

Recent advances in the treatment of pathogenic infections using antibiotics and nano-drug delivery vehicles

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Abstract: The worldwide misuse of antibiotics and the subsequent rise of multidrug-resistant pathogenic bacteria have prompted a paradigm shift in the established view of antibiotic and bacterial–human relations. The clinical failures of conventional antibiotic therapies are associated with lengthy detection methods, poor penetration at infection sites, disruption of indigenous microflora and high potential for mutational resistance. One of the most promising strategies to improve the efficacy of antibiotics is to complex them with micro or nano delivery materials. Such materials/vehicles can shield antibiotics from enzyme deactivation, increasing the therapeutic effectiveness of the drug. Alternatively, drug-free nanomaterials that do not kill the pathogen but target virulent factors such as adhesins, toxins, or secretory systems can be used to minimize resistance and infection severity. The main objective of this review is to examine the potential of the aforementioned materials in the detection and treatment of antibiotic-resistant pathogenic organisms.

Keywords: antibiotics, resistance, polymer, chitosan, gold, recombinant, targeted, pathogen

Introduction

Antibiotics are compounds that either stop bacteria from growing or kill them outright, depending on their ability to inhibit critical bacterial cellular processes. Despite the profound success achieved by the use of broad-spectrum antibiotics in the treatment of complex bacterial diseases, new and recurrent bacterial infections continue to impose significant challenges on global health care.^{1–5} Conventional broad-spectrum therapies kill native as well as pathogenic bacteria disrupting the indigenous microflora increasing the risk of infection recurrence and systemic infiltration of the host.^{6–8} Recent studies suggest a number of pathogens such as *Mycobacterium tuberculosis*, *Burkholderia pseudomallei*, *Staphylococcus aureus*, and *Listeria monocytogenes* resist free form antibiotics by persisting in macrophages and erythrocytes.^{9,10} Some of these persistors remain dormant and can go undetected by standard culture methods.¹¹ For the last fifty years identification of antibiotic resistant strains relied on selective culturing (blood cultures) and enzyme linked immune-sorbent assays (ELISA).¹² Selective culturing for the diagnosis of pathogens takes from 18 to 48 hours.^{13,14} Unfortunately, these techniques are not economically feasible in low-resource settings where resistance rates exceed 80%.^{15,16} In 2012, the WHO published a list of critically important antimicrobials for human medicine (Table 1). Taken together, these challenges highlight the need for alternative antimicrobial detection and treatment strategies.

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Table 1 WHO listing (third revision, 2012)¹⁶ of critically important antimicrobials for human medicine

Critically important	Highly important	Important	Unclassified
Cephalosporins (third and fourth generation) ^a	Cephalosporins (first and second generation)	Nitrofurantoin	Carbadox
Fluoro and other quinolones ^a	Penicillins		
Glycopeptides ^a	Pleuromutilins		
Macrolides and ketolides ^a	Streptogramins		
Glycylcyclines	Riminoferazines		
Aminoglycosides	Aminopenicillins	Aminocyclitols	lonophores
Carbapenems/penems	Amphenicols	Cyclic polypeptides	Bambermycins
Cyclic esters	Lincosamides	Nitroimidazoles	
Lipopeptides	Steroid antibacterials		
Macrolides and ketolides ^a	Streptogramins		
Monobactams	Sulfonamides		
Oxazolidinones	Sulfones		
Penicillins (natural aminopenicillins)	Tetracyclines		
Polymyxins			
Rifamycins			
Tuberculosis and other mycobacterial drugs			

Notes: ^aThe top four critically important antimicrobials are prioritized based on: 1) high absolute number of people affected by diseases for which the antimicrobial is the sole or one of few alternatives to treat serious human disease, and 2) high frequency of use of the antimicrobial for any indication in human medicine, since usage may favor selection of resistance.

In the last few decades, many studies have focused on developing rapid detection and efficient drug delivery systems to meet the challenge of antibiotic resistance. These delivery vehicles can be nano or micron in size and composed of liposomes, dendrimers, peptides, polymer, and inorganic materials.^{17,18} Many of them are compatible with and have shown to enhance the sensitivities of conventional laboratory and field diagnostic tests by several orders of magnitude. The engineering of these vehicles is now so advanced that single bacteria can be routinely detected by point-of-care (POC) devices.¹⁹

In addition to detection, nano-vehicles are also used to treat bacterial infections. A drug or drugs can be loaded to the exterior or interior of the vehicle via chemical conjugation, adsorption, and encapsulation and then delivered to the infection site. Antibiotics delivered in this way shield the drug from the resistant mechanisms of the pathogen. Moreover, if the vehicle itself exhibits antimicrobial activity, the concentration of antibiotic can be reduced further. Other advantages of this combinatorial approach include longer drug retention times, improved pharmacokinetic profiles, and minimization of adverse effects to the host. These advances come at a time when the dissemination of metallo- β -lactamases among Gram-negative bacteria is growing dramatically and the resistance to carbapenem and β -lactam antibiotics exceeds 50% in many patient groups.²⁰ As a result, several of these antibacterial vehicles are undergoing preclinical and clinical testing, while numerous others have recently been approved in the treatment of bacterial diseases^{21–30} by The US Food and Drug Administration such as silver and

titanium nanoparticles (NPs) for antibacterial skin lotions and sunscreens that are in commercial use.

In this review, five areas where biocompatible nanomaterials can be used to combat antibiotic-resistant pathogenic bacteria are discussed. These areas include 1) detection of antibiotic-resistant pathogens, 2) targeted antibiotic treatments, 3) combinatorial antibiotic delivery, 4) NP vaccines, and 5) antibiotic repotentialization. Advances made in these areas bring us ever closer to real-time antibacterial therapies that can respond to changes in bacterial susceptibility, colonization resistance, infiltration of the host organism, and pathogenic virulence. In this review, we highlight the innovative materials currently being developed to detect and treat antibiotic-resistant pathogens.^{31–36}

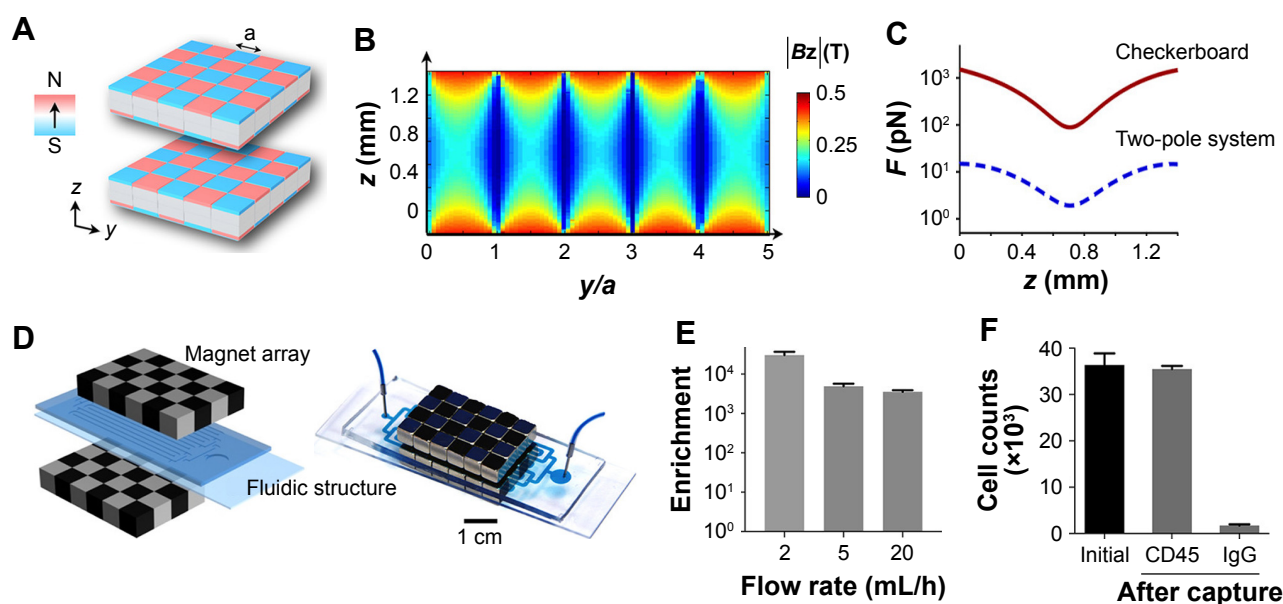
Pathogenic detection

The rapid identification of pathogenic bacteria in the early stages of therapy is crucial in preventing drug resistance. Among the myriad of molecular biology detection techniques available in modern day laboratories, those based on nucleic acid amplification technology such as whole genome sequencing, modern PCR, multiplex PCR (mPCR), quantitative PCR (qPCR), and droplet PCR are routinely used to distinguish resistant pathogenic strains. Real-time PCR (RT-PCR) or qPCR is capable of continuously monitoring changes in bacterial susceptibility.³⁷ However, RT-PCR is currently too expensive to be employed in resource-limited settings where cost-effective qualitative tests dominate the field.³⁸ In this regard, NPs can improve the sensitivity, accuracy, and reliability of qualitative tests, leading to better patient outcomes.

The first stage in identification involves sample preparation. This can take several days using gold standard techniques, which may be too long for critically ill patients who require administration of a specific antibiotic within 24 hours. Immunomagnetic separation is a rapid and highly facile extraction process that allows the researchers to concentrate microbial organisms from various clinical samples within hours.³⁹ In the last decade, magnetic nanoparticles (MNPs) particularly superparamagnetic nanoparticles (SPMNs) composed of magnetite (Fe_3O_4) or maghemite ($\gamma\text{-Fe}_2\text{O}_3$) are attracting a lot of attention due to their excellent magnetic properties, low cost, assay versatility, and higher capture efficiency.⁴⁰ In addition to sample preparation, SPMNs can be used directly or as part of multifunctional composite to improve the sensitivity (eg, $\text{Fe}_3\text{O}_4\text{-Ag}$, $\text{FeO}_4\text{-Au}$, and FePt-Ag) of disposable optical and electrochemical immunoassays. For example, in 2017, Kearns et al combined lectin-functionalized silver-coated MNPs with optically active antibody-coated silver NPs to isolate and detect three bacterial pathogens including methicillin-resistant *S. aureus* (MRSA) in an Eppendorf tube.⁴¹ In 2018, Cowger et al developed a novel susceptibility test called protein-adsorbed NP-mediated matrix-assisted laser desorption-ionization mass spectrometry. The whole procedure took <50 minutes and was able to distinguish between wild-type and drug-resistant strains of bacteria.⁴² In addition, Wei et al reported a portable multiplexed bar-chart SpinChip (MB-SpinChip) integrated with NP-mediated magnetic aptasensors for the

visual quantitative instrument-free detection of multiple pathogen. Using this MB-SpinChip, three major foodborne pathogens including *Salmonella enterica*, *Escherichia coli*, and *L. monocytogenes* were specifically quantified in apple juice with limits of detection of about 10 CFU/mL. Although significantly more expensive to construct than paper-based devices, MB-SpinChip may provide essential “real time” input during a potential pathogenic outbreak.⁴³

Antibody-labeled SPMNs are routinely used with standard and miniaturized MRI systems found in large hospitals and research laboratories. Diagnostic assays using only magnetic fields are simpler to use and better suited for the detection of bacteria in optically opaque media.^{44,45} In 2013, Chung et al used a magnetic-DNA probe in combination with a miniaturized nuclear magnetic resonance to detect the bacterial RNA of 13 species from patient specimens. The miniaturized micronuclear magnetic resonance system had a sample volume of 2 mL and capable of supporting rapid, high-throughput operations in POC settings.⁴⁶ More recently, Park et al used a chip magnetic capture, culture, and detection assay for on-site detection of antigen-presenting cells exposed to *S. enterica*. The magnetic capture, culture, and detection achieved high sensitivity, detecting *Salmonella*-specific antibodies secreted from as little as 50 hybridoma cells/mL of blood. The assay took a minimum of 19 hours, 5 hours less than conventional tests and could be performed with unskilled labor.⁴⁷ A schematic diagram demonstrating the capture and culturing stages of the assay is shown in Figure 1.



Using noninvasive imaging such as MRI to locate bacteria during the early stages of infection has the potential to reduce bacterial resistance and minimize organ damage to the host. Conventional MRI-based imaging has also been used to visualize *S. aureus* infections in endocarditis,⁴⁸ osteomyelitis,⁴⁹ and soft tissue infection models.^{50,51} Generally, these models detect inflammation rather than the causative agent of an infection. Although direct visualization of bacteria has been achieved using iron particle-labeled *S. aureus*,⁵² this technique is frequently limited by the lack of specificity. Ongoing work to create specific molecular imaging probes includes using iron NPs conjugated with IgG to detect intracellular pathogens, and particles coated with *M. tuberculosis* surface antibody for the specific detection of extrapulmonary mycobacterial infection^{53,54} have been reported.

For the past 3 decades, the optical and electrochemical properties of noble metal NPs, particularly AuNPs, have been extensively utilized by many fields of academic and industrial science.^{55,56} AuNPs exhibit a bright red maximum in the visible region of the optical spectrum; the maxima's bandwidth and intensity can be tuned by varying the shape, composition, and the distance between particles.⁵⁷ Aggregation of NPs induces interparticle surface plasmon coupling that results in a blue shift in the maxima. This colorimetric change has been utilized in numerous biological and NP immunoassays. It should be noted that the sensitivity for colorimetric sensors is low compared with fluorescence-based sensors, although the associated instrumentation is significantly cheaper. Moreover, many types of colorimetric sensors are designed to detect clinically relevant bacterial concentrations such as those found on human skin, although more sensitive methodologies are sometimes employed when the individual is suspected to be infected with a hypervirulent pathogen. For a comprehensive understanding of surface plasmon resonance in gold NPs, the review by Amendola et al is recommended.⁵⁸

Pathogenic bacteria were first detected using AuNP aggregation by Elghanian et al in 1997.⁵⁹ Since then, aggregation and dispersion of AuNPs have been widely explored for the detection of bacteria-specific DNAs, proteins, and live cells. In 2004, Storhoff et al used AuNPs to detect the *mecA* gene in MRSA genomic DNA samples.⁶⁰ The approach was effective in discriminating MRSA from methicillin-sensitive *S. aureus* strains. More recently, Chan et al also used AuNPs for direct colorimetric PCR detection of MRSA in clinical specimens, a total of 72 clinical samples were tested.⁶¹ The performance was comparable with real-time PCR assays but at lower cost per reaction. Similar technological approaches

have been applied in the detection of multidrug-resistant *M. tuberculosis* and hemorrhagic *E. coli*.⁶² Of particular interest is the application of these technologies in low-resource settings. In 2017, a working concept of an integrated paper microwell platform and an *M. tuberculosis* detection scheme based on AuNPs was demonstrated.⁶³ Moreover, continued improvements in micropatterned paper devices combined with digitized fluidics have led to an increase in the availability of affordable, accurate POC tests. There are several up-to-date reviews regarding the detection of antibiotic-resistant strains of pathogenic bacteria using POC.^{64,65} Unfortunately, the potential of AuNPs is yet to be realized in the clinic, as many nanodiagnostic tests are stuck in preclinical trials with few US Food and Drug Administration tests currently available to the patient.

In addition to iron-based and gold NPs, quantum dots (QDs), upconverting nanoparticles (UCNPs), and graphene oxide (GO) have also been used to detect bacteria.^{66,67} Fluorescent QDs and UCNPs exhibit greater sensitivity and specificity than nonfluorescent nanomaterials as only the signal originating from the nanomaterial is detected. Graphene can be combined with QDs to work as a quencher (silencing the fluorescence signal of QDs when both are in contact).⁶⁸ This property was utilized by Morales-Narváez et al in the fabrication of lateral flow biosensors.⁶⁹ Their system consisted of two lines of QDs deposited on paper (test and control). The first line was capable of capturing some bacteria (by means of antibodies attached on QDs). After dispensing the sample solution on the QD lines, a solution of GO was added. In the absence of bacteria, GO quenched the fluorescence of the lines. In comparison with traditional lateral flow biosensors, this simple referencing method prevented the formation of false positives as a negative sample always turned off both lines. More recently, Jin et al utilized the dissociation of UCNPs and fluorescence recovery to detect *E. coli* in food and water samples, exhibiting a detection range of 5–10⁶ CFU/mL and detection limit of 3 CFU/mL, respectively.⁷⁰ The novel UCNP-based fluorescence resonance energy transfer (FRET) aptasensor could be used to detect a broad range of targets from whole cells to metal ions.

Targeted antibiotic therapies

The microenvironment of a bacterial infection site is rich in physiological and molecular cues that can be utilized to activate drug release or facilitate the binding of an NP or vehicle to bacteria. Passive targeting (natural barriers), physical–chemical gradients (surface charge and pH), and active targeting are three ways in which targeted drug delivery

can be achieved.^{71,72} Passive targeting is possible due to the dysfunctional lymphatic drainage and elevated permeability that occurs at the infected site. This phenomenon can be exploited by nano and micron particles for targeted antibiotic delivery. In fact, the accumulation of both uncoated and PEGylated liposomes at soft tissue infected by *S. aureus* has been shown to correlate closely with particle size.^{73,74}

However, most infection sites are polymicrobial in nature.⁷⁵ For example, pulmonary infections often consist of combinations of Gram-negative and Gram-positive anaerobic or aerobic bacteria, such as *Klebsiella pneumoniae*, *E. coli*, *Pseudomonas aeruginosa*, and *S. aureus* including MRSA.^{75,76} Many of these bacteria present a negative surface charge under physiological pH and can be targeted using common ligands such as cationic or lipopeptides.⁷⁷ Cationic antimicrobial peptides and lipopeptides kill the microbes by interacting and disrupting bacterial cell membranes. Consequently, a range of vehicles incorporating natural and synthetic peptides have been developed for broad-spectrum applications.⁷⁹ Moreover, lipopeptides show remarkable growth inhibition activity of both Gram-positive and Gram-negative bacteria and fungus. It should be noted that there is a potential for electrostatic interactions and subsequent binding of cationic peptides with other charge modifying substances in circulation. Studies have shown potentially toxic in vitro and in vivo effects with the use of cationic lipids and polymer, including but not limited to cell shrinking and vacuolization of human cell lines.⁸⁰ Recently, Mitra et al showed that synthetic lipopeptides can exhibit good biocompatibility to different mammalian cell lines like HepG2, HeLa, and SiHa as well.⁸¹

Utilizing surface negative charge is one way to target the infection site; however, some pathogens can negate this approach by secreting lactic and acetic acid into the microenvironment.⁸² To overcome this problem, several groups have developed surface charge switching polymer micelles and NPs that target the negatively charged cell wall of MRSA at low pH.^{83,84} Recently, a zwitterionic pH-sensitive polymer, poly (N'-citraconyl-2-(3-aminopropyl N, N dimethylammonium) ethyl methacrylate) or P(CitAPDMAEMA), was used to inhibit the growth of *E. coli* and *S. aureus* under acidic conditions.⁸⁵ The polymer remained zwitterionic at physiological pH and exhibited low hemotoxicity and good biocompatibility. Surface charge switching micelles and NP have been synthesized containing pH-responsive poly-L-histidine,⁸⁴ beta-amino ester,⁸⁶ and numerous chemical functionalities.⁸⁷ In addition, polymers containing acid-degradable acetal/ketal functionalities have also shown

promising results for pH-responsive drug delivery systems for anticancer drugs.⁸⁸ With this in mind, Kalthapure et al recently developed a pH-responsive solid lipid NP with an acetal linkage to deliver vancomycin and inhibit the growth of methicillin-sensitive *S. aureus* and MRSA. The in vivo study showed that the amount of MRSA remaining in the skin of encapsulated vancomycin-treated mice was ~22-fold lower than those treated with the free form of the drug.⁸⁹

In addition to MRSA, many bacteria such as *Helicobacter pylori*, *Listeria*, *Salmonella*, *Campylobacter rectus*, *E. coli*, and *Shigella flexneri* are found in varying acidic conditions within the body (stomach [pH 1.0–2.0], vagina [pH 4.0–5.0], skin [pH 4.0–5.5], intestines [pH 5.0–8.0] and bladder [pH 4.5–8.0]).⁸⁴ The stomach mucosa is particularly vulnerable to the negative spiral bacterium *H. pylori*, which is etiologically associated with the development of gastritis, gastric ulcers, and associated carcinomas. The best results regarding the eradication of *H. pylori* have been achieved using a variety of antibiotic-loaded chitosan (CS) microparticles and NPs.^{90–93} Despite these successes, the physical–chemical requirements that determine NP assembly on different nanostructured *H. pylori* surfaces are still not resolved.⁹⁴ Recent work by Westmeier et al attempted to address this issue by studying the behavior of a library of model NPs, varying in size, surface functionalization, shape, and material on *H. pylori*.⁹⁵ The study showed that NP–bacteria assembly did not require specific functionalization or negative surface charge and that assembly in interfering medium was significantly influenced by the impact of low pH on the bacterial surface. Moreover, assembly of nonbactericidal silica NPs (25% surface coverage) was shown to attenuate the pathogenesis of *H. pylori* by probable inhibition of the type IV secretion system (Figure 2).

NPs have shown promise in treating stomach bacterial infections, but a significant challenge has been to develop antibacterial NPs that are suitable for the various pH gradients found in the gastrointestinal tract. One such material is the triblock copolymer poly(ethylene glycol)-block-poly (aminolated glycidyl methacrylate)-block-poly(2-(diisopropyl amino) ethyl methacrylate) (PEG-b-PAGA-b-PDPA), which was recently used as a pH-responsive micelle to target metastatic breast cancer.⁹⁶

In addition to negative charge and pH gradients, bacteria secrete many virulence factors including toxins, fibronectin proteins, collagen adhesins, and various enzymes (phosphatase, phospholipase, and lipase).⁹⁷ Thus, a simple way to target the infection site is to dope the surface of the antibiotic delivery vehicle with a complementary substrate or antibody.

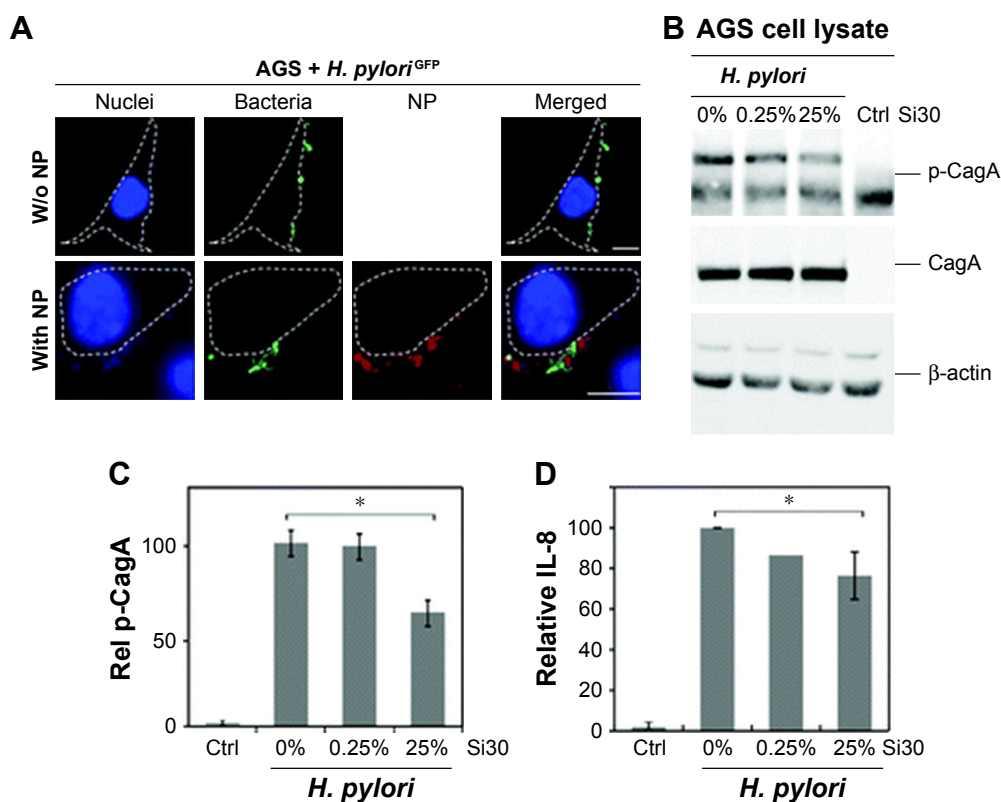


Figure 2 NP-coating attenuates *Helicobacter pylori* (*H. pylori*) pathobiology. Gastric cancer cells were infected with NP -*H. pylori*^{GFP} complexes estimated to maximally cover approximately 25% (Si30–25%: 1×10^8 bacteria, $600 \mu\text{g mL}^{-1}$ Si30; 10 min PBS) or 0.25% (Si30–0.25%: 1×10^8 bacteria, $60 \mu\text{g mL}^{-1}$ Si30, 10 min PBS) of the bacterial surface, respectively.

Notes: (A) Fluorescent microscope image demonstrating NP inhibition of the “hummingbird” phenotype. (B) Immunoblot analysis showing NP (Si30) attenuated type IV secretion system function and decreasing phosphorylated CagA levels. (C) β-Actin normalized phosphorylated CagA levels. (D) NP concentration-dependent decrease in IL-8 secretion. Westmeier D, Posselt G, Hahlbrock A, et al. Nanoparticle binding attenuates the pathobiology of gastric cancer-associated *Helicobacter pylori*. *Nanoscale*. 2018;10(3):1453–1463. Adapted by permission of The Royal Society of Chemistry.⁹⁵ Statistical significance was determined by using the Mann-Whitney test or paired t-test assuming significance at * $P=0.05$. **Abbreviations:** Ctrl, control; NP, nanoparticle.

As the enzyme metabolizes the substrate, it perforates the vehicle releasing the antibiotic near the infection site. In the last decade, various lipase or phosphatase-sensitive polymeric triple-layered nanogels have been used for the synchronized delivery of antibiotics at infection sites.^{98,99} Lipases can also be used to shield AMPs from nonspecific interactions with the host. For example, the lipase substrate poly(caprolactone-b-ethylene glycol) (PCL-b-PEG) was recently used to mask the dendritic polycation G-2. The hybrid dendrimer successfully killed >99.9% of inoculated bacterial cells at $\leq 8 \mu\text{g/mL}$, exhibiting good colloidal stability in the presence of serum and insignificant hemolytic toxicity even at $\geq 2,048 \mu\text{g/mL}$.¹⁰⁰ The design of these hybrid vehicles is now so refined that it is possible to treat a specific pathogenic strain. For example, Yang et al recently reported a unique intracellular antibiotic delivery NP, in which mesoporous silica NPs loaded with gentamicin were coated with (bacterial toxin)-responsive lipid bilayer surface shell and bacteria-targeting peptide ubiquicidin (UBI29-41). The inhibition of *S. aureus* growth in both in vitro and

in vivo of planktonic and intracellular infection, as well as a downregulation of inflammation-related gene expression in infected preosteoblasts or macrophages was observed.¹⁰¹

If the obligate or facultative bacterium escapes the infection site, the risk of infection recurrence increases. It is now accepted that bacteria escape by hiding (at least survive) in osteoblasts, erythrocytes, and phagocytic cells.⁹ These pathogens disrupt the microbicidal mechanisms of phagocytic cells by inhibiting the fusion of lysosomes with phagosomes prolonging their survival.¹⁰² Recent studies suggest that several pathogenic bacteria such as *M. tuberculosis*, *B. pseudomallei*, *S. aureus*, and *L. monocytogenes* are capable of this.¹⁰ Moreover, pathogens often use the cytoplasm of mammalian cells to exchange virulent and resistance genes.¹⁰³ One of the key mechanisms by which microbe’s requestor macrophages is by subverting the body’s most prolific nuclear receptors, the vitamin D receptor.¹⁰⁴ The impact of this subversion on melatonin homeostasis and the subsequent increase in intracellular infection are well known.¹⁰⁵ Unfortunately, the development of vitamin D receptor agonists such as olmesartan medoxomil has yet to be fully realized.¹⁰⁶

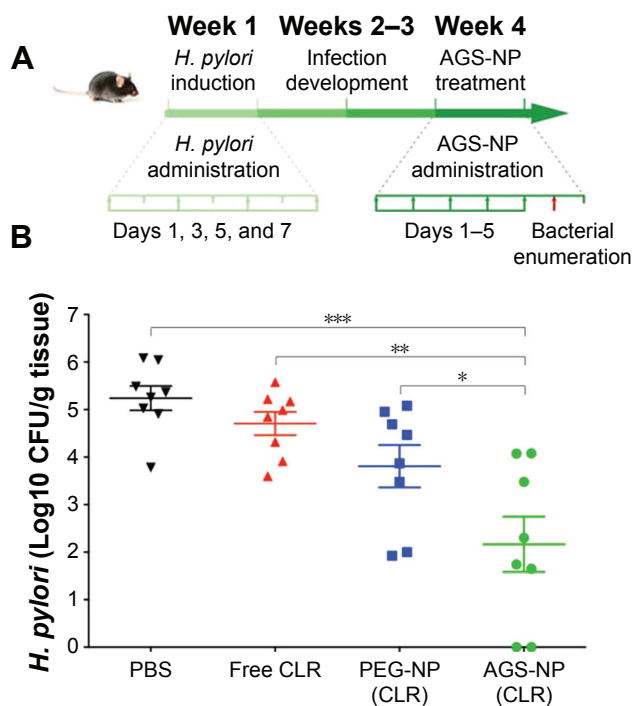
Once a macrophage becomes infected, the next step is to target the intracellular pathogen. As set out by Xiong et al,²⁰ the optimal activity of a given antibiotic against any bacterial-infected cell depends on the concentration of the active drug within the subcellular compartments, which is dependent on the drugs cellular retention, subcellular distribution, and activity. One way to achieve this is to deliver the antibiotic to the cell or infection site via a biodegradable vehicle. Over the past 2 decades, several biodegradable delivery systems composed of natural or synthetic polymers such as poly(lactide-co-glycolide) (PLGA),^{18,107,108} poly(lactide) (PLA),¹⁰⁹ albumin, and CS⁷⁸ have demonstrated the potential of this approach. The major sites of intracellular infection are the mononuclear phagocytic system tissues found in the liver and spleen. This natural barrier brings together infected cells and antibiotic delivery vehicles amplifying the therapeutic effect of the antibiotic. This amplification is often reported when the mononuclear phagocytic system is the primary foci of infection in an experimental model. For example, ampicillin-loaded polyisohexylecyanoacrylate NPs demonstrated a therapeutic efficiency >2 orders better than the free form of the antibiotic in vivo.¹¹⁰ Similar results have been obtained using gentamicin-loaded nanoplexes based on block copolymers of poly(ethylene oxide-b-sodium acrylate) and poly(ethylene oxide-b-sodium methacrylate).¹¹¹

Outside the liver and spleen, bacterial-infected macrophages are also found in the pulmonary cavities, eliminating these bacteria (active or dormant) remains one of the toughest challenges that developmental drug delivery systems currently face.¹¹² For example, current treatment of pulmonary tuberculosis (TB) infection involves prolonged oral administration of high systemic doses of combined frontline antibiotics like rifampicin (RFP), isoniazid, pyrazinamide, and ethambutol over a 6- to 8-month period.¹¹³ *M. tuberculosis* can survive in alveolar macrophages by inhibiting phagosome maturation and phagosome-lysosome fusion. Delivery of RFP via PLGA microspheres to alveolar macrophages resulted in a 19-fold higher concentration of RFP in cells infected with TB compared with the administration of the free drug in solution.¹¹⁴ Uptake of the antibiotic by the infected alveolar macrophages was dependent on the size and concentration of vehicles used and the population of macrophages encountered at the infection site. Using PLGA microspheres to extend the release of the drug to the primary site of infection is a promising treatment for TB as it can achieve the desired therapeutic effect in a shorter period.¹¹⁵ Moreover, treatment time can be further reduced if the RFP-loaded PLGA microparticles are administered

via intratracheal insufflation. When TB-infected guinea pigs were treated using the said procedure, their lungs showed significantly increased drug levels compared with intravenous and oral delivery of RFP.¹¹⁶

The rapid development of targeted therapies for TB has benefited from the unique surfaces of mycobacteria, making the targeting of the infection site easier. However, differentiating between two Gram species is more difficult.¹¹⁷ Yet, there are many common ligands (antibiotics included) that can be used to specifically target Gram-positive or Gram-negative pathogens.¹¹⁸ In the last 2 decades, various antibiotics such as vancomycin and amoxicillin have been conjugated to the surface of gold NPs,^{119,120} iron oxide NPs,^{121,122} porous silica NPs,¹²³ and surface dendrimers, resulting in the preferential binding of Gram-positive bacteria. Moreover, other small molecules such as lectins, single domain antibodies, and aptamers¹²⁴ can be conjugated to the surface of the delivery vehicle to target Gram-negative pathogens. For example, mannose-specific or fucose-specific lectins showed enhanced binding affinity to the carbohydrate receptors on *H. pylori*, while bacterium-based aptamer selection techniques have been utilized to target NPs to *Salmonella typhimurium*. In addition to these techniques, other groups have utilized cell-pathogen adhesion mechanisms to target opportunistic pathogens.¹²⁵ Recently, Angsantikul et al utilized clarithromycin (CLR) loaded PLGA NPs (100 nm) coated with gastric epithelial cell membranes AGS-NP (CLR) to treat *H. pylori* infection in mice. After 6 days, the bacterial burden in a gram of stomach mice tissue treated with AGS-NP (CLR) was ~3.08 orders of magnitude lower than control (Figure 3).¹²⁶

Another pathogen that resides within human macrophages is MRSA. Studies examining infected alveolar macrophages found that PEGylated and non-PEGylated liposomes loaded with vancomycin were capable of significant improvements in MRSA clearance.¹²⁷ Other types of delivery vehicles such as solid lipid NPs and PLGA loaded with vancomycin have shown similar improvements in MRSA clearance rates as well.¹²⁸ More recently, Pei et al combined the pH-sensitive properties of CS with PEGylated-PLGA to form a hybrid vehicle namely PpZEV. PpZEV NPs exhibited increased vancomycin release at acidic pH and significantly higher levels of uptake and MRSA clearance in infected macrophages when compared with free vancomycin and unmodified PLGA-NPs.¹²⁹ Macrophage uptake of antimicrobials can be further enhanced by attaching various ligands including O-stearoyl amylopectin, phosphatidylcholine, maleylated bovine serum albumin, and mannose onto the NP surface.¹³⁰ A summary of the various types of nanovehicles



used to improve the efficacy of antibiotics and the treatment of bacterial infections is shown in Table 2.

Increasing microbial susceptibility: combinatorial applications

The accumulated misuse of free-form antibiotics has led to the emergence of multidrug-resistant MDR strains. The accepted definition of MDR is coresistance to three or more classes of antimicrobial drugs.¹³¹ A serious concern is *Enterobacteriaceae* (avian *E. coli*), which is known to carry the New Delhi metallo-beta-lactamase gene (NDM-1), as well as genes that confer resistance to copper sulfate and quaternary ammonium disinfectants.¹³² At present, most antibiotic-resistant mechanisms do not apply to NPs whose mode of action is direct contact with the cell wall. Thus, combining antibiotics with nanomaterials should reduce bacterial resistance and improve the pharmacokinetic profile of the drug.

Liposomes composed of phospholipids and cholesterol have been used to deliver antibacterial drugs for several decades.¹³³ As a drug delivery system, liposomes offer several advantages. Using mice and guinea pig TB-infected models, Deol et al showed that RMP, INH, and PZA-encapsulated liposomes were significantly more effective in reducing the recovery times than their free drug

Table 2 Targeted antibacterial therapies

Delivery vehicle	Antibacterial agent	Pathogen	Reference
Monoolein, DOTAP, and monoolein LLC NPs	Rifampicin	<i>Staphylococcus aureus</i>	74
/	SYN-004, β-lactamase enzymes	<i>Clostridium difficile</i>	75
/	OFBT	Gram-negative bacteria, Gram-positive bacteria, and fungi	76
/	PDMAEMA-b-PTMA, QDMA-b-PTMA	MRSA	82
/	P(CitAPDMAEMA)	<i>Escherichia coli</i> , <i>S. aureus</i>	83
Peptidomimetic polyurethanes	/	<i>E. coli</i> , <i>S. aureus</i>	84
pH-sensitive lipids	Vancomycin	MRSA	85
Solid lipid nanoparticles	Vancomycin	MRSA	88
Chitosan/TPP	Amoxicillin	<i>Helicobacter pylori</i>	91
PLGA/UCCs-2 NPs	Amoxicillin	<i>H. pylori</i>	92
Silica NPs	/	<i>H. pylori</i>	95
Lipase-sensitive polyurethane micelles (PUM-Ag)	Ag NPs	<i>E. coli</i> , <i>S. aureus</i>	98
Lipid and peptide-coated mesoporous silica NPs	Gentamicin	<i>S. aureus</i>	101
		Infected preosteoblast, macrophage	
100-faceted Pd cubes,	/	Gram-positive bacteria	117
111-faceted Pd octahedrons	/	Gram-negative bacteria	
Amoxicillin-coated AuNPs	Complexed with gold	<i>S. aureus</i>	120
AGS-NPs	Clarithromycin	<i>H. pylori</i>	126
PpZEV NPs	Vancomycin	MRSA	129

Abbreviations: AGS, gastric epithelial cells; DOTAP, N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl sulfate; LLC, lyotropic liquid crystalline; NP, nanoparticle; OFBT, oligo-(7,7'-bifluorene-benzo[c][1,2,5]thiadiazole); pCitAPDMAEMA, poly(N'-citraconyl-2-(3-aminopropyl-N,N-dimethylammonium)ethyl methacrylate); PDMAEMA, poly(N,N-dimethylaminoethyl methacrylate); PLGA/UCC, poly (lactide-co glycolide/ureido-conjugated chitosan); PpZEV, PEG-PLGA and Eudragit E100, and chitosan; PUM, polyurethane micelles; QDMA, quaternary dimethylamino; TPP, tripolyphosphate.

counterparts.¹³⁴ Due to the physical and chemical instability of liposomes, many researchers have employed niosomes to maintain the physical–chemical properties of antibiotic combinations.¹³⁵ Niosomal delivery allows for the delivery of combinatorial antibiotics that are too toxic in their free form. For example, drug compounds such as bismuth derivatives and gallium desferrioxamine interfere with iron absorption and reduction of alginate, LPS, and the secretion of adhesion factors of *P. aeruginosa*.¹³⁶ In a recent study by Mahdiun et al, the combinatorial effect of tobramycin and bismuth ethanedithiol-loaded niosomes (Nio-TOB) on the quorum sensing and biofilm production of *P. aeruginosa* was demonstrated. The minimum inhibitory concentration (MIC) of TOB and Nio-TOB for most strains was 16.64 and 1.3 mg/mL, respectively.¹³⁷ *P. aeruginosa* uses the same uptake mechanisms for both gallium and iron.¹³⁸ Thus, targeting bacterial iron metabolism can arrest *P. aeruginosa* virulence. Simple gallium siderophore complexes such as gallium citrate have shown good antibacterial activity (1–5 mg/mL MIC) against *P. aeruginosa* and low drug resistance in vivo. In addition, gallium protoporphyrin IX has shown 100- to 1,000-fold increase in potency against various MRSA strains compared with gallium nitrate.¹³⁹ However, relatively low aqueous solubility, moderate toxicity, and low synthetic yields of Ga-protoporphyrin IX may have dampened enthusiasm for further development. Antibiotics can also be conjugated with bacterial siderophores increasing their membrane permeability, which in turn increases their antimicrobial activity. In 2017, Oh et al reported on a novel siderophore-conjugated cephalosporin, LCB10-0200. In accordance with Clinical and Laboratory Standards Institute methods, LCB10-0200 showed potent antibacterial activity against *P. aeruginosa* clinical isolates, including β -lactamase producing strains. Moreover, LCB10-0200 was more effective than ceftazidime in treating systemic, respiratory tract, urinary tract, and thigh infections caused by antibiotic susceptible and resistant strains of *P. aeruginosa* in mouse models.¹⁴⁰

Until recently, first-line therapies for *H. pylori* eradication consist of proton pump inhibitors or ranitidine bismuth citrate and CLR, plus amoxicillin or metronidazole.^{141,142} Proton pump inhibitors and amoxicillin are known to increase the risk of secondary infections such as *Clostridium difficile*, while combinations of metronidazole are frequently used to treat *C. difficile* disease. Moreover, CLR resistance is now at a level where continued use of mainstream CLR-based triple therapy is not recommended.^{143,144} The

clinical lifetime of CLR may have been prolonged if it had been encapsulated into a mucoadhesive particle. As early as 2008, several groups had tested various encapsulated triple combinations using NPs composed of CS, lectin-conjugated gliadin, and PGLA. Ramteke et al used lectin-conjugated NPs to simultaneously release amoxicillin, omeprazole, and CLR, resulting in a 94.83% eradication rate of *H. pylori* from infected rats.¹⁴⁵

As >40% of the global population harbor *H. pylori*, a new treatment strategy is urgently needed.¹⁴⁶ Fortunately, other types of eradication therapies such as liposomal linolenic acid (LipoLLA) have shown great potential in eradicating *H. pylori*. Unlike amoxicillin, LipoLLA kills both spiral and coccoid forms of the pathogen. However, a few studies suggested that certain carriers may cause adverse health effects by directly eliminating the gastrointestinal microbiota or by alternating their functions. For example, silver NPs could damage the gut microbiota even though silver is generally considered to have low toxicity. In a recent paper by Li et al, the gut microbiota of *H. pylori*-infected mice exhibited minor alterations in bacteria populations after LipoLLA therapy with a notable boost in the relative abundance of Proteobacteria and Firmicutes along with a decrease in *Verrucomicrobia* and *Bacteroidetes*.¹⁴⁷ On the contrary, conventional triple therapy led to the growth of *Enterobacteriaceae*, *Enterococcaceae*, and *Clostridiaceae* species in the mice.

It is becoming increasingly obvious that the composition of the gut microbiome must be taken into consideration during the development and administration of future antibacterial treatments. Sparing the gut microbiome using narrow-spectrum antibiotics (fidaxomicin) has been shown to correlate with a reduction in *C. difficile* recurrence compared with broad-spectrum antibiotics.¹⁴⁸ However, TB resistance is increasing although narrow spectrum drugs are the mainstay of current treatments. Moreover the oral-pulmonary microbiota is too small to act as a significant reservoir of antibiotic resistance, which suggests the administration of free form RFP, its rapid delocalization, and resulting gut metabolites have contributed significantly to an increase in its resistance.¹⁴⁹

Vaccination vehicles (toxins and antigens)

As well as killing bacteria, the large surface area of NPs can be used to capture bacterial toxins. Many pore-forming toxins (PFTs) such as listeriolysin O (LLO) *L. monocytogenes* and *Aerolysin* from *Aeromonas hydrophila* promote deacetylation

and dephosphorylation of histones, resulting in subsets of immune genes, such as CXCL2, MKP2, or IFIT3, becoming repressed thus predisposing the host to secondary infections. A radical but simple solution to accelerate the removal of active toxins from the host is to use erythrocyte membrane-coated polymer NPs or nanosponges.¹⁵⁰ The membrane of red blood cells (RBCs) serves as perfect stealth coating with high biocompatibility and immune evasion ability.^{151–153} Using the extrusion method, various nanomaterials such as PLGA, melatonin, inorganic NPs, gold nanocages, and QDs have been coated with RBC membranes. In all cases, the in vivo circulatory half-life of RBC-coated nanomaterials was superior to materials coated with PEG. In 2017, Zhang et al prepared RBC membrane-coated vancomycin nanogels using the cell membrane-templated polymerization method for the treatment of MRSA infections.¹⁵⁴ The redox-responsive hydrogel acted as the delivery vehicle for vancomycin, while the RBC membrane absorbed and neutralized the pore-forming toxin secreted by the bacteria. Compared with free antibiotics and nonresponsive nanogels, RBC-coated nanogels exhibited remarkable antibacterial

activity in vitro. Recently, Chen et al developed a broad-spectrum nanosponge by coating 100 nm PLGA NPs with human RBC membranes.¹⁵⁵ When tested with PFTs, melittin, α -hemolysin, LLO (*L. monocytogenes*), and streptolysin O (group A *Streptococcus*), the hRBC nanosponges completely inhibited toxin-induced hemolysis in a concentration-dependent manner. The inhibitory human nanosponge concentration (IC100) value for the four PFTs tested was 7.5, 16, 0.63, and 1.0 μ g/mL, respectively. The investigation showed that nanosponge sequestered toxins were less toxic not only to cells but also to live animals. Figure 4 shows a schematic structure of human nanosponge and its mechanism of neutralizing PFTs. In addition to α -hemolysin, MRSA also secretes other membrane-damaging toxins, including gamma-toxin, leucocidins, and phantom-valentine leucocidin. Such toxins can also be impregnated onto the surface of RBC-coated NPs. In 2017, Wei et al demonstrated a multitoxin laden nanotoxoid formulation using the aforementioned cell membrane-cloaked NPs.¹⁵⁶ The formulation was nonhemolytic and showed no observable toxicity upon subcutaneous administration in mice. Immunization with

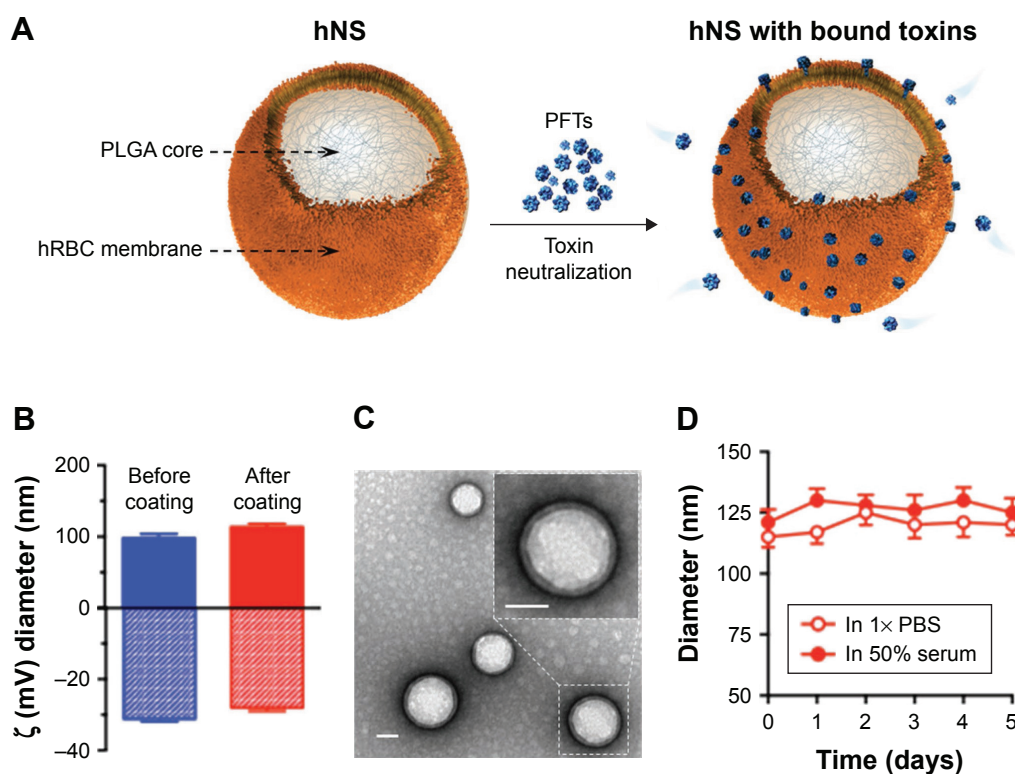


Figure 4 Structure of hNS.

Notes: (A) Schematic diagram depicting hNS and its mechanism of neutralizing PFTs. (B) Nanoparticle hydrodynamic size (diameter) and zeta potential (mV) ($n=3$) before and after membrane coating. (C) Transmission electron microscopy image of hNS. (D) Diameter stability of hNS over 5 days in different media ($n=3$). Image modified with permission from Chen Y, Chen M, Zhang Y, et al. Broad-spectrum neutralization of pore-forming toxins with human erythrocyte membrane-coated nanosponges. *Adv Healthc Mater.* 2018;7(13):e1701366. © 2018 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.¹⁵⁵

Abbreviations: hNS, human RBC nanosponge; PFTs, pore-forming toxins; PLGA, poly (lactide-co-glycolide); RBC, red blood cell.

the nanoformulation induced germinal center formation in the lymph node and increased antibody titers against three staphylococcal virulence factors. The vaccine was shown to be effective in reducing skin lesion formation and bacteria count in mouse model.

The unique capabilities of RBC membrane-coated NPs to detain toxins and induce an immunological response are well known.^{157–160} However, Gram-specific and mycobacterial outer membrane vehicles (OMVs) 50–250 nm can also be coated onto NPs. Given their small size, proinflammatory pathogen-associated molecule patterns, suitability for antigen-presenting cells uptake, and efficient lymph node drainage, OMVs are well suited to bacterial vaccine development. By marrying the virtues of synthetic NPs and OMVs, *E. coli* OMV-coated 30 nm gold NPs have been shown to induce fast dendritic cell activation and maturation in the lymph nodes of mice. The hybrid vehicles generated higher antibody titers with stronger avidity than those elicited by OMVs alone.¹⁶¹ In addition, the vaccinated mice showed elevated levels of IL-17 and interferon-gamma. The adjuvant activity of gold NPs has been used extensively in the development of antibacterial vaccines including *L. monocytogenes*, *Yersinia pestis*, *P. aeruginosa*, and *Streptococcus pneumoniae*.³¹ Multishell gold NPs have the potential to deliver bacterial antigens either through encapsulation or surface association. However, such investigations are usually conducted using cheaper biodegradable polymer NPs, such as PLGA NPs. For example, PLGA NP-encapsulated ovalbumin induced a higher and sustained antibody response than liposomes of similar size. It was also noted that the PLGA vaccine elicited a protective immunity against listeria infection with the highest bacterial clearance compared with liposomes. Antigen-coated polymer NPs have been shown to induce a strong cytotoxic CD8+ T cell response, whereas antigen encapsulation preferentially activates CD4+ T cells. However, several studies have shown that particle-encapsulated antigens induce CD8+ T cell as well, clearly further investigations on cellular processing of antigens is needed.^{162,163}

To obtain protective immunity, adjuvants that augment the immunostimulating activity are required. Adjuvants can be formulated in the same way as antigens by encapsulation, incorporation, or conjugation with a polymer NP. Akin to bacteria, adjuvants that activate pattern recognition receptors (PRRs) can be added to the surface of antigen-loaded NPs. There are three major families of PRRs: Toll-like receptors (TLRs), retinoic acid-inducible gene I-like receptors, and nucleotide-binding oligomerization domain-like receptors. The cooperation of multiple PRRs has been shown to contribute to host defense against bacteria. Critical to the control

of *S. typhimurium* is the synergy between TLR2 and TLR4, whereas the cooperation between active TLR2 and TLR9 plays an important role in controlling TB infection.^{164,165}

As previously highlighted, antibiotic administration via pulmonary insufflation has proved very effective in the treatment of TB. Insufflation was employed by Lu et al to deliver Ag85B antigen and trehalose-6-6-dibehenate-loaded PLGA NPs to TB-infected guinea pigs, resulting in anti-TB protection, albeit inferior to BCG vaccination.¹⁶⁶ A single toxin/antigen vaccine is often inadequate against MRSA or TB infection consequently researchers frequently adopt a triantigen approach. For example, three mycobacterial antigens, MPT-83, MPT-64, and Ag85B, were loaded into PLGA NPs by Cai et al and administered via intramuscular injection to mice. After vaccination, elevated cellular and humoral responses against all three antigens were observed. In particular, a single dose of the triantigenic formulation was comparable with the BCG vaccine.¹⁶⁷

Antibiotic re-potential and gene editing

NP coatings (Ag, Au, ZnO, CuO, TiO₂, etc) have been used to inhibit the growth of bacterial films on various surfaces (implants, dental materials, and wound dressings) for decades.¹⁶⁸ Toxicity issues surrounding implant failure are beyond the scope of this review as are the coselective properties of ZnO, CuO, and TiO₂ in animal feeds.^{13,23} In 2017, several in-depth reviews discussed the antibacterial mechanisms of free form inorganic NPs.^{23,34} More recently, work by Kadiyala et al showed that ZnO nanoparticles (ZnO-NPs) kill MRSA via ROS-independent mechanisms involving multiple metabolic pathways.¹⁶⁹ Many reports suggest that these inorganic nanomaterials are toxic to specific cell lines and organs.^{170–173} However, when used at very low concentrations with antibiotics to treat infections they are not.¹⁷⁴ A recent study by Panáček et al demonstrated a strong synergistic effect of antibiotics combined with AgNPs at very low concentrations demonstrating no cytotoxic effect on mammalian cells. Moreover, restoration of the susceptibility of a resistant *E. coli* strain to ampicillin was observed when ampicillin was combined with AgNPs. In addition, the reduced impact of fewer NPs on gut DNAases using the synergistic approach makes the uptake of resistant plasmids and transposons by live bacteria in humans unlikely.⁴

Much remains to be learnt about the exogenous and endogenous factors affecting antibiotic resistance. Changes in cell physiology can provide insight into the bacterial mechanisms contributing to antibiotic

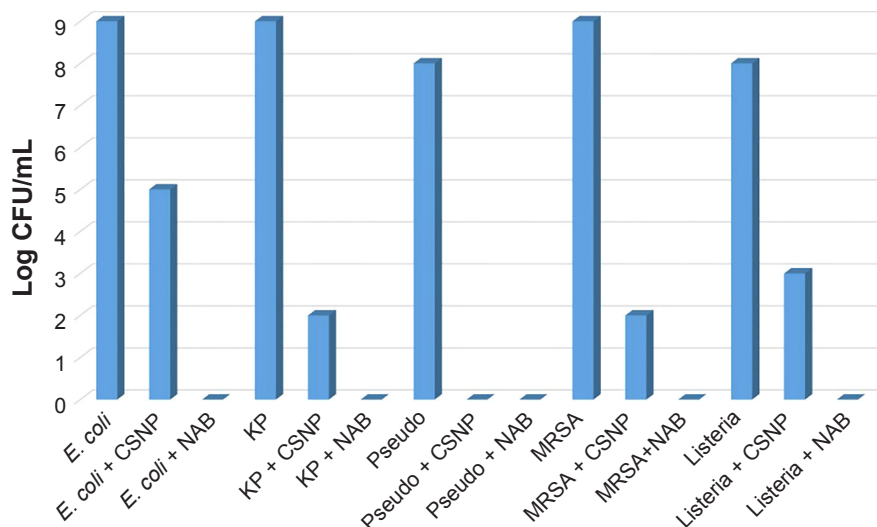


Figure 5 CFU assay.

Note: Image reproduced from Jamil et al¹⁸¹ with permission (Copyright 2016 Frontier Publishing).

Abbreviations: CFU, colony-forming unit; CSNP, chitosan nanoparticles; E. coli, *Escherichia coli*; KP, *Klebsiella pneumoniae*; MRSA, methicillin-resistant *Staphylococcus aureus*; NAB, nano-antibiotic; Pseudo, *Pseudomonas aeruginosa*.

tolerance. Sugar potentiation of β -lactam antibiotics is now well established with arabinose, glucose, mannitol, ribose, and lactose capable of increasing the sensitivity of infectious strains of *E. coli* to carbenicillin and cefuroxime by several orders of magnitude.^{175,176} In addition to primary metabolite potentiation, nontoxic (human host) diarylamidines such as pentamidine have displayed synergy with antibiotics used to treat Gram-positive bacteria, yielding effective combinations in vitro to colistin-resistant bacteria.^{177,178} In addition, repotentialization of secondary metabolites (antibiotics) using metabolizable sugars and a diarylamidine was recently used to kill *E. coli* O157:H7 via a ROS mechanism while repressing Shiga toxin production.¹⁷⁹ More recently, Milton et al¹⁸⁰ presented a new urea-containing class of 2-aminoimidazole-based adjuvants that potentiated colistin activity against colistin-sensitive *Acinetobacter baumannii*. Lead compounds enabled 1,000-fold reduction in the MIC of colistin in vitro. It could be argued that there are many naturally occurring antibacterial polymers (CS) that can repotentiate last line antibiotics just as well. For example, recent work by Jamil et al synergistically enhanced the bactericidal activity of β -lactam antibiotic cefotaxime with antimicrobial biopolymer CS, rendering it more effective against biofilm producing MDR pathogens.¹⁸¹ Figure 5 shows the superior antibiofilm activity of cefotaxime-loaded CS NPs compared with CS NPs. Finally, interfering RNAi has been shown to repotentiate current antibiotics by targeting resistance genes. Recently, Meng et al used lipid nanocarrier to deliver antisense oligonucleotides targeted to the *mecA* gene, which is known to play a role in β -lactam resistance.

Upon testing, they found it was possible to restore MRSA susceptibility to oxacillin in vitro and in vivo.¹⁸²

In the event that these combinatorial approaches fail to arrest antibiotic resistance in animal models, the last line of defense is gene editing. Using a combination of clustered regularly interspaced, short palindromic repeats (CRISPR) and Cas9 protein, this accurate method of gene editing was recently applied as an anti-MRSA strategy in a study by Bikard et al.¹⁸³ Using a delivery system known as a phagemid, it was possible to deliver the gene editing machinery to the MRSA resulting in a 10^4 -fold reduction in the number of viable colonies in vitro. However, challenges arise due to the wildly varying size and structure of different phagemids; moreover, delivery of the CRISPR–Cas9 package to intracellular microbes could mitigate off-target effects at both host cell and bacterial level. Current delivery strategies involve in capsulation of the phagemid with biological inert polymers, lipids, silica, and DNA target.^{184,185} To the best of our knowledge, there are no reports of RBC membranes being used to deliver CRISPR/Cas9 machinery to antibiotic-resistant pathogens.

Conclusion

In the last decade, advances in bionanoengineering have contributed to the development of highly sensitive bacterial detection technologies and sensors. Major efforts in refining the interior and surface chemistry of organic and inorganic drug carriers such as CS, PLGA, and gold have demonstrated excellent outcomes via the detection treatment and prevention of bacterial diseases, by specific targeting of pathogenic

bacteria, combinatorial delivery of antibiotics, and effective antibacterial vaccination strategies.

It is expected that advances in microbiology, digital microfluidics, and bionanoengineering will continue to bring improvements regarding the rapid isolation and detection of infectious pathogenic bacterial strains, improvements in cost-effective drug delivery systems, as well as tailored effective therapies in developed countries.

Unfortunately, the advantages of improved biodistribution and therapeutic outcomes using targeted nanomaterials have yet to be realized in developing countries where the need to combat antibiotic resistance is felt the strongest. Thus, the application of these advances will require communication and collaboration between scientists and clinicians from many different countries and fields of research.

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Disclosure

The authors report no conflicts of interest in this work.

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