Gold nanoclusters augment a fluoroquinolone lethality against biofilm associated persister cells

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Keywords: Gold nanocluster, biofilms, persister cells, fluoroquinolones, Pseudomonas aeruginosa
Abstract

Persister cells are an important medical concern, leading to the overuse of antibiotics and ultimately contributing to antimicrobial resistance. The use of an adjuvant that augments the antibiotic efficacy has yet to be explored for combating persister cells within biofilm infections. Here we demonstrate a paradigm shift in targeting bacteria in chronic infections by coadministration of a conventional antibiotic with a novel engineered gold nanocluster (AuNC@CPP). AuNC@CPP was found to reduce the minimum biofilm eradication concentration (MBEC) of ofloxacin up to 300-fold (from > 3000 μg/mL to 10 μg/mL). Compared to ofloxacin alone (FLOXIN®Otic), coadministration of ofloxacin with AuNC@CPP induces up to a 10,000-fold reduction in bacterial burden in a validated mouse model of chronic P. aeruginosa of ear infection mimicking chronic suppurative otitis media. The biocompatibility of AuNC@CPP encourages efforts for development as an antibiotic adjunct for combating bacterial biofilm infections.
Biofilm formation, a tactic used by bacterial pathogens to evade drug treatment and the human immune response, is a growing threat to human health with a total annual cost in the USA at around $94 billion \(^1,^2^3\). Persister, non metabolic cells that remain after treatment in chronic bacterial infections are responsible for antibiotic tolerance within biofilms and lead to recurrence of infections when the antibiotic is ceased\(^4^6\). Biofilms shield persister cells from the immune system by providing a physical diffusion barrier. Over time, with repeated failed antibiotic attack on persister cells, development of antibiotic resistance occurs \(^7,^8\). Clinically, biofilm-related infections including chronic suppurative otitis media (CSOM), catheter-associated urinary tract infections, gastrointestinal infections, chronic cutaneous wounds and cystic fibrosis lung disease\(^9^1^0\) are often attributable to Pseudomonas aeruginosa \((P.\ aeruginosa)\) and can be difficult to cure with current antibiotic strategies.

Antibiotic resistance of \(P.\ aeruginosa\) is linked with recurrent or recalcitrant infections and failed eradication \(^1^1\). To address this, it has been proposed to target both growing bacteria and non-growing persister cells for more effective treatment of persistent infections\(^1^2\). Huge efforts have been taken to use antibiotics in combination with adjuvants targeting important metabolic pathways contributing to drug resistance (i.e., permeablisers, lactamase inhibitors, efflux pump inhibitors, quorum sensing inhibitors, toxin inhibitors etc.)\(^1^3^1^5\). These antibiotic adjuvants require an active metabolism to exert their action, thus resulting in effective killing of growing bacteria but not against non-growing persister cells. Therefore, there is need for a new antibiotic adjuvant specifically aimed at eradication of biofilm associated persister cells.

Induction of reactive oxygen species (ROS) production, especially hydroxyl radicals \((\text{HO}^\bullet)\) is a common mechanism by which bactericidal antibiotics kill their target cells\(^1^6^1^7\). Persister cells, however, respond to ROS stress by not producing \(\text{HO}^\bullet\) radicals\(^1^8\), instead
superoxide dismutase (SOD) activity converts the superoxide radical (O$_2^•$) into hydrogen peroxide (H$_2$O$_2$), which is finally converted to water (H$_2$O) by catalase$^{19}$. Indeed, the antibiotic tolerance in $P.$ aeruginosa biofilm is mediated by antioxidant scavenger enzymes and reduced production of the pro-oxidant 4-hydroxy-2-alkylquinoline (HAQ)$^{20}$.

Here, we designed gold nanoclusters that can potentiate a standard of care fluoroquinolone (ofloxacin) against biofilm associated persister cells. As the result of this potentiation, the bacterial burden in the middle ear with effusion of a validated mouse model of CSOM was reduced up to 10,000-fold below the level of bacterial colonies obtained upon topical administration of ofloxacin alone.

**Ofloxacin fails to eradicate in vitro biofilm from otopathogenic $P.$ aeruginosa.**

The advantage of topical therapy is the ability to deliver higher concentration of antibiotics to the treatment site when compared with oral or parenteral antibiotics. Thus, topical antibiotic administration is a therapeutic strategy proposed to treat problematic bacterial pathogens in a biofilm$^{21}$. To challenge this hypothesis, we used two strains of $P.$ aeruginosa, including the antibiotic sensitive wild-type reference strain PA01 and a clinical otopathogenic strain isolated from a CSOM patient (PA CSOM). Biofilms were challenged with a fluoroquinolone as they are commonly used to treat infections caused by $P.$ aeruginosa and the current standard of care in CSOM$^{22-24}$, the only available antibiotic effective at killing both growing and non-growing cells$^{25}$, and they have no restriction to diffuse into $P.$ aeruginosa biofilms$^{26}$. To demonstrate that the diffusion barrier of bacterial biofilm (dense cell cluster and sticky biofilm matrix) plays a minor role in defense against fluoroquinolone attacks, we first compared the biofilm formation capacity of PA CSOM and PA01. We quantify the biofilm formation capacity by the normalized biofilm matrix value (crystal violet stained biofilm absorbance at 595 nm,
A595/ total cell growth, optical density at 600 nm, OD600). We found that PA01 is the higher biofilm producer (Fig. 1a). We also found that the minimum inhibitory concentration (MIC) of ofloxacin against PA01 and PA CSOM were 3.3 μg/mL and > 52.8 μg/mL, respectively (supplementary Fig. 1). Resistance to ofloxacin was defined as a MIC ≥ 8 μg/mL, suggesting that PA CSOM is a fluoroquinolone-resistant strain 27, 28.

After confirming that PA01 produces more biofilm than does PA CSOM, we used these strains to determine the minimum biofilm eradication concentration (MBEC) of FLOXIN®Otic against 48 h old biofilms using the commercially available MBEC Assay® (Innovotech Inc. Edmonton, Canada). This new technology is useful for predicting clinical failure and clinical success of antimicrobial therapy against biofilm bacteria29,30 In this assay, the MBEC is identified when incubated recovery media has an optical density at 650 nm (OD650) ≤ 0.1 or no regrowth of bacteria when spotted on Luria broth (LB) agar plates (supplementary Fig. 2). Our results show that ofloxacin has a MBEC of 750 μg/mL (about 227 x MIC) against PA01 biofilm (Fig. 1b). This finding is consistent with that of Masadeh and colleagues who reported a MBEC of 640 μg/mL against 24 h old strain P. aeruginosa (ATCC 27853) biofilm31. Contrary to the results for PA01, no eradication of the PA CSOM biofilm was obtained after treatment with ofloxacin at 3000 μg/mL, the concentration in the commercial preparation (FLOXIN®Otic). The incubated recovery media from all concentrations tested shown an OD650 > 0.1 (Fig. 1c). As shown in the photograph (Fig. 1d), the treatment of PA01 biofilm with ofloxacin at 750 μg/mL leaves no viable cells compared to bacterial growth in the recovery media of PA CSOM after treated with ofloxacin at 3000 μg/mL. Given that PA01 produces more biofilm than PA CSOM, yet has a much higher susceptibility to ofloxacin, we concluded that the diffusion barrier of biofilm likely plays a minor role in the defense against fluoroquinolones. Of note, the maximum
concentration of ofloxacin in otorrhea of CSOM patients with a persistent purulent discharge ranged from 405 to 653 μg/mL at 8 h after topical administration of FLOXIN®Otic\textsuperscript{32}. The lack of in vitro susceptibility for FLOXIN®Otic of PA CSOM biofilms suggest that the drug will not eradicate persister cells in ears infected with this clinical otopathogenic strain of \textit{P. aeruginosa}, leaving the patients at continuing risk of recurrence. Our results highlight that delivering a high concentration of ofloxacin by topical administration does not always lead to eradication of \textit{P. aeruginosa} biofilms.

The resistance of PA CSOM to ofloxacin does not correlate with high catalase activity.

To establish whether the survival of PA CSOM biofilms was due to tolerance or strains that had acquired high level resistance to 3000 μg/mL of ofloxacin, the planktonic logarithmic-phase culture of surviving persister cells was challenged with 100 μg/mL of ofloxacin (Fig. 2). To be considered effective, the antimicrobial drug needs to kill $\geq 99.9\%$ equivalent to 3 log\textsubscript{10} CFU/ml reduction compared to the initial inoculum (no antimicrobial treatment)\textsuperscript{33}. When re-grown in the absence of ofloxacin, persister cells of PA CSOM became sensitive to 100 μg/mL of ofloxacin-mediated killing (i.e., 3 log\textsubscript{10} CFU/ml reduction), providing conclusive proof that the survival cells were not resistant to 3000 μg/mL of ofloxacin.

Fluoroquinolones potentially induce formation of reactive oxygen species (O$_2$•$^-$ and H$_2$O$_2$), which damage DNA, lipids and proteins causing cell death\textsuperscript{34, 35}. O$_2$•$^-$ is not very reactive with biomolecules but it does react rapidly with another molecule of O$_2$•$^-$ to form H$_2$O$_2$, which is stable and could diffuse through the bacterial cell membrane to form HO• radicals via the Fenton reaction (H$_2$O$_2$ + Fe$^{2+}$ $\rightarrow$ HO• + HO$^-$ + Fe$^{3+}$). To cope with the destructive nature of this oxidative process, catalases are needed in \textit{P. aeruginosa} persister cells to protect them from fluoroquinolones eradication\textsuperscript{36}. Given the protective role of catalases in \textit{P. aeruginosa} biofilm
resistance to H$_2$O$_2$, we hypothesized that strong catalase activity could protect PA CSOM against ofloxacin-induced HO$^*$ radicals \textit{via} the Fenton reaction. Comparing PA01 and PA CSOM planktonic cells, it can be seen that catalase activity was similar in both \textit{P. aeruginosa} strains (supplementary Fig. 3). Moreover, 3% H$_2$O$_2$ fails to eradicate planktonic stationary phase cultures of PA CSOM and PA01 (supplementary Fig. 4). It can therefore be concluded that protection against oxidative stress is not the main reason why ofloxacin at 3000 μg/mL fails to kill PA CSOM, but has a significantly high antimicrobial activity against PA01.

**Gold nanoclusters augment the lethal action of ofloxacin against biofilms and persister cells**

The maximum achievable physiological concentration of ofloxacin in the middle ear mucosa of CSOM patients after topical administration of FLOXIN®Otic is 602 μg/mL$^{32}$. The PA CSOM biofilm was able to survive up to 3000 μg/mL ofloxacin, approximately five times the therapeutically achievable concentration. Because novel antibiotic development takes a decade or longer, a way to effectively use currently available ofloxacin by lowering its MBEC into a range clinically achievable, provides a potentially shorter path to development. We engineered a peptide-gold hybrid nanocluster (AuNC@CPP) that comprises a cell-penetrating peptides (CPP) Ac-YGRKKRRQRRR-(β-Ala)-(β-Ala)-(β-Ala)-Cys-CONH$_2$ and thiolated polyethylene glycol with a carboxyl termination (an efficient protecting ligand that confers good stability to AuNC@CPP in solution as well as in biological systems). UV-Vis spectrum of AuNC@CPP showed a monotonous decrease from UV into the visible but no surface plasmon resonance peak at 520 nm indicative of the formation of ultrasmall particles (core diameter ≤ 2 nm)$^{37}$ (supplementary Fig. 5). The absence of surface plasmon resonance peak demonstrates that AuNC@CPP are ultrasmall with a core diameter ≤ 2 nm. AuNC@CPP exhibits an emission peak at 438 nm (emission energy 2.8 eV). According to the correlation number of gold atoms, N, per
cluster with emission energy, AuNC@CPP is an Au8 nanoclusters. Estimating the AuNC@CPP diameter (D in Å) using Equation 1, revealed that the gold core is approximately 0.63 nm. Characterization of AuNC@CPP by agarose gel electrophoresis shown that AuNC@CPP are negatively charged, so they move toward the positive electrode (supplementary Fig. 5).

\[ N = \pi D^3 / 102 \]  

Equation 1

AuNC@CPP alone exhibits a MBEC of 1600 μg/mL against 48 h old PA01 biofilms (supplementary Fig. 6). However, both AuNC@CPP and CPP alone were not able to eradicate PA CSOM biofilm at the concentrations tested (supplementary Fig. 7). When combined with AuNC@CPP (800 μg/mL), the MBEC of ofloxacin against PA CSOM was >300-fold lower than the MBEC of ofloxacin on its own (> 3000 μg/mL). As shown in the photograph (Fig. 3), the treatment of 48 h old PA CSOM biofilm with AuNC@CPP (800 μg/mL) alone, ofloxacin at 3000 μg/mL and combination of AuNC@CPP plus ofloxacin at 5 μg/mL did not eradicate the biofilm and associated persister cells. Remarkably, a combination of AuNC@CPP (800 μg/mL) and ofloxacin (10 μg/mL) leaves no viable cells. Thus, biofilms formed by P. aeruginosa strain resistant to ofloxacin can be eradicated using AuNC@CPP plus ofloxacin within a clinical achievable concentration. Similarly, the treatment of 48 h old PA01 biofilm with AuNC@CPP (800 μg/mL) plus ofloxacin at 5 μg/mL leaves no viable cells (150-fold reduction in MBEC) (Fig. 3). The fractional biofilm eradication concentration index (FBEC index) was calculated to evaluate synergism between AuNC@CPP and ofloxacin. The FBEC index shown that synergism occurs when AuNC@CPP and ofloxacin are combined (Table 1). Together, these findings highlight that there is much to be gained with existing drugs by taking advantage of synthetic nanotechnology.
Biocompatibility assessments

We used an industry standard MTT viability assay to test the cytotoxicity of AuNC@CPP against adenocarcinomic human alveolar basal epithelial cells (A549 cells). We found that cells exhibited more than 90% viability upon direct exposure to AuNC@CPP at 3200 μg/mL for 24 h (supplementary Fig. 8). Furthermore, we showed that administration of AuNC@CPP is unlikely to be of concern for systemic toxicity or induction of gastrointestinal illnesses if the entire dose pass the Eustachian tube (i.e., canal that connects the middle ear to the nasopharynx) and is ingested. Indeed, 35 days after administration by oral gavage at a dose of 10 mg/kg (or 1000 μg/mL) daily for 14 days, no statistical significance in body weight loss was seen between placebo control (PBS) and AuNC@CPP or between sexes (supplementary Fig. 9). In addition, there was no noticeable change in fecal form during the observations. AuNC@CPP does not prompt significant change in hematologic, liver and kidney function (supplementary Table 1 and 2). There was no change in almost all organ-to-body weight ratio (Figure 4c-h). The heart-to-body weight ratio was significantly increased in male treated AuNC@CPP group (Figure 4e). As it did not corroborate the histopathological data, it is not considered toxicity. Light microscopic examination of sections of organs of PBS (control) and treated AuNC@CPP group showed a normal histology and absence of any gross pathological lesions (Fig. 4). Further studies of increasing time exposure and doses are necessary to determine if toxicity occurs at higher doses or if there is a no adverse effects limit.

Reduction of bacterial burden in CSOM mouse model of chronic P. aeruginosa infection

To determine the clinical relevance of this therapy adjuvant, the in vivo efficacy of Floxin®Otic was compared with that of Floxin®Otic plus AuNC@CPP. We have chosen CSOM as an in vivo model of P aeruginosa biofilm-related infections given its significant global burden as the
leading cause of hearing loss in children in developing countries, with an incidence of 31 million cases and prevalence of >300 million\textsuperscript{42,43,44}. CSOM currently has no cure with end stage disease resulting in surgery, not available where resources are often limited or non-existent\textsuperscript{45}. Since the evaluation of potential therapeutics should be performed in animal models that mimic the course of human disease, we recently developed and validated a mouse model of chronic \textit{P. aeruginosa} of ear infection mimicking CSOM\textsuperscript{46}. The primary endpoint of our in vivo study was the level of bacterial colonies from effusion of the middle ear 14 days after stopping treatment. The treatment regime was chosen to mimic that already prescribed in the clinic. The antimicrobial treatments were commenced 14 days after inoculations were performed to create CSOM. An 8 \textmu L drop was placed in the ear canal twice a day for 14 consecutive days. Since the AuNC@CPP concentration used for the in vivo study was 5.4 times lower than the MBEC (1600 \textmu g/mL) against PA01 biofilm, we performed a superiority study with three arms, including the placebo control group (phosphate-buffered saline, PBS), FLOXIN\textregistered Otic (24 \textmu g of ofloxacin) and combination (24 \textmu g of ofloxacin + 296 \textmu g of AuNC@CPP). Administration of FLOXIN\textregistered Otic showed less than 90\% reduction equivalent to < 1 log\textsubscript{10} in the bacterial burden compared to placebo control (6.98 \pm 1.45 Log\textsubscript{10} CFU/mL vs 7.69 \pm 0.26 Log\textsubscript{10} CFU/mL) (Fig. 5 and Table 1). This finding provides support for the idea that when treatment for a chronic infection has started at least 7 days after infection, antibiotics failed to reduce \textit{P. aeruginosa} burden\textsuperscript{47}. In contrast, a combination of FLOXIN\textregistered Otic and AuNC@CPP led to 99.999\% reduction in the bacterial burden compared to placebo control (2.54 \pm 0.51 vs 7.69 \pm 0.26 Log\textsubscript{10} CFU/mL and p < 0.05) (Fig. 5 and Table 1). To be considered effective, an antimicrobial chemotherapy needs to reduce more than 99.9\% equivalent to 3 log\textsubscript{10} bacterial reduction compared to the placebo control. Therefore, the current treatments for CSOM fail and FLOXIN\textregistered Otic did not maintain clinical
benefit once treatment is stopped. Combination of AuNC@CPP and fluoroquinolone could lead to new ways of effectively treating chronic *P. aeruginosa* of ear infection associated with CSOM. Further research is planned to determine optimal dose and schedule for maximum in vivo efficacy.

**Surface-bound hydroxyl radicals augment the lethal action of ofloxacin against biofilms**

H₂O₂ can be catalytically decomposed to form HO• radicals on gold nanocluster surface and form surface-bound HO• radicals. We make use of the blue reaction product (maximum absorbance at 652 nm) formed in the reaction between HO• radicals and peroxidase substrate 3,3,5,5-tetramethylbenzidine (TMB) to probe the formation of surface-bound HO• radicals. AuNP@CPP catalyzes the oxidation of TMB in the presence of H₂O₂ (supplementary Fig. 10), thereby confirming that AuNC@CPP acts as a catalyst to generate of surface-bound HO• radicals. To relate the formation of surface-bound HO• radicals with lethal action of AuNC@CPP plus ofloxacin combination, HO• radicals was scavenged with thiourea during the treatment of PA01 and PA CSOM biofilms. The addition of thiourea preserved the viability of biofilms (supplementary Fig. 11), indicating that the formation of surface-bound HO• radicals on gold nanocluster contributes to the killing of biofilms and associated persister cells. These two observations provides support for the hypothesis that stimulating HO• radicals production could eradicate persister cells⁴⁸. To determine whether the eradication in persister cells was specific to AuNC@CPP or more generally associated to peroxidase-like activity of metal nanoparticles, a comparative study using iron oxide nanoparticles (Fe₃O₄; 797146 Sigma-Aldrich) with a peroxidase-like function was conducted. Combination of Fe₃O₄ and ofloxacin was unable to eradicate PA CSOM biofilms (supplementary Fig. 12), confirming that the eradication in persister cells was specific to AuNC@CPP.
Discussion

The fraction of persister cells in biofilms is usually low (~0.01%), and they should be distinguished from the metabolically inactive bacteria, which constitute a large fraction of the biofilm. Nanotechnology approaches for treatment of biofilm-associated infections fall into two categories, antibiotic loaded nano-systems and nano-systems for disassembling bacterial extracellular matrix. The goal of the former strategy is to improve the bioavailability of conventional antibiotics. This study, as well as prior work suggests that increasing the dose of the drug has little effect on persister cells death. This may explain why many antibiotic loaded nano-systems fail to eradicate established biofilms. The latter nanotechnology strategy is derived from the idea that alteration of the biofilm matrix makes persister cells more vulnerable to conventional drugs. In fact, antibiotic treatments often fail to eradicate planktonic persister cells of P. aeruginosa. As a pertinent example, the use of PLGA nanoparticles loaded with ciprofloxacin, a fluoroquinolone, was able to eradicate 99.8% of established P. aeruginosa biofilms. However, 0.2% of the cell population remained intact and constitutes a main source of persister cells. Furthermore, dispersed biofilm cells represent a distinct stage in the transition from biofilm to planktonic lifestyles and are highly virulent against macrophages compared with planktonic, non biofilm cells. A reminder of the clinical risk in this approach is that in vivo dispersion of mobile biofilm bacteria has been shown to cause fatal sepsis in the absence of antibiotic therapy in a mouse wound model. If the persister cells, which retain their phenotype for days or weeks after withdrawal from biofilm, are not directly addressed the chronic infection cycle will continue.

Synergistic combination of two antimicrobial agents appear particularly attractive in the case of P. aeruginosa biofilms. Disadvantages of combination therapy is that if tolerance has
already emerged to one drug, the combination may end up promoting the transmission of resistance to a partner drug\textsuperscript{60}. Therefore, tolerance is an important factor to consider in designing combination treatments that prevent the evolution of resistance. The ability of citrate-capped silver nanoparticles (AgNPs) in combination with the aminoglycoside antibiotic tobramycin or aztreonam, to prevent the recovery of PAO1 biofilms in vitro has become an attractive nanotechnology strategy\textsuperscript{61, 62}. However, emergence of \textit{P. aeruginosa} resistance to AgNPs motivated the use of ultrasmall AuNC as an antibiotic adjuvant\textsuperscript{63}. The antibiotic-adjuvant combination has the advantage of preserving existing antibiotics. The current work advocates that antibiotic-adjuvant combination could be a potential therapeutic option to target persister cells. Several lines of evidence suggest that persister cells accumulate at least some fluoroquinolones-induced damage probably through the contribution of HO\textsuperscript{*} radicals generated by bactericidal antibiotics\textsuperscript{64, 65}. We reasoned that the fluoroquinolones treatment may be clinically significant against persister cells if a way is found to boost intracellular production of HO\textsuperscript{*} radicals. Under acidic pathological conditions, the surface-bound HO\textsuperscript{*} radicals generated from decomposition of H\textsubscript{2}O\textsubscript{2} on AuNC@CPP mediated persister cell death. We also found that Fe\textsubscript{3}O\textsubscript{4} with a peroxidase-like function could not augment the ofloxacin efficacy against \textit{P. aeruginosa} biofilms. This data suggests that peroxidase-like activity is an important, but probably not the only factor that increases the effectiveness of ofloxacin when combined with AuNC@CPP. The planktonic \textit{P. aeruginosa} persister cells were not eradicated upon exposure to 3\% H\textsubscript{2}O\textsubscript{2}-induced oxidative stress. Paradoxically, several authors have speculated that oxidative stress mediated by induction of ROS generation is the dominant antibacterial mechanism of action for ultrasmall AuNCs\textsuperscript{66, 67}. Therefore, in this study, a line of research was highlighted for further investigations if the correlation between ROS production and antibacterial capacity is the
cause of bacterial cell death. The exact biomolecular mechanisms of how AuNC@CPP interact with bacterial cells have not been studied in detail. We plan on investigating these mechanisms in future studies.

We are aware of several limitations of this study. In particular, the use of unique murine infection models to demonstrate the antibacterial capability in vivo. It will be essential to establish whether AuNC@CPP-ofloxacin combination is more effective than ofloxacin alone in mouse model of chronic \textit{P. aeruginosa} of lung infection mimicking Cystic Fibrosis\textsuperscript{68}. Since gold nanoparticles did not cause ototoxicity\textsuperscript{69}, the possibility that AuNC@CPP could induce ototoxic effects was not assessed in the present study and therefore requires further investigation.

In conclusion, we have established a synergistic antimicrobial agent through the combination of AuNC@CPP and a fluoroquinolone. We have demonstrated that the combination of AuNC@CPP and ofloxacin was highly effective in a mouse model of chronic \textit{P. aeruginosa} of ear infection mimicking CSOM. Taken together, the results demonstrate the opportunity that ultrasmall AuNC offer for rescuing antibiotics from tolerance in biofilms.

**Methods**

**Synthesis of cell penetrating peptide (CPP)**

Ac-YGRKKRRQRRR-(β-Ala)-(β-Ala)-(β-Ala)-Cys-CONH\textsubscript{2} (CPP) was synthesized using an ABI 433A automatic peptide synthesizer on a 0.25 mmol scale by standard fluorenylmethyloxycarbonyl (Fmoc) solid-phase peptide synthesis (SPPS) on a Novabiochem NovaPEG Rink Amide resin. Post peptide synthesis, an acetyl group was coupled manually onto the N-terminus by adding Oxyma Pure and acetic anhydride in N, N-dimethylformamide (DMF). The Peptidyl resin was washed with N, N-dimethylformamide (DMF) and dichloromethane (DCM) and then dried. The modified peptide was cleaved from the resin with a trifluoroacetic
acid (TFA)/Phenol/triisopropylsilane (TIS)/water cleavage cocktail. The peptide was purified by reverse-phase high performance liquid chromatography (RP-HPLC) using Waters system. The molecular masses were determined by MALDI-TOF using the Voyager-DE RP Biospectrometry Workstation instrument (supplementary Fig. 13).

Synthesis of AuNC@CPP

Gold nanoclusters were synthesized according to the method published previously with some modifications70. Freshly prepared aqueous solutions of HAuCl₄ (20 mM, 1 mL) and thiolated polyethylene glycol with a carboxyl termination (PEG-COOH, 18 mg, 2 mL) and CPP (18 mg, 2 mL) were mixed in water (13.4 mL). After that, an aqueous NaOH solution (1 M, 1.2 mL) was added to the mixture. A freshly prepared NaBH₄ solution (112 mM) was obtained by dissolving 43 mg of NaBH₄ in 2 mL of NaOH solution (1 M), followed by the addition of 8 mL of ultrapure water. After that, 0.4 mL of NaBH₄ solution was added into the solution, and the AuNC@CPP were collected after 24 h. After synthesis, the solutions were dialyzed (dialysis membrane, MWCO = 3000) for 2 days against Milli-Q water, which was changed every 8 h to remove the unconjugated thiol PEG-COOH and CPP. The resulting AuNC@CPP were lyophilized and dried completely before further use.

AuNC@CPP characterization

UV–vis spectroscopy: Absorption spectra of AuNC@CPP was recorded in the visible domain of the electromagnetic spectrum (400–800 nm) using an absorption spectrophotometer (spectramMax M2, Molecular Devices, Downington, PA). Aqueous suspension of AuNC@CPP (800 µg/mL) was put in a 96-well polypropylene microplate of 1 cm optical path length. The emission spectra of as-synthesized AuNC@CPP were measured at excitation wavelength λex =
The overall charge of AuNC@CPP was determined using agarose gel electrophoresis (0.75%) in 0.5 x TBE (60 mL, pH 8) running at 10V/cm for 1 h.

Bacterial strains, growth media, and conditions
We used two strains of *P. aeruginosa*, including antibiotic sensitive wild-type reference strain PA01 and a clinical otopathic strain isolated from CSOM patient (PA CSOM). In all experiments, bacterial cells were cultured in 10 mL of Luria-Bertani (LB) broth at 37 °C and were aerated at 225 r.p.m in 50 mL plastic tube (polypropylene). Exponential phase cultures were prepared as follows: a stationary overnight culture was diluted 1:1,000 in LB and incubated at 37 °C with aeration at 225 r.p.m. until optical density at 600 nm (OD$_{600}$) = 0.3 was reached.

Minimum inhibitory concentration (MIC)
The MIC values were determined by the 2-fold serial broth microdilution method. Therefore, both *P. aeruginosa* planktonic strains (PA01 and PA CSOM) were pre-cultured overnight on a LB agar plate and were then introduced at an initial inoculum of optical density OD at 600 (OD$_{600}$) = 0.2 into LB medium, in a 96-well polypropylene microplate and incubated at 37 °C for 24 h. Growth media contained defined concentrations of ofloxacin in decreasing 2-fold steps. Subsequently, the bacteria growth in presence of ofloxacin was evaluated by measuring the OD$_{600}$ in a microplate reader (spectramMax M2, Molecular Devices, Downington, PA). The lowest concentration of antibiotic that inhibited visible bacterial growth after 20 h of incubation at 37°C was defined as the respective MIC.

Quantification of biofilm formation
Biofilms were prepared as follows: stationary overnight cultures of PA01 and PA CSOM were inoculated into wells of 96-well microtiter plates containing 150 µl LB medium. Inoculated plates were incubated at 37 °C without shaking for 24 h. After incubation, the growth was confirmed as the optical density at 600 nm (OD$_{600}$) using a microplate reader (spectramMax M2,
Molecular Devices, Downington, PA), after which the LB was removed from each well and the wells rinsed with phosphate buffered saline (PBS) to remove planktonic cells. Crystal violet (CV) solution (0.1%, w/v) was applied and the microtiter plate was incubated at room temperature for 10 min followed by PBS washing to remove the excess CV. 10% acetic acid was added to each well to extract the CV stain. Absorbance of CV staining was read at 595 nm ($A_{595}$) using the microplate reader. Results were analyzed as the ratio of the CV staining (biofilm matrix, $A_{595}$) to the total cell growth ($OD_{600}$).

Biofilm eradication assay

Overnight cultures of either PA01 or PA CSOM were diluted 1:1,000 in fresh LB medium and 150 μL was added to wells in a MBEC Assay®Biofilm Inoculator with 96 wells. Biofilms were allowed to develop onto Peg lids for 48 h without shaking. The peg lids were gently rinsed to remove planktonic bacteria and incubated in a new MBEC Assay®Biofilm Inoculator with 96 wells containing a serial dilution of tested solution in PBS. These MBEC Assay®Biofilm Inoculator with 96 wells were incubated for 24 h. Then the Peg lids were washed and sonicated for 15 min in a new MBEC Assay®Biofilm Inoculator with 96 wells containing fresh medium (recovery media). Following incubation for 48 h, the optical density at 650 nm ($OD_{650}$) was read using a microplate reader (spectramMax M2, Molecular Devices, Downington, PA). Wells with an $OD_{650}$ of less than 0.1 is evidence of biofilm eradication. The MBEC value was defined as the lowest concentration of drug that eradicates the biofilm ($OD_{650} > 0.1$). To confirm the MBEC, 5 μL of the recovery media was spotted on LB agar plates and incubated at 37 °C without shaking for 48 h.

Fractional biofilm eradication concentration index (FBEC index)
To determine the interactions that occurred between ofloxacin and AuNC@CPP combination the FBEC was calculated. FBEC index = MBEC of agent in combination / MBEC of agent on its own. Synergy is defined as $\sum$ FBEC $\leq$ 0.5. The $\sum$ FBEC > 0.5 to 4.0 is defined as no interaction and > 4.0 is defined as an antagonistic effect.

Cell culture and in vitro cytotoxicity assay

Human lung adenocarcinoma cell line (A549) was maintained in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin. The cells were incubated in 5% CO$_2$ humidified at 37°C for growth. The cytotoxicity induced by AuNC@CPP was investigated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. A549 cells ($2 \times 10^4$/ml, 100 μl/well) were seeded in 96 well plates. After 24 h, the cell culture was exposed to concentrations of AuNC@CPP ranging from 0 μg/mL to 3200 μg/mL (AuNC@CPP were dispersed in DMEM). After 24 h of incubation, medium containing AuNC@CPP was removed, cells were washed with PBS and incubated with fresh cell culture medium for another 24 h. Then, 20 μl of the MTT (5 mg/mL) was added to each well and incubated for 4 h in 5% CO$_2$ humidified at 37°C. The medium was removed carefully and 200 μL of dimethyl sulfoxide (DMSO) was added to each well to dissolve formazan crystals. Absorbance of formazan was read at 595 nm using the microplate reader. A blank solution (0 μg/mL of AuNC@CPP) was tested and no cytotoxicity could be observed. Three independent experiments and 4 replicates were performed. Results were analyzed as the average of viability (% of the untreated control) ± Standard deviation (SD).

In vivo toxicity evaluation

All animal work was approved by Stanford University’s Administrative Panel on Laboratory Animal Care. The 10-12-week-old C57BL/6J mice were purchased from Jackson Laboratories.
(Sacramento, CA) and housed in Stanford University’s animal resources facility according to standard guidelines in which food and water were provided ad libitum with the room maintained in 12-hour dark/light cycles. C57BL/6J mice female (n = 4 each group) and male (n = 3 each group) were divided into 2 groups, control (phosphate-buffered saline, PBS) and AuNC@CPP. The treatments were administrated by oral gavage at a dose of 10 mg/kg (i.e., 1000 mg/mL) daily for 14 days. During the 35-day study, the body weights of animals were measured every two days. At 35 days of post-treatment, mice were sacrificed. Blood and organs were collected. All organs were preserved, fixed in 10 % neutral formalin buffer, processed into paraffin embedding, and stained with hematoxylin and eosin for pathology analysis using a light microscope. The microscopic analysis of tissue (histopathology), including esophagus, stomach, heart, kidney, spleen, pancreas, thymus, small intestine, colon, bladder, testis and ovary were carried out for potential histological alteration. Results of the organ-to-body weight ratio (relative organ weight, ROW) were analyzed as the ratio of the organ weight (mg) to the body weight of the animal at necropsy day (g). The following organs were examined for ROW: thymus, spleen, heart, kidneys, liver and testis. Blood samples were subjected to toxicity analysis. An inferior vena cava blood collection was performed at sacrifice. 150 µl of blood was placed in a K2EDTA tube for hematology analysis and the remaining blood sample was placed in a 1.5 mL Eppendorf tube for serum extraction. Serum was separated by centrifuging the blood to remove the cellular fraction for liver and renal function testing.

In vivo efficacy testing

A validated mouse model of P. aeruginosa CSOM to test potential therapeutics was used46. Briefly, PA01 was incubated at 37 °C with shaking. After 30 h of incubation, the stationary phase culture was treated with FLOXIN®Otic (final concentration of ofloxacin = 5 µg/mL) for 4
h to select for persister cells. After creating a subtotal tympanic membrane perforation, we inoculated persister cells (5 µL, 1.6 × 10^8 CFU/mL) into the middle ear cavity and the mice were allowed to rest with the ipsilateral ear up until recovery. Infection was allowed to develop for 14 days before commencement of antibiotic therapy. All therapies were delivered by placing 8 µL drop in the ear canal twice a day for 14 consecutive days. Therapy outcome was measurement of the bacterial colonies (CFU/mL) from effusion of the middle ear 14 days after stopping treatment.

Production of hydroxyl radical via the decomposition of hydrogen peroxide by AuNC@CPP

The oxidation of peroxidase substrate 3,3′,5,5′-tetramethylbenzidine (TMB) by hydroxyl radical from the decomposition of hydrogen peroxide (H₂O₂) catalyzed by AuNC@CPP was carried out. The hydroxyl radical is able to oxidize TMB into oxidized TMB (Ox-TMB) with an absorption peak at 652 nm. Thus, the level of hydroxyl radical can be indirectly determined by measuring the absorbance at 652 nm. 500 µL of TMB substrate solution and 500 µL of H₂O₂ (30% solution) were added into 1000 µL 10 mM phosphate buffer (pH 4) containing 800 µg/mL of AuNC@CPP. The mixed solutions were incubated for 30 min at room temperature and absorbance of blue colored product was read at 652 nm using a microplate reader (spectramMax M2, Molecular Devices, Downington, PA).

Hydrogen peroxide survival assay

Overnight cultures of PA01 and PA CSOM were diluted to a starting OD₆₀₀ of 0.04 in 1 mL potassium phosphate buffer supplemented with 3% H₂O₂ solution in 1.5 mL microcentrifuge
tubes. After 24 hours of incubation, the cell solution was washed three times with sterile potassium phosphate buffer and 10 µL was cultured overnight on an agar plate.

Measurement of catalase activity

Planktonic stationary phase cultures (PA01 and PA CSOM) were centrifuged at 8,000 × g for 5 min at 4°C, washed twice with 1 mL of ice-cold potassium phosphate buffer (pH 7.2), and then resuspended in 1 mL of potassium phosphate buffer and transferred to an Eppendorf tube for sonication. The sonicate was then centrifuged at 13,000 × g for 10 min at 4°C to remove unbroken cells and cell debris, and the supernatant was transferred to a fresh tube. Total protein concentration was determined by using the BCA protein assay with bovine serum albumin as a standard. Catalase activity was determined by using the colorimetric catalase assay kits according to the manufacturer. The catalase activity unit is defined as the enzyme activity that decomposes 1 µmol of H_2O_2 per min at room temperature.

Statistical Analysis

Data are represented as the mean ± s.d. Statistical analyses (details in figure legends) were calculated with GraphPad Prism Ver. 6 (GraphPad, San Diego, CA). A p-value of < 0.05 was considered statistically significant. Supporting information is available.

Acknowledgements

Funding support from the National Institute of Health, National Institute of Allergy and Infectious Diseases (R21), Stanford’s Maternal and Child Health Research Institute, the Stanford SPARK translational medicine program, the Department of Otolaryngology, Head and Neck Surgery at Stanford University and the Stanford Initiative to Cure Hearing Loss through generous gifts from the Bill and Susan Oberndorf Foundation. Z.C. was supported by Shandong
Provincial Key Research and Development Program No.2019GSF108268. X.C. was supported by Henan Province Health Commission Research and Development Program. No.2016015.

Author contributions: L.A.B. and P.L.S.M. designed research and L.A.B. provided the supervision and oversight of this study; L.A.B., Z.C., J.C., X.C. and B.B performed research; J.T. contributed on cell penetrative peptide synthesis; L.A.B. designed and prepared the AuNCs; Z.C., J.C., L.A.B., A.X and P.L.S.M. analyzed data and wrote the paper. All authors have given approval to the final version of the manuscript. We also declare that L.A.B. and P.L.S.M. are listed inventors on a provisional patent regarding the antibiofilm activity described in this paper.
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**Figures caption**

**Fig. 1** Ofloxacin fails to eradicate PA CSOM biofilms. a) Comparison of biofilm formed (A<sub>595</sub>/OD<sub>600</sub>) from *P. aeruginosa* wild-type PA01 with otopathogenic strain (PA CSOM). After a 48-h incubation, PA01 show greater biofilm formation (**p≤0.001**). Data are presented as mean ± s.d. (n= 5). b-c) Attempted eradication of PA01 and PA CSOM in vitro biofilms by ofloxacin. PA01 is eradicated at 750 μg/mL, whereas PA CSOM is not eradicated at 3000 μg/mL. Data are presented as mean ± s.d. (n= 3). d) Representative petri dish showing persister cells resuscitation following ofloxacin treatment. Values refer to the concentration of ofloxacin (μg/mL). MBEC of PAO1 = 750 μg/mL compared to PA CSOM not susceptible to maximum concentration of ofloxacin (3000μg/ml).

**Fig. 2** Cartoon depicting the flowchart to evaluate ofloxacin sensitivity to persister cells resuscitation in logarithmic-phase culture. (1) Surviving persister cells following the treatment of PA CSOM biofilm with ofloxacin at 3000 μg/mL were placed in fresh media and incubated at 37 °C and were aerated at 225 r.p.m. (2) Cells in logarithmic-phase (OD<sub>600</sub> = 0.3) were treated with either phosphate-buffered saline (PBS) or ofloxacin (100 μg/mL) for 24 h. (3) following the treatment, cells were plated to determine the number of bacteria per milliliter (CFU/mL). Data are presented as mean ± s.d. (n= 3). ****p≤0.0001 and **p≤0.01.

**Fig. 3** Potentiation of ofloxacin by AuNC@CPP against *P. aeruginosa* biofilms. Representative petri dish showing persister cells resuscitation following treatment of 48h-old PA01 and PA CSOM biofilms by ofloxacin, AuNC@CPP and their combination for 24 h. For each condition, the three spot on the petri dish are the recovery media from three independent experiment (n= 3). No growth of PAO1 or PA CSOM is seen with AuNC@CPP combined with ofloxacin. (PAO1 5 μg/mL and PA CSOM 10 μg/mL).

**Fig. 4** Oral administration of AuNC@CPP does not cause systemic toxicity. Representative histological photomicrograph of organs by H&E staining. There was no obvious morphologic change on the histological structure of tissues after daily oral gavage at a dose of 10 mg/kg daily for 14 days with AuNC@CPP and PBS. Fig. 5 In vivo efficacy in mouse model of chronic *P. aeruginosa* ear infection mimicking CSOM. Comparison of the number of bacteria per milliliter (CFU/mL) from middle ear effusion 14 days after the end of the following treatments: placebo control (phosphate-buffered saline, PBS), FLOXIN®Otic (24 μg of ofloxacin) and combination (24 μg of ofloxacin + 296 μg of AuNC@CPP). The CFU/mL from each mouse are plotted as individual points and error bars represent the deviation in CFU/mL within an experimental group. Φ indicates that no middle ear effusion was able to be sampled due to technical constraints. There was no difference between the PBS control group and the ofloxacin group. AuNC@CPP plus FLOXIN®Otic combination led to a 5 log reduction in bacteria 14 days after treatment. *p≤0.05 and Not significant (N.S).
## Tables

**Table 1** Fractional biofilm eradication concentration index (FBEC) for PA01 and PA CSOM

| Antibiotic     | MBEC of agent on its own (µg/mL) | MBEC of agent in combination (µg/mL) | FBEC index | Synergy is defined as $\sum$FBEC $\leq$ 0.5 |
|----------------|---------------------------------|--------------------------------------|------------|------------------------------------------|
| PA01           |                                 |                                      |            |                                          |
| FLOXIN®Otic    | 750                             | 5                                    | 0.0067     |                                          |
| AuNC@CPP       | 1600                            | 800                                  | 0.5        | 0.5067                                   |
| PA CSOM        |                                 |                                      |            |                                          |
| FLOXIN®Otic    | > 3000                          | 10                                   | < 0.0033   |                                          |
| AuNC@CPP       | > 6400                          | 800                                  | < 0.125    | < 0.1283                                 |

**Table 2** Comparison of the antimicrobial efficacy of the various treatments measured in number of bacterial colonies from an effusion of the middle ear two weeks after the treatment.

| Treatments                                      | Effusion (Log$_{10}$ CFU) |
|------------------------------------------------|---------------------------|
| PBS                                            | 7.69 ± 0.26 (n = 4)       |
| FLOXIN®Otic                                     | 6.98 ± 1.45 (n = 6)       |
| Combination (FLOXIN®Otic + AuNC@CPP)             | 2.54 ± 0.51 (n = 4)       |
Fig. 1
Before After

30

Logarithmic-phase of growth (PA CSOM)

Ofloxacin (100 µg/mL)
Fig. 3
Fig. 4
Fig. 5