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Catalytic antimicrobial robots for biofilm eradication

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Abstract

Magnetically driven robots can perform complex functions in biological settings with minimal destruction. However, robots designed to damage deleterious biostructures could also have important impact. In particular, there is an urgent need for new strategies to eradicate bacterial biofilms as we approach a post-antibiotic era. Biofilms are intractable and firmly attached structures ubiquitously associated with drug-resistant infections and destruction of surfaces. Existing treatments are inadequate to both kill and remove bacteria leading to reinfection. Here we design catalytic antimicrobial robots (CARs) that precisely and controllably kill, degrade and remove biofilms with remarkable efficiency. CARs exploit iron oxide nanoparticles (NPs) with dual catalytic-magnetic functionality that (i) generate bactericidal free radicals, (ii) breakdown the biofilm exopolysaccharide (EPS) matrix, and (iii) remove the fragmented biofilm debris via magnetic field driven robotic assemblies. We develop two distinct CAR platforms. The first platform, the biohybrid CAR, is formed from NPs and biofilm degradation products. After catalytic bacterial killing and EPS disruption, magnetic field gradients assemble NPs and the biodegraded products into a plow-like superstructure. When driven with an external magnetic field, the biohybrid CAR completely removes biomass in a controlled manner, preventing biofilm regrowth. Biohybrid CARs can be swept over broad swathes of surface or can be moved over well-defined paths for localized removal with microscale precision. The second platform, the 3D

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molded CAR, is a polymeric soft robot with embedded catalytic-magnetic NPs, formed in a customized 3D printed mold to perform specific tasks in enclosed domains. Vane-shaped CARs remove biofilms from curved walls of cylindrical tubes, and helicoid-shaped CARs drill through biofilm clogs, while simultaneously killing bacteria. In addition, we demonstrate applications of CARs to target highly confined anatomical surfaces in the interior of human teeth. These ‘kill-degrade-and-remove’ CARs systems could have significant impact in fighting persistent biofilm-infections and in mitigating biofouling of medical devices and diverse surfaces.

Summary

Magnetically-driven catalytic antimicrobial robots eliminate biofilm with precision and efficacy via synergistic ‘kill-degrade-remove’ mechanism.

Introduction

Biofilms, surface-associated bacterial communities, are notoriously difficult to treat and remove (1, 2). They form on abiotic surfaces (implants, catheters) and biotic surfaces (teeth, mucosal), often in difficult-to-access spaces, with deleterious effects including persistent infections and medical complications (3, 4). In addition, biofilms clog water lines, valves, pipes and crevices in industrial settings (2, 4). Biofilms contain bacterial cells surrounded by a matrix of extracellular polymeric materials, such as exopolysaccharides (EPS), which provide cohesion to the biofilm structure (2). These complex and mechanically stable scaffolds also act as barriers to antibacterial drugs, protecting bacteria within them (1, 4). The effective eradication of biofilms poses a significant technical and societal challenge. Current antimicrobial approaches are largely ineffective, as they fail to address simultaneously biofilm complex structural and biological properties associated with drug resistance (3), and their ability to rapidly re-establish themselves if biofilm debris and bacteria are not removed. Thus, new technologies are needed to effectively target the biofilm structure, kill the bacterial cells embedded within and remove the degraded products. Such a ‘kill-degrade-and-remove’ biofilm disruption approach may be achieved using robotics.

Robots, dynamic systems with controlled movement and force generation, have been explored in diverse applications ranging from drug delivery to precision therapeutics (5, 6). Magnetic robotic actuation is attractive since it allows tether-free controlled motion and enables a wide variety of robotic motility and locomotion strategies (7, 8). Under suitable conditions, magnetic fields easily and harmlessly penetrate most biological and synthetic materials, and can direct particle motion in confined spaces (5, 8). However, while robots have been developed for specific tasks such as targeted cargo delivery, manipulation of particles and cells, and multimodal locomotion (8, 9), applications for physical and chemical biofilm disruption have yet to be developed.

Here we develop catalytic antimicrobial robots (CARs) capable of performing multiple tasks for effective biofilm elimination by simultaneously targeting drug and mechanical resistance factors (Fig. 1a). The CARs’ catalytic activity chemically kills bacteria and degrades the biofilm matrix, and their magnetic actuation physically removes the biofilm from surfaces. These magneto-catalytic properties rely on iron oxide nanoparticles (NPs) (Fig. 1b) with

intrinsic enzyme-like (i.e. peroxidase) properties (10). These NPs catalyze hydrogen peroxide to generate reactive antibiofilm molecules (11, 12). Furthermore, iron oxide NPs in suspension or encapsulated in other structures can be directed to biofilm locations using magnetic fields (13, 14). With these principles, we present two small-scale robotic platforms that incorporate iron oxide NPs (Fig. 1c) to kill and clean biofilms on surfaces or in difficult-to-access, confined spaces. Robots drive along specific paths to precisely and controllably tear down and scrub away dead bacteria and biofilm debris. The ability to dictate the location of bactericidal action allows targeted biofilm disruption on surfaces or in inaccessible locations, and debris removal is central to the prevention of biofilm regrowth. The incorporation of the catalytic killing-degrading modality that acts in concert with magnetic properties introduces a conceptual framework for a robotics platform to achieve unprecedented ability to eradicate biofilms.

Results

Characterization and optimization of magneto-catalytic NPs

We developed robotic biofilm killing and removal platforms that exploit catalytic-magnetic NPs in a formulation designed to enhance their catalytic activity. Iron oxide NPs (213 ± 26.5 nm in diameter) catalyze hydrogen peroxide (H_2O_2) to generate free radicals that potentially kill biofilm embedded bacteria (10–12). These free radicals can also degrade the biofilm EPS matrix, albeit more slowly than their bactericidal action (15). The EPS degradation rate can be enhanced with EPS-degrading enzymes mutanase/dextranase that assist the breakdown of (α -1,3) and (α -1,6)-linked extracellular glucans in the biofilm (16). We thus formulated NP suspensions ($2000 \mu\text{g mL}^{-1}$) with 1% H_2O_2 and 1.75U/8.75U mutanase/dextranase for maximal efficacy of bacterial killing and EPS degradation (Supplementary Fig. S1). Confocal imaging reveals that entire clusters of bacteria were killed and the EPS-matrix was effectively dismantled after exposing the biofilm to the NP catalytic formulation (Supplementary Fig. S1). However, if not removed, biofilm debris serves as a reservoir of nutrients and facilitates attachment and growth of bacterial cells, reinitiating the biofilm life-cycle (2, 3). Thus, removal of the cellular and matrix debris is of paramount importance to persistent eradication of biofilms. This can be achieved by leveraging the magnetic properties of NPs to create catalytic antimicrobial robots.

To characterize the magnetic force on the dispersed NPs, their motion in a viscous medium is studied (Fig. 2a). A cylindrically shaped permanent magnet of diameter 2 mm was placed beneath the NP suspension ($200 \mu\text{g NP}$ in $100 \mu\text{L}$ of 50% glycerol), and the particle accumulation was recorded. The grey scale of the suspension (Fig. 2a1, a2) serves as a proxy for the particle concentration, where $x=0$ is the center of the magnet (details in Supplementary Fig. S2). The NPs accumulate above the magnet with pronounced peaks near the magnet's sharp edges, where the field gradients are highest. A balance of viscous and magnetic forces allows the magnetic force on a $2 \mu\text{m}$ diameter aggregate of NPs to be estimated to be $\sim 30\text{pN}$ (details in Materials and Methods). The particles form a cluster whose size depends on the concentration of NPs (Fig. 2b) and the movement of the permanent magnet defined by an automated displacement routine via a control algorithm for specific motion patterns (Fig. 2c). The control algorithms for this work are programmed

using open-loop control, in which motion patterns are preprogrammed based on modeling of system dynamics (details in Materials and Methods).

We then dispensed the same concentration of NP dispersion ($2000 \mu\text{g mL}^{-1}$ NP) over the biofilm-covered surface, and assessed the bioactivity and motion of the nanoparticles without and with catalytic activation. Time-lapse imaging revealed that the NPs (absent catalysis) on the biofilm fail to aggregate in the presence of the magnetic field. These NPs do not move in response to the magnet (Fig. 3a upper panel) as observed in glycerol using the same magnet. Rather, upon contact with the biofilm, the NPs adhered and were unable to form a cluster (Fig. 3a/3a1). Furthermore, NPs without catalytic activation neither killed bacteria nor degraded the EPS (Fig. 3b, Supplementary Fig. S1). Upon catalytic activation, however, NPs not only kill the bacteria and degrade the EPS, but also adhere to and interact with the biofilm debris to form a cluster in the presence of the magnet (Fig. 3a bottom panel/ 3a1, Supplementary Fig. S1). Quantitative analysis shows that the size of the cluster increases with time (Fig. 3a1), indicating a critical role of both catalytic activity and magnetic actuation for NPs aggregation. Furthermore, high-resolution confocal microscopy reveals that the NPs form an intriguing superstructure in this setting whereby micrometer scale, rod-like oriented NP aggregates are enmeshed with biofilm debris (Fig. 3b1, Supplementary Fig. S3) to form a bio-inorganic hybrid assemblage.

Biofilm removal by biohybrid Catalytic Antimicrobial Robots (CARs)

Motivated by these findings, the NP dispersions were used in a stepwise approach to “kill-degrade-and-remove” biofilms (Fig. 4a/4b). NPs suspended in a solution of H_2O_2 and enzymes were placed onto the biofilm. After catalytic disruption of the biofilm was initiated, the NPs were pulled toward the degraded biofilm-covered surface using a permanent magnet. Orthogonal confocal imaging showed that these rod-like biohybrid aggregates assembled across the biofilm thickness to form vertically-oriented bristles via magnetic field gradients (Fig. 4a). This cluster was then moved over the surface via a Siskiyou three-axis manipulator with 0.1 micron precision and point-to-point accuracy of 2 microns. During this process, the cluster morphs into a C-shaped aggregate (Fig. 4b1), to plow through and remove the biofilm (Fig. 4b2). Thus, the NPs become building blocks for robotic superstructures formed by directed assembly under the action of the magnetic field. The assembly formation relies on NPs catalytic properties to break down the biofilm and kill the bacteria, their interactions with the biofilm debris to allow adhesion between these elements, and their magnetic properties to allow the superstructure to form in geometries influenced by the dynamics of the external field (Fig. 4b/4b1). These biohybrid CARs constitute our first robotic platform.

Different biofilm cleaning modes can be selected by tuning the size of the biohybrid CARs. For example, a 150 mm^2 wafer covered with biofilm can be swept clean by forming a 3.5 mm^2 biohybrid CAR and driving it methodically over the substrate using a permanent magnet coupled to a micromanipulator (Fig. 4a/b). Initially, 100 μL (200 μg of NPs) aliquot of dispersed NPs suspension was pipetted over the entire biofilm surface. After 30 min of catalysis-mediated biofilm disruption, the magnetic field was applied. The biohybrid CAR formed within a circular magnetic field established in the plane of the biofilm for 1 minute.

This field pulled the NPs through the biofilm to the biofilm-glass surface (Supplementary Fig. S4). Thereafter, the magnetic field was cycled to move the biohybrid CARs in a circular pattern across the surface. During this process, NPs were continually recruited from the bulk, increasing the size/density of the biohybrid CARs as the experiment proceeded (Supplementary Fig. S4). By defining a trajectory that starts at the center of the workspace and progressively sweeps outward in a concentric manner, the biohybrid CARs clear the biofilm biomass away from the contaminated surface (Fig. 4b/4b2). The absence of bacteria over the surface after the biohybrid CAR's displacement was confirmed with lack of fluorescent signal from SYTO 9-labeled bacteria (Fig. 4b2 far right panel), indicating the ability of large-scale cleaning (at the centimeter scale). It is imperative that bacterial cells are killed, that the biofilm is degraded and that biofilm debris is removed to avoid regrowth of biofilm (1, 3, 4). Thus, we assessed whether biofilms could regrow following treatment with CARs by incubating the cleared surface in growth culture medium for additional 24 h (details in Materials and Methods). Neither viable bacterial cells nor biofilm regrowth was detected on surfaces treated with CARs (Fig. 4c; Supplementary Fig. S5), demonstrating exceptional efficacy of the robots for bacterial killing, and biofilm degradation and removal.

Smaller biohybrid CARs can remove biofilms along preferred paths, while leaving surrounding domains intact. This might be desirable, for example, to remove biofilms without damaging nearby host-tissues or to sample biofilms at specific pathological sites, in order to identify microbial composition. By placing a droplet of the dispersed NPs suspension near the location to be cleaned, the NP suspension can be concentrated near a focal point for highly localized biomass removal (Fig. 4d). To demonstrate this spatial targeting of specific biofilm sites, we generated specific patterns to displace a 2 mm² CARs biohybrid to remove biofilms following pre-determined trajectories, such as circle, square, and triangle (Fig. 4d1; Video S1). The biohybrid CARs clear paths for complete removal of biofilm-covered areas with micrometer-scale geometrical precision with distinctive patterns (Fig. 4d; 4d1; Video S1). High magnification confocal images confirm clearance of the cleaned area, delineated by sharp edges with distinct features such as curved contours, straight lines, and pointed patterns (Fig. 4d1 far right panels). Thus, the biohybrid CARs can be steered to sweep surfaces effectively clean of biofilm debris for broad swathes or localized removal through synergistic combination of catalytic and magnetic properties. The relatively large NPs used in this study are targeted toward topical use or for use on or in contaminated structures with difficult access. While these NPs have remanant magnetization, this property is not required for the biohybrid CARs. We have conducted additional experiments using Feraheme (ferumoxytol), an FDA-approved formulation (17) comprised of small dextran-coated iron oxide nanoparticles (<20 nm) with negligible remanant magnetization (Supplementary Fig. S6). Catalytic activation and directed motion of CARs based on these smaller NPs using magnetic field gradient are sufficient to breakdown and remove biofilms, although the mechanism of disruption and collection differs significantly from that with larger nanoparticles, which yield large aggregates and biohybrid superstructures. Nevertheless, the data collectively show that catalytic activity is key for bacterial killing and biofilm degradation, while programmed displacement of the CARs allowed them to collect debris as they proceeded along given paths. Further, the CARs

superstructure effectively removed biofilms, while the NPs without catalytic activation did not, indicating that magnetic properties alone are insufficient for complete biofilm removal.

Biofilm removal by small scale 3D molded CARs

Biofilms do not only form on accessible surfaces in exposed areas. Rather, they often form in inaccessible locations featuring confining geometries (catheters, water lines) and are challenging to eradicate, creating clogs that cause infection or block fluid flow. To address this need, NPs are embedded in soft, water-based agar hydrogel to form magnetically-driven 3D molded CARs to perform specific tasks such as to dislodge biofilms from the walls of cylindrical tubes or to drill biofilm clogs (Fig. 5). Printable and soft robotics enable the production of 3D structures with specific geometries with a single or few input commands (18, 19). Here we exploit a facile, single-step molding technique to fabricate customized 3D small-scale magnetic robots (20). This method allows incorporation of functional additives and materials without additional fabrication steps like those required in conventional 3D printing at concentrations that would interfere with processes that rely on UV activated cross linkers (21, 22). We incorporated NPs at high concentration (10% w/v) by filling millimeter-scale 3D-printed molds with a thermo-reversible gelifying agar polymer. We formed two shapes (Fig. 5a), including double helicoid CARs, formed from two helices wrapped around a central axis and vane-like CARs with fin-like structures around a central core. The first shape was inspired by the propulsion efficacy of helical propellers that can penetrate physical barriers (9), while the latter shape was motivated by the success of vane-shaped tools at disrupting biofilms (23) (Supplementary Materials).

Magnetic fields gradients can drive small-scale robots and control their locomotion (24). Furthermore, rotating magnetic fields generated by rotating permanent magnets or electromagnetic coils have been used to drive helical or screw-shaped small-scale robots through fluids (25, 26). By applying a magnetic torque, helical robots can be driven through soft tissues, gels, and blood clots to eliminate harmful blockages or to access interior spaces (27, 28). Here we use uniform-rotating magnetic fields generated by two pairs of nested Helmholtz coils (20) configured so that magnetic field strength and rotational frequency are controlled independently (Fig. 5b). In all experiments, robots are operated at the center of the workspace where fields are approximately uniform and measure 3.4 mT. Robots execute pre-programmed trajectories within a glass tube of length 450 mm and diameter 7 mm; the robot's position and velocity were captured by a high-speed CMOS camera (Materials and Methods).

Both robots are magnetized along their short axis (Fig. 5b1); this process relies on the NP remanant magnetization and the associated magnetic dipoles to achieve controlled rotation. As the magnetic field rotates, the robots realign with the magnetic field direction. The rotation of the vane CARs allows them to generate localized fluid shear stress and to scrub the walls of the tube (Supplementary Materials). Their translation relies on a magnetic field gradient along the axis, established by placing a permanent magnet at the end of the tube. The tube was filled with H_2O_2 /enzyme solution and incubated at room temperature for at least 5 minutes. Since the agar hydrogel used to form the 3D molded CARs is permeable to H_2O_2 , the reagent and NP catalyst within the robot form the bactericidal products

(Supplementary Fig. S7). As the rotating vane CAR is pulled through the tube, it makes direct physical contact with the biofilm and generates frictional and fluid shear stresses as it moves along the tube. The magnitude of the shear stress can be estimated based on the gap thickness and rate of rotation, and determined to be 0.062 Pa, in keeping with prior studies on removal of EPS-defective biofilms. (Supplementary Materials). Previously, we demonstrated that intact biofilms were only fractionally removed with high shear stress (1.78 Pa), while EPS-defective biofilms (EPS-digested or impaired EPS production) could be almost completely removed even with significantly lower shear force (0.1–0.2 Pa) (23, 29). Thus, disruption of EPS matrix is critical to efficiently dislodge biofilms. In this study, we showed that biofilms at the wall of glass tubing can be removed by the shear force (~0.06 Pa) generated by the vane-robot when the catalytic activity is present, consistent with our previous findings. By combined catalytic action and mechanical displacement, the rotating vane CAR driven into the tube can efficiently scrape and displace the degraded biofilm from walls, which forms a pile of debris (Fig. 5c1) that can be cleared by flushing the tubing with water (Supplementary Fig. S8).

The chiral geometry of the helicoid CAR enables forward motion from the applied magnetic torque (20) with a velocity proportional to the magnitude of the field and the rotation frequency (30) (Supplementary Fig. S9a). The helicoid CAR is propelled in a corkscrew-like fashion at an average velocity of 5 mm s^{-1} in the H_2O_2 /enzyme solution. This motion makes the helicoid CAR (Fig. 5a) suitable for drilling through biofilm occlusions and clearing biofilm clogs (Fig. 5d/d1; Video S2). To demonstrate this concept, we formed biofilms at the end of conical tubing to mimic clogging-plaque. Upon application of the rotating magnetic field, the helicoid CAR approached the clog, drilled through it, leaving a pile of biomass debris behind similar to a drill bit action (Fig. 5d1; Supplementary Fig. S9b; Video S2). The amount of removed biomass debris was plotted against various frequencies in Supplementary Fig. S8c, showing incremental increase of biomass debris with frequency. In addition, we demonstrated the ability of the helicoid CAR to remove occlusions and restore paths in biofilm-clogged tubes (Fig. 5d2).

To confirm that the biofilm eradication is due to the synergistic effects between the catalytic and magnetic properties of NPs, we have performed control experiments with the molded helicoid CAR without catalytic activation (Supplementary Fig. S10). Established biofilms present high physical resistance, making them difficult to mechanically disrupt them without the EPS degradation. Under identical conditions, the helicoid CAR without catalysis fails to remove the clog although biofilm disruption was observed as the robot cut through parts of the biomass from drilling. However, the bacteria cells released from physical drilling and those within the remaining biofilm clog, remained viable. In contrast, CAR with catalytic activation completely removed the biofilm clog and killed all bacteria, further underscoring the need for the degradation reaction as well as the robotic motion. It is noteworthy that mechanical disruption without bacteria killing could be detrimental in some settings, as live bacteria would be released, increasing the risk of re-colonization and reinstatement of biofilms. These results demonstrate that catalytic NPs laden robots with designed geometries can be propelled using magnetic torques to dismantle biofilms occluding tubes or on bounding surfaces, and simultaneously kill the dislodged bacterial cells.

We envision using CARs for effective treatment of biofilms on biotic and abiotic surfaces, a major unresolved problem that spans over several scientific fields (dentistry, medicine, engineering) causing infectious diseases and biofouling in medical devices (Fig. 6a). To illustrate practical applications, we demonstrate the potential use of CARs to access the interior of human teeth (Materials and Methods). By using aggregated NPs, we demonstrate application of the biohybrid CAR system to one of the most challenging anatomical areas of teeth, termed the isthmus; this is a narrow gap (on order of hundreds of micrometers in width) between the root canals where bacterial biofilms often form. Currently available methods are unable to physically access or disinfect this anatomical region. In our demonstration, we first place a droplet of the dispersed NPs suspension in the left side of the isthmus (Fig. 6b), and then we move them using a permanent magnet coupled to a micromanipulator as described earlier (Fig. 4d). We observed aggregated NPs readily traversing the isthmus as directed by the external magnetic field (Fig. 6b). Then, we demonstrate disruption of biofilms in the isthmus using fluorescently labeled biofilms by moving the biohybrid CARs in a back and forth motion (bottom middle panels in Fig 6b). For 3D molded CARs, we show actuation of a miniaturized 3D molded CAR through the canal of the tooth, another common location of dental biofilm formation (Fig 6d). Together, these examples illustrate feasibility and potential applications of controlled movement and specialized shapes to access, move and target harmful biofilms.

Discussion

In summary, we have introduced catalytic antimicrobial robots (CARs) as a novel modality for biofilm disruption and removal that can effectively address both the biological and mechanical traits associated with biofilm recalcitrance and reinfection, a property unattainable using current approaches. By leveraging iron oxide NPs for their catalytic activity to kill and degrade the biofilm and driving CARs with external fields to physically disrupt the biomass and its debris, effective biofilm removal with high precision can be achieved using untethered, automated routines. In the first platform, biohybrid CARs are assembled from nanoparticles and biofilm debris into superstructures that, when driven by an external magnetic field, plow through and completely remove biofilms, preventing its regrowth altogether. This platform is suitable for biofilm removal from accessible surfaces. In the second platform, 3D molded CARs with specialized shapes are designed to remove biofilms from difficult-to-access spaces. Specifically, vane-shaped CARs remove biofilms from enclosed curved walls, and helicoid-shaped CARs drill through biofilm clogs. Finally, we demonstrate a practical application of CARs to target the interior of the human teeth, accessing and removing biofilms in a difficult to reach anatomical region termed isthmus. This robotic approach further advances previous iron oxide nanoparticle-based modalities that solely exploit their magnetic properties (6, 7, 31). These modalities include magnetic actuation to induce hyperthermia for enhanced cell killing (32–34), to deliver antimicrobial drugs loaded into ferromagnetic NPs (13, 32, 35) or to cause topographical disturbances of ferrofluid-coated surfaces for disruption of algae accumulation (14). However, complete biofilm elimination can be achieved by exploiting simultaneously their catalytic and magnetic activation. Thus, we propose the synergistic ‘kill-degrade-and-remove’ mechanism that underpins this remarkable antibiofilm performance. Such function provides a conceptual

framework for a robotic platform that can be used across multiple disciplines to precisely and controllably eradicate harmful biological structures in medical and industrial settings, including catheters and water lines. Since the biohybrid CARs robots entrain the biomass, they can be also applied in diagnostics by retrieving microbial samples for compositional analyses. In addition, applications beyond biofilm disruption are possible as the inherent catalytic activity of iron oxide nanoparticles has been used to detect/visualize (36, 37) and promote killing of cancer cells (17). Although this proof of concept work is still in early phase, we envision a modular and scalable robotics platform using iron oxide nanoparticles as building blocks to impart the catalytic and magnetic properties to the CARs. Additional mechanistic studies shall elucidate the role of remanant magnetization and catalytic activity of different nanoparticle sizes for robotic assembly and biofilm removal on different surfaces and surface geometries. Further optimization and production are feasible due to facile surface chemical modification and functionalization as well as low cost synthesis of these nanoparticles at large scale (36), which can be adapted or complement bacterial targeting and toxin neutralization approaches as reported recently (38). Magnetic nanomaterials with intrinsic catalytic 'enzyme-mimetic' properties may have applications in other robotic systems that require biological functionalities and therapeutic properties.

Materials and Methods

Synthesis of iron oxide nanoparticles (NPs)

Iron oxide nanoparticles (NPs) were synthesized in a solvothermal system and characterized as previously described (10). Briefly, 0.82 g of FeCl_3 was dissolved in 40 ml of ethylene glycol. Then, 3.6 g of sodium acetate was added to the solution with vigorous stirring for 30 min. The mixture was then transferred to a custom-built 50 ml teflon-lined stainless-steel autoclave and incubated at 200°C for 12 h. After incubation, the iron oxide nanoparticle precipitate was collected, rinsed several times with ethanol and then dried at 60 °C for 3 h. The synthesized nanoparticles were characterized using scanning and transmission electron microscopy (SEM, JEOL 7500F; TEM, JEOL 2010F, JEOL USA, MA, USA). The peroxidase-like catalytic activity was tested via an established colorimetric assay using 3,3', 5,5'-tetramethylbenzidine (TMB) as substrate, which generates a blue color with specific absorption at 652 nm as a result of reaction with free-radicals generated by NPs in the presence of H_2O_2 (10).

Bacteria killing and EPS matrix degradation by NPs

Biofilms were formed on polystyrene dishes with inner circular glass bottom (MaTek dish, MaTek Corporation, Ashland, MA, USA). *Streptococcus mutans* UA159, a biofilm-forming model organism and a well-characterized oral pathogen, was grown in ultra-filtered (10-kDa cutoff; Millipore, MA, USA) tryptone-yeast extract (UFYTE) broth at 37 °C and 5% CO_2 to mid-exponential phase (39). Each MaTek dish was inoculated with $\sim 2 \times 10^5$ CFU of actively growing *S. mutans* cells per ml in UFTYE containing 1% (w/v) sucrose at 37°C with 5% CO_2 for 19 h. Screening for optimal concentration of NPs and enzymes was performed to select the most effective bactericidal and EPS degradation activity (as detailed in Supplementary materials and Supplementary Fig. S1) for a formulation designed to enhance the catalytic bioactivity. The optimized concentrations of NPs ($2000 \mu\text{g mL}^{-1}$) with H_2O_2

(1%, v/v) and enzymes (dextranase/mutanase; 8.75/1.75 U mL⁻¹) were used for the biofilm disruption experiments. High-resolution confocal fluorescence imaging and standard culturing method (number of viable cells by CFU determination) were performed to assess the bacterial killing and EPS degradation of the formulation of NPs with H₂O₂/enzymes (15, 39). NPs were topically applied onto the biofilm-covered surface followed by addition of 1% H₂O₂ (in 0.1 M NaOAc buffer at pH 4.5) and enzymes (dextranase/mutanase). For culturing method, total number of colony forming unit (CFU) per biofilm was determined following biofilm treatment. Briefly, the treated biofilms were removed and homogenized via water bath sonication followed by probe sonication (30 s pulse at an output of 7 W; Branson Sonifier 150, Branson Ultrasonics, CT, USA). Homogenized biofilm suspensions were serially diluted and plated onto blood agar plates using an automated Eddy Jet Spiral Plater (Neutec Group Inc, Farmingdale, NY, USA), and the numbers of viable cells were determined by CFU counting. For fluorescence imaging, SYTO 9 (485/498 nm; Molecular Probes, Carlsbad, CA, USA) and propidium iodide (535/617 nm; Molecular Probes, Carlsbad, CA, USA) fluorescent probes were used for labeling live and dead bacteria, while Alexa Fluor 647-dextran conjugate (647/668 nm; Molecular Probes, Carlsbad, CA, USA) was used for labeling EPS (39). Confocal images were acquired in the same field of view at 0 and 60 min using a Zeiss LSM 800 upright laser scanning microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) with a 20X (numerical aperture, 1.0) water immersion objective. Images were analyzed and processed using ImageJ and Amira 5.4.1 software (Visage Imaging, San Diego, CA, USA).

Modeling of magnetic flux density, and actuation and characterization of NP aggregates

In experiments with nanoparticles in suspension, NP aggregates were formed and actuated using a cylindrical permanent magnet through pre-programmed planar trajectories immediately below the surface. The permanent magnet was mounted to the arm of a Siskiyou four-axis nanomanipulator. The permanent magnet attached to the micromanipulator has a maximum magnetic flux density which measures 175 mT (Extech Instruments, MF100, Boston, MA, USA). In order to characterize the forces on the NP aggregate, we mathematically modeled magnetic flux density on a glass surface and observed NP aggregate motion within a model viscous fluid (glycerol). Using the maximum magnetic flux density of the permanent magnet along with its geometry, we developed a numerical simulation to compute the magnetic field using ANSYS software. Additionally, we applied 100 µL of NP suspension (concentration ranging from 250–2000 µg mL⁻¹ in 50 % (v/v) glycerol) over the glass surface, and exposed to a magnetic field generated by the permanent magnet for 300 s to characterize particle movement and size of the NP aggregate. Then, we moved the NP aggregate along pre-programmed trajectories coded in Python using custom libraries to confirm the proper actuation of the nanoparticles. Videos of the NP aggregate assembly and movement dynamics were captured using a PointGrey Flea3 Monochrome camera outfitted with a 6 mm Navitar lens at a video frame of 10 fps. Still images were also extracted and processed with Otsu's thresholding method using ImageJ. Then, the change in particle density and size of the clusters were quantified using MATLAB image processing libraries and ImageJ. We measured the rate of movement of a cluster of NPs in 50% glycerol (density 1126.3 kg m⁻³, dynamic viscosity 5 mPa s⁻¹) to approximate the maximum Reynolds number. The speed of a 2 µm cluster was 1.6 µm s⁻¹ in the region of

highest field gradient. The Reynolds number was on the order of 10^{-7} , well within the low Reynolds number regime. Thus, inertia was negligible and Stokes law was applicable. Given this velocity, the particle size and drag coefficient, the viscous drag force on a representative $2\text{ }\mu\text{m}$ NP aggregate was estimated, from Stokes law, to be $\sim 30\text{ pN}$. Since other forces were negligible, we estimated the magnetic force to be equivalent in magnitude to the drag force with opposite direction.

Furthermore, we also characterized the behavior of NP suspension and aggregate formation on the *S. mutans* biofilm. Biofilms were formed on the planar surface of a MaTek dish for 19 h as described in the previous section, and then treated with NP suspension. Biofilm cells were labelled with SYTO 82 (541/560 nm; Molecular Probes, Carlsbad, CA, USA), and the NP was detected via inherent reflectance optical property using confocal microscopy. To investigate NP motion and aggregation dynamics, experiments were conducted with either NP with catalysis or NP alone (without catalysis). One hundred microliters of enzymes solution (Dex/Mut; 8.75/1.75 U in 0.1M NaOAc pH 4.5 buffer) were applied on the biofilm surface, followed by 100 μL of NP suspension ($2000\text{ }\mu\text{g mL}^{-1}$ in 0.1M NaOAc pH 4.5 buffer) with 1% H_2O_2 and incubated at room temperature for 30 min. In NP alone, the same procedure was conducted using the same amount of NPs in NaOAc buffer without enzymes and H_2O_2 . After incubation, biofilms were exposed to a magnetic field generated by the permanent magnet for 120 s. Videos of the NP aggregate movement process on biofilms were captured using a Zeiss Axiovision fluorescence upright microscope system (Carl Zeiss Microscopy GmbH, Jena, Germany) or a Wild stereoscope (Wild Heerbrugg, Switzerland). Still images were processed and quantified using ImageJ. We also examined the detailed structural organization of the NPs in treated biofilms using a Zeiss LSM 800 microscope with a 20X (numerical aperture, 1.0) water immersion objective. NPs were detected via 405 nm laser with a 445/50 nm emission filter and biofilm cells were detected by 560 nm laser with a 590/60 emission filter. Images were processed and reconstructed by Amira and ImageJ. In addition, the bactericidal and EPS degrading activity of NP with catalysis or NP alone (without catalysis) was determined by culturing and fluorescence methods as described in the previous section. Biofilm analyses revealed that NP with catalysis kill bacteria and disrupt EPS, and the magnetic field assembles NPs and the biodegraded products into rod-like structures, which move in a controlled manner to generate a biohybrid catalytic antimicrobial robot.

Biofilm removal by biohybrid Catalytic Antimicrobial Robots (CARs)

Biofilms were formed on the planar surface of a MaTek dish as described above, and treated with catalytic NPs formulation. For large-scale (broad swathes) biofilm removal, 200 μg of NPs in 100 μL of 0.1M NaOAc pH 4.5 buffer solution with optimized H_2O_2 /enzymes was sequentially applied onto the biofilm surface. For precision cleaning, a small volume of concentrated NP suspension (100 μg in 20 μL) was applied in a specific location of the biofilm surface, followed by adding additional 80 μL buffer solution without disturbing the localized NP spot. After 30 min incubation, the NPs were exposed to magnetic field, and biohybrid CARs were locally assembled and driven along pre-programmed trajectories forming a biofilm-removing plow. Actuation in all experiments was performed via application of an external magnetic field programmed to achieve a desired motion pattern.

Sensing was achieved via a CCD camera, which can be used for visual feedback in a closed-loop system (40). The control algorithms for this work were programmed using open-loop control, in which motion patterns were preprogrammed based on modeling of system dynamics. The open-loop driving and control algorithms were chosen since these were simple and flexible, enabling a variety of locomotion with high precision. Videos of the removal process were captured using a PointGrey Flea3 Monochrome camera outfitted with a 6mm Navitar lens at a video frame of 10fps. After biofilm removal, the cleared area was imaged to confirm bacterial killing and complete removal of biomass from the surface using confocal fluorescence microscopy and culturing methods as detailed earlier. In addition, we investigated whether biofilm could regrow from the cleared area. After biofilm killing and removal by CARs treatment, we incubated the cleared area in UFYTE broth at 37 °C and 5% CO₂ for an additional 24 h. Biofilms treated with buffer or NP alone (without catalysis) were also incubated for 24 h to demonstrate bacterial viability and further biofilm growth in the absence of CARs. After incubation, biofilms were collected for confocal fluorescence imaging, quantification of the number of viable cells (CFU counting) and determination of total biomass (dry-weight).

Design of small scale 3D molded CARs

Robots were designed and fabricated using a 3D micro molding technique resembling injection molding method (20, 41). This process enabled direct fabrication of custom, shape-specific millimeter-scale robots which were composed of agar gel (Difco, BD Biosciences, Sparks, MD, USA) embedded with NPs. Briefly, two-piece negative molds were designed in SolidWorks and 3D printed using a ProJet 6000HD stereolithography printer. Two different robot shapes (helicoids and vanes) were designed for operation in cylindrical tubes. Agar gel was heated above 80°C, was vigorously mixed with NPs, and was poured into the mold. The resulting robot profiles solidified at room temperature. The final robot composition is 3% (w/v) agar and 10% (w/v) NPs. The smallest robots used in the tooth included 25% glycerol to reduce drying and warping prior to mold extraction. Robots were manually removed from the molds and pre-magnetized along their short axis with a neodymium iron boron (NdFeB) magnet. For the results presented in this work, CAR sizes are selected for given application spaces, including biofilm-coated tubes (7 mm diameter), or internal architecture of the tooth (canal: 1.5–1.8mm diameter).

Actuation and control of small scale 3D molded CARs

Small scale CARs produced via micromolding were driven using uniform, rotating magnetic fields which are generated using two pairs of nested Helmholtz coils as previously described (20). Each coil pair was driven independently using a programmable power supply and a custom program written in C⁺⁺. The magnetic field strength and rotational frequency were controlled independently. The robots were magnetized perpendicular to their long-axis or direction of travel. Rotating magnetic fields were applied to realign the robots. In all experiments, magnetic fields rotating at 2 or 4 Hz were applied, and robots were operated at the center of the workspace where fields were approximately uniform and measure 3.4 mT (Extech Instruments, MF100, Boston, MA, USA). In addition, a magnetic field gradient was applied using a permanent magnet to drive forward the vane CARs or to provide additional strength for the helicoid CARs to drill through the biomass clog. Robots executed pre-

programmed trajectories within a glass tube measuring 600 mm long and 7 mm in diameter. Experiments were recorded at 10 fps using a Point Grey Flea3 Monochrome camera mounted adjacent to the outer coils such that the entire length of the tube is in the field of view. In order to determine the robot's position and velocity, videos were processed using standard OpenCV libraries.

Biofilm removal by small scale 3D molded CARs

Pasteur Pipets (Pyrex Borosilicate Glass, VWR International, PA, USA) were cut to have specific dimensions: 1) 450 mm length and 7 mm diameter with open end (Type I); 2) 550 mm length with closed conical end (Type II). Then, each customized Pasteur Pipet was sterilized by autoclaving, and inoculated with approximately 2×10^5 CFU of *S. mutans* per ml in UFTYE containing 1% (w/v) sucrose at 37 °C with 5% CO₂. In order to form a biofilm which coats the walls of the tube, 2 ml of the inoculated culture medium was added to Type I tube. Biofilms were cultured for 42 h and the culture medium was changed at 19 h and 29 h. Alternatively, 200 µl of the inoculated culture medium was added to Type II tube to form biofilm clogs at the end of the conical portion of the tube. In addition, biofilms were also prepared to mimic clogs in the center of the tube. To achieve this, pre-formed biofilms that were cultured for 42 h on MaTek dishes were harvested and transferred to the open end of the Type I tube using a spatula to create a biofilm clog. Then, the occluded end of the tube was connected with transparent Tygon tubing (6.4 mm ID, 9.5 mm OD) (AAA00017, Cole-Parmer, Chicago, IL, USA). The biofilm was labelled with green fluorescent protein (SYTO 9) for visualization and quantification purposes. Helicoid and vane robots were driven along pre-programmed trajectories to clear biofilms from the inner tube wall surface and to ablate biofilm clogs. Videos of the removal process were captured using a frame rate of 10 fps (Point Grey Flea3 Monochrome, 6mm Navitar lens). Illumination of the experimental workspace was done with blue excitation LEDs (470 nm) along with an amber acrylic emission filter (McMaster-Carr), which enabled a fluorescence imaging of biofilms. Image frames were processed to determine the amount of biomass removed by the robot. Biofilm relative accumulation was determined by integrating the green channel value in the pixels of an increasingly rectangular area formed as the robot advances through the tubing. This was done using custom libraries for image analysis in Python (skimage, PIL).

Application of CARs in human tooth model

To illustrate practical application of CARs, we demonstrate the potential use of CARs to access the interior of human tooth using discarded extracted teeth. Two different preparations were used; i) cross-section for isthmus and ii) longitudinal section for root canal. To prepare the specimens, the crowns of single root teeth were removed at the cemento-enamel junction using a 0.6-mm-thick precision diamond saw (Isomet 5000; Buehler Ltd, Lake Bluff, IL) at 1000 rpm under water-cooling. Canals were enlarged using XP-Endo 3D Shaper (Brasseler USA, Savannah, GA) at 1000 RPM followed by Endosequence files size #40, #50, #60 (Brasseler USA, Savannah, GA). We then prepared root vertical sections by vertically sectioning the root segments using a 0.6-mm-thick precision diamond saw (Isomet 5000; Buehler Ltd, Lake Bluff, IL) at 1000 rpm under water-cooling. The canal space was then enlarged with Gates Glidden drill #6 (Tulsa Dentsply, Tulsa, OK) at 300 rpm under water-cooling to produce a canal space diameter of ~1.5mm.

Horizontal isthmus sections were prepared by sectioning the coronal segment of the mesial root of the first molar using a 0.6-mm-thick precision diamond saw, after visual confirmation of the presence of the isthmus, the canals on the horizontal sections were enlarged using Gates Glidden drill #6 (1.5 mm in diameter) at 300 rpm under water-cooling. Vertical and horizontal sections were then treated with 6% NaOCl followed by 17% Ethylenediaminetetraacetic acid solution (Vista Dental Products USA, Racine, WI) in an ultrasonic bath for 10 minutes each, followed by a 10 min bath sonication in sterile water to remove the residual effect of the chemicals. Specimens were then sterilized in an autoclave for 20 minutes at 121° C. To demonstrate movement of the CARs inside the isthmus specimen, the specimen was placed in a 2 ml of water in Matek dish, and 1 µL of NP suspension ($5000 \mu\text{g mL}^{-1}$ in 0.1M NaOAc pH 4.5 buffer) was topically placed at one canal end in the isthmus. Then, the sample was exposed to a magnetic field generated by the permanent magnet mounted on a four-axis nanomanipulator as described in the previous section. Then, videos of the NP movement in isthmus were captured using a Zeiss Axio Zoom.V16 fluorescence upright stereo zoom microscope system (Carl Zeiss Microscopy GmbH, Jena, Germany) with a 1X objective (numerical aperture of 0.25). To demonstrate biofilms disruption, isthmus samples were inoculated with $\sim 2 \times 10^5$ CFU of *S. mutans* or *E. faecalis* per ml in 24 well culture plate containing LMW media and 1% (w/v) sucrose at 37 °C with 5% CO₂ for 18h. The specimens were then washed in 0.89 % NaCl sterile saline solution using dip washing method to remove unattached cells. The samples were treated and exposed to a magnetic field as described in the previous section. We examined biofilm removal on the isthmus before and after CARs actuation using a Zeiss LSM 800 microscope with a 10X (numerical aperture, 0.4) air objective. Tooth was detected via 405 nm laser with a 445/50 nm emission filter and biofilm cells were detected by 488 nm laser with a 500/40 emission filter. Images were processed and reconstructed by ImageJ. To demonstrate movement of the CARs inside the vertical canal sample, miniaturized 3D molded helicoidal CARs were prepared using 1 mm molds. Since it was a water-based hydrogel, it dehydrated in ambient conditions prior to experiments resulting in a slightly smaller size of $\sim 700 \mu\text{m} \times 1.5 \text{ mm}$, which is consistent with our prior methods (20, 41). The specimen was placed horizontally in 3 ml of water in Matek dish and the robot was placed inside the canal, a cover glass was placed on top of the specimen to create a seal. The robot was driven using uniform, rotating magnetic fields which are generated using two pairs of nested Helmholtz coils as described in the previous section.

Statistical analyses

The data were analyzed by pairwise comparisons of multiple groups with regression models using ranked values. Kruskal-Wallis test (non-parametric and based on ranks) was used for two-group comparisons. The significance levels were set at 5%, and no adjustments were made for multiple comparisons. SAS statistical software, version 9.3 (SAS Institute, Cary, NC) was used to perform the analyses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Catalytic and magnetic iron oxide nanoparticles (NPs) as building blocks for small-scale robots designed for biofilm killing and removal.

(a) Diagram illustrating the challenges of biofilm removal due to EPS matrix that provide a protection against antimicrobials and mechanical stability (3, 4). (b) Diagram depicting the magnetic-catalytic NPs and their bacterial killing and EPS degradation mechanisms via reactive free radicals generated from hydrogen peroxide (H_2O_2) via peroxidase-like activity. The EPS degrading activity is enhanced by addition of mutanase/dextranase to digest extracellular glucans. (c) To kill and remove biofilms, catalytic-magnetic NPs in suspension serve as multifunctional building blocks to form Catalytic Antimicrobial Robots (CARs). In the first CAR platform, biohybrid CARs are assembled from NPs suspended in H_2O_2 and mutanase/dextranase solution by a permanent magnet attached to a micromanipulator, and

used to remove biofilms from accessible surfaces. The NPs form bristle-like structures that can be moved in a controlled manner for biofilm removal. In a second platform, catalytic-magnetic NPs are embedded into gels to form 3D molded CARs having specialized vane and helicoid structures. 3D molded CARs are designed to remove clogs and biofilms in confined and inaccessible locations.

Figure 2. Characterization of NPs in a model solution.

(a) Cylindrical magnet placement using a micromanipulator and magnetic flux density; (a1) NP cluster in suspension before and after magnetic actuation, and (a2) time-lapse quantification of NP cluster using MATLAB; (b) Surface area of the cluster vs suspension density of NP determined from still images 300s after magnetic flux application using an optical thresholding method; (c) Motion of NP cluster under magnetic control. Initially, a NP cluster with a circular footprint is formed, centered above the magnet. Suspended NPs move toward the NP cluster which is moved in a pre-programmed trajectory, clearing the solution.

Figure 3. Characterization and formation of biohybrid CARs.

(a) NP actuation in biofilm in presence of magnetic field. *Streptococcus mutans*, a biofilm-forming model organism and a bacterial oral pathogen, was used to form biofilms. NPs, absent catalysis, do not aggregate owing to adhesive interactions with the biofilm. Conversely, NPs with catalysis degrade the biofilm and move toward the magnet to assemble into a cluster. (a1) Quantitative imaging analysis shows the size of cluster in NP with and without catalysis (as shown in a). (b) Data from bacterial viability assays using fluorescence imaging and culturing methods. Green fluorescence indicates live bacteria and red indicates dead bacteria, and the number of viable cells as determined by colony-forming units (CFU) counting. The data show that NPs alone are devoid of antibacterial activity, whereas NPs with catalysis potentially kill bacteria. (b1) Biohybrid CAR superstructure is revealed by high resolution confocal microscopy. Absent catalytic activity, only small NPs assemblages form (top panel). With catalytic activity, biohybrid CARs form; they consist of a complex bio-inorganic hybrid assemblage comprising spatially oriented, rod-like, micron-size superstructures of NP enmeshed with bacterial mass and debris (bottom and right panels).

Figure 4. Platform 1: Biofilm removal using biohybrid CARs.

(a) Orthogonal view of biofilms treated with biohybrid CARs reveals the rod-like superstructure forming pillars (highlighted by white dashed lines) through the compromised biofilms. After incubation, biohybrid CARs are manipulated precisely using a magnetic field gradient to completely remove biofilms and biofilm debris including dead bacteria and degraded EPS from surfaces. (b) Diagram depicting cleaning of large areas of a biofilm coated surface by magnetically controlled sweeping of biohybrid CARs (after bacterial killing/EPS degradation); (b1) The biohybrid CAR morphs into C-shaped aggregate (see

arrows) as it moves over the surface to plow through and remove biofilm (labelled with SYTO9-green fluorescence). (b2) Fluorescent images indicating complete cleaning of an *S. mutans* biofilm (labeled in green) grown on a glass surface by using biohybrid CARs sweeping effect. (c) Biofilm-removed surfaces were incubated for additional 24 h with the biofilm growth medium (supplemented with 1% sucrose) to assess bacterial regrowth using confocal microscopy and culturing methods. There is no biofilm re-growth on biofilm-removed surfaces by CARs even after 24 h incubation. Control and NP treated biofilms were also incubated using the same conditions, both showing abundant bacterial cells (in green) and EPS (in red); quantitative analyses show high amounts of biomass and high number of viable cells. Quantitative data also confirm that there is no detectable biomass or viable cells with treatment by CARs. (d) The controlled movement of focused biohybrid CARs results in a precise removal of biofilms from surfaces with defined geometrical shapes. (d1) Fluorescent microscopy confirms the complete removal of bacteria from the surface.

Figure 5. Platform 2: Small scale CARs with functional shapes for specific biofilm disruption tasks.

(a) Model representations of vane-like and helicoid shaped CARs fabricated from 3D micromolding agar gel embedded with NPs. Both vanes and helicoids measure 5mm in diameter and 10mm long. The final robot composition is 3% (w/v) agar and 10 % (w/v) NPs.

(b) Helmholtz coil system used to drive 3D molded CARs through cylindrical tubes. Sinusoidal time-varying currents (I_x , I_z) are applied to each coil pair to generate uniform, rotating magnetic fields. Robots are driven using fields measuring 3.4mT and rotating at

4Hz. (b1) Robots are driven through the cylindrical tube with linear velocity $\mathbf{v}$, which is generated by applying a torque $\mathbf{T}$ to the robot body. The torque is generated when the magnetic dipole moment $\mathbf{M}$, seeks to align with the rotating field $\mathbf{B}$. (c) Schematic diagrams of biofilms on the wall of cylindrical tube, and wall cleaning by vane CAR. Vane CARs are used to clean the curved surfaces of glass tubes. Vane CARs are rotated with an applied magnetic torque $\mathbf{T}$, and are driven forward in the tube at a velocity $\mathbf{v}$ by applying a force using a magnetic field gradient. (c1) Vane CARs advance forward in the tube, sweeping the walls and generating a pile of debris behind them. The integrated fluorescence intensity from the biofilm debris increases with time behind the advancing robot. (Left) Accumulated biomass behind the vane CAR as it moves forward through the tube. (d) Schematic diagrams of biofilm clogs in cylindrical tubes, and drilling by helicoid CARs. Helicoid CARs are used to clean biofilms clogs at various locations within the glass tube. The robot is propelled along linear paths within glass tubes through an applied magnetic torque $\mathbf{T}$. Their forward propulsion is enabled by their chirality. (d1) Helicoid CARs advance forward in the tube to drill through the biomass clog. (Left) Accumulated biomass behind the helicoid CAR as it moves forward through the tube increases with time. The robot drills by overcoming the biofilm mechanical resistance associated with the conical shape of the biofilm (for $t < 30$ s for outer biomass removal and $t > 60$ s with additional force to penetrate the conical portion for the inner biomass removal) accumulated at the dead-end of the confined space. (d2) Helicoid CARs can also drill and restore biofilm-occluded paths. Fluorescent images showing the action and biofilm removal efficacy of vane-like (c1) and helicoid (d1/d2) robots: green color indicates *S. mutans* biofilms or clogs.

Figure 6. Potential applications for CARs platforms.

(a) CARs can be used to treat biofilms on biotic (e.g. teeth) and abiotic (catheter or implant) surfaces. (b) Demonstration of using CARs to access the interior of human teeth. Cross section of the tooth canal shows the isthmus, which is a narrow gap (300–600 micrometers in width) between the root canals. Longitudinal section (across the tooth length) shows the tooth canal. Biohybrid CARs can access isthmus, one of the most challenging anatomical areas of teeth, where bacterial biofilms are commonly found. Aggregated nanoparticles can readily transverse the isthmus as directed by the external magnetic field. Lower middle panels

show disruption of biofilms in the isthmus by CARS using fluorescently labeled biofilms.
(c) For 3D molded CARs, miniaturized 3D molded helicoidal robots can be magnetically actuated through the canal of the tooth, another common location of dental biofilm formation.