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Synergistic antimicrobial therapy using nanoparticles and antibiotics for the treatment of multidrug-resistant bacterial infection

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Abstract

Infections caused by multidrug-resistant (MDR) bacteria pose a serious global burden of mortality, causing thousands of deaths each year. Antibiotic treatment of resistant infections further contributes to the rapidly increasing number of antibiotic-resistant species and strains. Synthetic macromolecules such as nanoparticles (NPs) exhibit broad-spectrum activity against MDR species, however lack of specificity towards bacteria relative to their mammalian hosts limits their widespread therapeutic application. Here, we demonstrate synergistic antimicrobial therapy using hydrophobically functionalized NPs and fluoroquinolone antibiotics for treatment of MDR bacterial strains. An 8–16-fold decrease in antibiotic dosage is achieved in presence of engineered NPs to combat MDR strains. This strategy demonstrates the potential of using NPs to ‘revive’ antibiotics that have been rendered ineffective due to the development of resistance by pathogenic bacteria.

Introduction

Increasing antibiotic resistance in bacterial strains is a serious and growing threat to human health [1, 2], as multi-drug resistant (MDR) bacteria cause millions of infections each year. For instance, more than 40% of Staphylococcus aureus strains collected from hospitals are resistant to methicillin (methicillin-resistant S. aureus, MRSA) and in some cases even resistant to broad-spectrum antibiotics such as carbapenems and vancomycin [3–5]. Most cases of MDR infections require prolonged antibiotic treatment that are associated with extensive health-care costs and further contribute to increase in antibiotic tolerance in surviving bacterial cells [6]. Recent projections suggest that annual global deaths caused by MDR infections will reach 10 million by 2050 [7]. Immunity to traditional antibiotics will continue to increase, and the situation will become more dire as the number of strains of MDR bacteria increases [8, 9].

There have been considerable efforts to develop new antimicrobials by screening natural products, modifying existing antibiotics, and synthesizing antimicrobial peptides [10]. Synthetic macromolecules such as polymers that mimic host-defense peptides are frequently used as biocidal agents [11, 12]. More recently, nanoparticles (NPs) have emerged as promising weapons in our antimicrobial arsenal. Their antimicrobial efficacy is attributable to their large surface area enabling high synergy arising from multivalent interactions [13, 14]. For example, NPs functionalized with small molecule ligands exhibit broad-spectrum activity against bacteria [15, 16]. Additionally, NPs can address common antibiotic resistance mechanisms such as efflux pump...
mediated expulsion [17, 18]. Recently, Feldheim and coworkers showed that appropriate surface functionalization of AuNPs with small molecule ligands can regulate the expression of genes responsible for multiple antibiotic resistance in bacteria [16].

One strategy to enhance the efficacy of NPs against bacterial cells is to functionalize them with antibiotics. For example, NPs ‘capped’ with vancomycin and aminoglycoside exhibit enhanced antibacterial activities against resistant strains as compared to antibiotics and NPs alone [19, 20]. However, an alternative strategy is to use NPs in combination with existing antibiotics to combat MDR bacterial infections [21, 22]. We hypothesized that fine-tuning of NP surface chemistry could exhibit synergistic effect with antibiotics in combating MDR bacteria. We investigated the role of the surface chemistry of NPs in combination therapy by screening NPs with different functional groups in combination with antibiotics against resistant bacteria. Assessment of the structure activity relationship revealed that hydrophobic functionalized C10 and C12-AuNPs lowered the minimum inhibitory concentration (MIC) of fluoroquinolone antibiotics against MDR bacteria by 8–16 times. The synergy of this combination therapy was attributed to the ability of functionalized NPs to act as efflux pump inhibitors [22], confirmed by accumulation of ethidium bromide (EtBr) inside bacterial cells upon incubation with NPs. We also investigated the outer-membrane proteins (OMPs) of bacterial cells, which serve as their first line of defense [33]. Combination of NPs with antibiotics provides a complementary approach to target MDR bacteria while helping to avoid the regulatory issues associated with other bioconjugate systems, further improving current therapeutic strategies.

Methods

Synthesis and ligand functionalization of gold nanoparticles

We followed two-step method for the synthesis of AuNPs—(i) AuNP core was synthesized, followed by (ii) fabrication of NP core with ligands using place-exchange reaction. AuNPs with core diameter of ∼2 nm was synthesized using Brust–Schiffrin two-phase synthesis protocol [23]. For the synthesis of Au core, AuCl₄⁻ was transferred from aqueous phase to toluene using tetraoctylammonium bromide as phase transfer catalyst. The gold salt was then reduced using sodium borohydride (NaBH₄) in presence of 1-pentanethiol. Next, the pentane-thiol capped AuNPs were rigorously washed using ethanol to remove TOAB completely. These thiol-protected AuNPs are highly stable because of the strong thiol-gold interaction and they can be easily handled and characterized [24]. Subsequently, ligand functionalization of AuNP core was done using Murray place-exchange method [25, 26]. Pentane-thiol capped AuNPs (10 mg) and ligand of interest (30 mg) were dissolved in a mixture of 1 ml ethanol and 5 ml dry DCM. The solution was stirred under nitrogen flow for 72 h at room temperature. Next, under reduced pressure, the solvents were removed and the resulting precipitate was washed with hexane (10 ml, twice) and DCM (10 ml, thrice). Subsequently, the precipitate was dissolved in distilled water and dialyzed for 72 h (molecular cut-off weight 10 000 gm) to remove excess ligands/pentane-thiol [27]. Thereafter, the NPs were lyophilized and redispersed in deionized water. The particles were characterized by TEM and MALDI-MS spectroscopy as shown in figures S2 and S3 available online at stacks.iop.org/NANO/1/015004/mmedia [28].

Determination of MICs

MIC is the minimum concentration of an antimicrobial agent that inhibits visible growth of bacteria overnight. The MIC of fluoroquinolone antibiotics (ciprofloxacin and levofloxacin), CCCP Efflux pump inhibitor (EPI), and AuNPs for MDR E. coli was determined using a broth microdilution method as recommended by the Clinical and Laboratory Standards Institute. E. coli CD-549 was cultured in lysogeny broth (LB) medium at 37 °C and 275 rpm to stationary phase. The cultured bacteria were then harvested by washing and centrifuging cycles with 0.85% sodium chloride solution three times. The concentration of bacteria was determined by measuring the optical density at 600 nm. Bacterial solution (100 μl) was mixed with 100 μl serially diluted concentrations of antimicrobial agent (antibiotics or NPs) in a 96-well plate, yielding a final bacterial concentration of 5 × 10⁶ cfu ml⁻¹. The bacterial solution without antimicrobial agent was used as a growth control, whereas medium alone was used as a sterile control. All the assays were performed in triplicate, and at least two independent experiments were repeated on different days.

Checkerboard titrations for synergy testing

To assess possible synergy between antibiotics and NPs, we performed two-dimensional checkerboard titrations using a micro-dilution method. In 96-well plates, 2-fold dilutions of antibiotics against a range of 2-fold dilutions of NPs were used to determine the MIC of the combinations. The concentrations of NPs were varied from their MIC to 1/32th of their MIC, similarly the concentrations of antibiotics were varied from their MIC to...
Tryptic digestion and 1D LC-MS was determined by BCA assay. To determine the ability of NPs to act as Ethidium bromide accumulation assay in the dark for 30 min. To dilute the 8 M urea to 1 M before digestion, 430 μl of 2.5 min. The lysate was centrifuged for 15 min at 10 000 g at 4 °C in ice using a Soniprep sonicator resuspended again in 10 ml ice-cold 30 mM Tris HCl. The bacterial pellet was suspended in 20 ml ice-cold 30 mM Tris HCl (pH 7.2) and the suspension was again centrifuged at 10 000 g at 4 °C for 15 min. The pellet was resuspended again in 10 ml ice-cold 30 mM Tris HCl (pH 7.2). The suspended cells were disrupted by sonication in ice using a Soniprep sonicator (Misonix S-4000, USA) for 45 s with intermittent cooling every 45 s for a total of 2.5 min. The lysate was centrifuged for 15 min at 10 000 g at 4 °C. The membranes were collected by ultracentrifuge (OptimaTM L-100XP, Beckman, USA) at 50 000 g for 1 h at 4 °C. The obtained OM pellet was suspended in a small amount of 30 mM Tris HCl (pH 7.2) and stored at −20 °C. The concentration of OMPs was determined by BCA assay.

Ethidium bromide accumulation assay
To determine the ability of NPs to act as Efflux pump inhibitors, we used a previously reported procedure. The E. coli CD-549 strain was grown in LB medium until mid-log phase (OD₆₀₀ = 0.6). Next, bacteria were centrifuged at 16 000 g for 5 min and washed with PBS. OD₆₀₀ of bacteria was adjusted to 0.3. Subsequently, EtBr was added to the final sample at a concentration of 4 μg ml⁻¹ followed by addition of CCCP/NPs at ½ MIC concentrations and incubated for 30 min. Kinetics study of fluorescence obtained was measured using UV/Vis spectrophotometer at excitation and emission wavelength of 530 nm and 585 nm (respectively) at 25 °C for 30 min.

Extraction of bacterial OMPs

E. coli was grown with ½ MIC of engineered NPs in M9 medium for 18 h at 37 °C. Outer membrane proteins of MDR E. coli were extracted following previously reported protocols [34]. Briefly, overnight bacterial cultures were centrifuged at 10 000 g at 4 °C for 15 min. The bacterial pellet was suspended in 20 ml ice-cold 30 mM Tris HCl (pH 7.2) and the suspension was again centrifuged at 10 000 g at 4 °C for 15 min. The pellet was resuspended again in 10 ml ice-cold 30 mM Tris HCl (pH 7.2). The suspended cells were disrupted by sonication in ice using a Soniprep sonicator (Misonix S-4000, USA) for 45 s with intermittent cooling every 45 s for a total of 2.5 min. The lysate was centrifuged for 15 min at 10 000 g at 4 °C. The membranes were collected by ultracentrifuge (OptimaTM L-100XP, Beckman, USA) at 50 000 g for 1 h at 4 °C. The obtained OM pellet was suspended in a small amount of 30 mM Tris HCl (pH 7.2) and stored at −20 °C. The concentration of OMPs was determined by BCA assay.

Tryptic digestion and 1D LC-MS/MS analysis
30 μg of total proteins in 150 μl of 8 M urea in 50 mM ammonium bicarbonate were reduced and alkylated by adding 2 μl of 0.5 M Tris(2-carboxyethyl) phosphine (TCEP) and incubating at 30 °C for 60 min. The reaction was cooled to room temperature before alkylation by adding 4 μl of 0.5 M iodoacetamide at room temperature in the dark for 30 min. To dilute the 8 M urea to 1 M before digestion, 430 μl of 50 mM ammonium bicarbonate was added. Mass spectrometry-grade LysC/Tryptsin (Promega) was added (1:20 ratio) for overnight digestion at 30 °C using an Eppendorf Thermomixer at 700 rpm. Formic acid was added to the peptide solution (2%), followed by desalting by C18 TopTip (Item # TT200C18.96, PolyLC) and finally drying on a SpeedVac. Tryptic peptides were re-suspended in 150 μl of 2% acetonitrile in % 0.1 formic acid to bring the concentration to (0.2 μg μl⁻¹). 10 μl of total tryptic peptides (2 μg total) was utilized for 1D LC-MSMS analysis in triplicate runs by on-line analysis of peptides by high-resolution, high-accuracy LC-MS/MS, consisting of an EASY-nLC 1000 HPLC Acclaim PepMap peptide trap, a 50 cm–2 μm Easy-Spray C18 column, Easy Spray Source, and a Q Exactive Plus mass spectrometer (all from Thermo Fisher Scientific). A 230 min gradient consisting of 5%–16% B (100% acetonitrile) in 140 min, 16%–28% in 70 min, 28%–38% in 10 min, and 38%–85% in 10 min was used to separate the peptides. The total LC time was 250 min. The Q Exactive Plus is set to scan precursors at 70 000 resolution followed by data-dependent MS/MS at 17 500 resolution of the top 12 precursors.

2D LC-MS/MS analysis
Twenty ug total desalted protein digests were reconstituted in 1.5% acetonitrile in 100 mM ammonium formate pH ~ 10. A total of 2.5 μg was then loaded onto a first-dimension column, XBridge BEH130 C18 NanoEase (300 μm × 50 mm, 5 μm), using a 2D nanoACQUITY Ultra Performance Liquid Chromatography system (Waters corp., Milford, MA) equilibrated with solvent A (20 mM ammonium formate pH 10, first dimension pump) at 2 μl min⁻¹. The first fraction was eluted from the first-dimension column at 17.5% of solvent B (100%
acetonitrile) for 4 min and transferred to the second-dimension Symmetry C18 trap column 0.180  μm × 20 mm (Waters corp., Milford, MA) using a 1:10 dilution with 99.9% second-dimensional pump solvent A (0.1% formic acid in water) at 20 μl min⁻¹. Peptides were then eluted from the trap column and resolved on the analytical C18 BEH130 PicoChip column 0.075  μm × 100 mm, 1.7 μm particles (NewObjective, MA) at low pH by increasing the composition of solvent B (100% acetonitrile) from 1% to 6% in 2 min, then to 16% in 80 min, to 26% in 12 min, and finally to 38% in 2 min, all at 400 nl min⁻¹. Subsequent fractions were carried with increasing concentrations of solvent B. The following four first-dimension fractions were eluted at 20%, 23%, 27%, and 60% solvent B. The analytical column outlet was directly coupled to an Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific) operated in positive data-dependent acquisition mode. MS1 spectra were measured with a resolution of 60 000, an AGC target of 1e6, and a mass range from 350 to 1400 m/z. Up to 5 MS2 spectra per duty cycle were triggered, fragmented by collision-induced dissociation, and acquired in the ion trap with an AGC target of 1e4, an isolation window of 2.0 m/z, and a normalized collision energy of 35. Dynamic exclusion was enabled with duration of 20 s.

Protein identification and data analysis
The LC-MSMS raw data were submitted to Integrated Proteomics Pipelines (IP2) Version IP2 v.3 (Integrated Proteomics Applications, Inc.) with ProLucid as the search program [29] for peptide/protein identification. ProLucid search parameters were set up to search the UniProt ECOLI.CFT073.ATCC700928. UPEC (versus October 2015) protein fasta database including reversed protein sequences using trypsin for enzyme with the allowance of up to two missed cleavages, Semi Tryptic search with fixed modification of 57 Da for cysteines to account for carboxamidomethylation and precursor mass tolerance of 50 ppm. Differential search includes 16 Da for-methionine oxidation. The search results were viewed, sorted, filtered, and statically analyzed using DTASelect for proteins with protein FDR rates <2.5 [30]. Differential label-free proteomics data analysis was performed using IP2-Census, Protein Identification STAT COMPARE [31] with 1D LC-MS/MS in three technical replicates and one technical replicate of 2D LC-MS/MS dataset to discover lower-abundance proteins. The result was a label-free quantification analysis, with t-test and DAVID Bioinformatics Resources Functional Annotation 6.7 [32].

Mammalian cell viability studies
20 000 NIH 3T3 fibroblast cells (ATCC CRL-1658) were cultured in DMEM medium (ATCC 30-2002), supplemented with 1% antibiotics and 10% bovine calf serum. The cells are cultured at 37° C in a humidified atmosphere of 5% CO₂ for 48 h. Next, the cells are washed with phosphate-buffered saline (PBS) and different concentration of NPs in 10% serum containing media were incubated with the cells for 24 h at 37° C in presence of humidified atmosphere of 5% CO₂. The cells were washed with PBS (3 times) to remove excess NPs and cell viability was then determined using Alamar blue assays according to manufacturer’s protocol (Invitrogen Biosource). Washed cells were treated with 220 μl of 10% Alamar Blue solution in 10% serum containing media. The solution was incubated at 37° C under a humidified atmosphere of 5% CO₂ for 3 h. Subsequently, 200 μl solution from the wells was transferred in a 96-well black-microplate. The fluorescence reading was measured using a UV/vis spectrophotometer with excitation and emission at 560 and 590 nm respectively. Cell incubated without NPs were treated as 100% viable cells and the cell viability was calculated accordingly. These experiments were performed in triplicates.

Results and discussion
We have previously reported that positively charged 2 nm AuNPs bind to the surface of bacterial membranes, forming distinct aggregation patterns and causing cellular lysis [33]. Further modification of the NP surface monolayer with hydrophobic ligands elicited broad-spectrum activity against clinical MDR isolates [34]. These studies suggest that tuning NP surface chemistry can regulate their interactions with bacteria, in-turn affecting their efficacy in antimicrobial combination therapies. We screened a library of NPs (synthesis and characterization, figures S1–S3 in supporting information) with varying hydrophobicity of ligands to evaluate their efficacy in combination with antibiotics (levofloxacin and ciprofloxacin) using a checkerboard titration method.

We first evaluated the antimicrobial activity of different functional NPs by determining their MIC against uropathogenic E. coli (Escherichia coli, CD-549) as a model strain. MIC was determined using broth dilution method where 5 × 10⁵ cfu ml⁻¹ of bacterial cells were incubated with different concentration of AuNPs overnight [35]. The MIC for different NPs are listed in figure 1. The MIC of antibiotics (levofloxacin and ciprofloxacin) against E. coli (CD-549) was determined to be 512 mg l⁻¹.
To determine the potency of the combination of AuNPs and antibiotics, we performed checkerboard titration and calculated the fractional inhibitory concentration index (FICI) for both NPs and antibiotics (table 1) [36, 37]. The FICI values corresponding to TTMA and C6-AuNPs (≥0.5 and <4.0) indicate an additive response, whereas the FICI values for C10 and C12-AuNPs indicate a synergistic response (<0.5) with the antibiotics (more details of FICI calculations are provided in tables S1 and S2 of supporting information). Additionally, the concave curve obtained with microdilution checkerboard method in case of C10 and C12 AuNPs indicate synergistic response (figure 2) [38]. The MIC of antibiotics was decreased 8-fold (64 mg l⁻¹) in presence of C-10 and C-12 AuNPs at sub-MIC NP dosages (4 nM, 8 nM respectively).

After determining the most effective NPs for combination therapy, NP–antibiotic cocktails were further tested against uropathogenic clinical isolates of *Pseudomonas Aeruginosa* (CD-23) and methicillin-resistant *Staphylococcus aureus* (MRSA, CD-489). C10 and C12 AuNPs showed synergistic effect in combination with fluoroquinolone antibiotics resulting in upto 16-fold reduction in the MIC of antibiotics (figure 3). These results indicate the ability of functionalized NPs to antibiotic efficacy against both Gram positive (*S. aureus*) and Gram negative (*E. coli, P. Aeruginosa*) strains. More information regarding the checkerboard combinations is provided in the tables S3–S5 of supporting information and details of the strains used are given in table S6.

After establishing synergy obtained from the combination of NPs and antibiotics, we probed the effect of different AuNPs on MDR bacteria that could be relevant in establishing synergy with antibiotics. We investigated the ability of NPs to act as efflux pump inhibitors, as expulsion of antibiotics via efflux pumps is a

<table>
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<th>Combination</th>
<th>FICI</th>
<th>Effect</th>
<th>Fold increase in antibiotic efficacy</th>
</tr>
</thead>
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<tr>
<td>TTMA—levofloxacin</td>
<td>0.56</td>
<td>Additive</td>
<td>2</td>
</tr>
<tr>
<td>TTMA—ciprofloxacin</td>
<td>1</td>
<td>Additive</td>
<td>2</td>
</tr>
<tr>
<td>C6—levofloxacin</td>
<td>0.625</td>
<td>Additive</td>
<td>8</td>
</tr>
<tr>
<td>C6—ciprofloxacin</td>
<td>0.75</td>
<td>Additive</td>
<td>2</td>
</tr>
<tr>
<td>C10—levofloxacin</td>
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<td>Synergistic</td>
<td>8</td>
</tr>
<tr>
<td>C10—ciprofloxacin</td>
<td>0.25</td>
<td>Synergistic</td>
<td>8</td>
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<td>C12—levofloxacin</td>
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<td>8</td>
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<tr>
<td>C12—ciprofloxacin</td>
<td>0.375</td>
<td>Synergistic</td>
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major contributor to drug resistance in bacteria [39, 40]. We used ethidium bromide (EtBr), which is widely used as a substrate for efflux pumps in cells, to determine the ability of NPs to act as efflux pump inhibitors [41]. We first conducted kinetics study using carbonyl cyanide m-chlorophenyl hydrazine (CCCP) to determine influx and efflux of EtBr in bacteria. We observed that at sub-MIC dosages (0.5 × MIC) of CCCP (efflux pump inhibitor), accumulation of EtBr inside bacterial cells was significantly increased, as shown by the increase in fluorescence in figure 4(a). Subsequently, the ability of NPs to accumulate EtBr inside the bacterial cells was also tested. NPs at sub-MIC concentrations (0.5 × MIC) were added to bacterial cells followed by addition of EtBr...
(4 μg ml⁻¹) and the fluorescence kinetics (Ex: 530 nm and Em: 585 nm) was studied after 30 min’ incubation. Carbonyl cyanide m-chlorophenyl hydrazine (CCCP), a widely-used efflux pump inhibitor, was used as a positive control, and untreated cells were used as negative control [42]. We observed that hydrophobic NPs (C10 and C12) enhanced accumulation of EtBr inside cells similar to that of CCCP (figures 4(b)–(d)). Whereas, untreated cells showed high efflux of EtBr corresponding to their low fluorescence. Notably, less hydrophobic TTMA NPs also showed accumulation of EtBr inside cells but it was comparatively lesser than CCCP. Importantly, synergistic interaction of hydrophobic NPs as compared to additive interaction of TTMA NPs can be explained due to higher ability of hydrophobic NPs to inhibit efflux mediated exclusion in bacterial cells.

We next investigated the proteomic profiles of the bacterial membrane to further understand the effect of NPs on bacteria, since the bacterial membrane acts as the first line of defense against foreign attack [43]. We used E. coli as model strain for our proteomic studies, due to their high relevance in clinical studies. We extracted bacterial membrane protein from untreated bacterial cells and cells treated with C-10 and C-12 AuNPs using sarkosyl method, and further analyzed them by mass spectrometry [44, 45].

We identified two distinct mechanisms underlying the synergy achieved by the addition of engineered NPs—(i) deregulation of major efflux pump protein and (ii) downregulation of proteins responsible for regulating important cellular processes. The TolC·AcrAB efflux pumps are major contributors to antibiotic resistance in E. coli species [46]. Upon incubation with hydrophobic AuNPs, the expression of tolC is downregulated indicating suppression of efflux pumps. Furthermore bamA, bamD, and bamE proteins, crucial for assembly of tolC at bacterial outer membrane proteins are strongly deregulated, compromising the detoxification of the cell [47].

Secondly, we concluded that hydrophobic NPs also interact with multiple proteins to disrupt crucial cell-survival processes that can also contribute to enhancing the efficacy of antibiotics. For example, proteins such as kpsD and rfaQ, responsible for export of polysaccharides through the outer membrane and biosynthesis of lipopolysaccharides (respectively) were downregulated (figure 5(a)) [48, 49]. Additionally, Lam B, transporter of carbohydrates into cells was downregulated along with Tss, a nucleoside transporter protein [50]. Furthermore, downregulated proteins such as LamB, OmpC, Tss, OmpW, and NlpB were previously confirmed to contribute to antibiotic resistance in E. coli species (proteomics for C10 AuNPs in figure S5) [51].

We next investigated the biocompatibility of hydrophobic NPs by performing viability assays on mammalian cells. NIH-3T3 fibroblast cells were cultured and treated with AuNPs using previously reported methods.
protocols [24]. C10 AuNPs showed an IC_{50} of 560 nM, while more hydrophobic C12 AuNP had an IC_{50} of 300 nM. Additionally, after treatment with 100 nM of AuNPs (C10 and C12), NIH-3T3 fibroblast cells showed 80% (for C12) and 90% (for C10) cell viability, indicating the low toxicity of these NPs against mammalian cells. The observed specificity of NPs for bacterial cells can be attributed to bacteria’s more negatively charged cell surface as compared to mammalian cells. Moreover, the cholesterol present on mammalian cells helps stabilize the membrane and prevents toxicity from cationic AuNPs [24].

**Conclusion**

In summary, we report a strategy to use nanoparticles in combination with current antibiotics to combat MDR bacteria. In this system, primary synergy is generated by the particle blocking the efflux pump, with additional contributing mechanisms elucidated through proteomic analysis. Using this combination strategy, we were able to dramatically reduce the dosing requirements for the antibiotic. For these systems, choices of surface chemistry are central to the efficiency of the regimen, with hydrophobic cationic particles being particularly effective. Combining NPs with antibiotics is a modular approach, providing a strategy for ‘recycling’ currently ineffective antibiotics while circumventing some of the research and regulatory issues associated developing de novo antimicrobials.

**Acknowledgments**

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**Conflict of Interest**

The authors declare no competing financial interest.

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