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Complete List of Authors:	Miller, Kristen; University of South Carolina, Environmental Health Sciences Wang, Lei; University South Carolina, Chemistry and Biochemistry Benicewicz, Brian; University South Carolina, Chemistry and Biochemistry Decho, Alan; University of South Carolina, Department of Environmental Health Sciences

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REVIEW ARTICLE

Inorganic Nanoparticles Engineered to Attack Bacteria

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K.P. Miller,[a±] L. Wang,[b±] B.C. Benicewicz,[b] and A.W. Decho[a*]

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Antibiotics were once the golden bullet to constrain infectious bacteria. However, the rapid and continuing emergence of antibiotic resistance (AR) among infectious microbial pathogens has questioned the future utility of antibiotics. This dilemma has recently fueled the marriage of the disparate fields of nanochemistry and antibiotics. Nanoparticles and other types of nanomaterials have been extensively developed for drug delivery to eukaryotic cells. However, bacteria have very different cellular architectures than eukaryotic cells. This review addresses the chemistry of nanoparticle-based antibiotic carriers, and how their technical capabilities are now being re-engineered to attack, kill, but also non-lethally manipulate the physiologies of bacteria. This review also discusses the surface functionalization of inorganic nanoparticles with small ligand molecules, polymers, and charged moieties to achieve drug loading and controllable release.

1. Introduction

The seminal realization of the nano-world, and its potential to be manipulated at molecular and atomic scales developed over a half century ago.[1] However, largely due to a lack of technical capability, a time of obtuse contemplation followed. During this initial lull in nano-based research, the 1950s and 60s saw a surge of attention in the development of antibiotics – the wonder bullet cited to stop all bacterial disease. However, this phenomenon was quickly tempered by a rapid emergence of antibiotic resistance (AR) among pathogens, often in the form of multi-drug resistance (MDR). AR continues to grow today, and at present, the future utility of antibiotics remains questionable.

Recently, the surge in nanoresearch, coupled with dramatic increases in technical capabilities, has allowed the disparate fields of nanochemistry and antibiotics to come together. Traditionally, nanoparticles and nanomaterials have been developed as carriers to deliver anticancer and other forms of drugs to eukaryotic cells. Given this foundation, the delivery of antibiotics using engineered nanoparticles has become an emerging and realistic area of research. Nanoparticle-based antibiotic-carriers are now being re-engineered to attack and kill, but also non-lethally manipulate the physiologies of bacteria. Overcoming the problem of antibiotic resistance, however, requires a more in-depth understanding of interactions between the biology of microorganisms and the physical chemistry of nanoparticles. This understanding is necessary in order to ultimately target bacteria, and overcome their immense arsenal of defences. This review centers on fundamental, polymer-based, and time-release surface chemistry, which is currently being developed for nanoparticle delivery of antibiotics to bacteria. We also integrate approaches being developed to enhance the detection and quantification of bacteria within biological systems. Concurrent with this chemistry is a necessary overview of certain bacterial processes, as these directly and indirectly interact with the chemistry of nanoparticles.

2. Nanoparticles as Drug Carriers: A Brief Historical Perspective

Credited for conceiving the idea of a "magic bullet" to selectively target toxic organisms in the body, Paul Ehrlich inspired many pioneers of the nanoparticle field. In the 1950s and 60s, Peter Speiser's group worked on the development of polyacrylic beads ,[2,3] then microcapsules,[4] and eventually the first nanocapsules.[5] Their ultimate goal was to achieve sustained drug release from nanocapsules in the blood after intravenous injection.

Since that time, nanoparticles have been used for pharmaceutical and medical applications, mainly for cancer treatment[6] and enhancing the efficacy and targeting of cancer drugs.[7,8] This has allowed for the use of lower concentrations of highly-toxic drugs in an effort to reduce side effects.[9] More recently, and in conjunction with the important discovery that polyethylene glycol chains on nanoparticles prolong blood circulation and reduce liver uptake,[10] studies have been conducted on the ability of nanoparticles to cross the blood brain barrier, and target deep brain tumors or infections.[11-14] Innovative research conducted over the past 50 years has reinforced the roles of nanoparticles as drug delivery vehicles and has inspired the current diversity in nanoparticle research. Today, investigators focus their nanoparticle research on recognition, sensing, imaging, and delivery in biological systems with a broad range of core materials.

3. Direct and Indirect Antimicrobial Properties of Inorganic Nanoparticles

The unique physical-chemical properties of inorganic nanoparticles/nanomaterials have been utilized for hundreds of years.[15] More recently, certain types of inorganic NPs have been found to exhibit strong antimicrobial properties. However, their applications as antimicrobial agents are limited by their apparent toxicity to other biological systems (e.g. human cells). This section

examines major types of inorganic NPs, and discusses the chemistry of their toxic- and/or non-toxic properties, and antimicrobial mechanisms of action. Also provided is an overview of the methods used to determine the efficacy of inorganic nanoparticles as luminescent biosensors for generation of ROS, DNA damage, determinations of cell membrane integrity, preparations for electron microscopy, live/dead assays, Raman scattering, SDS-PAGE analysis of proteins, and gene expression, etc. in bacterial systems. Since some bacteria can protect themselves and survive potentially-lethal stressors by entering a 'viable but nonculturable' (VBNC) state, special techniques, alternative to traditional culturing methods, are required to evaluate the antimicrobial properties of inorganic nanoparticles. These approaches are also discussed.

3.1 Silver Nanoparticles

The antimicrobial properties of silver and related silver compounds have been utilized for hundreds of years. Silver nanoparticles, in addition to silver ions, metallic silver, silver nitrate, silver sulfadiazine, and silver zeolite, typically encounter minimal bacterial resistance and possess strong bactericidal properties.[16-19]

The antimicrobial effects of silver nanoparticles have been observed in Gram-positive bacteria, Gram-negative bacteria, and yeast.[20-24] Silver nanoparticles are effective at preventing growth of bacteria on surfaces of agar plates, but are comparatively less-effective in liquid medium, due to aggregation of colloidal silver in the presence of high salts and other media components.[23,25-26] These results have suggested that the antimicrobial ability of silver nanoparticles is dependent upon surface oxidation (by the nanoparticle) and particle dispersion. The size and shape of silver nanoparticles also play a major role in their ability to interact with the bacterial surface and release silver ions (Ag^+) into solution. Given their enhanced surface area, smaller, spherical nanoparticles (1-10 nm) are generally more effective at attaching to the surface of a cell membrane and disturbing permeability and respiration than larger nanoparticles.[25,27-28] Thus, reducing the size and increasing the surface area of nanoparticles provides a greater number of reactive groups, Ag^+ in this case, and is believed to enhance nanoparticle toxicity.[29-31] Small nanoparticles penetrate the bacterial cell membrane and interact with the thiol groups of proteins, preventing expression of ribosomal subunit proteins, deactivating the enzymes and cellular components essential to ATP production,[28] and by preventing DNA replication.[17,27]

The mechanisms of action of silver nanoparticles are not entirely understood, although recent research has demonstrated that silver ions affect essential bacterial cellular components such as cell membrane integrity, respiration, and ATP production (mentioned above). To date, however, there have been relatively few observations reported for microbial resistance against silver. Originally discovered in *Salmonella typhimurium*,[32] and then later in *Pseudomonas aeruginosa*, *Candida albicans*, and the environmental isolate *Acinetobacter baumannii*, silver resistance has been the result of both intrinsic and acquired genes.[33] The distinction between silver sensitivity and silver resistance is difficult to determine, however, because silver-resistant phenotypes are not consistent. Silver resistance may be incurred by plasmids, specialized rapid-efflux pumps, or genetic mutations that are repaired after silver pressure has diminished.[18,33-34]

Medical devices and wound dressings impregnated with silver nanoparticles have made a large impact on reducing infection rates in hospital settings;[35] however, the toxicity of these compounds on humans is not fully understood. Cytotoxicity of silver nanoparticles on mammalian cells has been observed *in vitro*, suggesting that silver nanoparticles may enhance the generation of reactive oxygen species[29,36] and damage DNA.[37-38] The potential for extensive DNA and cell damage is a precursor for carcinogenesis.[39] Although the primary condition currently associated with silver is the cosmetic ailment known as argyria (irreversible bluish-gray discoloration of the skin), further research is required before silver nanoparticles can be considered as a safe antimicrobial agent. A thorough review regarding the effects of nanoparticles on the cell life cycle is provided by Mahmoudi *et al.*[40] Chernousova and Epple provide a thorough review on the toxicity of silver on single and multicellular organisms, specifically focusing on the range of toxicities of the various forms of silver.[41]

3.2 Titanium Dioxide Nanoparticles

Titanium dioxide (TiO_2) is a naturally-occurring compound that has been used extensively in cosmetics, sunscreen, and food additives because of its highly refractive qualities. Additionally, TiO_2 nanoparticles have been utilized for their antimicrobial properties. When photoactivated by UV- or visible-light photons, TiO_2 catalyzes the cleavage of water into hydrogen and oxygen, and produces reactive oxygen species (ROS) in solution.[42] Currently, TiO_2 nanoparticles, which can be activated by weak UV light, are used in interior furnishings such as tiles and wallpaper in hospital rooms, air conditioning and purification units, wastewater/sewage purification systems, and pollution abatement strategies to reduce bacterial loadings.[43]

Several studies have been performed to determine the antimicrobial efficiency of TiO_2 nanoparticles. After photoactivation, TiO_2 nanoparticles were found to be highly-toxic to *Escherichia coli* and *P. aeruginosa*.[44-47] The degree of toxicity was directly related to the cell wall configurations and peptidoglycan content of the microorganism, as *E.coli* (Gram-negative) was more susceptible than *S. aureus* and *E. faecalis* (Gram-positive), which were more susceptible than the fungi *C. albicans* and *Aspergillus niger* (Figure 1).[48 49]

The mechanism of action responsible for killing microorganisms exposed to photoactivated TiO_2 is only partially understood. It has been theorized that the ROS generated through photoactivation are responsible for the antimicrobial efficacy of TiO_2.[50] ROS are able to damage the cell membrane and disrupt essential membrane-bound proteins,[51] in addition to creating single stranded or double stranded breaks in DNA, rendering it unable to be replicated.[52-53] The antimicrobial activity of ROS varies between environments and experiments; therefore the exact mechanism of action is not fully understood, nor agreed upon.[54] Macrophages of mammalian immune systems rely heavily upon ROS to eliminate pathogens from the body. However, some pathogenic microorganisms are exposed to ROS often, and have inherent defense mechanisms in place for protection. For example, *S. typhimurium* sequesters iron ions to protect their DNA from ROS, in addition to the expression of a Type III secretion system that prevents the host cell from reducing hydrogen peroxide (H_2O_2) to the more-deadly superoxide anion O_2^-.[55, 56] Additionally, *Salmonella enterica* possesses several antioxidant enzymes that detoxify ROS.[57,58] The inherent nature of photocatalysis and the creation of ROS limit the antimicrobial efficacy of titanium dioxide nanoparticles to aerobically grown organisms in water, air, or on surfaces.

3.3 Gold Nanoparticles

Colloidal gold has also been used by scientists and medical practitioners for well over a thousand years. In the present day, due to its exceptional ligand binding ability, spectroscopic detection, high contrast in electron microscopy, and general stability, gold nanoparticles are widely used in biological and chemical systems.

Extensive reviews on the syntheses and applications of gold nanoparticles have been published.[59-61]

Gold nanoparticles are readily taken up by host immune cells, thus providing the capability to deliver drugs to intracellular microbial pathogens.[62] The exceptional binding affinity of gold nanoparticles allows higher concentrations of complexed drugs to be delivered to an affected area without releasing high levels of free, toxic drugs into the broader system. Gold nanoparticles lack the inherent antimicrobial effects noted for silver and titanium dioxide nanoparticles, therefore the simple presence of gold nanoparticles in solution with antibiotics is not enough to enhance the efficacy of antibiotics.[63-64] However, antibiotics, when conjugated to gold nanoparticles confer an increased and more-targeted local concentration (of antibiotics), and help destroy microorganisms more efficiently than antibiotics alone, while reducing levels of toxic drugs in the system. For example, conjugation of ampicillin, streptomycin, and kanamycin to gold nanoparticles decreased their minimum inhibitory concentrations (MIC) to the bacteria *E. coli*, *Staphylococcus aureus*, and *Micrococcus luteus*. In addition to enhancing the efficacy of these drugs, conjugating the antibiotics to gold nanoparticles also made these drugs more stable and heat tolerant.[65]

3.4 Iron Oxide Nanoparticles

Iron oxide nanoparticles with a diameter size of 50-100 nm were first applied in magnetic resonance imaging more than 20 years ago.[66] Magnetic nanoparticles with a diameter of 10 nm have been used in cancer therapy due to the hyperthermia effect.[67] For drug delivery applications, magnetic nanoparticles have been utilized to carry doxorubicin, human serum albumin, and cottonseed oil for cancer treatment.[68] In addition, surface functionalization was conducted on magnetic nanoparticles with ligands and polymers to load drugs.[69-70] Recently, magnetic SiO_2 nanoparticles were used to kill pathogenic bacteria.[71] As drug delivery vehicles, magnetic nanoparticles possess the unique advantage of guiding the nanoparticles to a desired location and keeping them localized at the site using an external magnet.

3.5 Porous nanoparticles

The future of nanomedicine and drug delivery rests upon the ability of nanoparticles to complex a high concentration of drugs, release those drugs in a controlled and timely manner, control breakdown of the drug-nanoparticle matrix, have easily manipulated surfaces, and be detectable *in vivo*.[72] Porous nanoparticles, such as silicon, iron (III) carboxylate, and manganese oxide, are ideal carriers for drugs having the previously listed capabilities.[72-74] Silica nanoparticles have been widely applied as carriers for delivery of enzymes, antibiotics, and DNA.[75] The biocompatibility of silica nanoparticles makes them an especially ideal carrier for applications associated with the human body. As an important class of silica materials, mesoporous silicas have attracted huge interest since their initial synthesis by Mobil Corporation in 1992.[76] Mesoporous silicas have been widely utilized in the fields of catalysis and biomedicine[77-78] because of their uniquely large surface area, controllable particle size and pore size, uniform pore structure, and easy surface functionalization. Although not inherently antimicrobial, these nanoparticles have the potential to be designed to carry antibiotics to multidrug resistant infections. Currently, researchers are focusing on utilizing porous nanoparticles to deliver anticancer drugs into tumors with incredible accuracy[79-80].

Table 1 Synopsis of nanoparticle characteristics.

Material	Notable Characteristics	Toxicity
silver	naturally antimicrobial, easily aggregate	skin discoloration, cell and DNA damage
titanium dioxide	naturally antimicrobial, require photoactivation	cell damage via ROS
gold	High-binding affinity, stable, heat-tolerant, material; not antimicrobial	generally-nontoxic
iron	magnetic, surface functionalizable, material not antimicrobial	require strict size limitations to avoid toxicity
mesoporous silica	Highly-biocompatible, controllable particle size, ideal drug-carriers, material not antimicrobial	generally-nontoxic

3.6 Methods to determine efficacy of inorganic nanoparticles on bacteria

Techniques alternative to traditional culturing methods are required to judge the antimicrobial properties of inorganic nanoparticles due to the ability of bacteria to quickly change and persist. Therefore, bacterial cell culture techniques are frequently used in conjunction with other methods, such as luminescent biosensors, detection of reactive oxygen species, cell membrane integrity determination, and electron microscopy, to determine the efficacy of inorganic nanoparticles.

For example, UV-Vis spectroscopy (OD 600 nm) can be used to monitor changes in the growth rate of *E. coli* in response to silver nanoparticles.[26] Other studies have relied on traditional agar plates to count colony forming units (CFUs) during or after nanoparticle exposure. However, as an alternative to these methods, bioluminescent bacteria such as *Vibrio fischeri* and genetically-modified *E. coli* have been used in nanoparticle ecotoxicological studies. The *V. fischeri*–based bioluminescence "Flash Assay" is useful for screening highly turbid nanomaterials that would otherwise confound UV spectroscopy measurements.[81-84] In addition to bacterial biosensors that indicate the overall toxicity of inorganic nanoparticles, bacterial biosensors have also been developed to simply detect the presence of inorganic nanoparticles. Blinova and colleagues presented results using zinc- and copper-sensitive strains of bioluminescent *E. coli*. In this study, the bioluminescence of the genetically-modified *E. coli* increased proportionally with the increase in available zinc or copper.[85] As an alternative to biosensors, the physical appearance of bacteria during nanoparticle exposure has additionally been used to determine toxicity.

Using electron microscopy, the membrane organization of bacterial cells can be visualized to determine if the nanoparticle treatment induced cellular lysis or cell wall or cell membrane damage.[26,86] Light microscopy-based techniques are also used to determine cell viability after nanoparticle exposure. Bacterial membrane integrity can be assayed with a fluorescent LIVE/DEAD stain combination (Syto9/propidium iodide), and visualized using confocal scanning laser microscopy or flow cytometry.[78,87] In this assay, while all cells having either intact- or damaged-membranes take up Syto9 stain, only those with intact-membranes (i.e. live cells) will exhibit a strong Syto9-fluorescence emission signature. This occurs because cells with damaged membranes (i.e. considered dead) also take up and predominantly exhibit a propidium iodide fluorescence emission signature. Fluorescence stains can also be utilized to detect the burst of free radicals and reactive oxygen species from bacteria during nanoparticle-induced death. The oxidation of 2', 7'-dichlorofluorescin-diacetate (DCFH-DA) has

been used to quantitatively determine the formation of ROS by bacteria under a microscope.[88-90] These alternative techniques are important in their ability to determine cell viability without relying on cell growth-based approaches.

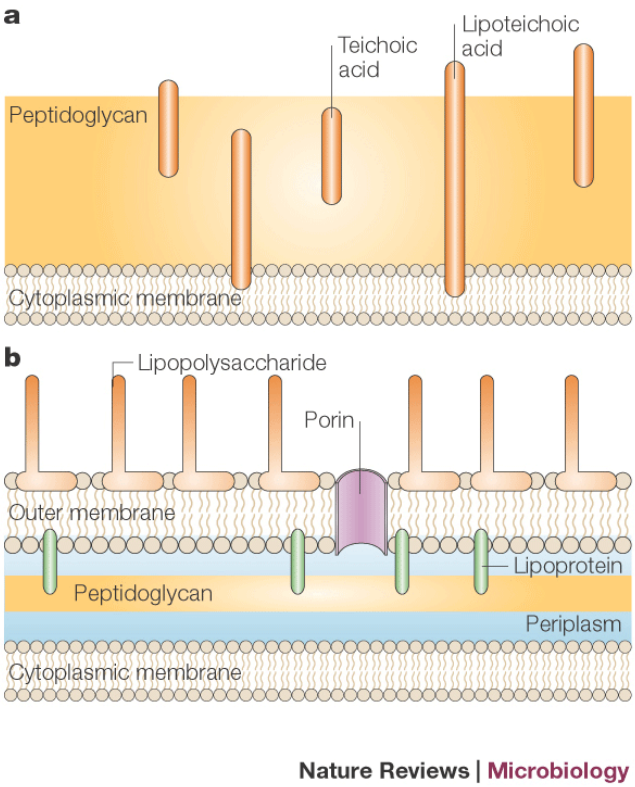


Figure 1 (a) The Gram-positive cell wall is composed of a thick, multilayered peptidoglycan sheath outside of the cytoplasmic membrane. Teichoic acids are linked to and embedded in the peptidoglycan, and lipoteichoic acids extend into the cytoplasmic membrane. (b) The Gram-negative cell wall is composed of an outer membrane linked by lipoproteins to thin, mainly single-layered peptidoglycan. The peptidoglycan is located within the periplasmic space that is created between the outer and inner membranes. The outer membrane includes porins, which allow the passage of small hydrophilic molecules across the membrane, and lipopolysaccharide molecules that extend into extracellular space. Image and caption reprinted with permission. [49]

4. Linking Antibiotics to Inorganic Nanoparticles: Challenges in Surface Chemistry Design

The application of nanoparticles as drug delivery vehicles has attracted considerable attention in past decades. Nanoparticles possess unique properties, such as mono-distribution of nanoparticle size, and thermal and magnetic properties.[91] In addition, their unique physicochemical properties, when compared to larger particles, provide the potential for nanoparticles to penetrate and reach areas of bio-membrane systems where dissolved molecules reach less-effectively. In the present section, we will explore how nanoparticle-based drug delivery vehicles can improve the solubility, pharmacokinetics and stability of free drugs.[92]

4.1 Surface Functionalization

Surface functionalization of nanoparticles is of great interest because of their potential applications in chemosensors, coatings, organic light-emitting devices (OLEDs) and biomedical engineering.[93-96] In the biomedical field, surface functionalization plays a critical role in tailoring the properties of nanoparticles for enhanced binding capabilities for therapeutic delivery,[97] selective recognition within biological systems,[98] and improved cellular internalization.[97]

4.1.1 Charged Moieties

Positive-charges, negative-charges, and zwitterionic moieties have been functionalized onto nanoparticle surfaces. Cationic compounds have been considered as important candidates for antimicrobial agents throughout the past twenty years. Among them, quaternary ammonium (QA)[99], alkyl pyridinium [158] and phosphonium-based compounds[100-101] are three main forms of these agents. QA compounds are the most important and commonly-used cationic agents used to kill bacteria. Dong and co-workers modified magnetic nanoparticles with poly(quaternary ammonium) (PQA) to kill *E. coli*, which retained a 100% biocidal efficiency over eight-cycles of usage of the nanoparticles.[102] Tiller and coworkers functionalized glass slides with poly(4-vinyl-N-alkylpyridinium bromide) to kill airborne bacteria on contact.[103] The surface attached pyridinium based PQA can also be functionalized onto inorganic nanoparticles to kill bacteria, which might have better performance because the graft density of the PQA on nanoparticles would be higher compared to flat surfaces. The antimicrobial properties of QA compounds are likely ascribed to their interactions with bacterial cell membranes, which subsequently results in disruption of the membranes.[104-106] Carmona-Ribeiro reviewed the specific functions of cationic materials when interacting with bacterial cell membranes and summarized several general steps for disruption of cell membranes.[107] We refer interested readers to the literature for detailed information in this field.

Many quaternary ammonium-based cationic polymers are prepared based on 2-dimethylaminoethyl methacrylate (DMAEMA).[108-111] The surface charge densities (usually greater than 10^{15} groups/cm^2) of PDMAEMA brushes determine their effectiveness in killing bacteria. The higher charge densities of surface-attached polymers typically exhibit stronger antimicrobial activities.[109] Most PDMAEMA-based QA compounds are prepared via the quaternization with alkyl halide, but viologen-quaternized PDMAEMA demonstrated a significantly-increased antimicrobial effect when compared to alkyl halide-quaternized QA compounds due to the enhanced cationic charged densities.[112] Other quaternary ammonium based cationic polymer materials have been developed in recent years. As a renewable material, rosin-based polymers containing multiple quaternary ammonium compounds have been developed to kill bacteria.[113-115]

QA compounds can also be used as drug delivery vehicles to load and release antibiotics. Lee *et al.* have demonstrated that mesoporous silica nanoparticles (MSN) can be functionalized with surface positive charges to deliver an anionic anti-inflammatory drug, sulfasalazine, with controllable loading, and release by changing pH value.[116] The positive charge surface was synthesized by a condensation reaction between trimethylammonium (TA)-silane and tetraethoxysilane (TEOS) of MSN. Sulfasalazine was loaded into the nanoparticle and remained in the framework of MSN under acidic conditions. It was then released by electrostatic repulsion from the gradually-formed negative surface charges that developed under neutral conditions (Figure 2).

Alkyl pyridinium-based polymers are usually prepared based on 4-vinyl pyridine. Quaternized poly (vinylpyridine) brushes were coated on glass surfaces to kill Gram-positive and Gram-negative bacteria with an effective charge density from 10^{12} to 10^{16} groups/cm^2.[117] Klibanov and coworkers have developed surface-immobilized *N*-hexyl-poly(vinylpyridine), *N*-hexyl,*N*-methyl-

polyethyleneimine (PEI), *N*-dodecyl,*N*-methyl-PEI as permanently microbicidal materials.[118]

Table 2. Overview showing different surface-functionalizations of nanoparticles.

Type of Ligands	Category	Description
1) Charged Moieties	Positive Charge	Quaternary ammonium (QA); Alkyl pyridinium; Phosphonium-based compounds
	Negative Charge	Anionic carboxylates in PEO-*b*-PAA
	Zwitterionic moieties	Sulfobetaine (SB); Carboxybetaine (CB)
2) Non-Charged Moieties	Small Molecule Ligands (SMLs)	Weakly-bound ligand (*e.g.*oleic acid) Strongly-bound ligand (*e.g.* phosphate- and silane-based)
	Polymer Ligands	Polyethylene-glycol (PEG), thermal-responsive poly(*N*-isopropylacrylamide) (pNIPAAm), pH responsive polymers (*e.g.* PAA), etc.

Surface functionalization with negatively-charged compounds has also been widely investigated for antimicrobial applications. It is reported that negatively charged nanoparticles are taken up by diffusion.[119] Surface attached anionic compounds can be employed as drug delivery vehicles to kill bacteria. Riffle and coworkers modified Fe_3O_4 nanoparticles with block copolymers PEO-*b*-PAA.[120] The unattached segments of PAA provide thousands of anionic carboxylates which was used to conjugate cationic aminoglycoside antibiotics via ionic complexation for therapeutic applications. The delivery vehicles can also be used to deliver moieties such as metal ions. Anionic poly(3-sulfopropyl-methacrylate) brushes have been prepared on Si/SiO_2 surfaces and employed to complex silver ions inside the brushes.[121] The surface-attached silver-containing brushes inhibited the growth of both Gram-negative and Gram-positive bacteria. The graft density of anionic polymer-silver ion complexes is expected to higher when grafting on inorganic nanoparticles. Thus, it could further improve the inhibition of bacteria growth.

Zwitterionic materials (also called inner salts) with one pair or multiple pairs of positive and negative charges in their structures have also been anchored on a variety of surfaces. Surface attached zwitterionic materials were shown to be resistant to bacterial adhesion and biofilm formation.[122] However, most of the applications of these surface-attached zwitterionic moieties are still used in the antifouling field. These anchored zwitterionic materials are found to be highly-resistant to protein adsorption.

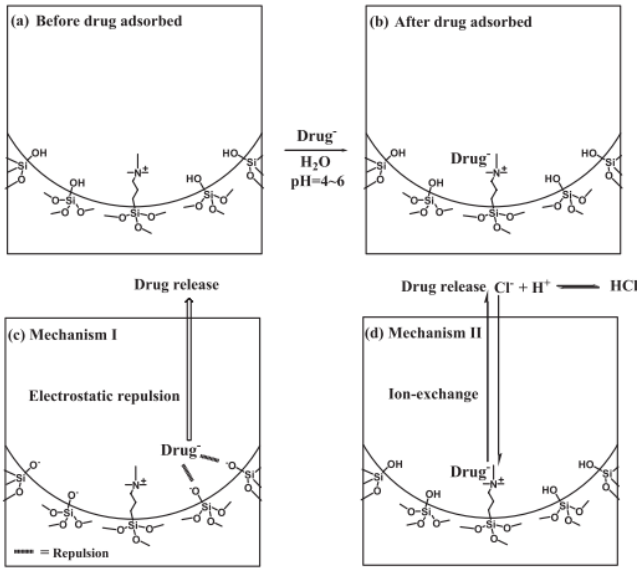


Figure 2 A schematic illustration showing the loading and release mechanism of an anionic drug in TA-modified MSN: (a) before- , and (b) after-drug adsorption; (c) electrostatic repulsion triggering drug release, and (d) ion-exchange triggered drug release. Reprinted with permission.[116]

The two main zwitterionic materials are based on sulfobetaine (SB) and carboxybetaine (CB). Thus, SB-based sulfobetaine methacrylate and CB-based carboxybetaine methacrylate materials have been widely investigated as antifouling materials.[122-131]

Surface functionalization using different charge moieties can be characterized by zeta-potential measurements.[132] This test reveals the electrical potential of a particle surface, which can be used to analyze its stability in solution. Generally, nanoparticles have been demonstrated to exhibit a stable dispersion in solution when the zeta potential is above ±30 mV. It is well known that this type of surface charge can inhibit aggregation of nanoparticles, thus surface modifications that introduce appropriate amounts of charges are an effective method to store nanoparticle suspensions. However, some functional group-coated nanoparticles, such as zwitterionic moieties or polymers, are exceptions.

4.1.2 Non-Charged Moieties

Small Molecule Ligands (SMLs)

Relatively small molecules represent an operational class of materials that have been widely used to modify the surfaces of nanoparticles. They provide several advantages, such as low molecular weights, easy coordination onto nanoparticles, and easy processing conditions. Compared to macromolecules, the relatively smaller size of these molecules makes surface functionalization with multiple ligands much easier. In the previous section, charged small-molecule compounds were reviewed in terms of surface modification. This section, therefore, will focus primarily on non-charged small-molecule ligands. A wide range of small molecule ligands have been coated onto the surfaces of nanoparticles for applications in biosensing, diagnosis, and drug delivery.[133-138] The ligands alter a nanoparticle's stability, hydrophobic/hydrophilic properties, zeta-potential, cytotoxicity, and interactions with cells.[139-141]

Small molecules provide a repulsive layer on a particle surface, which can enhance the stability of nanoparticles in suspension and minimize nanoparticle aggregations. Two factors should be considered while choosing SMLs for nanoparticle stabilization: 1)

the substrate particles, and 2) the dispersion solvent. Generally, silane-SMLs are used to modify SiO_2 nanoparticles, thio-SMLs are suitable to coat Au-nanoparticles and phosphate-based SMLs can be employed to functionalize iron oxide and TiO_2 nanoparticles. De and coworkers have summarized the surface functionalization of a variety of nanoparticles with corresponding SMLs.[142] In all of their nanoparticle surface functionalizations, SMLs were bound to surfaces via chemical absorption or physical absorption (hydrophobic/ hydrophilic interactions). The dispersion solvents consisted of organic solvents and water. Choosing appropriate solvents with a polarity close to that of the dispersion solvent is a necessary step while modifying particles.

Ligand-exchange is an important approach used to enhance the stability of nanoparticles in certain solvents. In this approach, strongly-bound ligands are typically used to replace weakly-bound molecules in order to accomplish a firm surface attachment. For example, oleic acid, a significant and commonly-used ligand for stabilizing metal oxide nanoparticles, is a weakly-bound molecule and is generally exchanged with phosphate- and silane-based ligands[143-145] for firmer attachment. Schadler and colleagues reported using a phosphate-azide ligand to replace oleic acid on TiO_2 and ITO surfaces,[146] followed by further functionalization via "click chemistry" on the new ligand.

Generally, oleic acid or oleylamine are added to stabilize magnetic iron oxide nanoparticles in the preparation process. However, this limits the surface functionalization of particles and reduces the dispersion of the particles in hydrophilic media (e.g. water). Thus, ligand-exchange is necessary step for further applications of magnetic iron oxide nanoparticles. Bronstein and co-workers used N-(6-aminohexyl)-aminopropyltrimethoxysilane to replace oleic acid on iron oxide nanoparticles to stabilize the particles.[147] Binder and co-workers employed 1,2-diols bearing ω-azido or ω-bromo ligands to replace octylamine or oleic acid on γ-Fe_2O_3 nanoparticles followed by post-functionalization of the new ligand to obtain fluorescent properties.[148] Sun and coworkers replaced oleylamine via ligand exchange to convert the nanoparticles from hydrophobic to hydrophilic for developing a more-stable dispersion in an aqueous environment.[149] Hatton and colleagues replaced oleic acid with various hydroxyl group-containing ligands.[150]

Ligand-exchange is also an important tool for the surface modification of nanocrystals. Murray and co-workers used nitrosonium tetrafluoroborate to replace oleic acid or oleylamine on nanocrystals in order to stabilize the nanocrystals in various hydrophilic solvents, and made the ligand-exchange reversible.[151] Talapin *et al.* used metal-chalcogenide complexes to exchange ligands on nanocrystals; an exchange that resulted in hydrophilic properties.[152-153] In addition, surface functionalization of nanocrystals via ligand-exchange has been extensively explored under a variety of conditions to obtain new resultant properties.[152-160]

Polymer Ligands

The surface coating of polymers on particles imparts new properties to the surfaces.[161-162] The coatings can be used to further manipulate the nanoparticle's stability in suspensions, the hydrophobic and hydrophilic properties, cytotoxicity, biocompatibility, and even interactions with cells. It also provides an additional platform to control the antibiotic loading and release from nanoparticles in drug delivery systems. In this section, a variety of common surface-modified polymers that were developed in recent years will be reviewed based on the class of the polymer.

Polyethylene glycol (PEG), a hydrophilic polymer, has been used to enhance the water solubility of materials, and as such, is a significant polymeric material that is widely-used in bioapplications owing to its unique properties of exceptional biocompatibility and non-toxicity.[163] Surface-coating with PEG can act as antifouling materials, as it prevents protein absorption and minimizes cell attachment.[163-164] Surface-anchored PEGs on nanoparticles have also been reported to enhance circulation time, improve tumor targeting, and increase stabilization in salt solution.[165-171]

Temperature-responsive poly(*N*-isopropylacrylamide) (pNIPAAm) is a unique polymer that alters its conformation predictably in response to changes in temperature. Surface anchored PNIPAAm shrinks and generates pores on nanoparticles allowing entrapped antibiotics and biomolecules to be released when the temperature is raised above a low critical solution temperature (LCST). The polymer swells and closes the surface pores to inhibit release when the temperature is below the LCST. These properties can be used to control drug release by adjusting the temperature.[172-173] PNIPAAm has a LCST around 32 °C in water, which is perfect for broad applications of drug delivery[174-175] and bio-separations.[176-177] Yavuz *et al.*[172] reported the controlled drug release of pNIPAAm-grafted Au-nanocage by adjusting a near-infrared laser to generate heat. The monomers NIPAAm and acrylic amide were polymerized by atom-transfer radical polymerization (ATRP) using a disulfide initiator (Figure 3). PDMAEMA is also a significant temperature-responsive polymer with a LCST around 45 °C in aqueous solutions while at pH 8.5. However, the PDMAEMA polymer is also pH-responsive due to the presence of multiple amino groups in its structures. Thus, the LCST is closely-associated with pH with a LCST of >50°C at pH 7.0 and no LCST at lower (i.e., acidic) pH.[178-179]

Since pH responsive polymers usually contain ionizable groups in their structures, they can be protonated and deprotonated under different pH conditions. Generally, there are two classes of pH responsive polymers, namely acidic- and basic-polyelectrolytes (i.e., polyacids and polybases, respectively). Representatives of polyacids are poly(acrylic acid) (PAA) and poly(methacrylic acid) (PMAA). Both contain multiple carboxylic acids, which can be used to chemically- or physically-bind small molecules. Using this approach, they have been coated on nanoparticles to kill bacteria as antibiotic delivery materials.[71,120,180-181] A major representative of polybase is PDMAEMA. The main applications of PDMADEMA are for preparing antimicrobial materials via quaternization. However, it has also been immobilized on various surfaces for biomedical applications due to the pH responsive surfaces.[127,182-183]

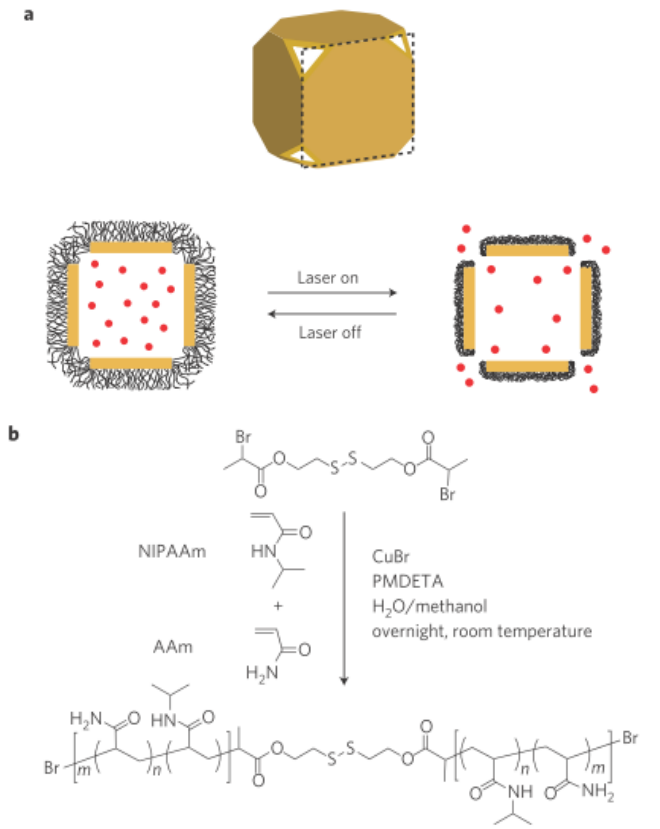


Figure 3 (a) A schematic illustration showing the controlled drug release of a pNIPAAm-grafted Au nanocage by adjusting a near-infrared laser to generate heat and release the entrapped contents; (b) Polymerization of NIPAAm and acrylic amide (AAm). Reprinted with permission.[172]

4.1.3 Polymer Preparation by Controlled Radical Polymerization

Generally, surface functionalization of nanoparticles with polymers has been achieved using two methods, namely, "*grafting from*" and "*grafting to*" techniques.[184] The "grafting from" technique provides a higher surface modification density when compared to the "grafting to" strategy. This is due to the gradually increasing steric hindrance of already-grafted polymer chains that occurs during the "grafting to" technique. Controlled/Living radical polymerization (C/LRP) has been employed in the "grafting from" technique due to its powerful application in the synthesis of well-defined and advanced structure polymers, such as block copolymers, branch polymers, and star-shape polymers. Among all the C/LRP techniques, nitroxide-mediated polymerization (NMP),[185] atom-transfer radical polymerization (ATRP),[186-187] and reversible addition-fragmentation chain transfer polymerization (RAFT)[188] have been extensively applied for the synthesis of complex polymer structures.[189-194] Surface functionalization of nanoparticles via controlled radical polymerization generates controlled chain length and polydispersity of polymers on surfaces.

The first report, to our knowledge, of surface functionalization using ATRP was in 1997 by Wirth and co-workers.[195] Acrylamide was polymerized via ATRP on benzyl chloride-attached silica surfaces. Then, Matyjaszewski and co-workers significantly expanded grafting polymers from surfaces via ATRP.[187,196-197] The first report of surface functionalization using NMP was in 1999 by Hawker and Russell on silicon wafers.[198] Since the invention of RAFT in 1998, it was first used to modify surfaces by Tsuji *et al.* in 2001.[199] They prepared the RAFT agent *in-situ* by conversion of a surface-supported ATRP initiator followed by surface-initiated RAFT polymerization of styrene. Brittain and co-workers employed azoundecylchlorosilane as an anchor-initiator to commence RAFT polymerization on silica particles.[200] Brittain *et al.* also employed a "click" reaction to anchor RAFT agents on silica particles to mediate the polymerization of styrene and methyl methacrylate.[201] Benicewicz and Wang employed a RAFT agent 4-cyanopentanoic acid dithiobenzoate (CPDB) to prepare a wide range of polymers from silica nanoparticle surfaces with a variety of graft densities of 0.01 - 0.68 chains/nm^2.[180-181] Thus, CRP methods have been tremendously important techniques for the "grafting from" method to prepare polymer grafted nanoparticles.[202-205]

CRP has also been widely applied in preparing a variety of free polymers, which are further used in "grafting to" strategies. Both "grafting to" and "grafting from" strategies have been demonstrated as effective methods to graft polymer brushes on surfaces, and have been reviewed by Benicewicz,[206] Brittain,[207] and Matyjaszewski.[208-209]

Normally, NMP requires high reaction temperatures and ATRP generates residual copper or other metals after polymerization, which are extremely difficult to completely remove. Thus, both of NMP and ATRP have not been widely applied on nanoparticle surfaces for biomedical applications. RAFT, generally employing mild reaction conditions without residue metal issues after polymerization, is adaptable to a variety of functional monomers.[180,199,202,210-211] Due to the advantages of the RAFT technique, it has been used for the surface functionalization of nanoparticles with lactose[212] and peptides,[213] and to deliver therapeutic agents[214] and siRNA.[215]

4.2 Controlling Specificity in Targeting Bacterial Cells

In recent years, and with increasing efforts to understand the human microbiome (*i.e.* bacterial communities inhabiting humans), it is now realized that the majority of bacteria inhabiting humans are non-pathogenic (*i.e.* commensal), and may even play essential roles in health.[216] Most antibiotics, however, target general bacterial processes (*e.g.* cell wall formation, protein translation, DNA replication), so even relatively 'narrow-spectrum' antibiotics can have lethal effects on many commensal forms; which in turn, are thought to negatively affect human health. Therefore, a major challenge in the future development of antibiotics will be in designing approaches that target only the pathogenic (*i.e.* infection-causing) forms, while leaving the majority of commensal bacteria intact.

One approach for targeting specific bacteria has been the use of antibodies. Antibodies are produced by the immune response of a mouse, rabbit, or rat,[217] and can be raised against specific proteins (antigens) located on the surface of the bacterium. The antibody, after purification, is typically conjugated to a fluorophore for detection, and then used to identify (via its fluorescence) specific bacteria with the antigen signature amongst a plethora of other species/strains in a complex mixture.

Nanoparticles, when coupled with antibodies, have been used as carrier vehicles for highly-sensitive biodetection of specific bacterial pathogens.[218-220] NP-based approaches allow single-cell detection because a single NP contains many fluorophore molecules. Efficient and sensitive detection of bacteria is necessary for maintaining food quality, and in environmental and biomedical applications. Traditional detection techniques, such as viable colony counting[221] and polymerase chain reaction (PCR) to detect pathogen genes, impose a high cost and are time-expensive. In comparison,

nanoparticles provide an excellent platform for sensitive and efficient detection because nanoparticle surfaces can be functionalized with specific antibodies to recognize biomarkers on bacteria. However, additional attention should be focused on the surface functionalization process because antibodies are typically expensive and require relatively long preparation times.

The relatively large size (*ca*. 150 kDa) of many antibodies limits their ability to disperse under many biological conditions, especially when conjugated to nanoparticles. Antibodies are Y-shaped glycoprotein molecules, and consist of both a framework (FR) and hypervariable (HV) region. The tail of the HV region provides the antibody with specificity for the binding antigen (*i.e*. the binding domain). A small section of this tail (containing the binding domain) has been used to facilitate the development of 13 kDa single-domain antibodies (sdAbs) that can be conjugated to quantum dots (QD) (<12 nm dia.). The sdAbs can be conjugated to QDs in a highly-oriented manner to enhance binding efficiency and be used as ultra-small diagnostic nanoprobes.[220,222] In general, antibody conjugated-nanoparticles have been used for highly sensitive detection of specific species within a complex mixture of bacteria.[218,223-226]

Conjugating antibodies to gold nanoparticles can allow for the targeting of specific microorganisms. Pissuwan and colleagues demonstrated that when antibodies were conjugated to gold nanoparticles, they selectively targeted and destroyed the parasitic protozoan *Toxoplasma gondii* after plasmonic heating.[227] The *in vitro* experiment with antibody-conjugated gold nanoparticles killed approximately 80% of the parasite, whereas the antibody or gold nanoparticles alone did not kill a significant number of protozoa. Norman and colleagues used a similar approach to target the bacterium *P. aeruginosa*.[228] In their experiment, near-infrared irradiation was used to photothermally heat gold nanoparticles attached to *P. aeruginosa*, resulting in membrane disruption and 78% cell death. Additionally, vancomycin conjugated gold nanoparticles demonstrated activity against vancomycin-resistant *Enterococci*, vancomycin-sensitive strains of *Enterococci* and other Gram-negative bacteria.[229]

Antibody conjugated-nanoparticles can also be designed as multifunctional platforms for targeted bacterial detection and destruction. When nanoparticles are equipped with magnetic properties (as discussed above), followed by conjugation with antibodies, they can be used for detection and/or separation of specific bacteria within a mixture of species.[219,224,230] For example, antibody conjugated-magnetic nanoparticles, can separate the bacteria *E. coli* and *Salmonella typhimurium*,[231] and allows a detection limit of 10^4-10^5 cells/mL. Magnetic nanoparticles have also been applied as sensors to enhance interactions between vancomycin coatings on nanoparticles and D-alanyl-D-alanine in bacterial cells, with a corresponding detection limit of 10^3 cells/mL. The antimicrobial enzyme lysostaphin was adsorbed on the surface of antibody-conjugated-nanoparticles specific to the bacterium *Staphylococcus aureus*.[232] Lysostaphin hydrolyzes the peptidoglycan linkages in the cell wall, which lyses and kills the bacterium.

The unique size effects and emerging surface functionalization toolboxes make nanoparticles an excellent platform to efficiently and sensitively detect bacteria. As discussed earlier in this review (see Section 4.1), the surfaces of nanoparticles can be engineered with polymers at finely-controlled densities to tailor the accessibility of conjugated moieties on the polymers. In this way, several different antibiotics can potentially be delivered by a single nanoparticle. This approach offers an exciting potential to increase the specificity of an antibiotic attack and to reduce destruction of helpful bacteria by antibiotics.

Finally, in order for nanoparticle-based approaches to operate efficiently, the nanoparticle-carrier must either penetrate the bacterial cell (as discussed below), or release its cargo at the cell surface. Approaches for the controlled release of conjugated molecules from nanoparticles have been developed using both pH-sensitive and infrared-sensitive ligands.

4.2.1 Entry of Nanoparticles into the Cell.

Certain antibiotics, owing to their mechanism of action, require entry into the cytoplasm in order to kill or inhibit bacterial cells. Together, the cell membrane(s) and cell wall provide a protective barrier that restricts the movement of molecules and ions into and out of the cell, as well as maintains the structural stability of the cell.[233] The outer membrane (OM) forms the outermost boundary with the extracellular environment of Gram-negative bacteria, while the cell wall accomplishes this in Gram-positive species. The OM and inner (plasma) membranes (IM) selectively allow smaller molecules (<600 Da) to diffuse through the lipid bilayer while preventing macromolecules larger than 1 kDa[234] from permeating the membrane without active transport.

Many studies have shown that the interaction of some metallic nanoparticles with bacteria results in cell lysis (and death).[83,235-236] In these studies, the nanoparticles used were relatively toxic and included ZnO, CuO, Ag, TiO_2, and Al_2O_3. In some cases, nanoparticles were observed within lysed cells, suggesting that the nanoparticles were taken up by cells and cell death occurred either during or shortly after uptake.

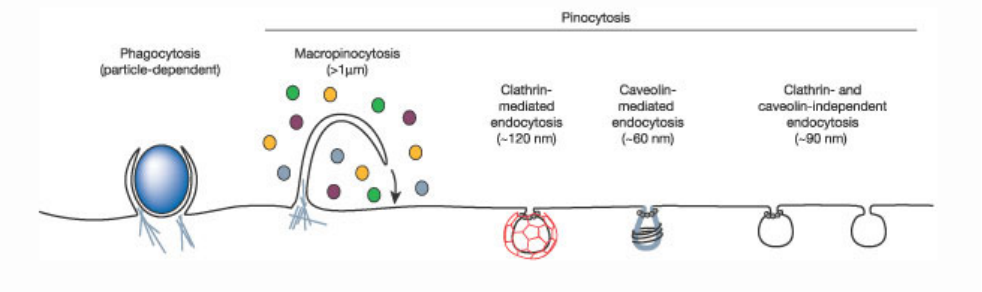
It is now realized, however, that bacteria may also take up very small particles into their cellular cytoplasm, without apparent lethal effects. For example, quantum dots (<10 nm diameter) were shown to be taken up via a purine-dependent mechanism.[237] Also, 10 nm diameter gold colloids have been shown to enter viable cells during the uptake of large proteins.[238] Although examples exist, particle uptake by bacteria is contrary to our current understanding, and is not well understood. Very few studies have noted either direct or indirect uptake.

In order for non-lethal particle uptake to occur, the process will likely require active transport, and the ability of the bacterium to hydrolyze a small portion of the cell wall, and then reform the wall without significant loss of intracellular contents. The interior of a bacterial cell, however, has a high turgor pressure relative to the immediate outside environment. Successful entry into a bacterium without cell death presupposes that the particle can cross through the cell membrane(s) and cell wall without lysis and significant loss of intracellular contents. How this might occur is currently unknown.

Some insight can be gained from the examination of the uptake of macromolecules into larger eukaryotic cells.[239-240] This process, called endocytosis, utilizes membrane vesicles to shuttle extracellular materials into the cell. There are two methods of endocytosis, classified as phagocytosis or pinocytosis.[241-242] Pinocytosis includes macropinocytosis, clathrin-mediated endocytosis, and caveolae-mediated endocytosis, as shown in the Figure 4.[243-244] These endocytosis pathways differ from each in the nature of the internalized materials, the size of vesicles, the type of cells, and the kinetics of the uptake process.[243] However, endocytotic-like uptake by bacteria has only been shown in one case, [238] and is not well understood at present.

Nanoparticles provide an excellent carrier to transport drugs[245] and siRNA[246] by endocytosis into the cytosol of eukaryotic cells. Without the nanoparticle carrier, these molecules would not be able to diffuse through the lipid bilayer. Interactions between nanoparticles and the cell membrane can be influenced by the size, shape, surface charges, and surface functional groups of nanoparticles. For example, Chithrani *et al.* reported that 50-nm Au

nanoparticles were more efficiently taken up by eukaryotic HeLa cells than 14-nm and 74-nm diameter Au nanoparticles.[245-246] Spherical nanoparticles[244] were reported to be internalized 500% more than rod-shape nanoparticles in eukaryotes. Nanoparticle size and shape have been reported to influence the binding of receptors on the cell membrane.[247]



Type of endocytosis	Brief description	Size of vesicle formed
1) Phagocytosis	Internalization of solid particles such as bacteria and yeast by specialized cells.	Dependent on the particles being engulfed.
2) Pinocystosis	Fluid-phase uptake of extracellular molecules. Multiple pinocytic pathways are possible (below).	(Below)
3) Macropinocytosis	Trapping of large fluid pockets by formation and enclosure of membrane protrusions.	>1 μm
4) Clathrin-mediated endocytosis	Concentration of transmembrane receptors and bound ligands in "coated pits" on the plasma membrane formed by the assembly of cytosolic proteins, the main assembly unit being clathrin.	≈120 nm
5) Caveolae-mediated endocytosis	Flask-shaped invaginations in the plasma membrane that mediate uptake of extracellular molecules into the cell by specific receptor binding.	≈50–60 nm

Figure 4 Major endocytosis pathways in eukaryotic cells. Reprinted with permission.[243-244]

The surface coating on nanoparticles can be designed to inhibit protein absorption and minimize nonspecific interactions with cell membranes. For example, neutral ligands such as PEG and zwitterions have been used to reduce inefficient targeting and potential agglomeration of nanoparticles at the cell surface.[248-250] Nanoparticles with surface negative charge moieties have been reported to have poor interactions with the cell membrane, which leads to very limited internalization.[119,251-252] In contrast, cationic nanoparticles have been widely demonstrated to bind with negative moieties on cell membranes and thus facilitate the movement of substrates across the lipid bilayer.[253] The mechanism of interaction between cationic nanoparticles and cell membranes was confirmed by AFM investigation,[254] and showed that the positive moieties destabilize the membrane and cause the formation of pores in defective areas of the membrane. Thus, in eukaryotic cells, the size, shape, surface charges, and surface functional groups of nanoparticles can influence the interaction between nanoparticles and the cell membrane. These factors will prove important when targeting intracellular bacterial pathogens.

4.3 Drug Loading

In order to address drug delivery approaches designed specifically for bacteria, it is useful first to examine the techniques used to engineer nanoparticles drug attachment. Generally, the two basic approaches used to attach drugs to nanoparticles include covalent and non-covalent binding. Both approaches possess specific advantages and disadvantages. Covalent binding offers a steady delivery platform, but usually requires pre-treatment of drugs. For example, Cheng and colleagues reported phthalocyanine-based photodynamic therapy (PDT) using drugs conjugated to gold nanoparticles.[255] The drug release and PDT efficacy are affected by the chemical bond between the drug molecules and the Au surface. Specifically, the labile amino adsorption triggers the drug release into HeLa cancer cells. In another study, Gu and co-workers reported the synthesis and improved activity of vancomycin-conjugated Au nanoparticles against vancomycin-resistant *Enterococci* (VRE) and Gram-negative bacteria.[229] Covalent binding between drugs and nanoparticles provides a secure method of binding that lessens drug pre-release or leakage. Covalent binding also allows for the drug release speed to be controlled by adjusting the breakage rate of the covalent bond. Usually, slow release drug delivery strategies employ the covalent binding method.

In non-covalent binding strategies, potential drug pre-release or leakage can be avoided by using stimulus functionalities on the nanoparticle shell to block or cover the drug releasing pores. Baeza and co-workers reported the preparation of poly(ethyleneimine)-b-poly(N-isopropylacrylamide) (PEI/NIPAM) coated mesoporous silica nanoparticles (MSN) with encapsulated iron oxide nanocrystals to deliver multiple drugs simultaneously (Figure 5).[79] The grafted block copolymer was designed to retain the drugs within the NP using a temperature-responsive control and by attaching proteins onto the grafted polymer shell via intermolecular interactions. The use of specific functionalities to block nanoparticle pores is an efficient method to prevent premature drug leakage.

In addition to drug entrapment, other barriers have been designed to minimize drug pre-leakage in non-covalently bound nanoparticles. Chen *et al.* reported a new magnetic drug delivery system that utilizes doxorubicin (DOX)-associated Fe_3O_4 nanoparticles coated with a PEG modified porous silica shell (Fe_3O_4-DOX/pSiO_2–PEG) to treat tumors.[256] The DOX-conjugated Fe_3O_4 nanoparticles were embedded in a silica shell and coated with PEG chains (Figure 6). After etching the thick silica shell, the Fe_3O_4-DOX/pSiO_2–PEG was 150 nm in diameter. The porous silica shell presents a physical obstacle that decreases the dissociation rate of DOX from the nanoparticle core.

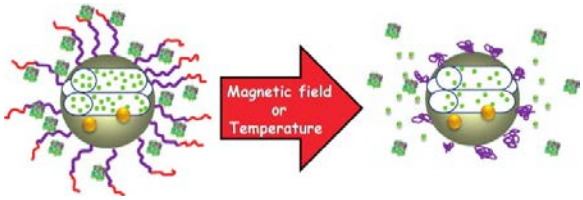


Figure 5 The schematic illustration of multidrug release by MSN-polymer nanocomposite. Reprinted with permission.[79]

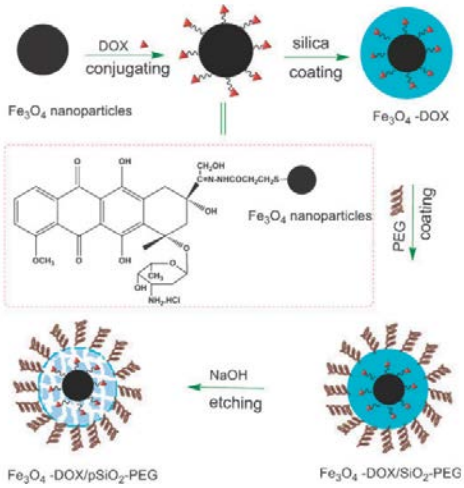


Figure 6 The schematic illustration of the synthesis of DOX-associated Fe_3O_4 nanoparticles coated with a PEG modified porous silica shell. Reprinted with permission.[256]

Like covalent binding, non-covalent binding provides a direct loading and release mechanism. However, drug pre-release or non-specific leakage is frequently noted. To counteract this problem, Mortera and co-workers prepared a mesoporous silica nanoparticle-based cage-like vehicle to deliver cysteine intracellularly.[73] The cysteine was encapsulated within the nanoparticle and only released when triggered by intracellular antioxidants. Similarly, Adeli *et al.* prepared a hybrid nanostructure with a gold core and a polyrotaxane shell.[257] Both cisplatin and DOX were associated with the hybrid nanoparticles, and drug release controlled by photothermal explosion. Similarly, Rotello and co-workers reported the synthesis of monolayer-functionalized gold nanoparticles with a hydrophobic alkane thiol core and a hydrophilic shell.[80] The hydrophilic shell consisted of a tetra (ethylene glycol) (TEG) component, end-capped with a zwitterionic group. Hydrophobic drugs were entrapped in the hydrophobic region of the nanoparticle surface monolayer via nonspecific binding and released into cancer cells by membrane-induced diffusion.

4.4 Controlled Drug Release

A variety of release strategies of nanoparticle drug delivery vehicles have been reported, and are controlled by pH, light, temperature, enzymes, or magnetic fields.[74,80,255,258-261] The drug release strategies are directly dependent on the drug binding methods. For those carriers with covalent binding between nanoparticles and drugs, a low pH solution will hydrolyze the covalent bond. For example, Zhang and co-workers prepared doxorubicin conjugated Fe_3O_4 encapsulated in thermo-responsive dextran-g-poly(N-isopropylacrylamide-co-N,N-dimethylacrylamide) and that conjugated to a 3-mercaptopropionic acid hydrazide-functionalized nanoparticle via an acid-labile hydrazone bond (Figure 7).[262] At a temperature above the lower critical solution temperature (LCST), drug release was controlled in a mild buffer solution. Alternatively, UV (256 nm) and visible (530 nm) light was used to switch the confirmation of the polypeptide backbone of diarylethene-containing cyclic peptidomimics.[263] Enhanced antimicrobial activity was seen with the 'open' form of the compound, and completely lost in the 'closed' form. Carriers that are pH-responsive also provide a controlled drug release for non-covalently bound nanoparticle and drugs. He and co-workers reported a one-pot self-assembly strategy to prepare a MSN-based drug delivery vehicle whose release is stimulated by pH change (Figure 8).[264]

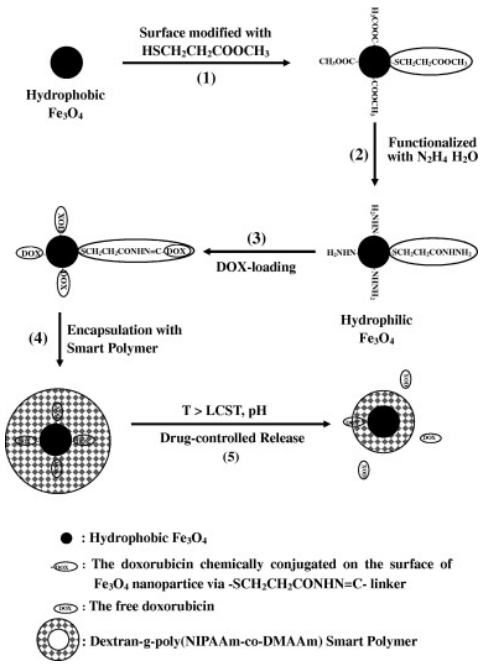


Figure 7 The schematic illustration of the synthesis of core-shell drug delivery vehicle. Reprinted with permission.[262]

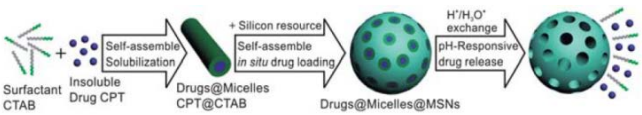


Figure 8 A schematic illustration showing the one-pot self-assembly strategy for synthesis of drugs@micelles@MSNs. Reprinted with permission.[264]

5. Overcoming the Bacterial Barriers of Infections: Nanoparticles as Antimicrobial Delivery Vehicles

Using NPs as antimicrobial delivery vehicles (ADVs) offers an extraordinary potential to control bacterial infections. Most bacterial infections occur as attached *biofilms*, where cells are embedded within a protective matrix of extracellular polymers (EPS) that are secreted by the bacteria.[265] Nanoparticles have shown a strong ability to complex to biofilms, and more specifically the EPS matrix of biofilms, both in natural environments[266-267] and under laboratory conditions.[268-269] In order to treat biofilm-based infections with NPs, their physical and chemical properties, primarily size and surface chemistry, must be designed to overcome several major hurdles within a biofilm.

First, NPs must be able to penetrate the EPS matrix in order to reach and destroy the cells enveloped within a biofilm. The NP size will limit its ability to diffuse through the EPS, while the NP surface properties will dictate the amount of interaction between the EPS polymers and the NPs. Therefore, in order to inhibit a biofilm infection, the corona surrounding a NP must be tailored to facilitate diffusion through the biofilm EPS to resident bacterial cells.[270] Although there is little research in this area, it has been noted that diffusion of NPs through biofilms is affected by the viscosity of the EPS, the variability of cell density, the bulk fluid flow of water, and the external mass transfer resistance (stagnant liquid layer on surface of biofilm that slows penetration of solutes) in three dimensions. However, the movement of nanoparticles through EPS is a poorly understood phenomenon. This section will discuss how NPs are impacted by these factors, as related to bacterial biofilms

5.1 The biofilm state: a primary hurdle for infection control

Biofilms have been recognized to play a major role in human health over the past 50 years, as the occurrence of bacterial infections has shifted from acute infections, such as cholera and diphtheria, to chronic infections, such as MRSA and *P. aeruginosa*.[265,271] Chronic bacterial infections are generally found in the biofilm state, allowing them to evade the immune system and persist through antibiotic treatment.[272] Such biofilm-based diseases are common and include infectious kidney stones, bacterial endocarditis, cystic fibrosis airway infections, periodontitis, and indwelling medical device infections.[273-274] A major difficulty in treating chronic bacterial infections lies in the biofilm matrix (i.e. EPS) in which the bacteria reside. Generally, antibiotics that are able to eliminate planktonic forms of bacteria may require 100- to 1000-fold higher concentrations to defy the heterogeneous complexity of a biofilm matrix, and even then the infection often will persist. Biofilm-associated cells possess a number of special adaptations, collectively called '*insurance effects*', which serve to enhance their survival and persistence against stressors such as antibiotics.[275]

As seen in Figure 9, the biofilm matrix provides nutrients, protection, and hydration to a dense community of bacteria. A significant amount of research has been devoted to understanding the role and development of the biofilm matrix (for a thorough review, see Flemming & Wingender).[271] In response to environmental conditions, the integrity and function of the biofilm changes to ensure survival and persistence of the bacteria. The complexity of the matrix is due to its many components, which include proteins, lipids, polysaccharides, glycoproteins, glycolipids, membrane vesicles, nucleic acids, ions, and, of greatest abundance, water.[276] EPS also contains many sorption sites[277] for potential interactions with nanoparticles and charged molecules. The plasticity of the biofilm matrix allows it to fulfil the roles of protector, electron donor/acceptor, anchor, and retainer of nutrients, enzymes, energy, and genetic material under dynamic conditions.[271] A recent study has suggested that the EPS of the biofilm matrix contributes to an osmotic gradient in the matrix and drives biofilm spreading.[278] In addition to a complex composition, biofilms also exhibit heterogeneity in bacterial species and gene expression.[265,279]

While occasionally biofilms can consist of a clonal population of one bacterial species, they most often occur as a population of many, diverse bacterial species.[280] Under both circumstances, gene expression varies throughout the biofilm in response to environmental conditions, resulting in localized areas of specific cell activity.[281] For example, the bacterium *P. aeruginosa* modifies gene expression to control alginate production and motility in response to

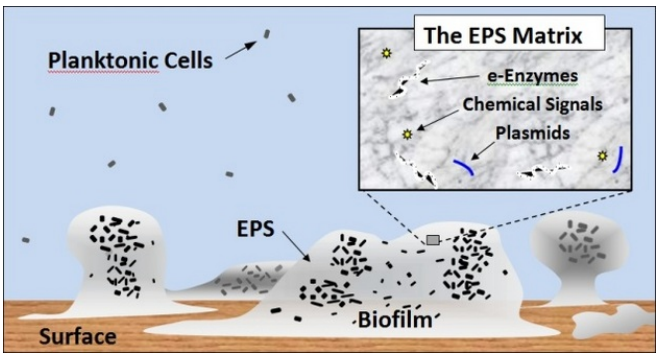


Figure 9 A biofilm is composed of attached microbial cells encased within a matrix of EPS, which surround and protect cells. The EPS matrix is typically composed of polysaccharides, proteins, lipids, and extracellular DNA (eDNA). Localized within the EPS matrix (see figure inset) are extracellular enzymes (e-Enzymes), small pieces of DNA carrying specific genes, and chemical signals. A biofilm may extend from just a few to hundreds of micrometers above a surface, but is equipped with many inherent adaptations that are not present in planktonic cells. Reprinted with permission.[282]

biofilm aggregation.[283] Even within the EPS itself there is much heterogeneity in its physical structure[284] owing to the existence of microdomains,[285-286] which may contribute to diffusive transfer. Specific extracellular polysaccharides (e.g. PsI) are now related to attachment and biofilm formation in *P. aeruginosa*.[287-288] Similarly, *S. aureus* has been shown to regulate key structural biofilm components via gene regulation throughout biofilm development.[289] The ability to display multiple spatiotemporal phenotypes throughout biofilm formation is a survival technique used by bacteria to colonize surfaces and establish an infection. The microenvironment created by the gradient of matrix components throughout the biofilm allows bacterial cells to thrive and persist under adverse conditions once the biofilm has been established. An excellent review of biofilm initiation and development is provided by Hall-Stoodley *et al*.[265]

5.2 Challenges for Inhibition of Biofilms using Engineered Nanoparticles

It is reasonable to assume that diffusion is a requirement for substantial biofilm growth (i.e. how else can nutrients (to cells) and wastes (from cells) move within a biofilm?). Using time-lapsed confocal scanning laser microscopy, the time of penetration for a 10 kDa molecule to reach the center of a cell cluster was estimated to be 3 minutes.[290] The inherent complexity of a bacterial biofilm makes it easy to underestimate nanoparticle mobility and difficult to standardize diffusion coefficients. Diffusion models must consider the 1) viscosity of the EPS, 2) the variability of cell density, 3) the bulk fluid flow of water, 4) interactions of the solute with the EPS (*i.e.* diffusion reaction constraints), 5) the sizes (and volumes) of water spaces between EPS molecules, and 6) the external mass transfer resistance (stagnant liquid on top layer of biofilm that slows penetration of solutes) in three dimensions.

5.2.1 The EPS matrix hurdle: a physical barrier to nanoparticle diffusion

Both simple and complex models have been developed to determine the rate at which molecules and/or nanoparticles of a particular size can permeate through and diffuse within a biofilm of a particular viscosity and composition.[291-293] The rapidly developing field of nanotechnology offers an alternative method to treating biofilm infections, and functionalized nanoparticles have the potential to

efficiently deliver antimicrobial compounds to microbial cells within a biofilm.

Diffusion through the EPS matrix is a complex process. Currently, it is thought that small molecules diffuse through both pure water and EPS at approximately the same rate. The EPS matrix consists of mostly free water that is immobilized within pore spaces between a framework of polymeric molecules.[294-295] Given that small molecules and nanoparticles often move freely through the water, the limiting factor in their movement through biofilms is directly related to interactions with the EPS matrix polymers. Many diffusion rates of small molecules have been measured in various types of biofilms with relative values ranging from 0.9 to 0.2, when compared with diffusion through pure water.[296-300] Using our knowledge of small molecules in biofilms, the movement of nanoparticles in biofilms can be studied.

Currently, a key criterion in the design of nanoparticles intended to penetrate a bacterial biofilm is the size and charge of the nanoparticle. Recent studies have shown that nanoparticles are able to diffuse through a biofilm; however, the rate of diffusion has been directly related to the size of the nanoparticle.[290-293,296,298,301-305] As such, a final hurdle involves the design of nanoparticle size and surface properties that will facilitate their penetration into a biofilm matrix.

Several publications have suggested that the connectivity and size of pores within a biofilm influences the speed and penetration of nanoparticles. Hindrances such as a porous biofilm, local accumulation of nanoparticles on bacterial cells and large macromolecules, the nonspecific adsorption of nanoparticles to freely diffusing species, abiotic particles, and gas all play a role in preventing efficient diffusion of nanoparticles.[292] Studies have been conducted to show that larger nanoparticles diffuse slower through biofilms because they get trapped in pores, cell aggregates, and the general biofilm matrix more than their smaller counterparts.[301-302] Peulen *et al.* demonstrated that 57 nm, 92 nm, and 135 nm nanoparticles had little success with penetration in a dense biofilm.[292] They concluded that the effective pore size in loose biofilms is approximately 50 nm, which translates to efficient diffusion for nanoparticles near 10 nm in diameter. Hidalgo *et al.* showed that nanoparticles of up to 70 nm could penetrate a bacterial biofilm; however, they also showed that nanoparticles measuring less than 30 nm were most effective at homogenously filtering through the matrix.[304] Steric hindrance by the biofilm matrix does not play a major role in nanoparticle diffusion, and therefore does not influence the rate at which nanoparticles diffuse through a biofilm.[298] Currently, a key criterion for designing nanoparticles that will effectively penetrate and target bacterial cells within a heterogeneous biofilm will be size manipulation.

5.2.2 Enhanced antibiotic resistance in biofilms: physiological and genetic

The phenotypic, genotypic, and physical complexities of a bacterial biofilm present many challenges to modern medicine. In general, bacterial antibiotic resistance occurs as a consequence of genetic mutation, acquisition of antibiotic resistance genes, and/or horizontal gene transfer.[306-307] Antibiotic resistance and antibiotic resistance genes have been present in bacterial genomes throughout their evolution. A study from 2004 showed that serine β-lactamase (an enzyme capable of inactivating β-lactam antibiotics) genes originated nearly 2 billion years ago, and have been present on plasmids for millions of years.[308] Most antibiotic producing organisms carry genes that encode antibiotic resistance to ensure self-protection;[309-310] such genes are usually found in the same gene cluster as the antibiotic synthesis genes.[311-312] A thorough review of the presence and role of antibiotic resistance genes in natural environments is provided by Allen *et al.*[313]

In addition to genomic manipulations, mechanisms of antibiotic resistance within a biofilm are also related to restricted antibiotic penetration,[314-318] decreased bacterial growth rates and metabolism,[318-321] quorum sensing and induction of a biofilm-specific phenotype,[67] induction of stress response genes,[322-323] and an increase in the expression of efflux pumps. These mechanisms are referred to as non-inherited resistance, and are intrinsic phenotypic characteristics of bacteria in a biofilm or structural obstructions of the biofilm matrix.[306,324-325] A detailed analysis of antibiotic resistance in biofilms has been reviewed by Høiby *et al.*[326]

Kirby *et al.* developed a method to assess the contribution of the physical structure to the phenotypic resistance of biofilms, without genetic or chemical methods that could be confounded by pleiotropic effects.[324] Their study demonstrated that during high cell densities, planktonic cultures exhibited similar levels of antibiotic resistance as biofilm cells; however, the cells released from biofilms were individually more susceptible to antibiotics that target the cell membrane components (colistin) or depend on membrane function for uptake (gentamicin, streptomycin) than their planktonic counterparts. They suggest that the cell membrane of biofilm bacteria may be more sensitive to antibiotics than the cell membranes of planktonic bacteria. Their results indicate that both cell membrane physiology and the structure of the EPS matrix plays a role in antibiotic resistance within biofilms.

The diverse nature of biofilms also enhances resistance to antibiotics, as seen when mixed biofilms of *Candida albicans* and *S. aureus* more effectively resist vancomycin than either as mono-biofilms or mono-planktonic cultures.[327] Social interactions are essential for successful multicellular complexity.[328] The ability of bacteria to interact within a biofilm allows the cells to maintain a more-stable environment that confers antibiotic resistance via horizontal gene transfer,[329-330] the sharing of common resources,[331-332] and the regulation of core sets of genes.[333] The physiological heterogeneity of a bacterial biofilm makes it extremely pliant and adaptable, and incredibly difficult to combat and eliminate. However, this complexity also offers a variety of targets for researchers to exploit.

A recent review by Yang *et al.* discussed current approaches that are used to eliminate bacterial biofilms.[334] The review focused on biochemical approaches such as antimicrobial agents and peptides, physiochemical approaches such as modifying industrial surfaces with anti-adhesive and microbicidal agents, biological approaches such as inhibiting biofilm formation with the use of natural products found in mixed culture biofilms, and approaches that directly interfere with structural development and differentiation within a biofilm such as eliminating EPS production or inhibiting the ability of bacteria to socially interact via quorum sensing inhibition. In an effort to combine these many approaches, the development of functionalized nanoparticles has been investigated.

Quorum sensing is a form of chemical communication used by bacteria, often to establish infections, develop biofilms, and enhance virulence.[326,335-336] Non-cytotoxic methods of controlling such bacterial infections, such as through quorum sensing manipulation, may help weaken an infection whilst reducing pressure on antibiotic resistance.[96,337-338] A recent study utilized functionalized nanoparticles to target bacterial quorum sensing *in vitro*.[96] In this study, beta-cyclodextrin functionalized silica nanoparticles were used to quench quorum sensing signal molecules and down-regulate quorum sensing genes. Beta-cyclodextrin is a non-specific binding agent of N-acylhomoserine lactones, a common quorum sensing signal molecule.[96,339] This technology lays the groundwork for the attachment of highly specific quorum quenching agents to

nanoparticles, and demonstrates how nanoparticles can enhance the quenching ability of compounds.

While the formulation of nanoparticles is well established and continues to be improved upon, their applications to target bacteria and the exact mechanisms of action are less well understood. A recent study by Decho, Benicewicz, and colleagues found that when complexed to SiO_2 nanoparticles, the common antibiotic penicillin-G is effective in killing penicillin-resistant strains of bacterial pathogens, including strains of methicillin resistant *Staphylococcus aureus* (MRSA), at low total concentrations.[340] The authors termed this effect the 'grenade hypothesis' and postulated that each nanoparticle delivers a concentrated package of antibiotic to a given cells, perhaps overwhelming its resistance mechanism (*e.g*. beta lactamase enzymes), which are normally used defensively against the antibiotic. While this hypothesis remains to be confirmed, the use of nanoparticles as antibiotic-delivery vehicles (ADVs) is an emerging area of exploration.

6. Conclusion

Nanoparticle-based delivery of antibiotics is being proposed, and developed, as a highly-efficient means to deliver antibiotics and target bacterial infections. It offers the possibility to deliver high concentrations of antibiotics, and thus overwhelm bacterial antibiotic resistance strategies. The future efficacy of nanoparticle-based delivery of antibiotics, however, rests upon the ability of nanoparticles to localize a high-concentration of drugs, reach a pre-determined target with high specificity and efficiency, then release those drugs in a controlled and timely manner, and without toxic effects to *in vivo* host cells.

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Notes

[a] Department of Environmental Health Sciences, Arnold School of Public Health, University of South Carolina, Columbia, South Carolina 29208, USA
[b] Department of Chemistry and Biochemistry, College of Arts and Sciences, University of South Carolina, Columbia, South Carolina 29208, USA
[±]KPM and LW contributed equally.
*To whom correspondence should be addressed.
Phone: 803 777-6584
Fax: 803-777-3391
E-mail: awdecho@mailbox.sc.edu

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