Superparamagnetic Iron Oxide-Encapsulating Polymersome Nanocarriers for Biofilm Eradication

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ABSTRACT

The rising prevalence and severity of antibiotic-resistant biofilm infections poses an alarming threat to public health worldwide. Here, biocompatible multi-compartment nanocarriers were synthesized to contain both hydrophobic superparamagnetic iron oxide nanoparticles (SPIONs) and the hydrophilic antibiotic methicillin for the treatment of medical device-associated infections. SPION co-encapsulation was found to confer unique properties, enhancing both nanocarrier relaxivity and magneticity compared to individual SPIONs. These iron oxide-encapsulating polymersomes (IOPs) penetrated 20 μm thick *Staphylococcus epidermidis* biofilms with high efficiency following the application of an external magnetic field. Three-dimensional laser scanning confocal microscopy revealed differential bacteria death as a function of drug and SPION loading. Complete eradication of all bacteria throughout the biofilm thickness was achieved using an optimized IOP formulation containing 40 $\mu\text{g/mL}$ SPION and 20 $\mu\text{g/mL}$ of methicillin. Importantly, this formulation was selectively toxic towards methicillin-resistant biofilm cells but not towards mammalian cells. These novel iron oxide-encapsulating polymersomes demonstrate that it is possible to overcome antibiotic-resistant biofilms by controlling the positioning of nanocarriers containing two or more therapeutics.

KEYWORDS: biofilm; polymersome; SPION; *Staphylococcus epidermidis*; antibiotic-resistance; nanomedicine.

INTRODUCTION

Biofilms are highly dynamic communities of immobile bacteria, typically attached to a surface or to each other, and surrounded by a protective extracellular polymeric matrix comprised of polysaccharides, proteins, nucleic acids, and lipids.¹ This unique microenvironment provides bacteria with conditions conducive to the development of innate resistance to both antimicrobial compounds and host response factors.¹⁻⁴ Bacteria within biofilms can be as much as 1000 times more resistant to antibiotic drugs,⁵ and when disseminated from the site of the biofilm, can produce recurrent antibiotic-resistant infections elsewhere in the body.^{6,7} This problem is so widespread that biofilm infections have been estimated to account for 60% of all infectious diseases treated in the Western world.^{8,9}

Biofilms are most frequently found on indwelling devices, such as catheters, ventilators, orthopedic implants, and cardiac implants,^{10,11} and thus medical devices are a primary source of biofilm introduction and dissemination. The treatment of these device-associated infections is particularly challenging, with as many as 50% of implanted devices requiring surgical removal once the biofilm has formed.¹²⁻¹⁴ Antimicrobial therapy alone is rarely effective, and when combined with surgical intervention, is often prolonged.^{8,15,16} New antimicrobials are becoming increasingly difficult to develop and have failed to keep pace with the emergence of resistant bacteria,¹⁷ and thus alternative strategies to combat device-associated infections are desperately needed.

Superparamagnetic iron oxide nanoparticles (SPIONs) have been shown to exhibit antimicrobial properties, and have been proposed as a potential magnetically positionable

antibacterial treatment.^{18–23} The clinical utility of individual SPIONs, however, is highly limited. The antibacterial properties of SPIONs are inversely proportional to their size, with smaller nanoparticles exhibiting more potent antimicrobial activity.^{24–27} In contrast, SPION magneticity, and the accompanying ability to control SPION positioning using magnetic fields, is proportional to nanoparticle size.²⁸ Thus, in a clinical setting, a physician would be forced to trade antibacterial efficacy for targeting ability. Additionally, SPIONs used for clinical applications should ideally be capped with hydrophobic agents such as oleic acid to take advantage of superior structural and magnetic properties compared to their hydrophilic counterparts.^{29,30} Such particles, however, cannot be easily dispersed in aqueous biological fluids. As a result, SPIONs are generally ineffective as an antibacterial monotherapy.

To overcome the challenges of SPION- and antibiotic-based penetration into biofilms, we have developed a biocompatible, multi-compartment polymersome formulation containing multiple hydrophobic SPIONs and hydrophilic methicillin for the treatment of medical device-associated infections (Figure 1). The relaxivity, magneticity, and biofilm penetration capacity of these iron oxide-encapsulating polymersomes (IOPs) were analyzed and compared to free SPIONs. The therapeutic efficacy of different IOP formulations was assessed in clinically relevant, methicillin-resistant *S. epidermidis* biofilms. Biofilm integrity and particle penetration was visualized and quantified using a combination of microscopy and colorimetric assays. Treatment cytotoxicity was assessed using both bacterial and mammalian cells. The results of these studies show that IOPs have great translational potential as magnetically positionable nanocarriers for the treatment of drug-resistant biofilms.

Figure 1. Current strategies for biofilm treatment using SPIONs and/or antimicrobials.

IOP = iron oxide-encapsulating polymersome; SPION = superparamagnetic iron oxide nanoparticle.

RESULTS

Hydrophobic SPIONs self-assemble inside polymersomes

A self-assembling, biocompatible amphiphilic diblock co-polymer composed of methoxypoly(ethylene glycol)_{5,000} and poly(*D*)-(*L*)-lactic acid_{50,000} (mPEG-PDLLA 5,000:50,000 Da) was utilized to synthesize mPEG-coated polymersomes with a hydrophobic bilayer

containing SPIONs (Figure 2a). Using a stirred atomization technique adapted from ³¹, hydrophobic 5 ± 2.5 nm SPIONs were dispersed via ultrasonication into a mPEG-PDLLA solution and then injected through an atomizer into an actively stirring aqueous buffer containing 250 $\mu\text{g/mL}$ methicillin. The resultant polymersomes were dialyzed against PBS, resulting in a highly stable translucent orange solution which showed no evidence of particle settling following storage. Transmission electron microscopy (Figure 2b) revealed uniform, spherical polymersomes with clusters of SPIONs embedded inside. These SPION clusters were limited to the particle margins rather than the central core, indicating that the SPIONs were embedded in the membrane bilayer as previously described for silver-embedded polymersomes.³¹

Figure 2. Synthesis of iron oxide-encapsulating polymersomes. (a) 5 nm monodisperse hydrophobic SPIONs are combined with mPEG-PDLLA co-polymer in organic solvent and ultrasonicated to create a uniform suspension (1). This organic phase is injected through an atomizer into an actively stirring aqueous phase containing PBS and methicillin (2). Finally, the mixture is dialyzed against pure PBS to remove the organic solvent and unencapsulated drug (3). The resulting polymersome solution is highly stable and translucent orange (4). (b)

Representative transmission electron micrograph of hydrophobic SPIONs embedded inside the polymersome bilayer. SPION = superparamagnetic iron oxide nanoparticle.

SPION encapsulation confers unique physical properties

Several nanoparticle characterization methods were employed to determine the relative advantage of co-encapsulating multiple SPIONs within a single polymersome (Figure 3). Dynamic light scattering (Figure 3a) revealed highly reproducible particles with a narrow size distribution of 83 ± 6 nm and a neutral (-1 ± 3 mV) surface charge. In order to determine the SPION loading efficiency, polymersomes were synthesized with 4 different SPION starting concentrations, dissolved in concentrated HCl, reacted with chromogen Ferene S, and quantified for total iron content using spectrophotometric measurements. The SPION encapsulation efficiency (Figure 3b) was measured to be $39 \pm 3\%$ and was linear ($R^2 = 0.999$) over the range of starting iron oxide concentrations (75 - 250 $\mu\text{g/mL}$) studied. IOP relaxivity (Figure 3c, d), measured using 7 Tesla magnetic resonance imaging (MRI), was dependent on iron oxide loading. IOPs containing 25 - 75 $\mu\text{g/mL}$ SPIONs showed enhanced r_2 relaxivity compared to free SPIONs, ideal for T_2 -weighted imaging, while IOPs containing ≥ 100 $\mu\text{g/mL}$ SPIONs had both low r_1 and r_2 relaxivity. Interestingly, there appeared to be a window of loading in which co-encapsulated SPIONs enhanced nanocarrier relaxivity rather than behaving like a single large SPION, as predicted by Strijkers and Nicolay.³² IOP magneticity (Figure 3e) was quantified using a novel absorbance-based pull-down assay. IOPs loaded with different concentrations of SPIONs each displayed a characteristic pull-down time (τ) when exposed to the same neodymium magnet. Interestingly, τ was exclusively determined by the number of SPIONs

encapsulated per IOP (Figure 3e) and was independent of the IOP dilution. IOPs loaded with 100 $\mu\text{g/mL}$ SPIONs displayed a τ of 5.2 minutes compared to those with 25 $\mu\text{g/mL}$ SPIONs (which required ~ 100 minutes for complete pull-down). All IOP formulations significantly outperformed the free nanoparticles ($p < 0.01$), which displayed a τ of ~ 13 hours.

Figure 3. Physical and magnetic properties of iron oxide-encapsulating polymersomes. (a) IOP size distribution measured by dynamic light scattering. (b) SPION encapsulation efficiency as measured using a Ferene S colorimetric assay. IOP relaxivity r_1 (c) and r_2 (d) at 7 Tesla, as measured using magnetic resonance imaging. (e) Magneticity of IOP formulations containing different concentrations of SPIONs as quantified using an absorbance-based magnetic pulldown

assay. (f) IOP penetration into live biofilms following 24 hr incubation in the presence and absence of an external magnetic field, visualized by Prussian blue staining for iron content. (g) Prussian blue staining of biofilms treated with IOPs containing different concentrations of SPIONs for 4 hrs under an external magnetic field. All IOP treatments were diluted to contain the same absolute concentration of iron to allow for side-by-side comparison. Data is shown as mean $\pm$ SD. IOP = iron oxide-encapsulating polymersome; SPION = superparamagnetic iron oxide nanoparticle.

SPION encapsulation enhances the efficiency of biofilm penetration

The ability of IOPs to penetrate clinically relevant bacterial biofilms was tested using the *Staphylococcus epidermidis* (*S. epidermidis*) strain RP62a, a clinical isolate³³ capable of forming robust, highly organized methicillin-resistant biofilms on device surfaces.³⁴ Biofilms grown on glass coverslips were treated for 24 hours with IOPs containing 100 $\mu\text{g/mL}$ SPIONs, with and without an applied magnetic field, and then stained with potassium ferrocyanide (Prussian blue stain for Fe^{II}) and pararosaniline (magenta stain for cellular material). In the absence of any applied magnetic field, minimal iron penetration was observed, appearing as small, irregular patches of blue that increased in size and frequency with IOP concentration. When IOPs were delivered in the presence of a neodymium magnet, biofilm staining produced a uniform blue color, suggestive of effective biofilm penetration (Figure 3f). To determine the influence of SPION loading, IOPs containing different concentrations of encapsulated SPIONs were used to treat live biofilms for 4 hours (Figure 3g). IOP solutions were individually diluted to contain the same absolute concentration of iron to allow for a side-by-side comparison of Prussian blue staining. Biofilm penetration was found to increase with increased SPION loading, thus IOPs

containing 100 µg/mL SPIONs, and dilutions thereof (Table 1), were used for all subsequent experiments.

Table 1. IOP dilutions tested for anti-biofilm activity and cytotoxicity.

Dilution	mPEG-PDLLA (µg/mL)	SPION (µg/mL)	Methicillin* (µg/mL)
1× stock	1000	100	50
0.4×	400	40	20
0.2×	200	20	10

*Only for formulations containing drug.

IOPs have potent anti-biofilm activity

The minimum inhibitory concentration (MIC) of free methicillin towards *S. epidermidis* RP62a was determined to be 2µg/mL for planktonic bacteria, and 500µg/mL for bacteria grown in biofilms, indicating that this strain is highly resistant to methicillin in biofilm form. The anti-biofilm activity of 24 hour IOP treatment was examined using laser scanning confocal microscopy of bacterial biofilms stained with a Filmtracer™ LIVE/DEAD Biofilm Viability kit (Figure 4). A small neodymium magnet was placed underneath a portion of each individual biofilm to selectively pull IOPs from the supernatant into the biofilm. Tile scans (1.6 mm x 1.6 mm) acquired midway through the biofilm were used to identify the regions of cell death, visualized by the presence of red bacteria (Figure 4a). Untreated controls were characterized by a homogenous biofilm comprised almost entirely of live (green) cells with no localized evidence of cell death above the magnet. Biofilms treated with IOPs containing saline (no drug) showed

partial bacteria death in areas exposed to the magnetic field. Biofilms treated with IOPs containing methicillin showed extensive bacteria death within the boundary of the magnetic field (dashed line) and very little bacteria death outside, demonstrating that an external magnetic field is required to reach the full therapeutic potential of the IOPs. Bacterial viability within the magnetic field was quantified from z-stack images collected at 0.6 μm intervals. Representative 3D reconstructions are shown in Figure 4b and Videos 1-5. Bacterial viability, plotted as a function of biofilm depth revealed that the ratio of live to dead bacteria decreased with treatment, and that this bacteria kill was accompanied by a decrease in biofilm density, particularly in the upper layers (Figure 4c). Concentrated IOPs (0.4 $\times$) with no drug were found to kill a large proportion of bacteria in the lower biofilm layers, but were less efficient in the upper layers. In contrast, IOPs containing methicillin killed bacteria throughout the full biofilm thickness. The IOP formulation containing 40 $\mu\text{g/mL}$ SPION and 20 $\mu\text{g/mL}$ of methicillin was sufficient to completely eradicate methicillin-resistant *S. epidermidis* biofilms as assessed by confocal laser scanning microscopy.

Figure 4. Anti-biofilm activity of iron oxide-encapsulating polymersomes. LIVE/DEAD staining of biofilms treated with different dilutions of IOPs (1× stock = 100 µg/mL SPION; 50 µg/mL methicillin) for 24 hrs. Live (green) and dead (red) bacteria were imaged using laser scanning confocal microscopy. (a) Tile scans collected halfway through the biofilm show concentration-dependent bacteria death within the boundary of the external applied magnetic field (dashed line). (b) 3-D reconstructions of z-stacks collected across the biofilm thickness inside the magnetic field. (c) The percentage of biofilm volume occupied by live (green) and dead (red) bacteria as a function of biofilm depth (0 µm = bottom) as quantified from image slices. IOP = iron oxide-encapsulating polymersome; SPION = superparamagnetic iron oxide nanoparticle.

IOPs are selectively toxic to bacterial cells

IOP cytotoxicity was assessed using both mammalian and bacterial cells. Different IOP formulations and dilutions were tested for 24 hours on human dermal fibroblast cells using an MTS assay, both in the presence and absence of the applied magnetic field (Figure 5a). The results showed no significant change ($p < 0.05$) in cell viability under all treatment conditions using a paired student's t-test, indicating that the IOP formulations were not toxic to fibroblasts. Bacterial cytotoxicity was assessed in methicillin-resistant biofilms by quantifying the fraction of live to dead bacteria in confocal images over the full biofilm volume (Figure 5b). All IOP treatments produced an overall reduction in biofilm volume, however, neither SPIONs alone, nor drug-free IOPs, succeeded in eliminating more than 60% of the live cell content. For IOPs containing drug, the treatment efficacy was dependent on the IOP concentration, with only IOPs containing ≥ 40 µg/mL SPION and 20 µg/mL of methicillin producing complete eradication of

methicillin-resistant *S. epidermidis* biofilms. Assessment of biofilm ultrastructure using scanning electron microscopy (SEM) revealed that bacteria cell death following IOP treatment was accompanied by decoherence of the extracellular polymeric matrix (Figure 5c). In contrast, untreated controls revealed a highly organized biofilm, comprised of numerous layers of densely concentrated cocci connected by long strands of biofilm matrix spanning between healthy bacteria.

Figure 5. Cytotoxicity of iron oxide-encapsulating polymersomes to mammalian and bacterial cells. (a) Viability of human dermal fibroblast cells as assessed using a MTS assay following 24 hr IOP treatment in the presence and absence of an external applied magnetic field. Data is shown as mean $\pm$ SD. (b) The total volume and relative fraction of live-to-dead bacteria in biofilms treated with IOPs for 24 hr with an applied magnetic field, as quantified using laser scanning confocal microscopy. (c) Representative transmission electron micrographs of treated biofilms and surrounding extracellular polymeric matrix. IOP = iron oxide-encapsulating polymersome.

CONCLUSIONS

For an anti-biofilm treatment to be clinically relevant, complete eradication of the infection is necessary. *Staphylococcus* bacteria possess a doubling time of 20-40 minutes, so if only a fraction of the cells are killed, treated biofilm structures will regrow rapidly.³⁵ While previous studies have reported a statistically significant reduction in biofilm viability using SPIONs, in many cases this has been achieved using particle concentrations in excess of 1 mg/mL,¹⁸⁻²⁰ which would be difficult to replicate *in vivo*. Here, we show that SPIONs, when co-encapsulated with an antibiotic, can eradicate antibiotic-resistant infections when delivered directly into the biofilm.

Previous studies have proposed that metallic nanoparticles, ranging from silver to various metal oxides can have bactericidal activity.^{18,19,23} Combining metallic nanoparticles with β -lactam antibiotics has been proposed as potential mechanism for overcoming antibiotic drug resistance,³⁶ but this has only been shown to be effective for silver nanoparticles to date.³¹ Importantly, co-encapsulating silver nanoparticles with ampicillin resulted in synergistic antimicrobial activity against *Escherichia coli* grown in suspension.³¹ Based on previous studies of SPION and antibiotic delivery into biofilms (summarized in Figure 1), we hypothesized that drug penetration would be as important as, or more important than, drug concentration. For this reason, we sought to create magnetically positionable nanocarriers that would expose multiple layers of biofilm to a high concentration of methicillin. The resulting IOPs displayed the capacity to effectively penetrate intact biofilms and render these drug-resistant biofilms sensitive to

treatment. Both methicillin³⁷ and SPIONs²² are known to potentiate ROS generation, and this may account for the high degree of therapeutic efficacy achieved here.

Co-encapsulating multiple SPIONs within single polymersomes proved critical for both positioning IOPs inside biofilms and providing anti-biofilm activity. Synthesizing IOPs containing a sufficient concentration of SPIONs allowed these particles to be pulled with high efficiency into biofilms on physiologically relevant time-scales. IOPs were as much as 150 times faster at migrating under a magnetic field than free SPIONs, meaning that it would be possible to use much weaker and shorter duration magnetic fields to position IOPs. Interestingly, diluting the IOP solution does reduce its therapeutic efficacy, indicating that the packing of the SPIONs within IOPs, as well as the absolute number of SPIONs introduced into the biofilm, are both important for reaching full therapeutic potential.

In conclusion, IOPs show great promise as magnetically positionable drug delivery vehicles. The co-encapsulation of multiple hydrophobic SPIONs into a single nanocarrier confers unique physical properties that render IOPs ideal for penetrating and treating biofilms. In contrast to other approved and experimental therapies, IOPs are capable of treating the entire biofilm thickness following a single drug administration. Given the importance of treating biofilms rapidly, uniformly, and efficiently, the nanocarrier described here has high translational potential as an entirely new approach for treating drug-resistant biofilms.

MATERIALS AND METHODS

Iron oxide polymersome (IOP) synthesis

IOP nanoparticles were synthesized through a two-phase self-assembly process. Briefly, 100 μ L of oleic acid-coated iron oxide nanocrystals (5 ± 2.5 nm, 25 mg/mL in chloroform; Ocean NanoTech, San Diego, CA) were resuspended in 1 mL of tetrahydrofuran (THF; Sigma-Aldrich, St. Louis, MO) under nitrogen gas. 10 mg of the methoxy poly(ethylene glycol)-poly(*D*)-(*L*)-lactic acid co-polymer (mPEG-PDLLA, MW $\sim$ 5,000:50,000 Da; Akina, West Lafayette, IN) was subsequently dissolved in the SPION suspension and dispersed via ultrasonication. This organic phase was injected through a syringe atomizer (LMA, San Diego, CA) at a rate of 0.5 mL/sec into 10 mL of a rapidly stirring solution of methicillin sodium salt ($\geq$ 85%, 250 μ g/mL; Sigma-Aldrich, St. Louis, MO) in 0.01 M phosphate buffered saline (PBS; Sigma-Aldrich, St. Louis, MO). After 30 sec of stirring, the speed of the solution was gradually reduced to a halt. The mixture was allowed to dialyze (Spectra/Por Float-A-Lyzer G2, 50 kDa MWCO; Spectrum Labs, Rancho Dominguez, CA) for 48 hrs against pure 0.01 M PBS.

IOP loading

Iron oxide loading was varied by changing the starting SPION concentration in the organic phase (range 75 - 250 μ g/mL) during polymersome self-assembly. Iron oxide loading was quantified using a Ferene S colorimetric assay (Sigma-Aldrich, St. Louis, MO). IOPs and embedded SPIONs were dissolved by 30 min of ultrasonication in 5 M hydrochloric acid (HCl) and neutralized using sodium hydroxide (NaOH). 10 μ L of this solution was added to 90 μ L of iron assay buffer and 5 μ L of iron reducer (reducing Fe^{3+} to Fe^{2+}). Finally, 100 μ L of iron probe solution was added, yielding a blue Fe^{2+} -Ferene S complex which was quantified using

spectrophotometry according to the manufacturer's instructions. Antibiotic drug loading was quantified as previously described.³¹

IOP characterization

IOP morphology was visualized using transmission electron microscopy (TEM; JEM-1010, JEOL, Peabody, MA). Nanoparticles were adsorbed onto 300-mesh copper-coated carbon grids (Electron Microscopy Sciences, Hatfield, PA) and negatively stained with 1.5% uranyl acetate (Electron Microscopy Sciences, Hatfield, PA). IOP size distribution and zeta potential were measured using dynamic light scattering (DLS; 90Plus Zeta, Brookhaven Instruments, Holtsville, NY) and software provided by the manufacturer. IOP relaxivity was measured following serial dilution of four IOP solutions containing different concentrations of iron oxide (25 - 100 $\mu\text{g/mL}$ encapsulated SPIONs) using a Bruker Biospec 7.0T/20-cm USR horizontal magnet (Bruker, Billerica, Massachusetts, USA) equipped with a 20-G/cm magnetic field gradient insert (ID = 12 cm, Bruker) and a 300 MHz, 30 mm mouse coil (Animal Imaging Research, LLC, Holden, Massachusetts, USA).

IOP magnetic pull-down assay

To determine the rate at which magnets pull down IOPs containing different concentrations of SPIONs, a cylindrical neodymium magnet (NdFeB Grade N52, 1/4" dia. x 1/8" thick, 5233 Gauss thru thickness; K&J Magnetics, Pipersville, PA) was placed in the bottom of each cuvette containing 2 mL of IOP solution. The optical density of the solution (OD) was measured at 1-2 minute intervals using a spectrophotometer (SpectraMax M3, Molecular Devices, Sunnyvale, CA) until the OD reached a plateau, typically 4 – 24 hours. As a control, OD curves were

obtained for hydrophilic dextran-coated iron oxide magnetic nanoparticles (5-10 nm, Ocean NanoTech, San Diego, CA) diluted to contain an equivalent iron oxide concentration (25 - 100 µg/mL). The resulting OD curves were fitted using a GRG Nonlinear solver to an exponential decay equation of the form:

$$OD = A \cdot \exp(-kt) + B$$

where OD is the optical density, t is time, and A, B, k are constants. The characteristic pull-down time was calculated as the inverse of the rate constant k .

Biofilm growth and treatment

Staphylococcus epidermidis strain RP62a (ATCC® 35984™; American Type Culture Collection, Manassas, VA) was hydrated and streaked for isolation on tryptic soy agar (Sigma-Aldrich, St. Louis, MO). For all bacteria experiments, a single colony was selected and used to inoculate 5mL of tryptic soy broth media (TSB; Sigma-Aldrich, St. Louis, MO), which was grown on a shaking incubator for 18 hours at 37 °C and 200 rpm. The resulting bacterial suspension was then serially diluted in TSB supplemented with 2% fetal bovine serum (FBS; American Type Culture Collection, Manassas, VA) to a cell density of 10^7 colony forming units (CFU)/mL, and 500 µL of this solution was seeded into each well of an 8-well Nunc™ Lab-Tek™ Chambered Coverglass slide (Thermo Fisher Scientific, Waltham, MA) for optical studies. Robust, mature biofilms were allowed to colonize on the surface of the coverglass under static conditions for 48 hrs at 37 °C and 5% CO₂. Biofilm treatment was performed by slowly aspirating no more than 400 µL of media (to avoid biofilm disruption), and then adding 200 µL fresh media plus 200 µL treatment solution (diluted as needed in 0.01 M PBS). Control wells received 200 µL of 0.01 M PBS as substitute for the treatment solution. For experiments

requiring an applied magnetic field, 8 square neodymium magnets (NdFeB Grade N52, 1/4" x 1/4" x 1/8" thick, 4933 Gauss thru thickness; K&J Magnetics, Pipersville, PA) were aligned with 8 individual coverglass chambers and mounted in a custom-milled polytetrafluoroethylene (PTFE) block. The coverglass was secured to the PTFE block and incubated at 37 °C and 5% CO₂ for the treatment duration. In order to determine minimum inhibitory concentration (MIC) for methicillin, biofilms and planktonic bacteria were treated with increasing concentrations of free drug (0-600 µg/mL). After 24 hours, biofilms were solubilized and 10 µL drops were plated on tryptic soy agar. MIC was defined as the lowest drug concentration to yield no colony growth.

IOP biofilm penetration

IOP penetration into live biofilms was analyzed using Prussian blue to stain for iron content. Following 4 – 24 hr treatment with IOPs, with and without the application of external magnetic fields, the biofilms were washed, heat-fixed for 60 min at 60 °C, treated with 2% (w/v) potassium ferrocyanide (Sigma-Aldrich, St. Louis, MO) in 0.6 mM hydrochloric acid (HCl; Sigma-Aldrich, St. Louis, MO) for 10 min, and washed. The biofilms were counterstained with 0.02% (w/v) pararosaniline hydrochloride (Sigma-Aldrich, St. Louis, MO) for 5 min and washed. The stained biofilms were photographed using an inverted widefield microscope (Eclipse TS100; Nikon, Tokyo, Japan) equipped with a color camera (PL-B625CU; PixelINK, Ottawa, Canada).

Confocal microscopy

Biofilm viability was visualized using confocal microscopy. Treated biofilms were washed with 0.85% sodium chloride (NaCl; Sigma-Aldrich, St. Louis, MO), incubated for 30 min with 250 µL of 0.85% NaCl containing 10 µM SYTO® 9 and 60 µM propidium iodide (Thermo

Fisher Scientific, Waltham, MA), and examined live using a laser scanning confocal microscope (LSM 700, 63× 1.4 N.A. Plan Apo oil objective; Zeiss, Oberkochen, Germany). Z-stacks were acquired at 0.6 µm intervals across the full biofilm thickness. The area occupied by live (green) and dead (red) bacteria was quantified image by image across the biofilm volume from binary images using Fiji v2.0.^{38–40}

Mammalian cell cytotoxicity

A MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI) were used in order to assess IOP cytotoxicity to normal human dermal fibroblasts (NHDF-Neo-Der Fibroblasts FGM-2, CC-2509; Lonza Group Ltd, Basel, Switzerland). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific, Waltham, MA), supplemented with 10% fetal bovine serum (FBS; American Type Culture Collection, Manassas, VA) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA). Fibroblasts ($\sim 25 \times 10^4$ cells/cm²) were seeded for 24 hr into chambered coverglass pre-treated with 5 µg/cm² fibronectin (Corning, Corning NY). Cells were treated with IOPs as described for bacterial biofilms, after which 400 µl of media was replaced with a 1:5 mixture of MTS reagent to media. After 3 hr incubation, the cell supernatant was transferred to a 96-well plate in triplicate and read at 490 nm with a spectrophotometer (SpectraMax M3, Molecular Devices, Sunnyvale, CA).

Scanning electron microscopy

For visualization of biofilm surface topography, biofilms were grown for 48 hr on sterilized squares of ACLAR® 33C (7.8 Mil thickness; Electron Microscopy Sciences, Hatfield, PA) and

processed using a method adapted from Erlandsen et. al.⁴¹ Briefly, biofilms were treated for 24 hr, washed in PBS, and fixed at 4 °C for 24 hr in 0.1 M sodium cacodylate buffer (Electron Microscopy Sciences, Hatfield, PA) containing 2.5% glutaraldehyde (Sigma-Aldrich, St. Louis, MO), 0.15% alcian blue (Electron Microscopy Sciences, Hatfield, PA), and 0.15% safranin O (Sigma-Aldrich, St. Louis, MO). The fixed biofilms were washed with 0.1 M sodium cacodylate buffer, infiltrated with 1% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA) for 2 hours, and dehydrated to 100% ethanol. The biofilms were dried using a critical-point dryer (Samdri-PVT-3D; Tousimis Research Corporation, Rockville, MD), mounted on specimen stubs, sputter-coated with 10 nm platinum (Cressington 208; Cressington Scientific Instruments, Watford, UK), and examined using a scanning electron microscope (S4800 SEM; Hitachi Ltd, Tokyo, Japan). Micrographs were acquired under high vacuum using an accelerating voltage of 3.0 kV.

Statistical analysis

Statistical significances were analyzed by student's *t*-test or one-way analysis of variance with $p \leq 0.05$ considered statistically significant. All data was expressed as mean $\pm$ SD.

SUPPORTING INFORMATION

Video 1. 3D reconstruction of live (green) and dead (red) bacteria in *S. epidermidis* biofilms treated with PBS for 24 hours in the presence of an external magnetic field. (AVI)

Video 2. 3D reconstruction of live (green) and dead (red) bacteria in *S. epidermidis* biofilms treated with IOPs containing 20 µg/mL SPIONs and 0 µg/mL methicillin for 24 hrs in the presence of an external magnetic field. (AVI)

Video 3. 3D reconstruction of live (green) and dead (red) bacteria in *S. epidermidis* biofilms treated with IOPs containing 40 µg/mL SPIONs and 0 µg/mL methicillin for 24 hrs in the presence of an external magnetic field. (AVI)

Video 4. 3D reconstruction of live (green) and dead (red) bacteria in *S. epidermidis* biofilms treated with IOPs containing 20 µg/mL SPIONs and 10 µg/mL methicillin for 24 hrs in the presence of an external magnetic field. (AVI)

Video 5. 3D reconstruction of live (green) and dead (red) bacteria in *S. epidermidis* biofilms treated with IOPs containing 40 µg/mL SPIONs and 20 µg/mL methicillin for 24 hrs in the presence of an external magnetic field. (AVI)

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Author contributions

B. G. conceived of the IOP concept, performed all experiments, interpreted the data, and wrote the initial manuscript; I. G. assisted with data acquisition, data analysis, and initial manuscript

preparation; S. S. was PI of the major supporting grant; A. V. provided scientific guidance, scientific mentorship, and edited the manuscript; T. W. provided overall intellectual guidance to the authors, supervised the interpretation of the results, and served as the primary mentor for B. G.

Competing financial interests

The authors declare no competing financial interests. The intellectual property described is protected by USPTO patent application 14/656261.

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ABBREVIATIONS

Technical abbreviations

IOP: iron oxide-encapsulating polymersome

OD: optical density

SPION: superparamagnetic iron oxide nanoparticle

Staphylococcus epidermidis: S. epidermidis

Chemical abbreviations

mPEG: methoxy poly(ethylene glycol)

MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

PBS: phosphate buffered saline

PDLLA: poly(D)-(L)-lactic acid

TSB: tryptic soy broth

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