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In the search for novel solutions to antibiotic resistance, are nanoparticles the next big thing?

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In the search for novel solutions to antibiotic resistance, are nanoparticles the next big thing?

By

Christopher Thomas Culek

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this creative component. The Graduate College will ensure this creative component is globally accessible and will not permit alterations after a degree is conferred.

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1. ABSTRACT

Multi-drug resistant and more virulent strains of bacteria are a serious concern for microbiologists and medical practitioners. Many common disease causing organisms are becoming resistant to multiple classes of antibiotics. Recent research has given us a greater understanding of the issues with bacterial adaptations to drugs, multi-drug resistance, biofilms, and infection site microenvironments. The new understanding of bacterial resistance has led to experimentation for new and potentially more efficacious drug delivery systems. One such delivery system is nanoparticles. Nanoparticles offer a way to target antibiotic treatment to the site of infection. This feature provides treatment with two major benefits: increased antibiotic efficacy, and reduced antibiotic toxicity.

2. INTRODUCTION

The prospect that disease causing bacteria are rapidly developing resistance to our current repertoire of antibiotics should raise the alarm of every health care provider and citizen in the country. Paradoxically, the widespread use, and misuse, of antibiotics in healthcare and agriculture have resulted in the rapid rise of drug resistant strains of bacteria. Unfortunately, the consequence of nearly a century of antibiotic use is now culminating in the emergence of multidrug resistant strains of bacteria. Government and non-government organizations such as the U. S. Centers for Disease Control and Prevention (CDC), the World Health Organization (WHO), and the Pew Charitable Trust have become advocates to raise public awareness of the urgency to discover new drug delivery methods, such as nanoparticles, new antibiotics, and for more prudent stewardship of existing antibiotics.

2.1 Scope of the Issue and Current Trends

The CDC reports that the United States experiences more than two million antibiotic-resistant bacterial infections a year, 23,000 people die as a direct result of those infections, and many more people die from health conditions that were complicated by an antibiotic-resistant infection.\(^1\) Almost 250,000 people require hospital care each year for *Clostridium difficile* (C. difficile), an opportunistic nosocomial infection and an urgent drug resistant species. In most of these infections, the use of antibiotics was a major contributing factor leading to the illness. Of the people infected by C. difficile, at least 14,000 die in the United States. The CDC report estimates that 50% of prescribed antibiotics are unnecessary or not optimally effective as prescribed.\(^1\)

The agricultural industry and veterinary medicine are also significant users of antibiotics.\(^2\) Estimates ranging from 40 to 80 percent of all antibiotics sold are for use in agriculture, and 96% of those sales are over the counter. The FDA now tracks medically important antibiotic use in food animal production. Historically, antibiotics have been routinely added to food and water to prevent, control, and treat disease, and to promote growth. Beginning in 2017, this practice is now allowed only under the authorization of a veterinarian.\(^3\)

The 2013 CDC report was intended to raise awareness on the most significant disease causing bacteria. They highlight 18 species of bacteria that pose a risk due to antibiotic resistance, and ranked these 18 species into three categories based on several criteria including rate of incidence, economic cost, transmissibility, available antibiotic treatment, barriers to prevention. This is summarized in Figure 1 (page 2).
Figure 1. A thorough list of the many drug resistant bacteria facing healthcare today categorized by the danger they present. The classifications were based on several criteria including: rate of incidence, economic cost, transmissibility, available antibiotic treatment, barriers to prevention.¹

The Pew Charitable Trusts produced a fact sheet based on recent CDC data.² Figure 2 (page 3) shows the recent trends for antibiotic prescriptions in the United States. Although overall antibiotic use remained fairly static, the classes of antibiotics being prescribed are changing. Physicians prescribe more broad-spectrum antibiotics while narrow spectrum antibiotic use is decreasing. Broad-spectrum antibiotics target a wide array of bacterial pathogens. They include glycopeptides, beta-lactam beta-lactamase inhibitor combinations, carbapenems, fluoroquinolones, macrolides, and third- and fourth-generation cephalosporins. Narrow-spectrum antibiotics are effective in treating a limited and targeted group of pathogens. They include penicillins, aminoglycosides, and first- and second-generation cephalosporins. This trend is concerning because broad-spectrum antibiotics show increased risk of drug-resistant infections.³ By affecting non-targeted commensals in the body, antibiotics increase drug resistance of these organisms. Also, most of these antibiotics can be toxic with serious side effects. Broad-spectrum antibiotics are not needed for bacterial infections that can be treated with narrow-spectrum drugs like penicillin. Antibiotic stewardship efforts are being implemented to guide practitioners to prudent use of appropriate antibiotics in hospital, outpatient clinics, and veterinary practice settings.
The data presented in this introduction highlight a limitation of traditional antibiotics, which are losing efficacy against bacteria. This report will discuss how bacterial resistance becomes a problem and how bacteria are able to defend themselves from antibiotics. It will discuss how researchers are using nanoparticles to overcome the issues of dose vs toxicity, targeting, and resistance mechanisms.
3. ANTIBIOTIC RESISTANCE

The CDC defines antimicrobial resistance as the result that occurs when from microorganisms change in order to reduce or eliminate the effectiveness of drugs, chemicals, or other agents used to cure or prevent infections. The interesting takeaway from this definition is the co-dependent nature of antibiotic resistance and antibiotics. Antibiotic resistance cannot propagate without the use of antibiotics, and antibiotic use does not occur without propagating antibiotic resistance. Thus, antibiotic consumption has contributed to the emergence of antibiotic resistance in various bacterial genera. In fact, some antibiotic-resistant bacterial strains are named for the drug which gave their rise. Well-known examples include Methicillin-Resistant Staphylococcus aureus (MRSA), which emerged in the 1960s, and Vancomycin-Resistant Enterococci (VRE) which emerged in the 1990s. Unfortunately, they remain a public health concern even as more antibiotic resistant organisms emerge, continuously adding to the pool of antibiotic resistant genes.

The development of resistance is linked to how often antibiotics are used and the genetic variability of the bacteria. Because many antibiotics belong to the same class of medicines, resistance to one specific antibiotic agent can lead to resistance to the whole class. Resistance that develops in one organism or location can spread rapidly and unpredictably, through the processes of horizontal gene transfer and affect antibiotic treatment of a wide range of infections and diseases. Drug-resistant bacteria circulate in populations of human beings and animals, through food, water and the environment, and transmission is influenced by trade, travel, and both human and animal migration. The result is a pool of antibiotic resistance genes and their precursors in pathogenic and non-pathogenic bacteria, which is known as the resistome. Antibiotic resistance genes are composed of four different types: resistance genes found in pathogenic bacteria, resistance genes found in antibiotic producing organisms for their own protection, cryptic resistance genes which are phenotypically silent DNA sequences not typically expressed during the lifecycle of bacteria and that do not obviously confer resistance due to low level of expression, and precursor genes that may evolve into full resistance genes under the appropriate selective pressure.

Bacterial antibiotic resistance is classified into three categories. Intrinsic resistance comprises the inherent properties provided by the microorganism, such as the semipermeable cell wall, efflux pump, and enzymes that degrade antibiotics. Acquired resistance occurs when previously susceptible bacteria become resistant by incorporating new genetic material through horizontal gene transfer or mutation. Adaptive resistance is the temporal ability to cope with antibiotics through rapid development of resistance. It emerges when populations of bacteria are subjected to gradual increases of antibiotics and quickly reverses when antibiotics are removed. Adaptive resistance requires epigenetic inheritance and heterogeneity in the population. It is likely a combination of epigenetic processes such as methylation and the variability in expression in methylated genes account for adaptive resistance.

3.1 How Bacteria Develop Resistance

Bacteria develop resistance by genetic change through mutation, horizontal gene transfer, epigenetic changes, and genetic variation or heterogeneity.

Changes in the bacterial genome that occur through mutation or horizontal gene acquisition lead to changes in the proteins expressed by the bacteria. A challenge to pathogen survival is the need to overcome the defenses of the target host and antibiotics. These defenses cause
stress, such as oxidative stress, that damage the pathogen’s genome. Bacteria engage in horizontal gene transfer, which benefits the bacteria because it allows the exchange of DNA to repair genomic damage. Horizontal gene transfer is the acquisition of foreign DNA from plasmids, transposons, integrons, and naked DNA. It is one of the most important drivers of bacterial evolution. Plasmid-mediated resistance is the transfer of antibiotic resistance genes which are carried on plasmids and transferred between bacteria, even between species, via conjugation. Phage mediated transduction is the transfer through viruses. Bacterial transformation involves the transfer of DNA from one bacterium to another through the surrounding medium. The DNA is incorporated into the bacterial chromosome by recombination.

3.2 Antibiotic Resistance Mechanisms

Antibiotic resistance is accomplished by several mechanisms that prevent access of the antibiotic to its target or alter the antibiotic target in order to render the antibiotic useless. These mechanisms include: reduced drug permeability across the bacterial cell wall, increased antibiotic efflux from the microbial cell, antibiotic inactivation by microbial enzymes, overproduction of the target enzyme, acquisition of alternative metabolic pathways to those inhibited by the drug, and modification of antibiotic targets. In addition, bacteria that exist as biofilms or intracellular pathogens benefit from an additional barrier that blocks antibiotics.

3.2.1 Reduced permeability to antibiotics

Many antibiotics act on an intracellular target. In order to reach these intracellular targets, antibiotics must pass through the cell membrane via channels or porins. There are several protein families that can affect the ability of antibiotics to cross the cell membrane. This form of antibiotic resistance is referred to as reduced permeability or decreased antibiotic penetration, and it results in reduced internalization of antibiotics. Clinically relevant drugs with intracellular targets that are affected by reduced permeability include: beta-lactams, fluoroquinolones, aminoglycosides, and tetracyclines. The major cause of reduced permeability is altering membrane bound porin molecules. Porin molecules permit the transport of hydrophilic substances across the outer membrane and the cytoplasmic membrane. They are altered in three ways to increase resistance: shifting the type of porins expressed, changing the level of porins expressed, and impairing porin function.

Outer membrane porins (Opr) are a broad family of porins found in gram-negative bacteria that offers a variety of substrate selectivities. An important example is OprD found in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. In *P. aeruginosa*, OprD allows uptake of basic molecules such as imipenem. According to a study by Yan et al, a four-nucleotide base-pair insertion to the oprD gene resulted in a conformational change in the OprD channel that reduced its permeability to carbapenem antibiotics. Additional outer membrane porins include OmpF and OmpC. OmpF allows passage of beta-lactam antibiotics into the periplasmic space. When under antibiotic stress, *Escherichia coli* reduce the expression of OmpF. Therefore, reduced permeability of beta-lactams limits their effect against *E. coli*. In a similar way, OmpC is down-regulated when subjected to antibiotic stress. A plethora of other bacteria also have reduced beta-lactam permeability due to porin changes.

Sugar porins are a family of transporter molecules that specialize in transporting sugars across the outer membrane of Gram negative bacteria, but also permit the transport of tetracyclines and fluoroquinolones. LamB variants in *E. coli* reduce permeability to these drugs as explained
MltA-interacting protein (MipA) in \textit{E. coli} is a transporter of aminoglycosides and fluoroquinolones. Often this porin is lost in resistant bacterial strains.\textsuperscript{13}

### 3.2.2 Antibiotic efflux

Reduced permeability often works together with another form of resistance permitted by efflux pumps. Efflux proteins pump material, including antibiotics, from inside the cell to the external environment. The effect of antibiotic efflux is reduced concentration and efficacy of intracellular antibiotics. There are a variety of protein families that convey drug resistance through efflux pumps.

Major facilitator superfamily (MFS) is a large and diverse superfamily of secondary active transporters. In gram-positive bacteria, this family of efflux pumps work as monomeric antiporters. Meanwhile, in gram-negative bacteria, they may be paired with other outer membrane pumps. A major group of MFS transporters are the tet family proteins which offer resistance to tetracycline.\textsuperscript{14}

Several other classes of efflux pumps include: small multidrug resistance transporters (SMR), ATP-binding cassette transporters (ABC), the resistance-nodulation-division proteins (RND), and multidrug and toxic compound extrusion transporter (MATE) as demonstrated in Figure 3 (page 7). The SMR efflux pump family is a secondary active transporter family. Important examples of this family include EmrE in \textit{E. coli} and Smr-2 in \textit{S. aureus} and \textit{P. aeruginosa}.\textsuperscript{15} RND proteins are a broad family of proteins, which include some secondary transporters. Important examples include the AcrAB-TolC system in \textit{Enterbacteriaceae} and the MexAB-OprM system in \textit{P. aeruginosa}.\textsuperscript{16} Researchers at Iowa State University have recently demonstrated the transport dynamics of an important multidrug RND efflux pump in \textit{Campylobacter jejuni}.\textsuperscript{17} This process includes the synchronized motion of three subunits along with independent conformational changes for each subunit. MATE transporters utilize a cationic gradient to pump out constituents. They are effective at removing quinolones.\textsuperscript{18} Finally, the ABC transporters are a large and diverse family of primary active transporters that includes several efflux pumps, and provide multiple drug resistance.

### 3.2.3 Enzymatic antibiotic inactivation

Most antibiotics catalyze or block an action with high specificity for a target molecule. Alteration of the antibiotic through enzymatic modification is another way in which bacteria can acquire resistance. A well-known example of this form of resistance is β-lactamase. This enzyme offers resistance to β-lactam antibiotics such as penicillins and cephalosporins through breaking the cyclic structure of the antibiotic.\textsuperscript{19} Aminoglycosides are also commonly inactivated by chemical reactions like acylation and phosphorylation.\textsuperscript{19} There are several known enzymatic modifications bacteria have employed to gain antibiotic resistance including: glycosylation, hydrolysis, phosphorylation, acylation, and hydroxylation.

### 3.2.4 Changes at the antibiotic target

Antibiotic resistance through changes at the antibiotic target includes overproduction of the target enzyme, acquisition of alternative metabolic pathways to those inhibited by the drug, and modification of antibiotic target sites through enzymatic or mutational alterations. For example, mutations to ribosomal RNA can reduce the binding affinity for antibiotics such as tetracycline and aminoglycosides.\textsuperscript{20} Vester et al provides a good summary of rRNA mutations that allow
resistance to macrolide antibiotics. Mutations to penicillin-binding protein permit resistance to the several β-lactam antibiotics. There are also mutations to topoisomerase that provide resistance to quinolones. The consequences of these mutations are demonstrated in Figure 4 (page 8). Beyond simple mutations, bacteria can bypass the effects of antibiotics by producing functionally similar but structurally different proteins. Sulfonamid resistance, for example, works in this way.

Figure 3. The various efflux pump classes and the antibiotics they're effective against.

### 3.2.5 Physical barriers for drug evasion

Biofilms and intracellular life are two ways bacteria can evade antibiotic treatment. The bacteria that exist in biofilms produce an extracellular matrix that provides a barrier to antibiotic treatment and host immune response, thus creating a protective niche in the body. In fact, biofilms confer up to 1000 times more resistance to antibiotics than planktonic bacteria. Several clinically relevant biofilm-forming bacteria include: *P. aeruginosa*, *S. aureus*, and *Enterococci*. A common component of biofilms is extracellular DNA, including DNA that encodes resistance genes, which is shared among the bacteria existing in the biofilm. Additionally, a fraction of bacteria in biofilms exist as “persisters.” These bacteria are in a non-reproductive, non-growing state that permits insensitivity to antibiotics. When treating a biofilm with antibiotics, persisters often outlast the antibiotic treatment and prolong the infection when they reactivate. This leads to chronic infections resistant to treatment.

The other major physical barrier some bacteria utilize to evade antibiotic treatment is intracellular life in host cells. The intracellular lifestyle often occurs in phagocytic cells where bacteria such as *Listeria* and *Brucella* have evolved ways to avoid degradation in the phagolysosome, and subsequently proliferate in the phagocytic cell. Additionally, using antibiotics against intracellular pathogens requires the antibiotic to accumulate in both the phagocytic cell and the intracellular compartment of the phagocyte at sufficiently high concentrations. Even then the bacteria may express resistant genes. Unfortunately, this makes treatment of these organisms difficult, and offers a high incidence of antibiotic treatment failure.
3.3 Limitations of Antibiotic Treatment

Antibiotics attack bacteria by targeting biochemical reactions, structural features, or both. The effects of antibiotics are either bactericidal if they kill their target or bacteriostatic if they inhibit cell division. Antibiotics work best when they target features specific to bacteria, thereby limiting unwanted side-effects to the host. However, no drugs are perfect and most if not all antibiotics carry the risk for adverse effects in the body. The prevalence of adverse effects increases as the dose of antibiotic increases, which is sometimes necessary to defeat antibiotic-resistant bacteria.

The limitation of antibiotics is their toxicity, lack of targeting, and ineffective treatment against resistant bacteria. The problem created by these three limitations lies at the heart of the impending threat bacteria pose to modern health, and is exactly where nanoparticles can most contribute.

4. NANOPARTICLES

Many advocates for nanoparticles, and nanotechnology in general, predict that they are on the precipice of mainstream medicinal use. Nanoparticles have the potential for more effective drug treatment through targeted drug delivery, intrinsic antimicrobial activity, and functionalization to overcome antibiotic resistance.

4.1 General Characteristics

Nanoparticles are incredibly small. The nanoscale, as demonstrated in Figure 5 (page 9), ranges from 1-1000 nanometers, or between the size of a typical bacterium down to the
diameter of a few adjacent silicon atoms. At this small size, interesting physical and biological features arise. In the context of medicine, nanoparticles are most useful at less than 100 nm, which allows passage through capillary fenestrations around the site of infection. Nanoparticles can take various shapes like round or filamentous.

Figure 5. Nanoparticles of various chemical compositions fit comfortably in the nanoscale range. Several types of nanoparticles are highlighted in section 4.2.

4.2 Types of Nanoparticles

The term nanoparticles refers to any particle in the nanoscale, but composition of those particles can be quite varied. To assist in categorizing nanoparticles, they have been classified into groups based on the chemical compositions. Several review articles summarize the classification of nanoparticles into the following groups: liposomes, solid-lipid nanoparticles, polymeric nanoparticles, polymeric micelles, dendrimers, and several types of inorganic nanoparticles made of gold, silver, silica, iron-oxide and others.

Liposomes are small lipid colloids that consist of a central aqueous space surrounded by a lipid bilayer with diameter ranging between 20-100 nanometers. Typically, liposomes near the 20-nanometer range are unilamellar micelles, whereas those near the 100-nanometer range are bilayered vesicles. Liposomes are self-assembling in aqueous solution, and are often
prepared by sonication. Being phospholipids, liposomes are more biocompatible than other nanoparticles. However, they may leak their loaded cargo, and experience uptake and removal by phagocytic cells.

Solid-lipid nanoparticles are another lipid-based nanoparticle composition that uses lipids that exist as solids around body temperature, for example acetyl palmitate or salts of myristic acid. They are also relatively biocompatible, and can be tailored for controlled cargo release for various environments. They are also more stable that their liposomal counterparts. The limitation of solid-lipid nanoparticles is their tendency to form gels and low loading capacity for antibiotics.

Polymeric nanoparticles are matrix polymers with hydrophilic and hydrophobic regions. Synthetic polymeric nanoparticles are typically made of polyesters like polylactide, polycaprolactones, or polyacrylates. They are also comprised of various other organic compounds. At Iowa State University, polyanhydride nanoparticles are a major focus of research. Polymeric nanoparticles are also formed from natural substances like albumin, alginate, or chitosan. These particles are stable in the body and can be tailored to degrade and release their cargo in specific environments. They also are associated with reduced ability to adjust dosage and are difficult to handle. Antibiotics can be attached to the surface of the nanoparticle, or dispersed through the particle during polymerization.

Another class of polymeric nanoparticles are polymeric micelles, which are formed from block copolymers or polymers with two distinct blocks. One block forms the hydrophobic inner shell while the second block forms the hydrophilic outer shell. Drugs like antibiotics are either physically encapsulated or otherwise attached through covalent bonding. These nanoparticles can effectively package hydrophobic cargo and accumulate at the target with enhanced permeability, but they also have low drug incorporation stability which could result in loss of the cargo before reaching the target site.

In addition to the organic-based nanoparticles there are a variety of inorganic nanoparticles. The most prominent of these are gold, silver, iron-oxide, and other metal nanoparticles. Like organic nanoparticles, these nanoparticles can be modified to have a variety of cargo-loading, cytotoxic, and other characteristics. These nanoparticles can be smaller with greater loading capacity and higher antimicrobial activity than other nanoparticles. However, they have lower biodegradability and are harder to excrete from the body. Therefore, inorganic nanoparticles tend be more toxic than their organic counterparts.

4.3 Functionalization

One of the most important features of nanoparticles is their so-called “functionalization,” in which they are engineered to demonstrate specific features. Nanoparticles have been functionalized for many of purposes including drug delivery. One form of functionalization that is a focus of this paper is surface functionalization. Surface functionalization involves the conjugation of various chemical features to the surface of the nanoparticle. In practice, this means chemists can attach different ligands to the nanoparticles. This is perhaps the most exciting feature of nanoparticles, because of its powerful effect on biodistribution. Researchers have attached various ligands to nanoparticles including various small molecules, dendrimers, polymers, antibodies and other biomolecules. Several of these ligands are highlighted in Figure 6 (page11). Attaching ligands to the surface of nanoparticles offers two benefits for drug delivery. The first is providing nanoparticles a way for “biosensing,” or specific recognition of the
target biomolecule. For treating infections, this is usually a bacterial receptor. The second advantage is offering nanoparticles a form of “stealth,” or preventing particle capture by the immune system. Together, these features increase delivery of antibiotics to the infection, reduce random dispersal of antibiotics in the body, and increase the half-life of circulating antibiotic loaded nanoparticle. Several examples of this process are shown later in this report.

![ACTIVE TARGETING OF DRUG LOADED NANOPARTICLE](image)

**Figure 6.** Several forms of surface functionalization. Section 5.2 highlights recent research involving these various ligand types.  

5. NANOPARTICLES APPLICATIONS IN ANTIBACTERIAL DRUG THERAPY

The progress in nanoparticle research to fight antibacterial drug resistance is summarized in several recent comprehensive review articles, including those by. These reviews indicate the potential benefits of nanoparticle treatment including their controlled distribution of payload drugs, targeted delivery of antibiotics to the site of infection, exploitation of the microenvironment for controlled antibiotic release, and their potential against intracellular pathogens and biofilms.

5.1 Drug Distribution

The physical properties of nanoparticles such as size, shape, hydrophilicity, and zeta-potential affect their distribution in the body. These features are an advantage over traditional delivery methods. For example, orally administered antibiotics rely on high concentration with random distribution throughout the body to reach the intended target tissues in adequate concentration. This form of uncontrolled delivery means much of the administered drug accumulates in uninfected host tissues. The previously mentioned physical properties of nanoparticles can be tailored to encourage delivery of nanoparticles to the site of infection, while avoiding accumulation in non-infected tissue.
Zaidi et al notes the importance of particle size in determining the distribution of nanoparticles in the body. Small nanoparticles are distributed throughout the body more rapidly and evade macrophages more efficiently than large particles. Small nanoparticles are also more efficiently loaded with antibiotic than large nanoparticles. These phenomena allow smaller nanoparticles to deliver more antibiotic more quickly to the site of infection than larger nanoparticles. However, nanoparticles smaller than five nanometers can be rapidly cleared by the kidneys, and thus lose distribution to the body. Also, smaller nanoparticles lose some selectivity in drug distribution as they can accumulate in more areas of the body beyond just the site of infection.

The shape of the nanoparticle is an important variable for the rate of clearance and phagocytosis. Studies have shown that particles of the same size, but different shapes have different distribution. Filamentous particles exist in circulation 10 times longer than spherical nanoparticles with similar composition highlighting the major role shape plays in the circulating half-life of a particle. Additionally, particle shape plays a major role in phagocytosis by influencing the actin structures needed for internalization. Shape seems to play at least as important if not more important role in phagocytosis than size.

Two other important physical properties include surface features such as hydrophilicity, and zeta-potential. These physical properties affect the way nanoparticles interact with cell membranes, proteins, and each other with consequences that include protein adsorption, endocytosis, and distribution in the body or cell. For example, increasing the hydrophilicity of a nanoparticle reduces its opsonization by antibodies, which prevents clearance by cells in the liver or spleen. On the other hand, increasing hydrophobicity promotes uptake by cells and therefore less distribution in the body. Zeta-potential is an electrokinetic property that measures the resistance of colloidal particles to coagulate. Having similarly charged nanoparticles can increase their zeta-potential, thus reducing their coagulation to each other while promoting their attraction to cell membranes of opposite charge. Zeta-potential above +40 mV are known to alter bacterial cell membrane permeability by acting as detergents, causing osmotic damage and cell death.

5.2 Drug Targeting

Perhaps the most exciting and important prospect of nanoparticles is their potential for targeted drug delivery. Selectively targeting antibiotics to the site of infection through delivery by nanoparticles offers a way to avoid drug delivery to unintended locations and the toxicity associated with it. At the same time, targeted delivery can potentially increase the efficacy of antibiotics, and reduce the emergence of drug resistance. Nanoparticles accomplish targeted drug delivery through passive or active mechanisms.

Passive mechanisms rely on changes that occur around the site of infection, such as increased vascular permeability, as a route for targeting. Bacterial components, like lipopolysaccharides or lipoteichoic acid, accumulate at the site of infection and trigger inflammation. This inflammatory response promotes increased vascular permeability by allowing gap widening, and barrier dysfunction as the vasculature dilates. These features, along with reduced lymphatic drainage, promote the enhanced permeation and retention effect as shown in Figure 7 (page 13). Nanoparticles can take advantage from this naturally occurring process for their own accumulation at the site of infection through manipulation of several features like size or shape that increase permeability in the locally dilated vasculature. Once reaching the site of infection the nanoparticles degrade and release their antibiotic payload. Laverman et al provide
evidence for this passive mechanism. They showed that liposomes, including PEGylated liposomes, accumulate in soft tissue infected by *Staphylococcus aureus*. Scintigraphy from their study is shown as Figure 8 (page 14).

Active targeting expands on the principles of passive targeting for further control of drug delivery. Active targeting utilizes specific features of the bacterial cell surface, such as charge or ligand receptors, as foci for accumulation. Many bacteria maintain a negatively charged surface. Nanoparticles with a positive charge use electrostatic potential in order to accumulate on the bacteria. Positively charged peptide nanoparticles may be used for direct toxicity to infections while reducing toxicity to other tissues. In one particularly interesting example, zwitterionic gold nanoparticles react to the pH microenvironment around an infection and switch to a positive charge. While positively charged, the nanoparticles aggregate to the bacterial cell wall. Active targeting is also accomplished by conjugating nanoparticles with ligands that directly bind with pathogens. For example, vancomycin is a ligand that strongly attaches to the surface of gram-positive bacteria by binding to alanine moieties in the cell wall. It also has some affinity for the receptors on the surface of Gram negative bacteria. Researchers have conjugated vancomycin to iron oxide and gold-based nanoparticles, which showed increased accumulation near the site of infection. Another example of nanoparticle based drug targeting used silica nanoparticles conjugated to vancomycin. Lectins can also be used as a ligand to actively target bacteria. In a study involving *Helicobacter pylori*, mannose or fucose-specific lectins were able to successfully target carbohydrate-based receptors on the bacterial cell surface.

Figure 7. Bacteria-induced Enhanced Permeability Effect for accumulation of nanoparticles at the site of infection. The inflammatory process creates a localized area of increased fluid accumulation, which nanoparticles exploit for therapeutic effect.
The selective attraction between antigens and antibodies offers another option for drug targeting, such as antibodies against endotoxins to target Gram-negative bacteria or antibodies against lipoteichoic acid to target Gram-positive bacteria. Black et al developed inorganic nanoparticles functionalized with anti-bacterial antibodies, and observed effective targeting to bacteria. Conjugating inorganic nanoparticles with antibodies greatly increased their targeting and efficacy. Dai et al used nanoparticles modified with Salmonella antibodies and were able to achieve targeting and low toxicity to the host.

There are several other molecules which can allow active targeting to bacteria, such as phage tail-spikes and aptamers. Studies using these molecules have shown strong attraction to *Salmonella typhimurium* and *Mycobacterium tuberculosis*. They may emerge as effective ligands for nanoparticles in the near future.

An interesting development in targeted drug delivery uses a technique called molecular imprinting in which cavities are developed into the nanoparticles. The cavities act as receptors for features on the bacterial surface like lipopolysaccharides. This is achieved through a process called inverse microemulsion polymerization, and has been used to target lipopolysaccharides on *Pseudomonas aeruginosa*. Researchers have attempted to target beta-lactamase using the molecular-imprinting technique as well.

### 5.3 Microenvironment Responsiveness

The microenvironment offers a variety of unique characteristics that drug delivery systems can respond to. Chen et al highlights several of those features, such as enzymes secreted by bacteria and pH. Bacteria secrete enzymes such as lipases that are concentrated around infection. Researchers used this idea to develop polyphosphodiester nanoparticles, sometimes called nanogels, that are degraded by lipases. Lipases are also utilized by polymeric nanoparticles linked to fatty acid esters or anhydrides. Other bacterial enzymes, like hyaluronidase, are being used in a similar way. Ji et al showed that hyaluronidase degrades nanoparticles into reactive oxygen species that are effective in dispersing biofilms.

A common feature of bacterial infections, especially those associated with a biofilm, is anaerobic glycolysis. The pH of the microenvironment is lowered by anaerobic glycolysis performed by bacteria. Some nanoparticles have been developed to respond to changing pH.
For example, researchers developed mesoporous silica nanoparticles loaded with b-lactam and further surrounded by a pH responsive metallic shell. At the site of infection, the shell degrades due to the lowered pH, and leaves the highly porous nanoparticle, which rapidly releases its antibiotic load.\textsuperscript{54} pH stimuli have been used to control drug release in the gastrointestinal tract, to intracellular lysosomes, or to the microenvironment surrounding bacteria. In one example, bismaleimide was used as a link to block pores in the nanoparticle from opening, but will move when protonated by a more acidic environment. The altered form of bismaleimide allowed pores to open and the antibiotic to be released.\textsuperscript{31} In another example, polymeric nanoparticles made of poly(L-histidine), polylactide-polyglycolide, and polyethyl glycol, switch from a negative charge to a positive charge when subjected to more acidic conditions. The switch promoted electrostatic binding to bacteria and delivery of antibiotics to the site of infection.\textsuperscript{55} Figure 9 summarizes this process.

![Figure 9](image_url)

**Figure 9.** An example of environmental responsiveness to promote accumulation of nanoparticles at the site of infection. Functionalized polymeric nanoparticles promote selective attraction to bacteria by exploiting electrostatic potential. The switch is promoted by the low pH that accompanies bacterial infection.\textsuperscript{56}

### 5.4 Liposomes

The interaction that occurs between nanoparticles and bacteria during drug delivery is dependent on the chemistry of the nanoparticle. The fusion mechanism between liposomes and bacteria is controlled by the electrostatic and hydrophobic forces that attract liposomes to the bacterial membrane. Passive fusion of liposomes occurs through the “stalk mechanism.” The liposome forms an hour-glass shaped stalk that promotes fusion and reorganization with the bacterial membrane. This mechanism is influenced by the composition of the liposome.\textsuperscript{34}
Controlled fusion of liposomes is accomplished using a trigger. Like other nanoparticles, liposomes use chemical interactions or a ligand as their trigger. For example, conjugating polyethylene glycol to the liposome increases its hydrophilicity and inhibits spontaneous fusion with cell membranes of host cells. Kirputin et al developed pH-sensitive liposomes. The liposome contained disulfide linkages connecting polyethylene glycol to a lipid called dioleylphosphatidylethanolamine. The disulfide linkages were pH sensitive, lysing around pH 5.5. The result was detachment of polyethylene glycol. The remaining liposome was then able to fuse to bacterial cells or phagocytes using the stalk mechanism. In another study, researchers developed a ligand that mimicked an antigen present on gastric epithelial cells. The ligand was recognized by a receptor expressed by Helicobacter pylori. The tight interaction between the ligand and bacterial receptor promoted liposomal fusion with the bacterium and subsequent release of antibiotic.

### 5.5 Nanoparticles as Antibiotics

Nanoparticles, especially metal-based nanoparticles, are intrinsically antibacterial. There are several mechanisms through which nanoparticles can achieve this action. According to Wang et al the leading causes of direct antibacterial action from nanoparticles are oxidative stress, metal-ion release, or other non-oxidative mechanisms. These mechanisms are highlighted in Figure 10.

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**Figure 10.** The toxicity of metallic nanoparticles against bacteria through three mechanisms: oxidative stress, metal ion release, and non-oxidative stress.

Oxidative stress is the most important of these three mechanisms. Various inorganic nanoparticles are reducing agents and convert the oxygen species of the microenvironment into reactive oxygen species. Reactive oxygen species increase bacterial membrane permeability and eventually erode membrane integrity. Additionally, intracellular reactive oxygen species
promote expression of oxidative proteins that result in death of the bacteria. Nanoparticles produce several types of reactive oxygen species. Magnesium and calcium based nanoparticles are associated with superoxide radicals (O$_2^-$), while zinc-oxide based nanoparticles produce hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals (·OH). Metal-based nanoparticles also attack bacteria by releasing metal ions that disrupt protein function in the cell, but this is thought to be a minor contributor to antimicrobial action.

An important non-oxidative mechanism is covalent bonding to the cell wall. This mechanism alters cell metabolism by restraining vital proteins, for example the proteins involved in the electron transport chain. However, this antibacterial activity is not very effective against gram negative bacteria.

5.6 Overcoming Resistance Mechanisms

As previously mentioned, efflux pumps are an effective way for bacteria to survive antibiotic treatment. Current research is developing ways to overcome this mechanism of resistance using chemicals like piperine. Piperine is an alkaloid chemical sometimes found in traditional medicines. In one study, a strain of methicillin-resistant _S. aureus_ that utilized efflux pumps were subjected to nanoparticles loaded with gentamycin. Two kinds of nanoparticles were given as treatment: bare liposomes or liposomes containing piperine. The bacteria were also given ethidium bromide, which fluoresces when bound to nucleic acids inside bacteria. Data from the experiment showed increased fluorescent activity and increased bactericidal activity in the piperine loaded liposomes versus the bare liposomes, allowing the researchers to conclude that piperine blocked the efflux pumps. These nanoparticles reduced efflux pump mediated antibiotic resistance in _S. aureus_

Nanoparticles offer a protective barrier between degradative enzymes and the antibiotic thereby preventing inactivation of the drug. Alipour et al used liposomes to encapsulate tobramycin and polymyxin B. These polycationic compounds are prone to inactivation by polyanionic endotoxins like lipopolysaccharides and lipoteichoic acid or by other extracellular components like mucin. During their _in vitro_ study, free antibiotic and liposome-containing antibiotic were incubated in several different cultures of polyanionic compounds. After incubation, the potency of each drug was tested. Both the free and packaged antibiotic showed reduced efficacy by the polyanionic environment as a function of concentration. However, the concentration of polyanionic compounds needed to inactivate free antibiotic was much less than that needed for antibiotic loaded into a liposome. The researchers further confirmed their data by applying the antibiotic to _P. aeruginosa_ from the sputa of a cystic fibrosis patients. The sputa provide a polyanionic environment that could inactivate the antibiotic. They found the packaged antibiotic to have four-fold higher activity than the free antibiotic, and was particularly helpful for polymyxin B efficacy.

5.7 Nanoparticle Applications to Biofilms

Nitric oxide has shown to inhibit biofilm formation and break up previously formed biofilms. The review by Diab et al highlighted a couple ways nitric oxide is being delivered to biofilms for this purpose. In Jadeleza et al, liposomes were loaded with isosorbide mononitrate, and used to release nitric oxide in a slow, controlled manner in a biofilm. The drug release ablated a biofilm of _S. aureus_. In another study, nitric oxide was released by polymer nanoparticles and prevented planktonic bacteria from attaching to a biofilm of _P. aeruginosa_ through stimulating phosphodiesterase activity. This action effectively inhibited biofilm growth.
Another approach is using nanoparticles to penetrate the biofilm. Several studies have been done to compare the penetration of antibiotics loaded into nanoparticles and soluble antibiotic. In Alipour et al the researchers used polymyxin B loaded into liposomes. Using microscopy methods, the researchers measured drug penetration into *P. aeruginosa* biofilms over a period of 16 hours. Polymyxin B showed improved penetration compared to soluble drug. A similar experiment was conducted using amikacin against *in vivo* respiratory infections of *Pseudomonas*. Although the free amikacin was unable to affect the biofilms, amikacin loaded into liposomes were able to penetrate and destroy biofilms. The researchers accounted this improved action to better drug penetration and sustained drug release by the liposomes.

5.8 Targeting Nanoparticles to Phagocytes

Nanoparticles offer a solution to persistent intracellular bacteria by targeting antibiotics to phagocytic cells. As Zaidi et al note, nanoparticles target phagocytes by promoting phagocytosis. Once inside the phagocytic cells, the nanoparticles release their antibiotic payload. A recently published study from Iowa State used polyanhydride nanoparticles to target *Brucella melitensis* in murine macrophages. When comparing soluble rifampicin against the nanoparticle loaded rifampicin, the nanoparticle outperformed. By 72-hours post infection, the macrophages given nanoparticle treatment had zero colony-forming-units of *Brucella*. Meanwhile, the soluble treated bacteria still managed over 10000 colony-forming-units by 72-hours post infection, and were growing in population. This data suggests that treatment using soluble antibiotic selected for resistant strains, while nanoparticle treatment was able to fully remove the infection. In this way, the authors concluded that nanoparticles could provide increased efficacy and dose sparing for intracellular antibiotic treatment. Their data is summarized in Figure 11.

![Graph](image)

Figure 11. Data from the Lueth et al *in vitro* study. "Soluble" represents free rifampicin. 20:80 CPH:SA and 20:80 CPTEG:CPH are two different polyanhydride nanoparticle compositions.
Passive and active targeting to phagocytes occurs by using opposite features as those for targeting to infection. For example, rather than the positive charge that promotes attraction of a nanoparticle to bacteria, a net negative charge of the surface of the nanoparticle promotes internalization by phagocytic cells.\textsuperscript{32,25} Also, rather than using hydrophilicity to promote distribution of the nanoparticle in the body, increasing hydrophobicity promotes internalization by phagocytes.\textsuperscript{26} Certain ligands attached to the surface of nanoparticles increase internalization of the nanoparticles by phagocytic cells.\textsuperscript{36} Nanoparticles featuring mannose, amylopectin, or animal derived albumin can enhance nanoparticle uptake by phagocytic cells.\textsuperscript{31} Antibodies could also potentiate internalization by phagocytic cells.\textsuperscript{25} Promoting nanoparticle internalization would improve antibiotic efficacy against intracellular organisms.

6. CONCLUSION

The emergence of antibiotic-resistant strains of bacteria is outpacing the development of new antibiotics, with new antibiotics quickly encountering resistance within a few short years of development. This issue is having a significant impact on health care, and will only become worse with the emergence of stronger multiple drug resistant strains of pathogenic bacteria. Our best chance to counter this growing concern is through breakthrough innovations, such as nanotechnology. The application of nanotechnologies is becoming a driving force behind ongoing changes in the antimicrobial field. Nanoparticles show promise as a drug delivery approach to defeat bacterial defenses through passive and active targeting, anti-biofilm action, microenvironment responsiveness, and inherent antimicrobial action. Perhaps continued improvement to the nanoparticle approach may bypass the issue of antibiotic resistance altogether.

In addition to these advantages, new innovations in nanoparticle technology like stimulus-response and other novel modes of killing mechanisms are being developed. Simultaneous real-time detection and therapy, known as theranostic nanoparticle technology, may demonstrate effective antibiotic treatment. Additionally, nanoparticles can be applied ways beyond drug delivery. As highlighted in Figure 12 (page 20), nanoparticles have many applications in preventative medicine by coating prosthetics or other medical implants and devices that often result in nosocomial infections.

Nanoparticles' rapid emergence as an antibiotic delivery method has elicited some concerns. There must be thorough examination of their long-term safety, and assessments of the biocompatibility between nanoparticles and the human body prior to large clinical studies. Further study of the retention and clearance, pharmacokinetics, biodistribution, and immune surveillance on the many varieties of nanoparticles is a challenge that will require the development of efficient \textit{in-vitro} and \textit{in-vivo} screening technologies. Despite the concerns, continuing research into production methods, composition, and payloads means nanoparticle technology will play a more significant and expanding role in our fight against bacterial infection.
Figure 12. The potential applications of nanoparticles.
REFERENCES


