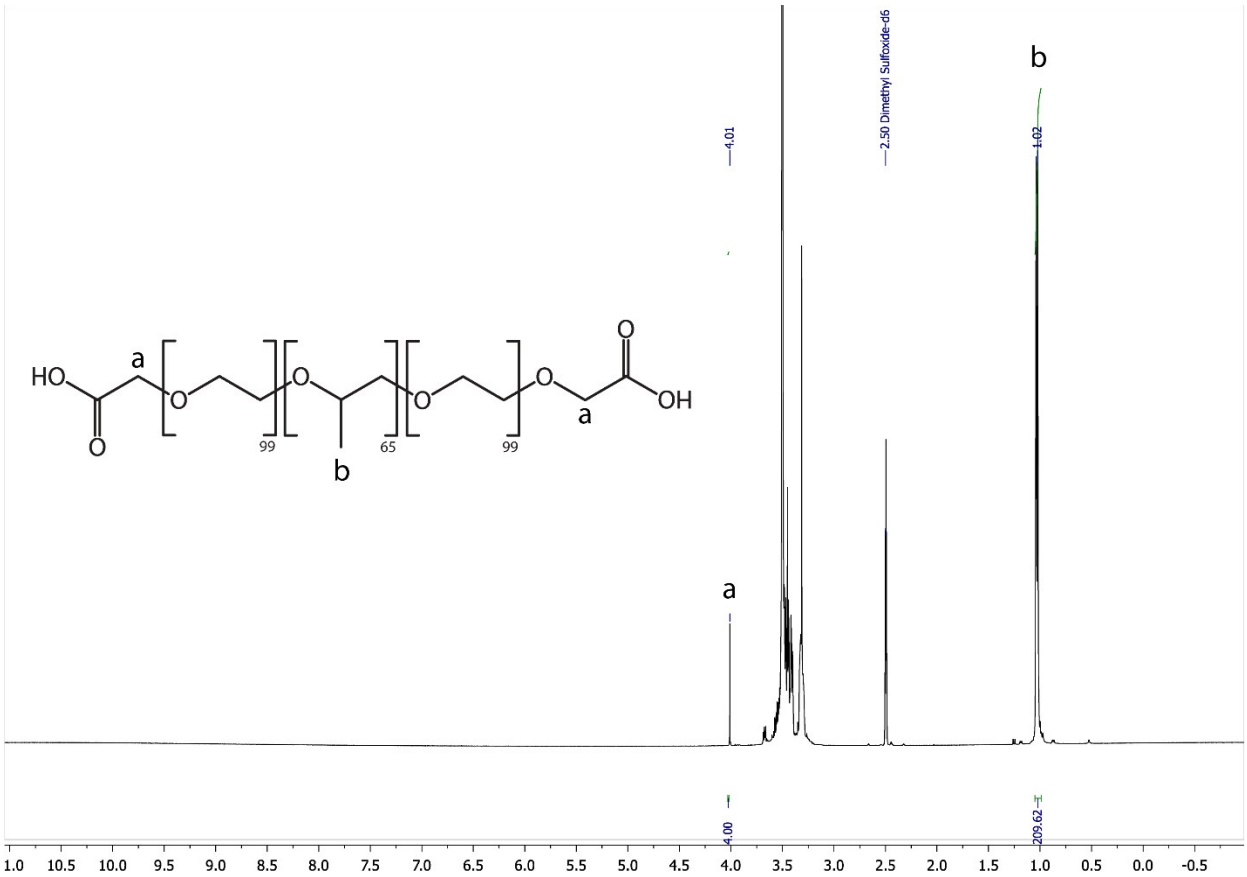

Figure S1. F127-$(COOH)_2$ Spectra. ^{1}H NMR (400 MHz, DMSO-d_6, 298K): $\delta = 4.01$ (s, OC$\boldsymbol{H_2}$COOH), 3.20–3.60 (m, -$CH_2C\boldsymbol{H_2}$O- of PEG and -C$\boldsymbol{H_2}$CHO- of PPG), 1.02 (d, C$\boldsymbol{H_3}$ of PPG).

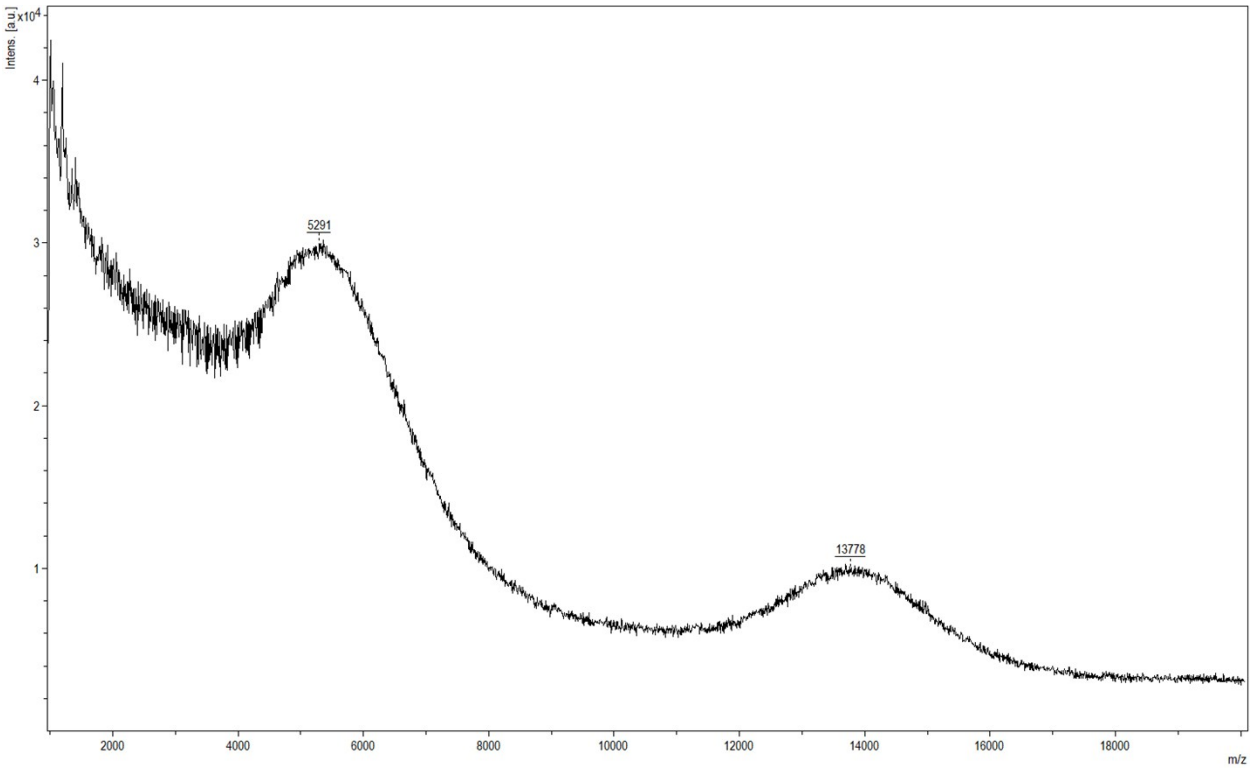

Figure S2. MALDI-TOF of F127-$(COOH)_2$. Expected average m/z for singly charged species: 12,628. Observed average m/z for singly charged species: 13,778. The larger observed average m/z compared to the expected and broad distribution is consistent with previously reported MALDI-TOF analysis of Pluronic polymers.[3]

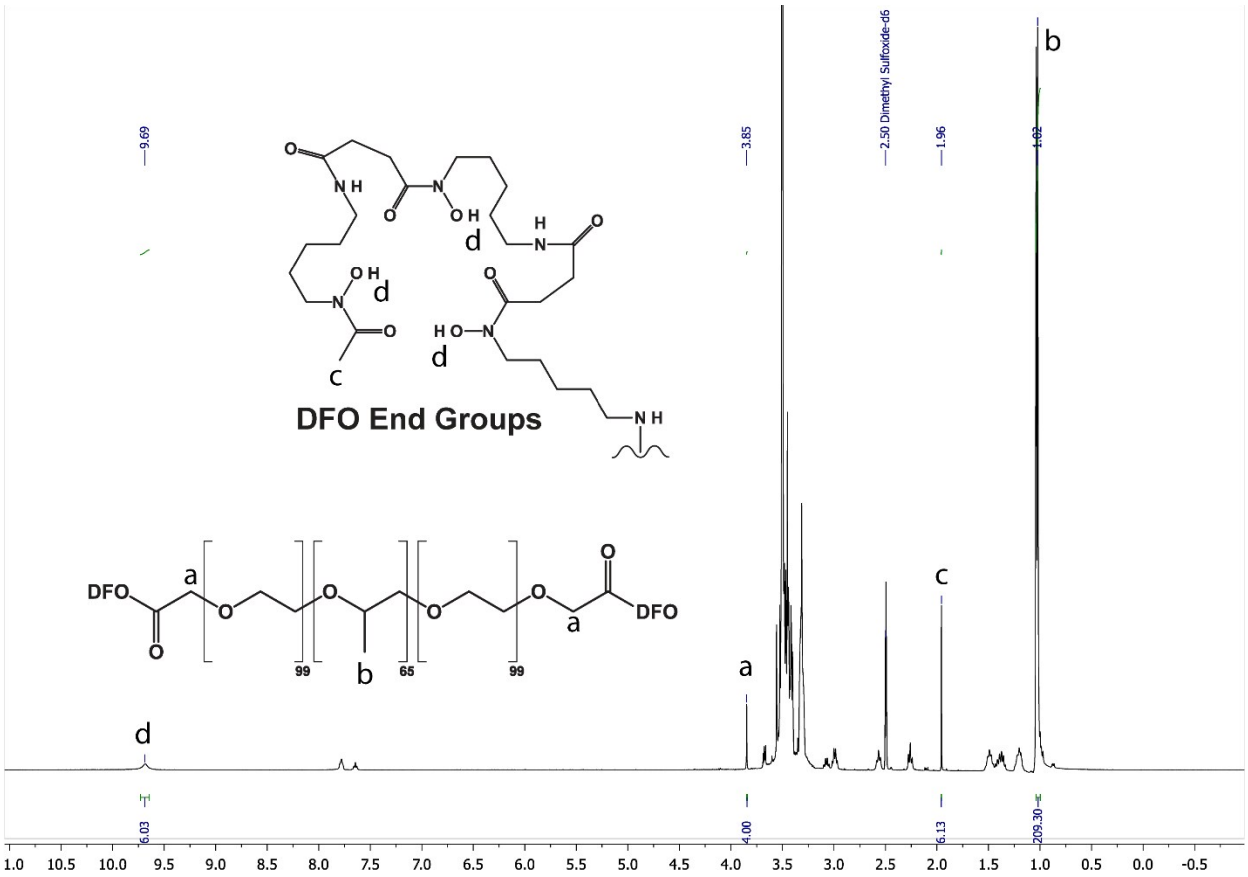

Figure S3. F127-$(DFO)_2$ Spectra. ^{1}H NMR (400 MHz, DMSO-d_6, 298K): 9.60-9.80 (br, NO***H*** of DFO), 7.60-7.80 (br, N***H*** of DFO), 3.85 (s, OC$\boldsymbol{H_2}$CONH of PEG), 3.20–3.60 (m, $CH_2C\boldsymbol{H_2}O$ of PEG and C$\boldsymbol{H_2}$CHO of PPG), 2.90-3.10 (m, C$\boldsymbol{H_2}$-NHCO of DFO), 2.50- 2.60 (m, C$\boldsymbol{H_2}$-CO of DFO), 2.20-2.30 (m, C$\boldsymbol{H_2}$-NH of DFO), 1.96 (s,C$\boldsymbol{H_3}$ of DFO), 1.10-1.50 (m, C$\boldsymbol{H_2}$ of DFO), 1.02 (d, C$\boldsymbol{H_3}$ of PPG).

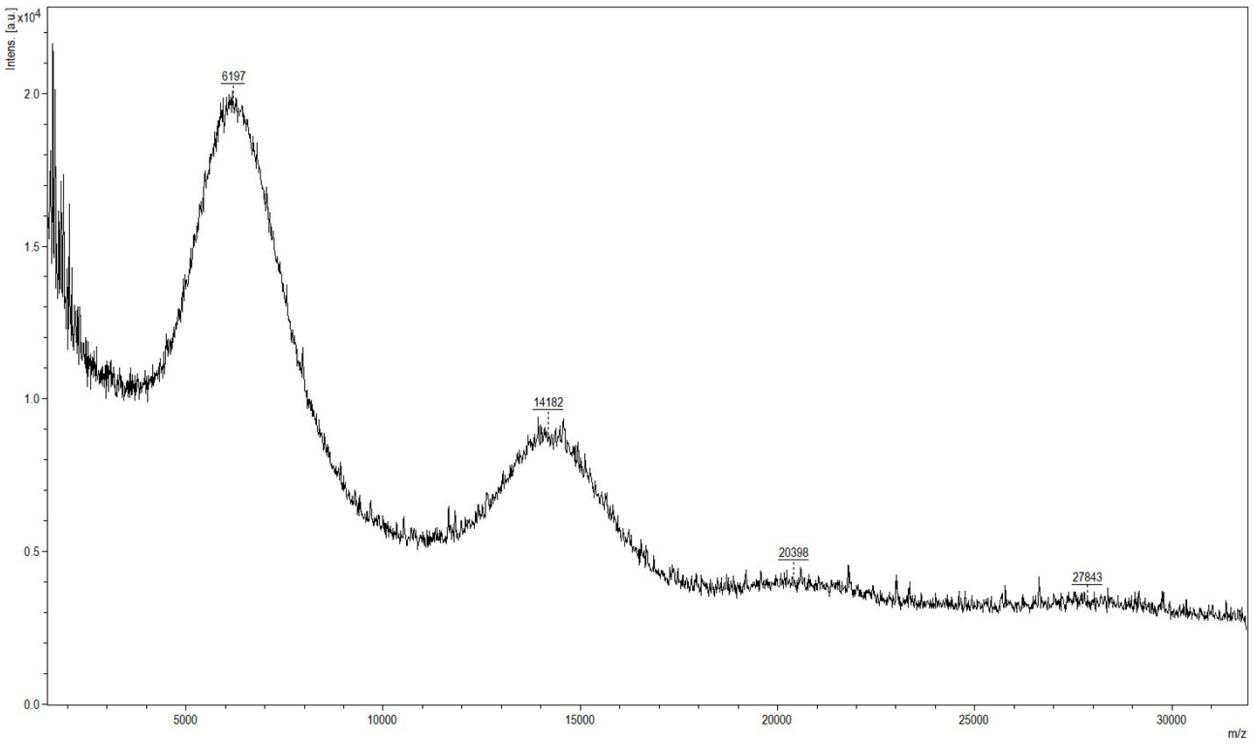

Figure S4. MALDI-TOF of F127-$(DFO)_2$. Expected average m/z for singly charged species: 13,714. Observed average m/z for singly charged species: 14,182. The larger observed average m/z compared to the expected and broad distribution is consistent with previously reported MALDI-TOF analysis of Pluronic polymers.[3] Conjugation of DFO to F127-$(COOH)_2$ results in a noticeable increase the apparent molecular weight of the polymer.

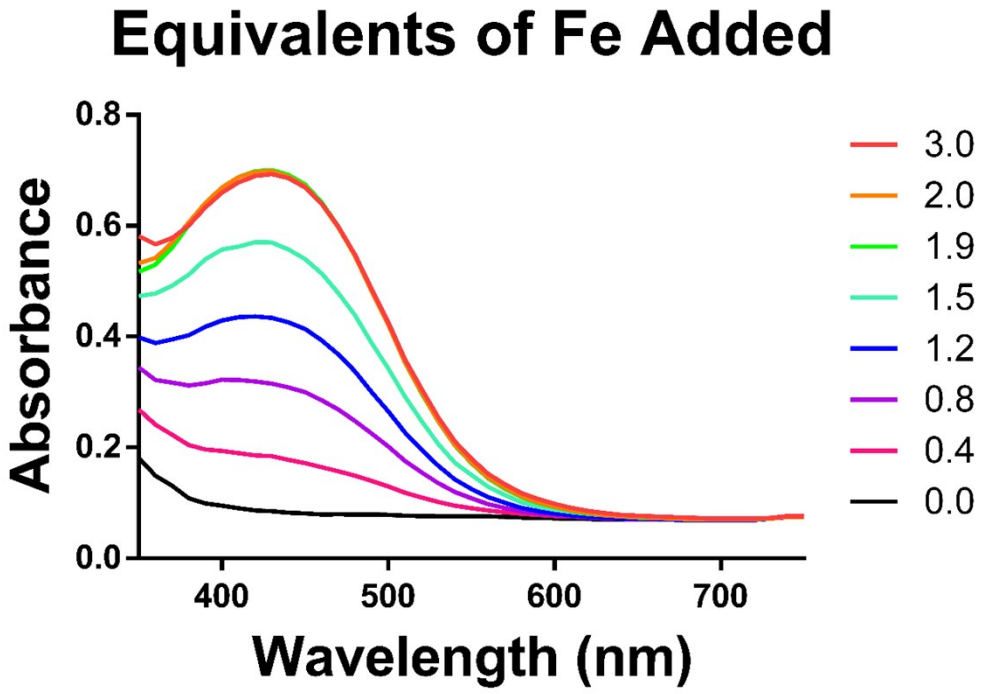

Figure S5. Titration of F127-$(DFO)_2$ with Fe^{III}. Increasing concentrations of Fe result in an increase in the A430 due to formation of DFO:Fe complexes. Maximum absorbance is measured after adding 1.9 eq. Fe due to all F127-$(DFO)_2$ being converted to F127-$(DFO:Fe)_2$; additional iron does not increase the A430 further, indicating an average ratio of 1.9 DFO end groups per F127-$(COOH)_2$ polymer.

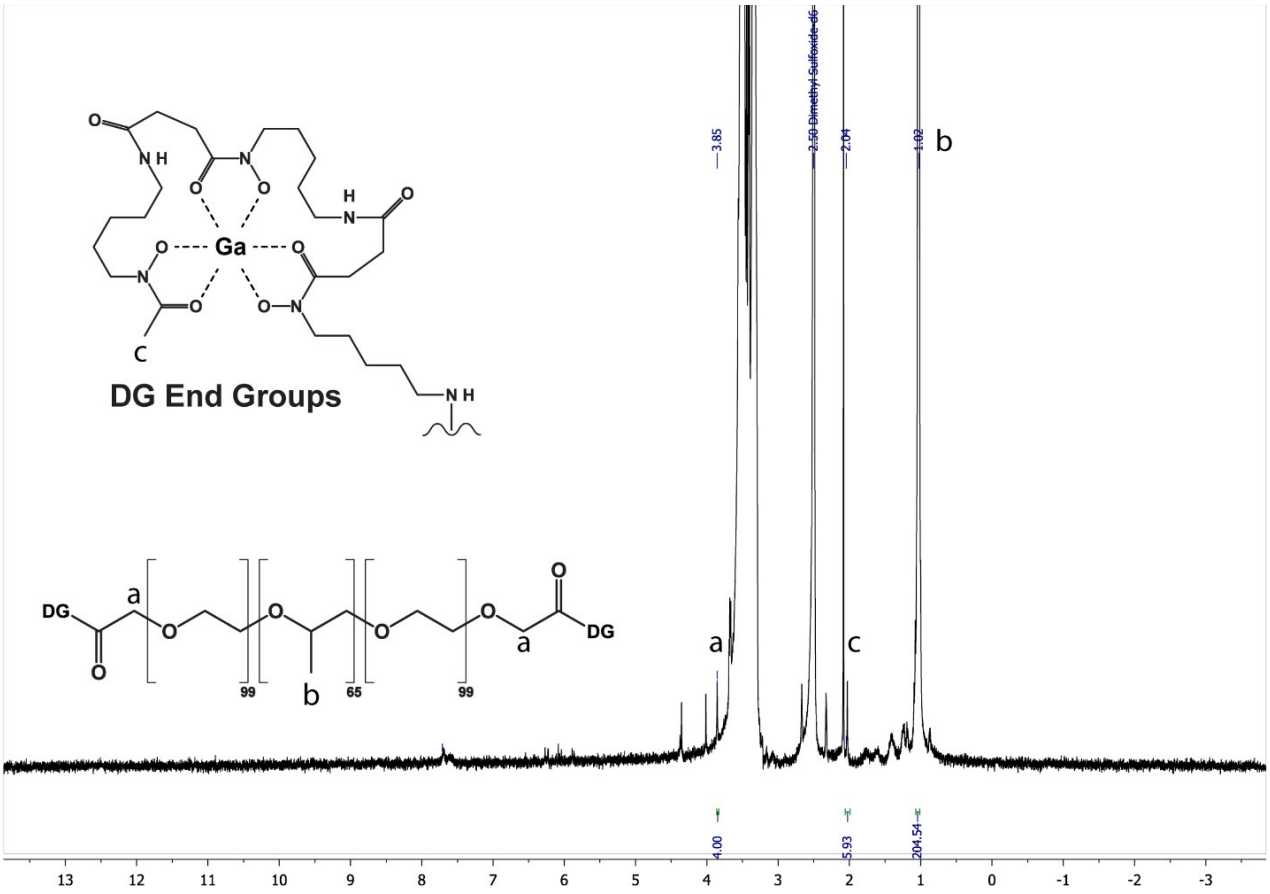

Figure S6. F127-$(DG)_2$ Spectra. ^{1}H NMR (400 MHz, DMSO-d_6, 298K): 7.60-7.80 (br, N***H*** of DFO), 3.85 (s, OC***H*****$_2$**CONH of PEG), 3.20–3.60 (m, CH_2C***H*****$_2$**O of PEG and C***H*****$_2$**CHO of PPG), 2.90-3.10 (m, C***H*****$_2$**-NHCO of DFO), 2.50- 2.60 (m, C***H*****$_2$**-CO of DFO), 2.20-2.30 (m, C***H*****$_2$**-NH of DFO), 1.96 (s,C***H*****$_3$** of DFO), 1.10-1.50 (m, C***H*****$_2$** of DFO), 1.02 (d, C***H*****$_3$** of PPG). Disappearance of hydroxamic acid protons (9.60-9.80, br, NO***H*** of DFO) indicates formation of DFO:Ga complex.

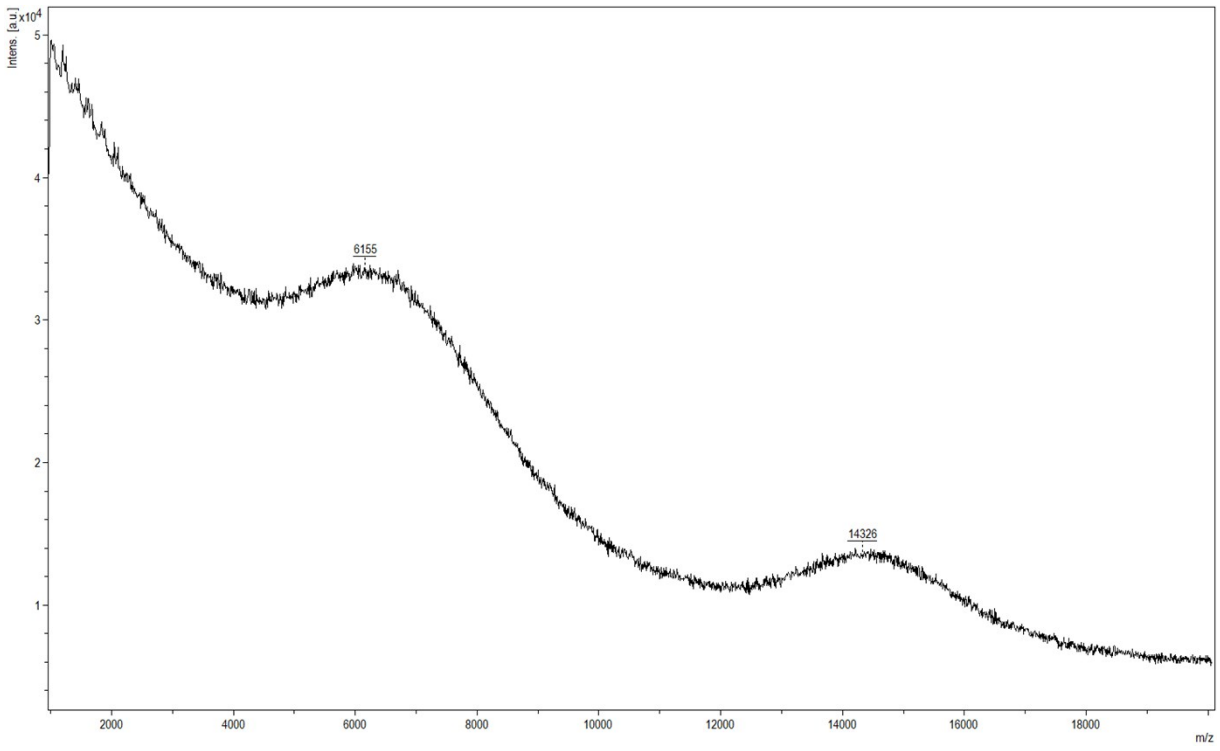

Figure S7. MALDI-TOF of F127-$(DG)_2$. Expected average m/z for singly charged species: 13,847. Observed average m/z for singly charged species: 14,326. The larger observed average m/z compared to the expected and broad distribution is consistent with previously reported MALDI-TOF analysis of Pluronic polymers.[3] Complexation of F127-$(DFO)_2$ to F127-DG_2 results in a 144 g/mol increase in apparent mass, which is approximately equivalent to the addition of two Ga atoms.

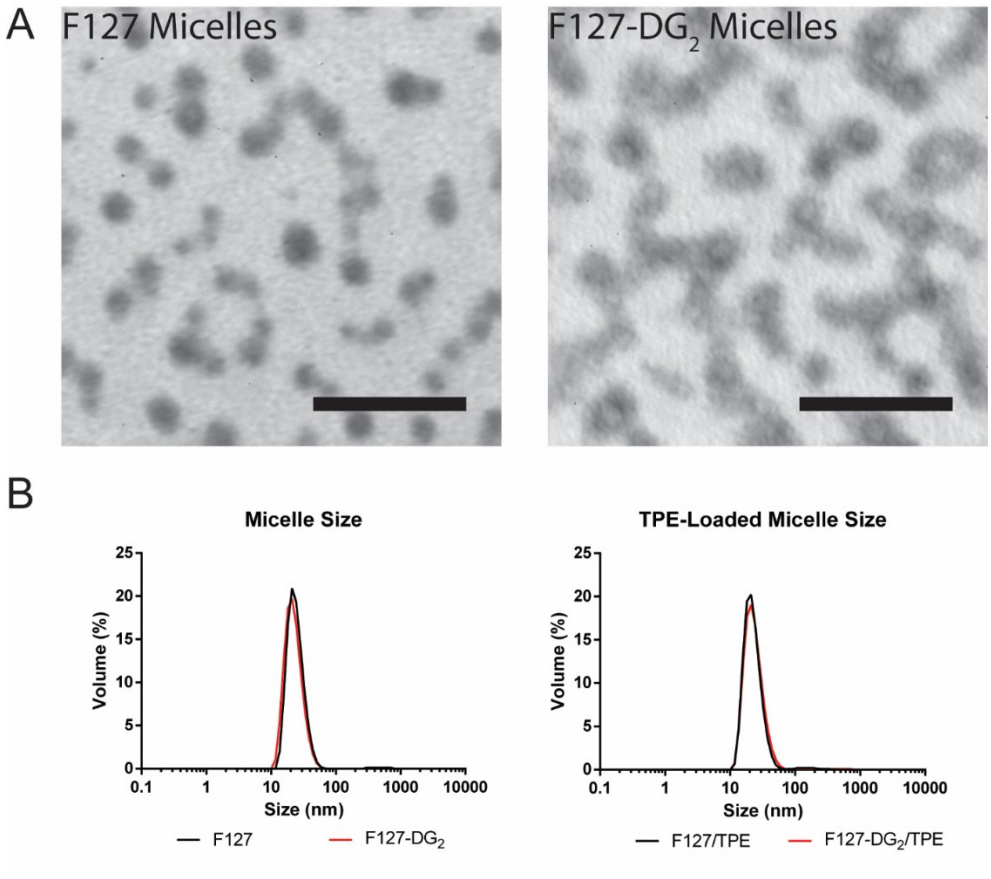

Figure S8. Characterization of F127 and F127-DG_2 micelles. (A) TEM imaging of F127 and F127-DG_2 micelles, scale bar represents 100 nm. (B) Dynamic light scattering of F127 micelles in PBS at 37°C. Modification of F127 micelles (21.6 nm) to F127-DG_2 micelles (24.4 nm) results in a slight increase in size, while loading with TPE to generate F127/TPE and F127-DG_2/TPE results in 23.2 nm and 24.8 nm micelles, respectively.

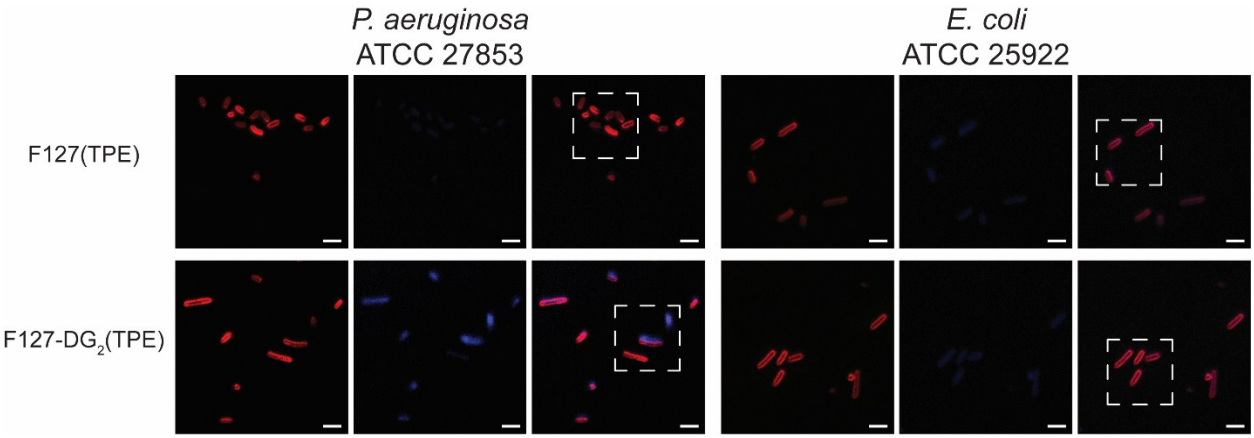

Figure S9. Targeted F127-DG_2/TPE micelles exhibit greater binding to the OM of *P. aeruginosa* than untargeted F127/TPE micelles based on increased TPE fluorescence (blue). Scale bar represents 2 μm.

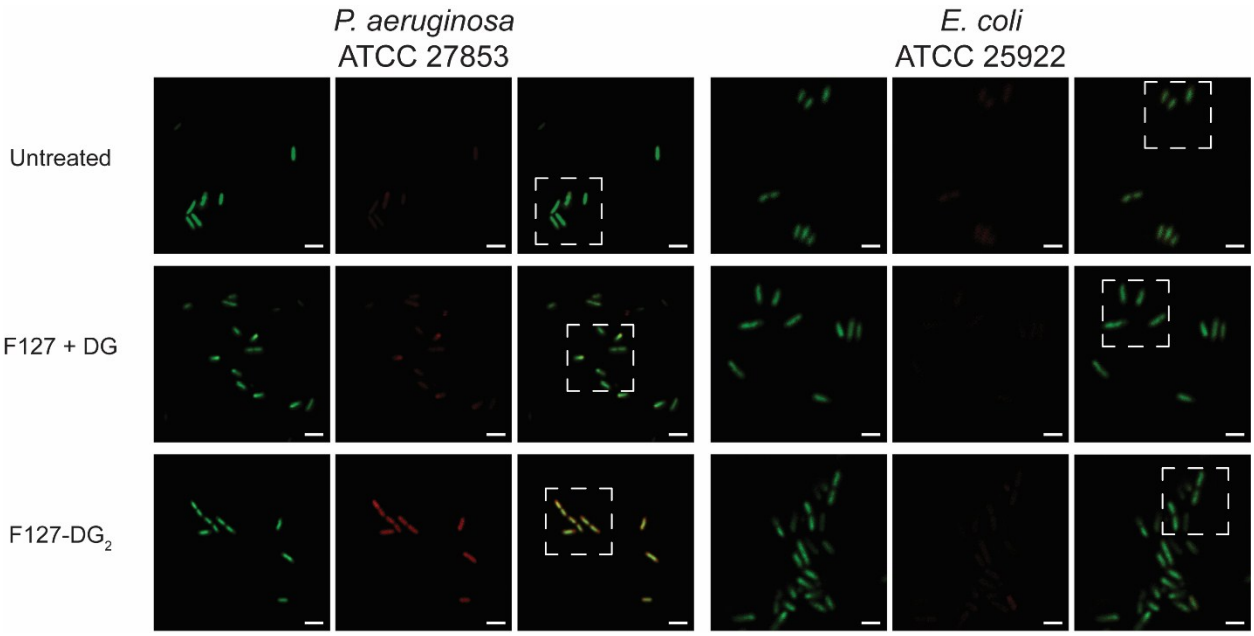

Figure S10. OM permeabilization with F127-DG_2 (64 μM) results in greater HI accumulation (red) in *P. aeruginosa* than unmodified F127 (64 μM) + DG (128 μM), while E. coli OM permeability is unchanged. Positive control staining performed with SYTO13 (green). Scale bar represents 2 μm.

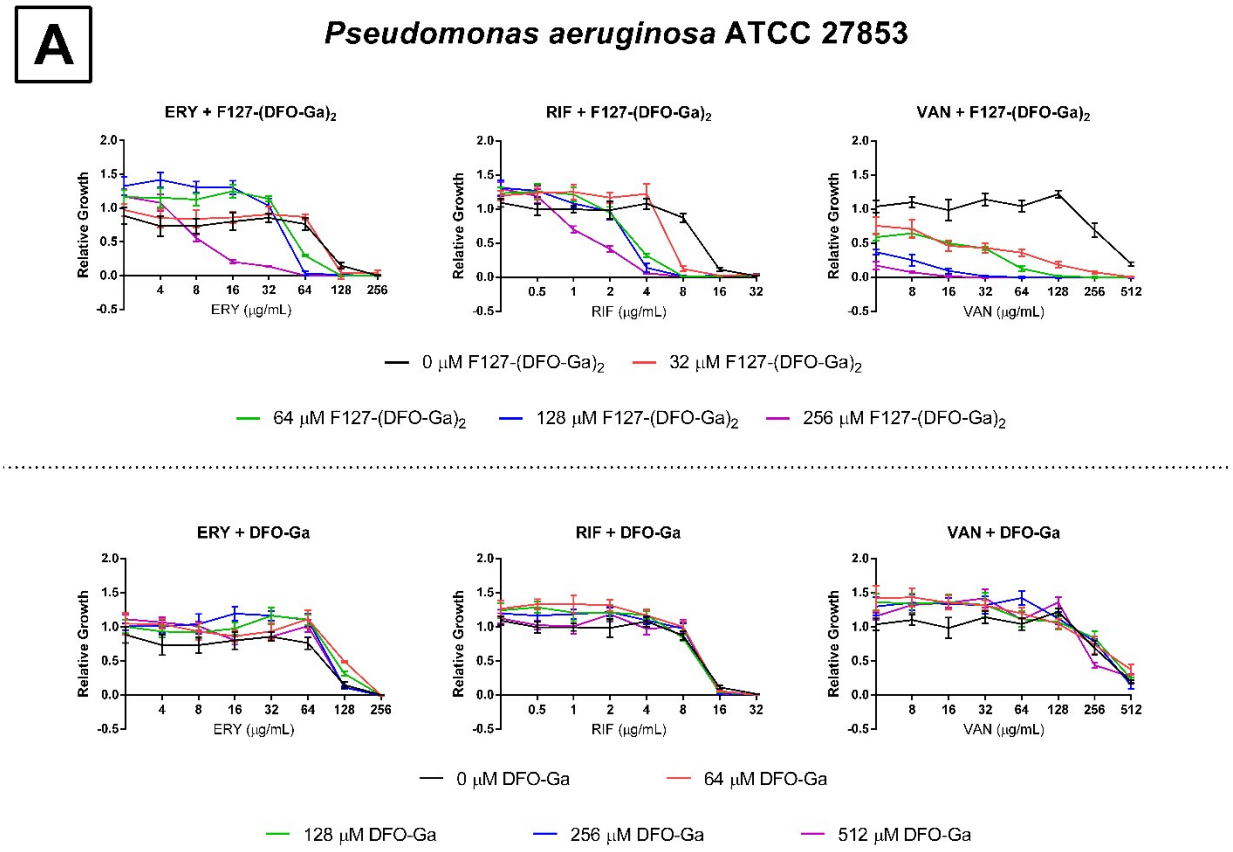

Figure S11. Dose response curves for ERY, RIF, or VAN combined with either F127-(DFO-Ga)$_2$ or free DFO-Ga plus unmodified F127 against *P. aeruginosa* strain ATCC 27853.

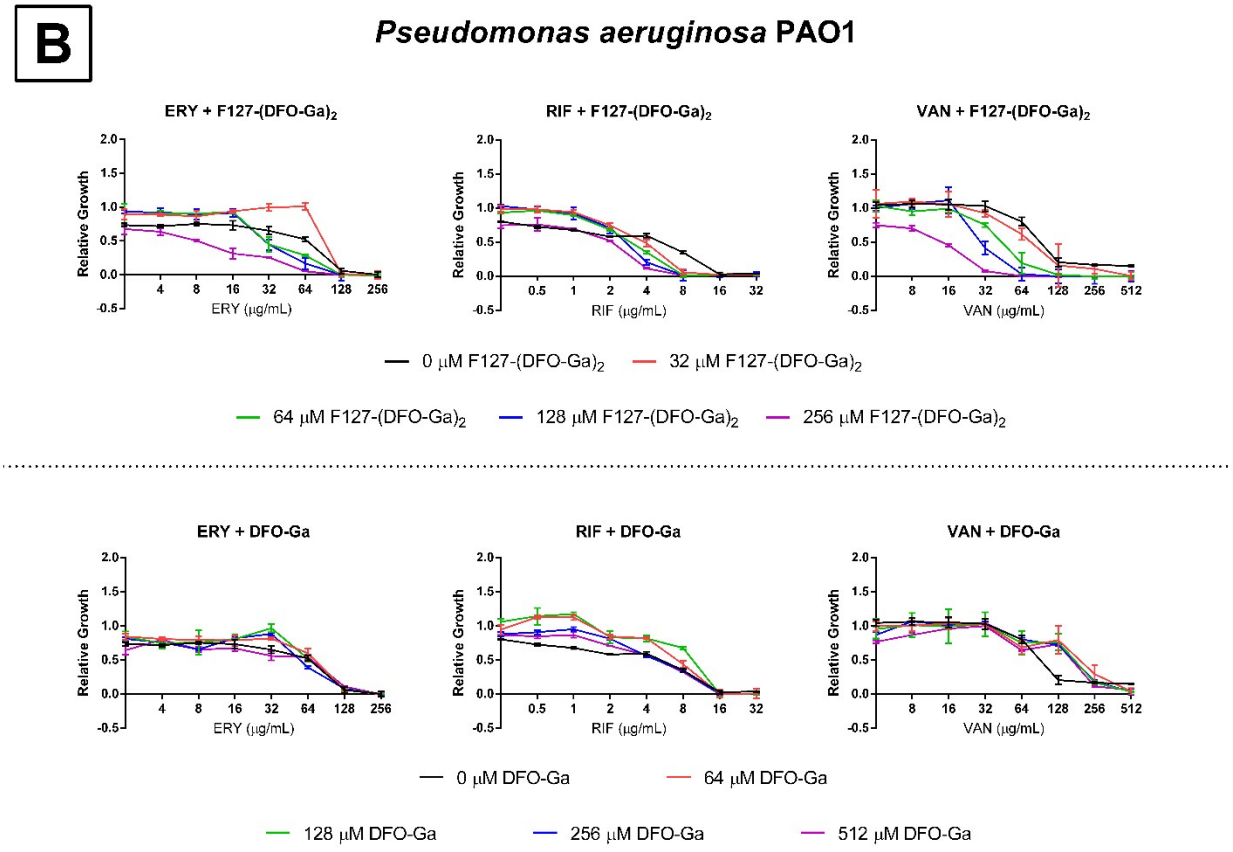

Figure S12. Dose response curves for ERY, RIF, or VAN combined with either F127-(DFO-Ga)$_2$ or free DFO-Ga plus unmodified F127 against *P. aeruginosa* strain PAO1.

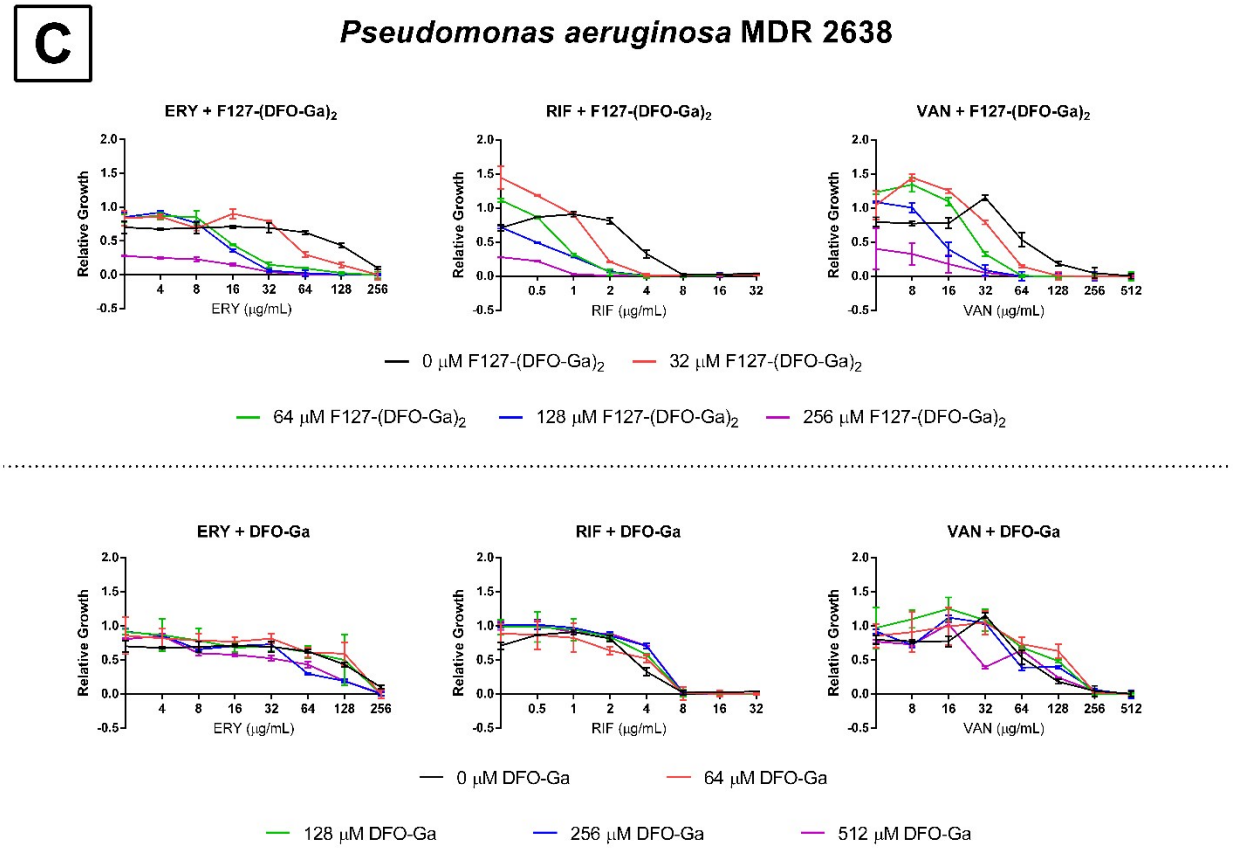

Figure S13. Dose response curves for ERY, RIF, or VAN combined with either F127-(DFO-Ga)$_2$ or free DFO-Ga plus unmodified F127 against *P. aeruginosa* strain MDR 2638.

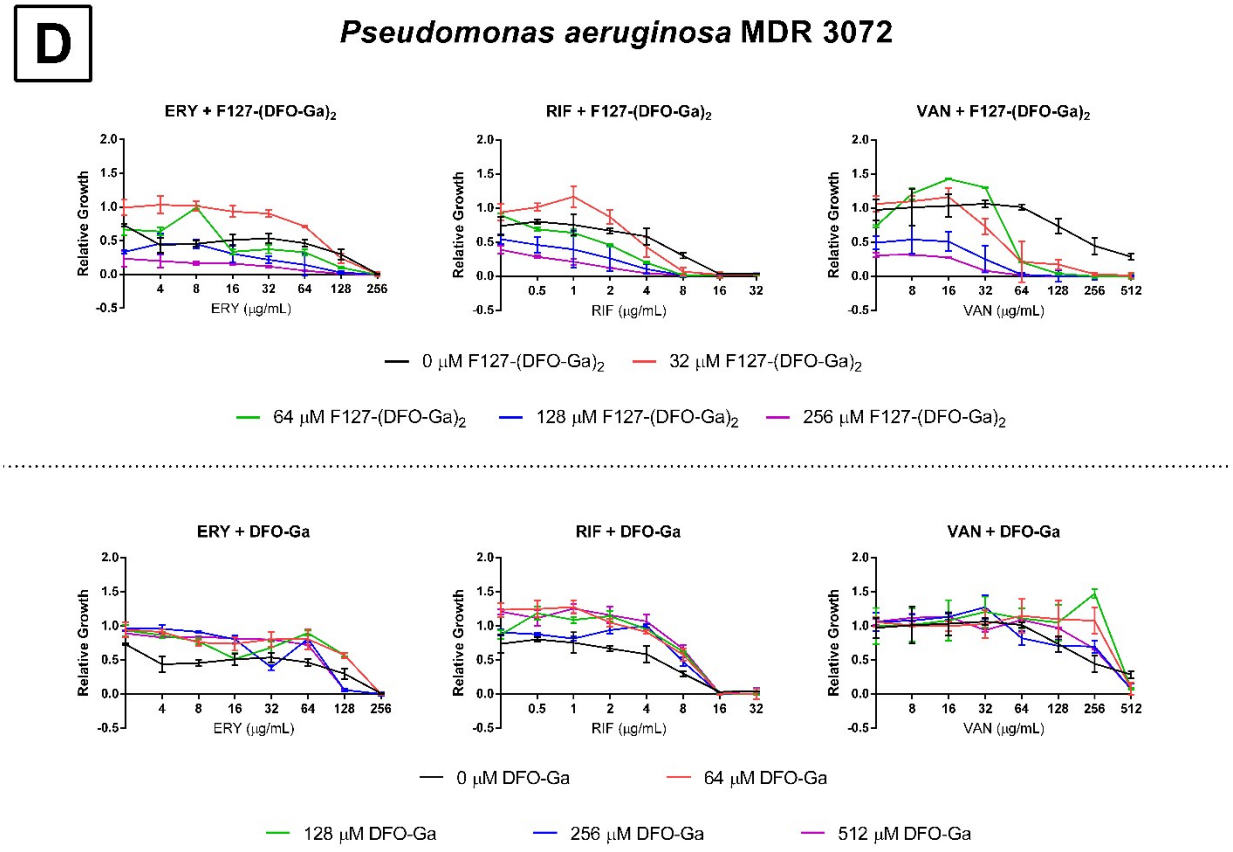

Figure S14. Dose response curves for ERY, RIF, or VAN combined with either F127-(DFO-Ga)$_2$ or free DFO-Ga plus unmodified F127 against *P. aeruginosa* strain MDR 3072.

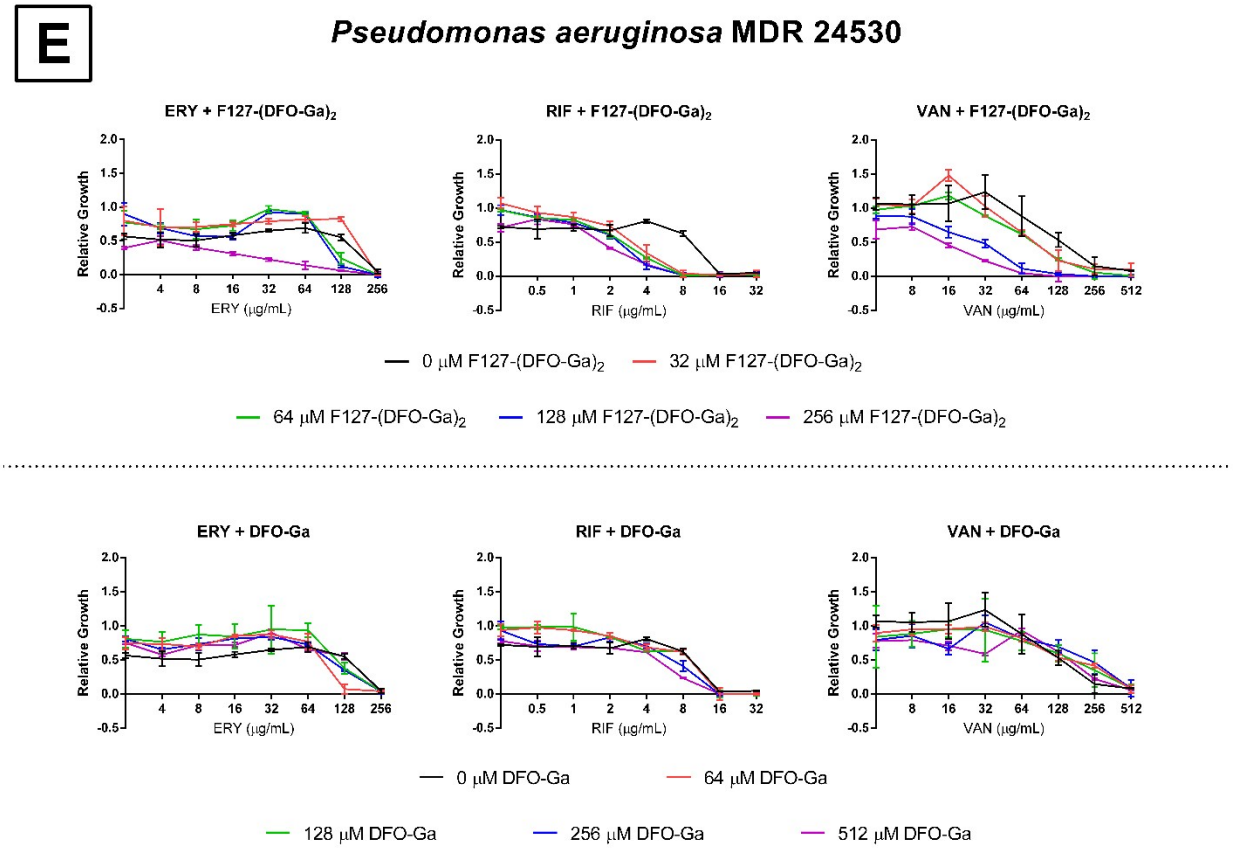

Figure S15. Dose response curves for ERY, RIF, or VAN combined with either F127-(DFO-Ga)$_2$ or free DFO-Ga plus unmodified F127 against *P. aeruginosa* strain MDR 24530.

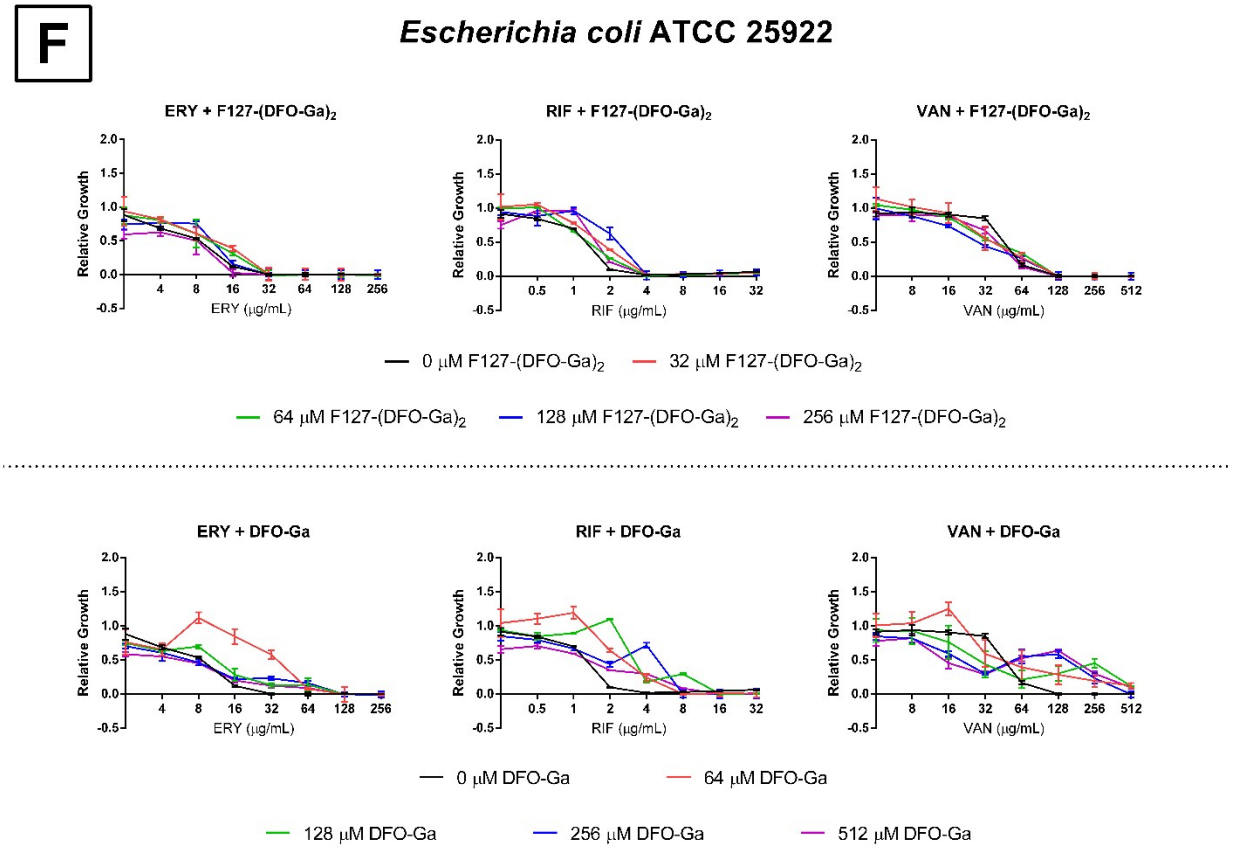

Figure S16. Dose response curves for ERY, RIF, or VAN combined with either F127-(DFO-Ga)$_2$ or free DFO-Ga plus unmodified F127 against *E. coli* strain ATCC 25922.

References:

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