Review Article

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Antibacterial nanomaterials: Upcoming hope to overcome antibiotic resistance crisis

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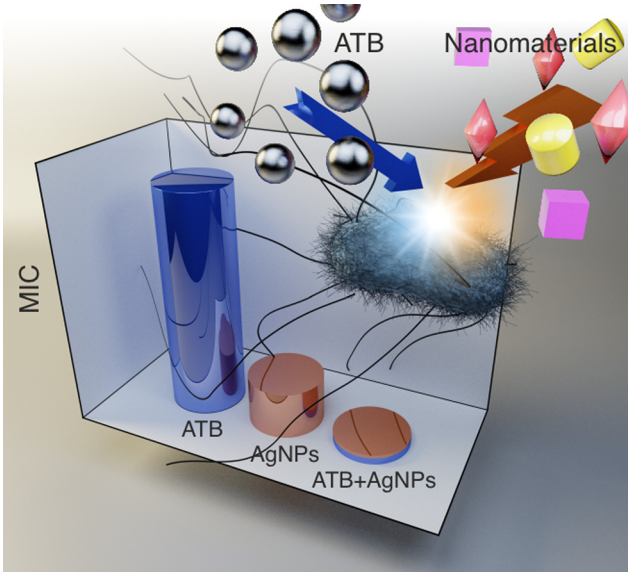
Abstract: When combined with nanomaterials, antibiotics show antibacterial activity against susceptible and resistant bacterial strains at significantly lower concentrations. Unfortunately, to date, no research study has examined the effect of the antibiotic mode of action and mechanism of bacterial resistance on the effectiveness of combined antibacterial treatment with nanomaterials. Therefore, in this review, we performed a thorough analysis and critical evaluation of previously published data related to the combined antibacterial effect of antibiotics with nanostructured materials with a targeted focus on relationships between antibiotic's modes of action and bacterial resistance mechanisms for relevant nanomaterials and their impact on the resulting synergistic effects. Following thorough data analysis and critical discussion, we have discovered and are the first who present that antibiotic's mode of action and bacterial resistance mechanism determine the final effectiveness of combined antibacterial treatment with nanomaterials. We therefore conclude that only certain combinations of nanomaterials with antibiotics can lead to the enhancement and restoration of the antibacterial effectiveness of antibiotics against certain resistant bacteria. Moreover, the recently occurring development of bacterial resistance towards nanomaterials is also discussed together with a possibility of how to prevent it. All discovered findings provide a new view and perspective on this issue helping to navigate further approaches to combat the antibiotic crisis.



Graphical abstract

Keywords: antibiotics, bacteria, modes of action, nanoparticles, resistance, silver

1 Introduction

Bacterial infections still represent a serious and increasing therapeutic problem despite exponentially increasing knowledge in all fields of medicine and considerable improvements in both diagnostic and therapeutic medicine. The main reasons are: (i) the endogenous character of a large proportion of bacterial infections, that is, pathogens originating from the human microflora; (ii) increasing resistance to the effect of antimicrobial drugs; (iii) increasing numbers of immunocompromised patients and persons with artificial materials; and (iv) a high frequency of invasive diagnostic and therapeutic procedures.

Antibacterial agents, as currently known, have been used for more than 75 years. Despite their boom in the

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1960s and 1970s, documented by the development and implementation of numerous novel drugs, they remain a major issue. Today's medicine even faces a real threat of antimicrobials losing their effectiveness against bacteria, and thus their ability to treat bacterial infections. According to a September 2016 statement by the UN General Assembly, it may be estimated that if bacterial resistance continues to increase at the same rate as before, untreatable infections caused by multidrug-resistant bacteria will be the most common cause of death by 2050 [1]. The increasing resistance of bacterial pathogens to antibacterial agents raises the possibility of a return to the *no-antibiotic era*, in which adequate drugs will be unavailable to treat bacterial infections with the etiological role of multidrug-resistant bacteria. According to the European Antimicrobial Resistance Surveillance Network (EARS-Net) interactive database, the high percentages of isolates with resistance to key antimicrobial groups reported from many European countries are of great concern and represent a serious threat to patient safety. For invasive bacterial infections, prompt treatment with effective antimicrobial agents is especially important and is one of the single most effective interventions to reduce the risk of a fatal outcome. Moreover, due to the emergence and spread of bacterial resistance by a mechanism based on the takeover of genetic material from resistant bacterial cells *via* recombination processes, an unstoppable spread of resistance to antibiotics occurs, regardless of their consumption [2]. Concerns about the approaching end of the nearly 80-year era of classic antibiotics caused by increasing bacterial resistance are more than justified and it is high time to adequately address this issue at all possible levels, including the development of new antimicrobial drugs effective against multidrug-resistant bacterial pathogens. At the present time, developing novel antibacterial drugs is not very popular and despite serious threats caused by bacteria (multidrug-resistant strains, newly emerging pathogens, bioterrorism), most big pharmaceutical companies have completely abandoned the development of antibacterials. Among others, this is mainly due to economic factors since higher profits may be earned by developing drugs against other types of diseases (hypertension, cancer, AIDS, *etc.*). The economic calculations must include considerable competition in the market and strict drug approval regulations. Finally, the process of developing novel antibacterials is also technically demanding and time-consuming.

One option for overcoming bacterial resistance is the combination of selected penicillin antibiotics (*e.g.*, ampicillin, amoxicillin, or piperacillin) with bacterial β-lactamase inhibitors (clavulanic acid, sulbactam, or tazobactam) [3]. However, numerous bacterial species exhibit markedly increased resistance against such combinations of antibiotics with other substances that block the defined bacterial resistance mechanism [4]. Thus, if a novel, developmental, antimicrobial drug is to be effective at conquering bacterial resistance, it must act at several cellular levels and not at a specific level, unlike traditional antibiotics. An option to overcome biofilm formation and bacterial resistance is restoring the antibacterial effects of antibiotics by their combination with novel nanostructured antibacterial substances. Nanomaterials have emerged as novel antimicrobial agents for the treatment and prevention of infectious diseases with demonstrated efficacy against resistant bacteria due to their high surface area to volume ratios resulting in higher ratios between atoms on the surface and atoms inside of materials in comparison with corresponding bulk materials [5–7]. The nanostructured antibacterial materials include metal or nonmetal nanoparticles (NPs) such as silver, gold, copper, bismuth, and selenium, and metal oxide NPs such as ZnO, TiO_2, CaO, MgO, Fe_2O_3, or Al_2O_3 NPs. Most of these nanostructured materials show antibacterial effects themselves through nonspecific activity, which can limit the development of bacterial resistance. Silver and its compounds including nanoscale silver materials represent well-known and highly effective antibacterial substances and thanks to that silver in various forms (metallic silver, silver salts, and colloidal silver) has been used as an effective antibacterial agent for many centuries. Silver NPs can inhibit the growth of pathogenic microorganisms including highly resistant strains at very low concentrations of units of ppm showing no cytotoxicity to mammalian cells [8–11]. Gold, copper, and copper oxide NPs themselves show lower antibacterial activity compared to silver NPs, but they significantly enhance the antibacterial effects of antibiotics in mutual combinations or in combinations with other metal NPs [12–15]. Copper oxide NPs cause bacterial leakage of the cellular content of methicillin-resistant *Staphylococcus aureus* and show high activity against staphylococcal biofilm formation [16]. Chemically (redox reaction) or biologically (using microbes) synthesized selenium NPs mainly inhibit staphylococcal bacteria including bacterial biofilm formation and slightly less Gram-negative *Escherichia coli* [17–19]. Bismuth NPs exhibit antibacterial properties just at relatively high concentrations (1 mM). Their antibacterial properties are limited by their low solubility in water, although it can be increased by chelation with dimercaptopropanol, for example [20]. Titanium dioxide and zinc oxide NPs show antibacterial activity against both Gram-negative and Gram-positive bacteria thanks to photocatalytic properties and the generation of reactive oxygen species (ROS), which can damage the bacterial membrane,

DNA, and other bacterial functions, resulting in cell death [21,22]. In addition, zinc oxide NPs are compatible with human skin cells so they can be used as a coating material for medical devices and textiles that come into contact with the human body, and therefore act as wound dressing material [23]. Magnesium and calcium oxide NPs exhibit strong antibacterial effects with minimum inhibitory concentrations of 6 and 100 mg L^{-1}, respectively, thanks to the production of ROS and their high alkalinity [24,25]. Aluminium oxide exhibits poor antibacterial properties and needs to be used in really high concentrations exceeding 1,000 mg L^{-1} [26].

Recently, several studies have indicated that nanostructured materials and especially silver, gold, copper, bismuth, and other NPs may strengthen the antibacterial effects of conventional antibiotics at low doses of both antibiotics and nanobased compounds. This finding clearly suggests that it is possible to find an effective combination of an antibiotic with nano-based compounds, resulting in a synergistic antimicrobial effect allowing efficient inhibition of bacterial pathogens using significantly lower doses as compared with the antibiotic alone [27–30]. High synergistic antibacterial effects of silver NPs even at a concentration below 1 mg L^{-1} in combination with antibiotics have been reported [31–35]. Such low concentrations do not exert cytotoxic effects on human cells or blood as was proved in earlier studies [36–38]. More importantly, restoring of susceptibility of resistant bacterial strains to antibiotics through the synergistic effect in combination with silver, gold, and TiO_2 NPs has been reported [37,39–44]. In other words, antibiotics that originally were totally ineffective show bactericidal effects against multidrug-resistant bacterial strains when combined with metal and metal oxide NPs. This constitutes a great perspective for nanostructured materials as antibacterial agents; combining antibiotics with nanomaterials provides one potential approach to an effective fight against the unresolved problem of increasing resistance of pathogenic bacteria against traditional antibiotics.

On the other hand, bacteria can resist the antibacterial effect of metal cations and oxyanions by, among others, energy-dependent active efflux of toxic ions [45]. Given the constant changes of bacterial genomes and their ability to adapt to negative conditions, it is apparent and predictable that bacteria are able to counter the antibacterial effects of metal and metal oxide NPs even though, unlike classic antibiotics, NPs show a multilevel mode of action that makes the development of bacterial resistance more complicated and difficult but cannot prevent it completely. Recently, Graves *et al.* reported that bacteria can easily develop resistance to silver NPs due to relatively simple genomic changes [46]. On the contrary, Panacek *et al.* and Gunawan *et al.* reported silver resistance in *Escherichia coli* strains, which is not due to changes in the bacterial DNA. Gunawan *et al.* found that *Bacillus subtilis* has a natural ability to adapt to cellular oxidative stress induced by Ag^+ leaching upon prolonged exposure to silver NPs supported on crystalline TiO_2 [47]. Panáček *et al.* stated that the Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* can develop resistance to silver NPs after repeated exposure by flagellin production, which triggers the

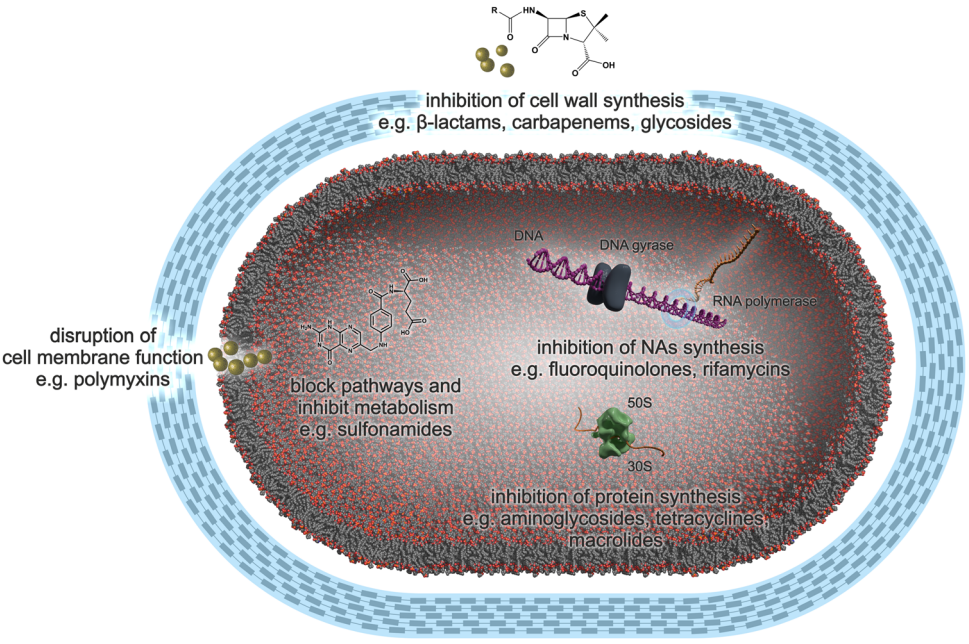

Figure 1: Mechanisms of action of antibiotics.

aggregation and destabilization of silver NPs [48]. Zhang *et al.* reported that *Escherichia coli* develops adaptive resistance to ZnO NPs after several days' exposure to the NPs, consisting of changes in the shape of the bacteria and the expressions of membrane proteins [49].

2 Antibiotics and bacterial resistance

Antibiotics are classified as antibacterial substances with bacteriostatic (inhibiting bacteria) or bactericidal (killing bacteria) properties. They are classified into several groups based on their mode of action, chemical structure, or spectrum of activity. Generally, the bacteriostatic or bactericidal effects of antibiotics are based on affecting bacterial growth processes and bacterial functions. On the other hand, an individual antibiotic with an appropriate mode of action usually acts upon one specific target site of the bacterial cell. Based on the mode of action (Figure 1), we recognize antibiotics inhibiting bacterial cell wall synthesis (*e.g.* β-lactams or glycopeptides), disturbing the cell membrane (polymyxins), inhibiting nucleic acid synthesis (fluoroquinolones), proteosynthesis (tetracyclines, aminoglycosides, or macrolides), and folic acid synthesis (sulfonamides) [50].

In order to exert its antimicrobial action, an antibiotic has to go through a few steps. First, it must enter the bacterial cell (influx), and then remain stable or be activated and accumulated to inhibitory concentrations. Afterwards, it can locate and interact with its target and perform antimicrobial action. Changes to any of these steps result in bacterial resistance to the antibiotic, no matter its mode of action, chemical structure, or spectrum of activity [21].

Bacterial resistance to antibacterial agents may be understood as the ability of the bacterial population to survive the effect of a defined concentration of a particular antibacterial. However, it is necessary to distinguish (i) natural (primary) resistance, that is, the resistance of bacterial species that are outside the range of effects of that antibacterial agent; an example of natural resistance is the absence of a target structure for a particular antibacterial; and (ii) acquired (secondary) resistance, that is, a change from an originally susceptible bacterium to a resistant one. The mechanisms of resistance to the effects of antibacterial agents may be characterized as follows: (i) production of bacterial enzymes that disrupt or modify the structure of antibacterials; (ii) changes in the permeability of the bacterial wall and cytoplasmic membrane; (iii) modification of antibacterial target sites; and (iv) increased elimination of an antimicrobial from bacterial cells (bacterial efflux) [51–53] (Figure 2).

The latter presents a more serious problem as its full extent cannot be defined in advance and needs to be detected by relevant microbiological tests. These require some time, which may be a problem, particularly in case of severe bacterial infections. Antibiotic therapy must be initiated as soon as possible, or immediately after the diagnosis is made, due to higher mortality of patients in whom adequate antibiotic therapy was delayed [54].

It should be stressed that the development and spread of bacterial resistance must be seen as natural processes that cannot be fully prevented but may be used and influenced, both positively and negatively. The majority of resistance mechanisms in bacteria developed long before the first modern antibacterial agents were used for treatment. This is probably determined by the fact that most antibacterials are derived from compounds commonly

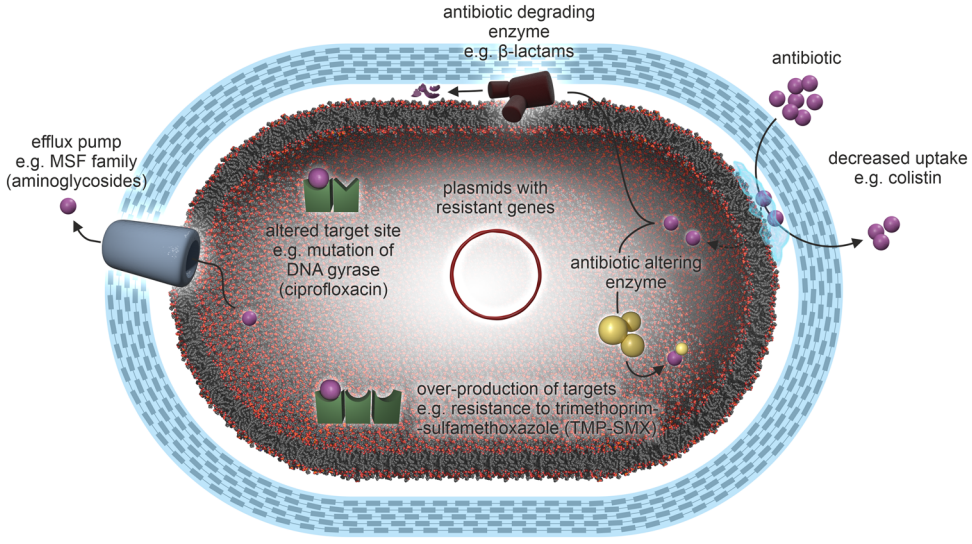

Figure 2: Mechanisms of bacterial resistance to antibiotics.

produced by other microorganisms. Resistance mechanisms usually do not occur by accident and suddenly but wait for conditions that allow them to succeed in the bacterial population. A typical example may be the resistance of *Staphylococcus aureus* to β-lactam antibiotics. After penicillin was introduced into treatment, strains resistant to penicillin occurred rapidly. In the early 1940s, less than 1% of *Staphylococcus aureus* strains in English hospitals were resistant to penicillin. The rate increased to as much as 60% in 1946 [55].

The most important causes for the development of bacterial resistance are changes in bacterial genotype. These may be defined by chromosomal mutations followed by a selection of resistant cells (chromosomal resistance) that may be negatively influenced by the selection pressure of antibacterial drugs, that is, antibiotic therapy itself. However, a more important mechanism is a process based on the transfer of genetic material through recombination processes, that is, conjugation, transformation, and transduction (extrachromosomal resistance). Therefore, a very worrying possibility must be considered that an imaginary threshold has been crossed and resistance of numerous bacteria to broad-spectrum antibiotics "lives its own life" through the transfer of mobile genetic elements encoding resistance, for example, production of broad-spectrum β-lactamases. The threshold means a certain level of resistance genes circulating in the bacterial population that are horizontally transferred by recombination processes (mainly conjugation), causing the unstoppable spread of resistance to antimicrobials independently of their consumption [56]. Bacterial resistance is not a theoretical microbiological term but a reality with serious negative clinical impacts.

Many studies have been published that document higher mortality and shorter survival of patients with infections caused by multidrug-resistant bacteria compared to infections caused by susceptible strains of the same species. For example, Rello *et al.* reported 86% mortality of patients with ventilator-associated pneumonia due to methicillin-resistant strains of *Staphylococcus aureus* as compared with 12% in the case of *Staphylococcus aureus* isolates susceptible to methicillin/oxacillin. Tumbarello *et al.* found that the mortality of patients with bloodstream infections caused by enterobacteria with positive production of broad-spectrum β-lactamases reached 60% in case of inadequate antibiotic therapy but only 19% if antibiotic therapy was effective. Kang *et al.* documented a difference in 30-day mortality from infections caused by *Pseudomonas aeruginosa* between adequate initial antibiotic therapy (28%) and delayed initiation of effective treatment (43%). Herkel *et al.* showed a statistically significant difference in mortality between adequate and inadequate antibiotic therapy of ventilator-associated pneumonia. The mortality rates were 27% for patients receiving adequate therapy and 45% for inadequate therapy, meaning that bacterial pathogens were resistant to initial antibiotic treatment [54,57–59].

The development of bacterial resistance is unstoppable and will continue despite all measures taken. One possible solution is undoubtedly nanotechnology, for example, a combination of existing antibacterials with silver NPs. These are highly active against numerous bacteria including multidrug-resistant strains, for example, methicillin-resistant strains of *Staphylococcus aureus*, vancomycin-resistant enterococci, and enterobacteria producing broad-spectrum β-lactamases.

3 Antibacterial nanomaterials and their mechanism of action

The mode of action of metal and metal oxide NPs is not fully described and clear yet, but for relevant metals, several modes of action have been proposed (Figure 3). Each of the NPs, regardless of the chemical composition, is able to fight bacteria by various mechanisms and such a multilevel mode of action makes the development of bacterial resistance much more difficult. Besides, NPs are able to deliver antibiotics to the bacteria, while acting as a drug carrier, which results in drug potency enhancement and limits overall drug exposure. The most commonly applied ways of how NPs fight a wide range of pathogens are disruption of the cell wall, cytoplasmic membrane, and production of ROS leading to oxidative stress, followed, to a lesser extent, by enzymatic inhibition, changes in gene expression, and protein deactivation [5,60,61].

NPs are accumulated on the surface and create "pits" in the bacterial wall. Therefore, they are able to penetrate the cell wall, causing changes to the cell membrane, structural damage, and cell death [62]. The bactericidal effect of positively charged ions released by NPs is enhanced by binding with the negatively charged surface of bacteria (carboxyl, phosphate groups) in a process known as biosorption [5,60]. Besides that, electrostatic binding to the cell wall leads to membrane depolarization, change of membrane potential, and loss of its integrity, resulting in interruption of energy transduction and cell death [22]. However, thanks to the thick peptidoglycan layer of Gram-positive bacteria, penetration of NPs into bacteria is harder and, therefore, NPs interact with the bacterial surface only [63,64].

Oxidative stress is induced by ROS, which has strong positive redox potential. ROS are induced by respiratory

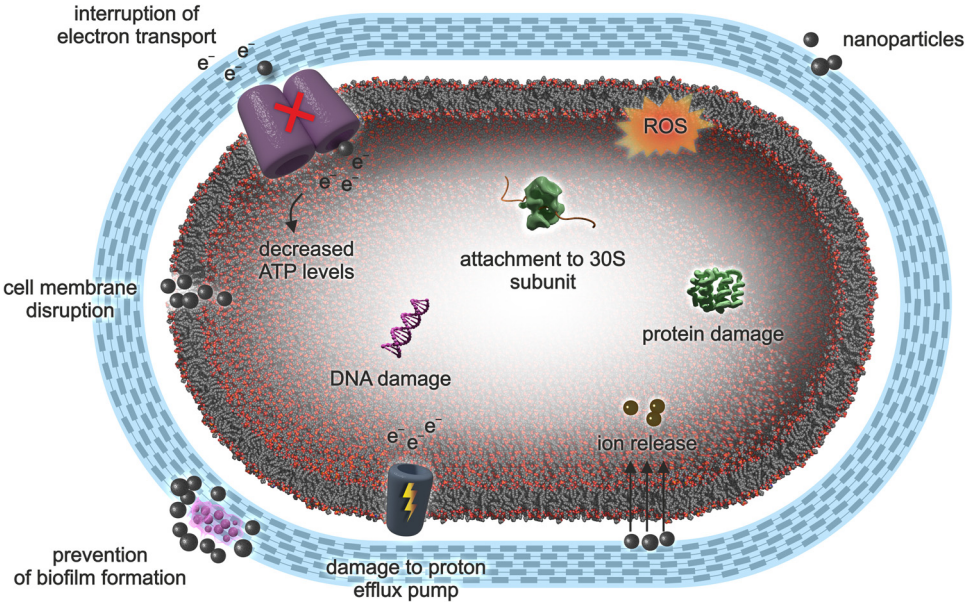

Figure 3: Mechanisms of action of nanostructured materials.

chain disruption or by NPs themselves [65]. ROS include hydrogen peroxide (H_2O_2), singlet oxygen (O_2), hydroxyl radical ($^{\cdot}OH$), and superoxide radical (O^{2-}). Different combinations of ROS are produced by different NPs, resulting in different antimicrobial properties. For example, Ag and Cu NPs generate all types of ROS, whereas MgO NPs only produce the superoxide radical and ZnO NPs produce a combination of hydrogen peroxide and the hydroxyl radical only [5]. ROS production occurs during the basic mechanism and it is caused by defects and vacancies in the crystal [66]. Normally, the production and the removal of ROS are balanced. However, under high levels of stress, there is excessive production of ROS, which changes the permeability of the cell membrane and causes bacterial damage [60,67,68]. The production of ROS is mediated by various mechanisms. The photocatalytic hypothesis is based on the irradiation of NPs with energy greater than the band gap, which leads to the stimulation of electrons in the valence band and their transition to the conduction band, resulting in a hole in the valence band and the production of highly reactive reactants on the surface of and inside the material. Intracellular and extracellular ROS can disrupt the cell membrane by lipid oxidation, easily producing free radicals [69,70]. Thanks to its thickness and negatively charged surface, the cell wall structure of Gram-positive bacteria is more difficult to penetrate, which slows down the penetration of oxygen radicals such as OH^-. Besides oxidative stress, ROS can cause cell damage to macromolecules, leading to lipid peroxidation, alteration of protein, inhibition of enzymes, and RNA or DNA damage. Significant antimicrobial effects *via* production of ROS can be observed in the case of Ag [71–73], ZnO [74,75], TiO_2 [76–78] and iron oxide [79] NPs.

3.1 Silver NPs

The mechanism of action is not fully understood, which complicates the understanding of interactions between NPs and bacterial cells. However, all existing data suggest that Ag NPs exhibit various antibacterial mechanisms in parallel and bind non-specifically to a wide variety of targets. By doing so, they disturb many aspects of the cell metabolism, which makes the development of resistance towards them much more difficult [80,81]. It is thought that silver NPs serve as a reservoir for silver ions released *via* the oxidative dissolution process [82,83]. NPs

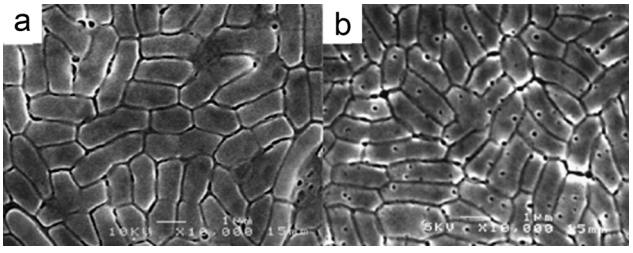

Figure 4: Scanning electron microscopy images of (a) native *E. coli* cells and (b) cells treated with silver NPs created "pits." Reproduced with permission [92]; Copyright 2004, *Journal of Colloid and Interface Science*.

are then able to adhere to the negatively charged bacterial cell wall and create "pits" (holes) in it (Figure 4b), which leads to depolarization and collapse of plasma membrane potential [62,84]. As a result, the cytoplasmic contents flow out and the cell membrane becomes more permeable, making penetration of NPs into the cells and their interaction with intercellular components much easier [85–87]. A released Ag^+ ion inhibits the site between cytochrome α2 and b-cytochromes in the respiratory chain. NPs can also interrupt the cellular respiration process, inhibit the cytochrome in the electron transport chain or denature the 30 S ribosomal subunit (prevent protein translation) [60,88]. The second mechanism proposes the production of ROS at the cell membrane, which leads to DNA replication damage, destruction of biomolecules and contributes to oxidative stress. Furthermore, silver NPs bind easily to thiol, amino, and phosphate groups, which are important parts of DNA, peptides, and enzymes. Interaction of NPs with those groups can therefore inactivate enzymes, change protein expression and interrupt metabolic processes, which might lead to damage or inhibition of DNA/RNA replication and cause irreversible bacterial damage and cell death [84,85,89,90]. The antibacterial mechanism involved in the synergism is the production of hydroxyl radicals and degradation of the function and protective factors, which leads to reduction of the antibiotic concentration and decline of bacterial viability [91].

3.2 Gold NPs

The most common antibacterial action of gold NPs is through inhibition of tRNA binding to the ribosome during DNA transcription or attachment of the NPs to the bacterial cell wall, changing its potential and leading to the adenosine triphosphate level decrease, leakage of cell contents, and cell death [93,94]. Gold NPs are more effective against Gram-negative bacteria, which is due to easier incorporation of the NPs into the bacteria [95,96]. Since gold NPs might have a ROS-independent mechanism, they seem to be safer for mammalian cells [97].

3.3 Titanium dioxide NPs

Thanks to their photocatalytic properties, TiO_2 NPs are able to kill bacteria just by simple UV illumination, which induces the generation of ROS, leading to oxidative stress, DNA, lipid, and protein damage [77,98]. However, even without illumination, TiO_2 NPs keep their antibacterial properties. In this case, NPs adsorb on the surface and interact directly with the cell wall, resulting in the loss of membrane integrity [99]. Doping by other metal NPs enhances their antibacterial properties and helps them fight bacteria [76,100–102].

3.4 Zinc oxide NPs

Similar to TiO_2 NPs, zinc oxide NPs exhibit strong photocatalytic properties. After UV irradiation or even without it, they produce ROS that might further cause inhibition of DNA replication, protein denaturation, or cell membrane disruption, resulting in high antibacterial effects [75,103]. Another proposed mechanism of action is zinc ion release followed by accumulation of ZnO NPs on the bacterial membrane (by electrostatic forces), therefore interrupting transmembrane electron transport or entrance into the cell causing enzymatic inhibition, DNA or mitochondrial damage, which all lead to inhibition of bacterial growth and cell death [104–106]. Recently, Kadiyala *et al.* suggested a mechanism of action related to energy metabolism alteration within the cell resulting in increased pyrimidine biosynthesis (especially uridine monophosphate biosynthesis), carbohydrate metabolism, and decreased amino acid synthesis. This mechanism also explains higher antibacterial activity against *S. aureus* in comparison with *E. coli*, because *E. coli* does not require uridine for anaerobic growth [107,108].

3.5 Iron oxide NPs

The mode of action of iron oxide NPs is through the dissolution of metal ions, which interact with the bacterial cell, penetrate the membrane and interfere with electron transfer or through the formation of ROS, which damage DNA and proteins [5,109,110].

3.6 Platinum NPs

Like other metal NPs, platinum NPs diffuse through the cell wall and cytoplasmic membrane and induce ROS generation, DNA damage, accumulation of cells during the S-phase of the cell cycle, and consequently, cell death [111–113].

3.7 Copper and copper oxide NPs

Copper NPs and their subsequent ion release can cause morphological changes and interact with the cell membrane

and decrease its transmembrane electrochemical potential, which affects the membrane integrity and causes cellular death [114]. Besides, they are able to generate ROS, which might result in mitochondrial damage, lipid peroxidation, and DNA damage. Copper oxide also produces ROS, which is followed by DNA degradation or membrane disruption leading to damage of vital enzymes and cell death [115–120]. In the presence of CuO NPs, the expression of key proteins is changed, which has a major influence on bacterial denitrification and other metabolic processes such as active transport and electron transfer [65,121].

3.8 Selenium NPs

Selenium oxyanions released from selenium NPs can disrupt the cell wall or produce ROS able to react with thiol groups present in the cell; together, they produce superoxide radicals that result in oxidative stress. Another mechanism of action might be disruption of intercellular adenosine triphosphate concentrations or depolarization/disruption of the bacterial membrane, which has a negative effect on cell division and membrane transport and leakage of the cytosolic content, respectively [122–124].

3.9 Magnesium oxide NPs

Antibacterial properties of magnesium oxide depend not only on the size of particles but also on pH, while high pH damages the cell membrane and causes bacterial cell death [115,125–127]. MgO NPs also generate ROS on the surface and damage the cell wall, which results in intracellular contents leakage and cell death [128–130].

4 Synergistic effect of antibiotics in combination with antibacterial nanomaterials

4.1 Synergistic activity against antibiotic-susceptible bacteria

In the previous section, the antibacterial activity of various metal or metalloid NPs and their compounds including their mechanism of action at different bacterial cellular levels have been reviewed and discussed. Additionally, nanostructured materials may also be applied in combination with antibiotics to enhance their antibacterial effects at significantly lower concentrations of both NPs and antibiotics. Many scientific papers have described synergistic effects of metal and metal oxide NPs in combination with antibiotics resulting in increased antibiotic activity and decreased nanoparticle toxicity to mammalian cells. The current state of the art on this synergistic activity has been repeatedly summarized [65,109,115,131]. However, most of the synergies reported in those reviews were observed using antibiotic-sensitive bacteria. The synergic effects were usually examined by either the disc diffusion method or the microdilution method (Figure 5). The microdilution method provides information on the level of synergy by determining the fractional inhibitory concentration (FIC), enabling to specify the enhancement of antibacterial activity as synergistic, additive, indifferent, and antagonistic effects. On the contrary, the disc diffusion method does not enable quantifying the synergistic effect in principle, and it is difficult to differentiate between synergistic and additive, indifferent or antagonistic effects.

To date, synergistic effects of antibiotics combined with metal and metal oxide NPs have been predominantly evaluated against antibiotic-sensitive bacteria. Most of the experiments determining synergistic effects were conducted on silver [33,35,133–140] and gold NPs [34,141–144]. However, synergistic effects have also been evaluated using other metal and metal oxide NPs showing antibacterial properties such as copper [28,145,146], titanium dioxide [147,148], and zinc oxide [30,64,149]. The synergy of silver NPs has been deeply studied in combination with antibiotics of various modes of action and chemical structures, for example, antibiotics inhibiting protein synthesis (aminoglycosides), cell wall synthesis (β-lactams and carbapenems), nucleic acid synthesis (quinolones), and antibiotics disrupting the cytoplasmic membrane (polymyxins). In this particular case, synergistic effects of silver NPs combined with β-lactams (ampicillin, methicillin, penicillin), glycopeptides (vancomycin), quinolones (ciprofloxacin), sulfonamides (trimethoprim), aminoglycosides (amikacin, gentamicin, kanamycin, streptomycin), macrolides (erythromycin), and tetracyclines (tetracycline) [33,34,39,133–135,138,139,150,151], have been confirmed against a wide range of both Gram-negative and Gram-positive bacteria. Silver NPs enhance antibiotic activity at very low concentrations ranging from tens to several units of ppm, which is beneficial to nanoparticle toxicity because low silver concentrations do not show toxic effects on mammalian cells and humans. Synergistic effects of gold NPs in combination with different antibiotics against sensitive bacteria have been observed in almost all cases including both Gram-negative and Gram-positive bacteria. For example, high synergistic effects of gold NPs combined with meropenem against *Acinetobacter baumannii* [143] and

amoxicillin and streptomycin against *Staphylococcus aureus* and *Escherichia coli* [34] at concentrations ranging from 1 to 16 mg L^{-1} gold have been reported. In the case of bismuth NPs, synergistic effects have only been evaluated using antibiotics inhibiting nucleic acid synthesis (fluoroquinolones). Enhancement of antibacterial activity was only shown for ciprofloxacin combined with bismuth NPs against *Klebsiella pneumoniae* [27]. Copper NPs enhance the antibacterial activity of antibiotics at concentrations ranging from 20 to 50 mg L^{-1} depending on the antibiotic or bacterial strain. For example, synergistic effects of copper NPs combined with ampicillin, amoxicillin, ciprofloxacin, and gentamicin have been observed against various bacteria such as *Bacillus subtilis, Escherichia coli,* and *Salmonella typhimurium* [28]. Zinc oxide NPs at concentrations ranging from 30 to 80 mg L^{-1} have been combined with fluoroquinolones (norfloxacin, ofloxacin) [30] or β-lactams (cephalexin, ceftriaxone, cefotaxime) [152] in order to highly enhance their activity against both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria. Titanium dioxide NPs have been studied as a potential antibacterial agent in combination with streptomycin, with increased antibacterial properties being shown against *Klebsiella pneumoniae, Salmonella typhimurium, Escherichia coli,* and *Staphylococcus aureus*.

4.2 Synergistic activity against antibiotic-resistant bacteria

It must be noted that all the mentioned results on high synergistic effects of metal and metal oxide NPs have been conducted on bacteria sensitive to antibiotics. On the one hand, those experiments are crucial to prove the ability of NPs to enhance the antibacterial properties of antibiotics. On the other hand, such research is relatively unnecessary, insignificant, and senseless as there is no need to increase the effectiveness of antibiotics reliably shown to fight bacteria. Enhancing and restoring the effects of antibiotics only make sense in the case of antibiotic-resistant bacteria that currently complicate the treatment of bacterial infections, increasing patient's mortality. Therefore, the following parts of this review are concerned with the synergistic effects and enhancement of the antibacterial activity of antibiotics combined with nanomaterials against antibiotic-resistant bacteria, which is important and significant to explore possible ways of overcoming bacterial resistance. All data are summarized in Table 1 and Figure 6. Moreover, relationships between antibiotic modes of action, bacterial resistance mechanisms, and types of nanostructured materials and their impact on the final synergistic effects are discussed below.

Enhancement of antibacterial activity of various antibiotics combined with silver [39–44], gold [13,153–156], and TiO_2 NPs [147] against resistant Gram-positive and Gram-negative bacteria has only been studied so far. Generally, bacteria can resist the antibacterial effects of antibiotics primarily (naturally) or secondarily (through genetic mutations or a gene transfer from other bacteria) (see the Antibiotics and Bacterial Resistance section). Enhancement of antibacterial activity of antibiotics against primarily resistant bacteria was only investigated in combination with silver NPs, whereas in the case of secondarily resistant bacteria, the synergistic effects of antibiotics combined with silver, gold, and TiO_2 NPs have been evaluated.

4.2.1 Silver NPs

4.2.1.1 Primarily resistant bacteria

Silver NPs are currently among the most studied biologically active metal NPs, with most experiments on synergistic effects against resistant bacteria being conducted exclusively with silver NPs. More importantly, the combination of silver NPs with antibiotics may result in increased antibacterial activity and restored activity of currently ineffective antibiotics against resistant bacteria [8].

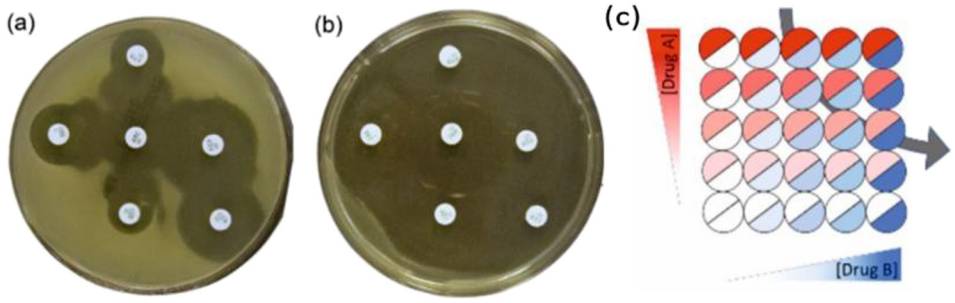

Figure 5: Disc diffusion method showing antibacterial activity of (a) an antibiotic alone and (b) in combination with Ag NPs. Reproduced with permission [37]; Copyright 2016, *Colloids and Surfaces B: Biointerfaces*. (c) Microdilution checkerboard method for simultaneous MIC and FIC determination. Reproduced with permission [132]; Copyright 2018, *Journal of Visualized Experiments*.

Table 1: Summary of antibacterial effects of antibiotics combined with silver, gold, and TiO_2 NPs against resistant bacteria

NPs	R	Mode of action	Antibiotic class	Antibiotic	No enhancement	Enhancement
Ag	Primary resistance	Cell wall synthesis	β-Lactams	Ampicillin	—	*A. baumannii* *E. cloacae* *K. pneumoniae* *P. aeruginosa*
			Glycopeptides	Vancomycin	*A. baumannii* *E. coli* *P. aeruginosa*	*E. coli*
	Secondary resistance	Cell wall synthesis	β-Lactams	Imipenem	—	*A. baumannii* *B. subtilis* *E. faecalis* *E. coli* *K. pneumoniae* *M. luteus* *P. aeruginosa* *S. aureus*
				Meropenem	—	*K. pneumoniae*
				Amoxicillin		*A. actinomycetemcomitans* *A. baumannii*
					A. pleuropneumoniae *E. coli* *S. gordonii* *S. oralis*	*A. pleuropneumoniae* *E. aerogenes* *E. coli* *S. aureus* *S. mutans* *S. sonnei* *P. aeruginosa*
				Penicillin	—	*A. pleuropneumoniae* *S. aureus* *S. mutants*
				Ampicillin	*P. aeruginosa*	*A. actinomycetemcomitans* *A. baumannii* *E. coli* *E. faecium* *M. pneumoniae* *S. typhimurium* *S. sonnei* *S. aureus* *S. gordonii* *S. oralis* *S. mutants*
				Cefotaxime	—	*E. coli* *K. pneumoniae*
				Ceftazidime	*A. baumannii*	*E. coli* *K. pneumoniae* *S. mutants*
				Cefpodoxime	*E. faecalis* *S. gordonii*	*A. actinomycetemcomitans*
					S. oralis	*S. mutants*
				Cefuroxime	*E. faecalis* *S. gordonii* *S. oralis*	*S. mutants*
			Glycopeptides	Vancomycin	*B. subtilis* *E. faecalis* *M. luteus* *S. aureus*	*S. aureus* *S. mutants*
		Cell membrane	Polymyxins	Colistin	—	*A. pleuropneumoniae* *P. multocida*

(*Continued*)

Table 1: *Continued*

NPs	R	Mode of action	Antibiotic class	Antibiotic	No enhancement	Enhancement
		Protein synthesis	Aminoglycosides	Amikacin	*A. baumannii*	*A. baumannii*
						E. cloacae
						E. coli
						E. faecium
						K. pneumoniae
						M. pneumoniae
						P. aeruginosa
						S. typhimurium
						S. sonnei
						S. aureus
				Gentamicin		*A. baumannii*
						A. actinomycetemcomitans
						A. pleuropneumoniae
						B. subtilis
						E. faecalis
					A. baumannii	*K. pneumoniae*
					E. coli	*M. luteus*
					E. faecalis	*P. aeruginosa*
					S. gordonii	*P. multocida*
						S. typhimurium
						S. oralis
						S. epidermidis
						S. mutants
						S. sonnei
						S. aureus
				Neomycin	—	*S. typhimurium*
				Kanamycin	—	*A. baumannii*
						E. aerogenes
						P. aeruginosa
						S. typhimurium
				Clyndamycin	*E. coli*	*A. actinomycetemcomitans*
					E. faecalis	
					P. aeruginosa	*S. aureus*
					S. aureus	*S. gordonii*
					S. oralis	
				Erythromycin		*A. actinomycetemcomitans*
					E. coli	*P. aeruginosa*
					E. faecalis	*S. aureus*
						S. gordonii
						S. oralis
				Chloramphenicol	—	*A. actinomycetemcomitans*
						P. aeruginosa
						S. oralis
				Tetracycline	*S. oralis*	*A. actinomycetemcomitans*
						S. gordonii
		Folate synthesis	Sulfonamides	Trimethoprim	*A. baumannii*	—
					B. subtilis	
					E. coli	
					E. faecalis	
					K. pneumoniae	
					M. luteus	
					P. aeruginosa	
					S. aureus	
		NA synthesis	Qinolones	Ciprofloxacin	*A. baumannii*	
					E. faecalis	*A. baumannii*

(*Continued*)

Table 1: *Continued*

NPs	R	Mode of action	Antibiotic class	Antibiotic	No enhancement	Enhancement
					E. coli	*A. actinomycetemcomitans*
					M. luteus	
					P. aeruginosa	*B. subtilis*
					S. epidermidis	*K. pneumoniae*
					S. gordonii	*S. mutants*
					S. oralis	
Au	Secondary resistance	Cell wall synthesis	β-Lactams	Amoxicillin	—	*S. aureus*
				Methicillin	*S. epidermidis*	—
					S. haemolyticus	
				Cefotaxime	—	*E. coli*
						K. pneumoniae
						S. aureus
				Ceftriaxone	—	*E. coli*
						K. pneumoniae
						S. aureus
			Glycopeptides	Vancomycin	*S. haemolyticus*	*E. faecalis*
						E. faecium
						S. aureus
						S. epidermidis
						S. mutants
		Protein synthesis	Aminoglycosides	Gentamicin	—	*S. epidermidis*
		NA synthesis	Quinolones	Ciprofloxacin	—	*E. coli*
						K. pneumoniae
						S. aureus
						S. epidermidis
						S. haemolyticus
				Levofloxacin	—	*E. coli*
						K. pneumoniae
						S. aureus
				Nalidixic acid	—	*S. epidermidis*
						S. haemolyticus
				Rifampicin	*S. epidermidis*	*S. epidermidis*
						S. haemolyticus
TiO_2	Secondary resistance	Cell wall synthesis	β-Lactams	Penicillin G	—	*S. aureus*
				Ampicillin		
				Cloxacillin		
				Oxacillin		
				Amoxycillin		
			Glycopeptides	Vancomycin		
			Cephalosporins	Cefotaxime		
				Ceftazidime		
				Cephalexin		
		Protein synthesis	Aminoglycosides	Amikacin		
				Gentamycin		
				Streptomycin		
			Azalides	Clarithromycin		
			Lincosamides	Clindamycin		
			Macrolides	Erythromycin		
		NA synthesis	Fluoroquinolones	Ciprofloxacin		
				Norfloxacin		
			Sulfonamides	Tetracycline		
				Cotrimoxazole		
				Rifampicin		
				Sulphazidime		
				Chloramphenicol		
			Quinolone	Nalidixic acid	*S. aureus*	—

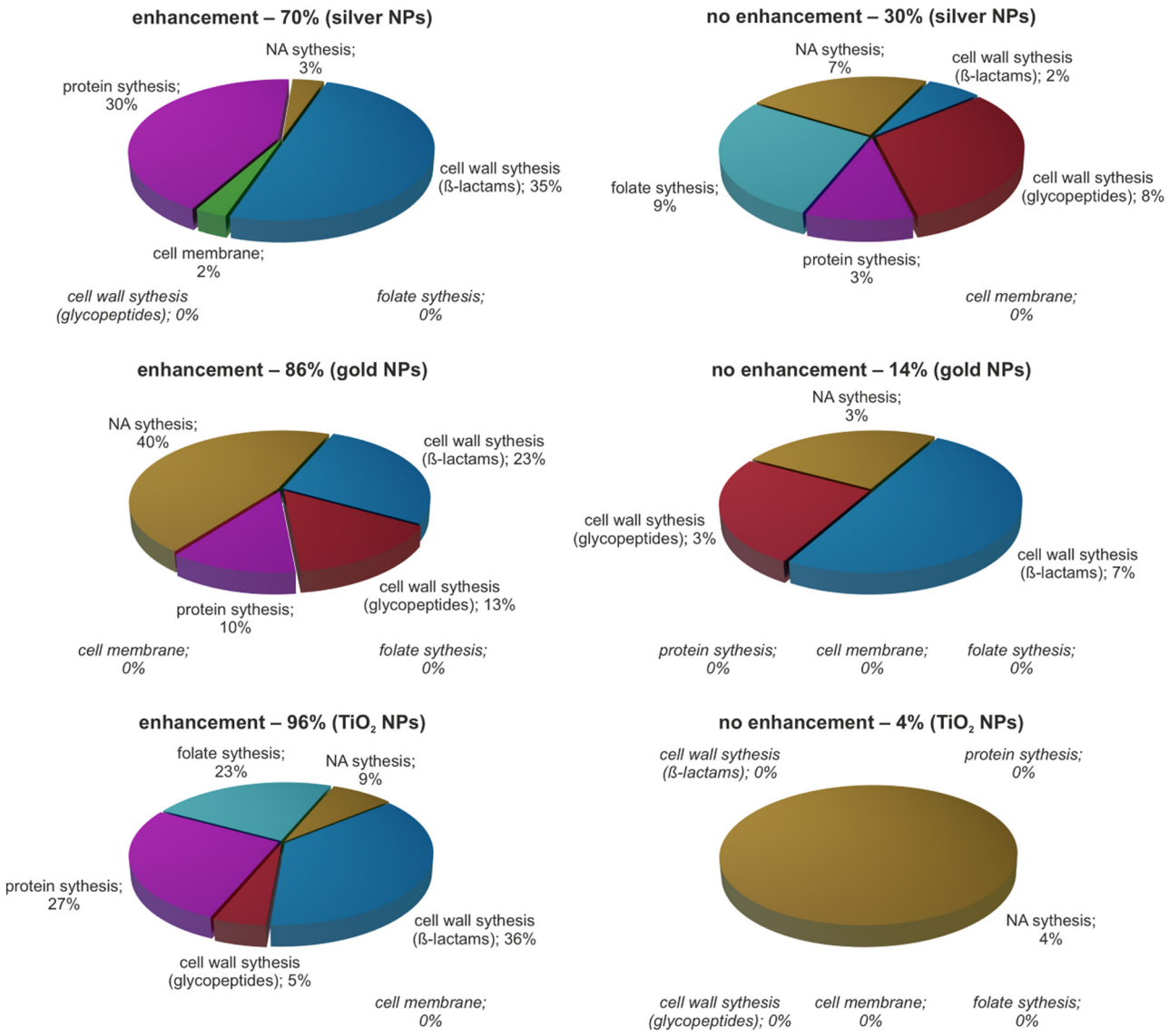

Figure 6: Percentages of enhancement and indifferent effects (no enhancement) of antibiotic classes divided based on the mode of action against resistant bacteria.

Synergistic enhancement of antibacterial activity using silver NPs against *Acinetobacter baumannii, Enterobacter cloacae, Klebsiella pneumonia*, and *Pseudomonas aeruginosa* primarily resistant to ampicillin and *Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Escherichia coli* primarily resistant to vancomycin has been studied by Lopez-Carrizales *et al.* [39] and Naqvi *et al.* [42].

Ampicillin and vancomycin belong to a group of antibiotics inhibiting cell wall synthesis, albeit through different molecular mechanisms. Ampicillin inhibits the synthesis of peptidoglycan, a building unit of the cell wall, through binding to a penicillin-binding protein (enzyme transpeptidase reforming the peptide cross-links) involved in peptidoglycan synthesis in both Gram-negative and Gram-positive bacteria. Vancomycin binds to the monomers of *N*-acetylmuramic acid and *N*-acetylglucosamine, building blocks of peptidoglycan, preventing the transpeptidase from acting on these newly formed blocks and thus cross-linking of the peptidoglycan layer, but only in the case of Gram-positive bacteria. Nevertheless, all tested bacteria naturally resist the effects of ampicillin and vancomycin. In the case of ampicillin, all tested bacteria naturally produce β-lactamases, enzymes changing the configuration of ampicillin molecules; this prevents binding to the target site (penicillin-binding protein) and inhibits peptidoglycan synthesis. Vancomycin is not active against Gram-negative bacteria due to the different mechanisms by which Gram-negative bacteria produce their cell walls and various factors

related to entering the outer membrane of Gram-negative organisms.

Synergistic effects and restored antibacterial effects of antibiotics have been observed for all experiments with ampicillin (a β-lactam antibiotic targeting cell wall synthesis) against all tested resistant bacteria (*Acinetobacter baumannii*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*), as reported by Lopez-Carrizales *et al.* [39] It is probably thanks to silver NPs that can disrupt the bacterial cell wall and cytoplasmic membrane easily. Cell wall disruption leads to the leakage of the periplasmic contents (including β-lactamases) out of the bacteria. As a result, the concentration of β-lactamases in the periplasm decrease, and the bacteria are not able to resist the ampicillin action. After that, the antibiotic may bind to the target site and exert its antibacterial activity. Silver NPs can also damage several molecular systems of efflux pumps working at the cell wall and cytoplasmic membrane. Therefore, antibiotic molecules cannot be pumped out of the bacteria, reach the minimum inhibitory concentration within the cell, and the antibiotic can become effective again.

Unlike ampicillin, the synergistic effects of silver NPs combined with vancomycin have not been demonstrated in all of the cases. Naqui *et al.* reported that silver NPs combined with vancomycin do not show enhancement of antibacterial activity against *Acinetobacter baumannii* and *Escherichia coli*. In the case of *Pseudomonas aeruginosa*, the vancomycin inhibition zone slightly increased from 14 to 17 mm when the drug was combined with silver NPs. However, such an increase is insignificant and cannot clearly confirm the enhancement of antibacterial activity. Considering the mechanism of primary resistance of Gram-negative bacteria involving the lacking target site for vancomycin, it may be expected that silver NPs cannot enhance the antibacterial effects of antibiotics. If there is no specific target site to bind an antibiotic in bacteria, its antibacterial activity cannot be enhanced. On the other hand, Kaur *et al.* and Ma *et al.* described the enhancement of antibacterial effect in *Escherichia coli*, where the vancomycin was bounded to the nanoparticle surface, so this interaction was able to provide effective delivery of the antibiotic to the bacteria, where both silver ions and vancomycin interacted with the cell membrane and inhibited bacterial growth [157,158].

4.2.1.2 Secondarily resistant bacteria

Synergistic effects of silver NPs with antibiotics and their ability to restore the activity of antibiotics against bacteria showing acquired (secondary) antibiotic resistance have not been studied as extensively as in antibiotic-sensitive bacteria [39–44]. Overall, approximately 100 experiments evaluating the synergistic effects of antibiotics with different modes of action and chemical structures combined with silver NPs against various resistant bacteria have been performed. This is a relatively small number compared to more than 700 experiments on the synergistic effects of silver NPs combined with antibiotics against antibiotic-susceptible bacteria. Most of the tested resistant bacteria involved Gram-negative strains causing the most problematic and difficult-to-treat infections in humans. Twenty percent of all tested resistant bacteria with described resistance mechanisms originated from public strain collections and 80% of resistant strains were isolated from human clinical materials; for these, unfortunately, any description of resistance mechanisms is missing. As for the mode of action, 43% of the reported antibiotics are protein synthesis inhibitors, 27% cell wall synthesis inhibitors, 25% nucleic acid inhibitors, and 5% cytoplasmic membrane disruptors.

The synergistic effects of β-lactam antibiotics (cell wall synthesis inhibitors) combined with silver NPs have been reported by Sharma *et al.*, Panáček *et al.*, and Ono *et al.* [37,159–161] The best outcomes have been observed with imipenem and meropenem (carbapenems). In the case of imipenem, the synergistic effects have been seen for all the tested resistant bacteria (Gram-negative *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and Gram-positive *Bacillus subtilis*, *Enterococcus faecalis*, *Micrococcus luteus,* and *Staphylococcus aureus*). Meropenem combined with silver NPs showed higher antibacterial activity against *Klebsiella pneumoniae*. In the case of Gram-negative bacteria, the mechanisms of resistance to carbapenems usually involve plasmid-mediated carbapenemase production and loss of porin channels [159]. A Gram-positive strain of *E. faecalis* is known for resistance to imipenem through carbapenemase production and also for overproduction of peptidoglycan strengthening the cell wall [160]. All these mechanisms of resistance to imipenem and meropenem can be overcome by the effects of silver NPs located in the cell wall (peptidoglycan overproduction) and periplasmic space (carbapenemase production). Silver NPs may interact with porin channels and peptidoglycan on the surface of the bacteria, disrupt and penetrate the cell wall, allowing the antibiotic to get inside and be effective again. Moreover, disrupting the cell wall and outer membrane may result in carbapenemase leaking out of the bacterial cell and decreasing its activity inside the periplasmic space.

Enhancing antibiotic effects with silver NPs has also been proven for amoxicillin, ampicillin, penicillin, ceftazidime, and cefotaxime, that is, β-lactam antibiotics inhibiting cell wall

synthesis. Silver NPs enhance the antibacterial effects of amoxicillin against *Acinetobacter baumannii*, *Aggregatibacter actinomycetemcomitans*, *Enterobacter aerogenes, Pseudomonase aeruginosa, Streptococcus mutans, Shigella sonnei,* or *Staphylococcus aureus* [140,162,163]. They also act synergistically or additively against *Actinobacillus pleuropneumoniae* and *Escherichia coli,* depending on the particle size. Ipe *et al.,* on the other hand, described an antagonistic effect in *Streptococcus oralis* and no enhancement in *Streptococcus gordonii* [163]. Surprisingly, larger silver NPs (25 nm) showed slightly stronger synergistic effects than smaller silver NPs (8 nm). A size-dependent final synergistic effect was also observed for penicillin against *A. pleuropneumoniae*; in this case, however, better antibacterial synergistic effects were obtained if penicillin was combined with smaller silver NPs. Besides, synergistic effects have been observed against *Staphylococcus aureus* and *Streptococcus mutants* [139,140]. Silver NPs additively or synergistically enhance effects of ampicillin against *Acinetobacter baumannii, Aggregatibacter actinomycetemcomitans, Enterococcus faecium, Escherichia coli, Salmonella typhimurium, Shigella sonnei, Staphylococcus aureus*, *Streptococcus mutants, Streptococcus gordonii,* and *Streptococcus oralis*. Enhanced antibacterial activity was reported in all of them, but the one described by Ipe *et al.*, no enhancement was observed [39,41,140,162–165]. Panacek *et al.* reported synergistic or additive enhancement of ceftazidime and cefotaxime combined with silver NPs against *Escherichia coli* and *Klebsiella pneumoniae*. The reason why silver NPs enhance the antibacterial activity of penicillins against resistant bacteria in the same way as in combination with carbapenems is the similar mechanism of bacterial resistance, that is, β-lactamase production and decreased uptake that can be overcome by effects of silver NPs. Singh *et al.* reported the enhancement of the antibacterial properties of ceftazidime against *Streptococcus mutants*. On the other hand, no effect has been observed in the case of *Acinetobacter baumannii* and the strain remained resistant even after the addition of silver NPs. Although these results are slightly inconsistent with the theory, no assumptions should be made based on a single negative result [140]. In the same way, there is not enough data available to evaluate the link between bacteria and their synergistic effect with antibiotics cefpodoxime and cefuroxime. Enhancement of their antibacterial effect was observed in *Streptococcus mutants and Aggregatibacter actinomycetemcomitans* but was not in *Enterococcus faecalis, Streptococcus oralis,* and *Streptococcus gordonii* [163].

Synergistic effects of vancomycin, a cell wall synthesis inhibitor, combined with silver NPs have not been observed for Gram-positive *Enterococcus faecalis*, *Staphylococcus aureus*, *Bacillus subtilis,* and *Micrococcus luteus*. Only a very slight increase in the inhibition zone from 14 mm to 17 mm for vancomycin alone and in combination with silver NPs, respectively, was observed against *Micrococcus luteus*. The reason why antibacterial effects cannot be enhanced by silver NPs can be found in the resistance mechanism of Gram-positive bacteria, which commonly involves a chemical change of the target site. The mechanism of acquiring vancomycin resistance in Gram-positive bacteria involves alteration of the peptidoglycan synthesis pathway. This includes the conversion of D-alanyl-D-alanine to D-alanyl-D-lactate or to D-alanyl-D-serine, leading to normal transpeptidase-mediated cross-linking of peptidoglycans in the cell wall. This resistance mechanism involving chemical changes of the target site is probably difficult to overcome for silver NPs. On the other hand, some authors [139,140,157,158] reported the increased antibacterial activity of silver NPs combined with vancomycin against *Staphylococcus aureus* and *Staphylococcus mutants*. Resistance to vancomycin is likely to be caused by a mechanism other than the one described above. The mechanisms are not described in any of the studies but since the synergistic effects were reported, we assume that bacterial resistance was acquired by a genetic mutation, accompanied by cell wall thickening and reduction of the cell's negative charge as previously described for *Staphylococcus* species [166,167]. Although vancomycin alone is unable to penetrate the bacterial cell wall, in combination with silver NPs, synergistic effects can be observed, resulting from the ability of NPs to penetrate the cell wall and create pits in it [92]. This allows vancomycin to get inside the bacteria and bind to the usual binding site.

Additive effects have been observed for colistin (a polymyxin antibiotic targeting the outer and cytoplasmic membranes) in combination with silver NPs against *Actinobacillus pleuropneumoniae* and *Pasteurella multocida*. Colistin is a polycationic peptide having both hydrophilic and lipophilic moieties. These cationic regions interact with bacterial lipopolysaccharides of the outer membrane by displacing magnesium and calcium bacterial counter ions in the lipopolysaccharide. Hydrophobic/hydrophilic regions interact with the cytoplasmic membrane just like a detergent, solubilizing the membrane in an aqueous environment. The most documented mechanisms of colistin resistance in bacteria involve *mcr*-1 gene-mediated modification of the lipid A subunit of the outer membrane by phosphoethanolamine and 4-amino-4-deoxy-L-arabinose residues by the enzymatic activity of diphosphate-glucose dehydrogenase, Ara4N biosynthetic enzymes, and lipid A phosphoethanolamine transferase. The altered lipid A has a much lower negative charge and affinity for colistin and related polymyxins, resulting in reduced activity and uptake of the antimicrobial substance. In addition, multidrug efflux systems can

also be responsible for polymyxin resistance in bacteria. Silver NPs are able to enhance the antibacterial activity of colistin by disrupting the outer membrane and cell wall, allowing colistin to penetrate them (*i.e.*, silver NPs open doors to colistin) and target the cytoplasmic membrane. As the mechanisms of action of silver NPs and colistin are similar (disruption of the outer membrane, cell wall, and cytoplasmic membrane), their combinations result in increased antibacterial activity.

Aminoglycosides such as amikacin, kanamycin, neomycin, and gentamicin represent a group of antibiotics inhibiting protein synthesis in bacteria. Amikacin was successfully evaluated for its synergistic effect in combination with silver NPs against *Acinetobacter baumannii*, *Enterobacter cloacae*, *Enterococcus faecium*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* in a study by Lopez-Carrizales *et al.* [39] and against *Acinetobacter baumannii, Salmonella typhimurium*, and *Shigella sonnei* in a study by Singh *et al.* [140]. Deng *et al.* reported that kanamycin and neomycin combined with silver NPs showed synergistic effects against *Salmonella typhimurium* [40]. For kanamycin, Ramirez and Tolmasky proved enhancement against *Acinetobacter baumannii*, while Singh *et al.* proved it for *Enterobacter aerogenes, Pseudomonas aeruginosa*, and *Salmonella typhimurium* [140,168]. Synergistic effects have been confirmed for gentamicin combined with silver NPs against *Bacillus subtilis*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus oralis, Streptococcus mutants, Staphylococcus epidermidis, Pasteurella multocida, Salmonella typhimurium, Shigella sonnei, Acinetobacter baumannii, Actinobacillus pleuropneumoniae, and Aggregatibacter actinomycetemcomitans*. On the contrary, synergistic effects have not been confirmed in the case of gentamicin combined with silver NPs against *Streptococcus gordonii, Escherichia coli, Enterococcus faecalis*, and *Actinobacillus baumanii* [42,43,140,163,169]. Among those widely studied aminoglycosides, Ipe *et al.* also studied synergistic effect between silver NPs and clindamycin, erythromycin, chloramphenicol, and tetracycline, respectively. In most of the resistant strains, the antibacterial effect has been enhanced but there were always some strains, where it was not enhanced. Unfortunately, the mechanism of resistance was not described in any of the strains, so no assumptions can be made based on the available information (Table 1).

Aminoglycosides act primarily by inhibiting the bacterial protein synthesis through binding to the 30 S and 50 S subunits of prokaryotic ribosomes. In addition, they also disrupt the bacterial cell walls of Gram-negative bacteria. Passage of these highly polar molecules across the outer membrane of Gram-negative bacteria is a self-promoted uptake process involving drug-induced disruption of Mg^{2+} bridges between adjacent lipopolysaccharide molecules. Bacteria can resist the antibacterial effects of aminoglycosides by four different mechanisms including reduced uptake and decreased cell permeability, alterations at the ribosomal binding sites, or production of aminoglycoside-modifying enzymes. Although silver NPs enhance the antibacterial effects of aminoglycosides against most tested resistant bacteria, the enhancement was not proven in some cases (*Escherichia coli* and *Acinetobacter baumannii*). Enhanced or indifferent effects of silver NPs combined with gentamicin against resistant bacteria possibly depend on the resistance mechanisms of bacterial strains. Some of them, such as reduced uptake and decreased cell permeability can be overcome by disrupting the outer membrane and cell wall by the effects of silver NPs. On the other hand, alterations at the ribosomal binding sites and production of aminoglycoside-modifying enzymes can be difficult to overcome by silver NPs. Unfortunately, the exact resistance mechanisms were not described in all tested bacteria resistant to gentamicin, and therefore, it is not possible to clearly determine which can be overcome and which cannot.

Silver NPs were evaluated for their synergistic effects in combination with trimethoprim, an antibiotic inhibiting folic acid synthesis (sulfonamide antibiotic). Trimethoprim acts on bacteria by blocking the production of tetrahydrofolic acid from dihydrofolic acid by binding to and reversibly inhibiting the required enzyme, dihydrofolate reductase. Thus, sulfamethoxazole and trimethoprim block two consecutive steps in the biosynthesis of nucleic acids and proteins essential to many bacteria. Bacterial resistance to trimethoprim is mostly acquired by a chromosomal mutation that results in the production of the enzyme dihydrofolate reductase, which is less vulnerable to trimethoprim inhibition. Due to irreversible mutation, bacterial resistance was not overcome by silver NPs for any of the tested bacteria *Acinetobacter baumannii*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Micrococcus luteus*, *Staphylococcus aureus*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa* [42].

Quinolones are antibiotics whose antibacterial action involves inhibition of nucleic acid synthesis. Although ciprofloxacin is the only antibiotic from this wide group evaluated for synergistic effects in combination with silver NPs, a wide range of resistant bacteria has been included. The reported results on the synergistic effects of ciprofloxacin in combination with silver NPs are rather inconsistent. Enhancement of antibacterial activity was shown for *Bacillus subtilis*, *Klebsiella pneumoniae*,

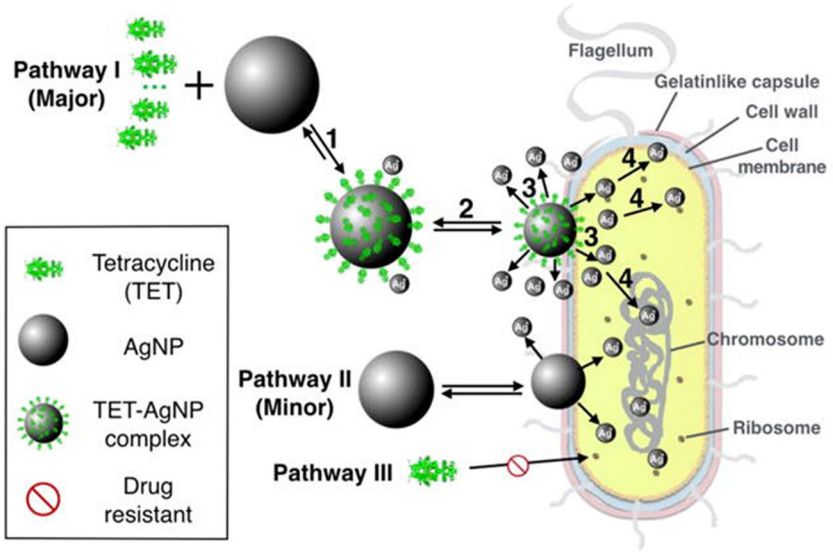

Figure 7: Synergistic antibacterial pathway I of silver NPs with tetracycline against resistant Salmonella leading to cell death and inefficient pathway III due to antibacterial resistance. Reproduced with permission [40]; Copyright 2016, Environmental Science and Technology.

Aggregatibacter actinomycetemcomitans, Streptococcus mutants, Acinetobacter baumannii, and *Escherichia coli* [37,42,163]. On the contrary, indifferent effects were determined for *Acinetobacter baumannii*, *Enterococcus faecalis*, *Escherichia coli*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis, Streptococcus oralis,* and *Streptococcus gordonii* [42,44,163].

Ciprofloxacin is a broad-spectrum antibiotic active against both Gram-positive and Gram-negative bacteria. It functions by inhibiting DNA gyrase and topoisomerase IV, a type II topoisomerase [170], necessary to separate bacterial DNA, thereby inhibiting cell division. Three mechanisms of resistance to quinolones are currently recognized: mutations that alter the drug targets' bacterial topoisomerases and DNA gyrase and mutations that reduce drug accumulation and efflux resistance mechanisms. The reason why synergistic effects have or have not been proven is probably similar to that for gentamicin combined with silver NPs. The final effect depends on the resistance mechanisms of bacterial strains. While some of them such as reduced uptake and decreased cell permeability can be overcome by disturbing the outer membrane and cell wall *via* silver NPs, others such as changing the target site probably cannot.

Silver NPs may facilitate the interaction of antibiotics with cells in numerous ways. For example, silver NPs may help antibiotics penetrate into the bacterial cell by changing membrane permeability; alternatively, both can cooperate to disrupt the cell wall. In the case of β-lactam antibiotics, silver NPs may decrease the activity of β-lactamases produced by bacteria by allowing their leakage after cell wall disruption. The cells can be damaged and weakened by the simultaneous action of antibiotics and silver NPs, leading to cell death. Hwang *et al.* suggested that this synergism is associated with the generation of hydroxyl radicals, alteration of protective cellular functions, and anti-biofilm potential [138].

The synergism between silver NPs and antibiotics (Figure 7) can be explained by the binding reaction between them [40,139]. Namely, the amino and hydroxy groups of an antibiotic are bonded to the nanoparticle *via* chelation, which results in the creation of a conjugate in which the silver core is surrounded by antibiotic molecules [162]. Silver NPs are then selectively attracted to the cytoplasmic membrane consisting of glycoproteins and phospholipids, so that the NPs act as drug carriers transporting the antibiotic near the cytoplasmic membrane (1), resulting in an enhanced contact with the cell wall and increased concentrations of the antibiotic and silver close to the cell membrane (2). The local increase in the silver ion concentration near the bacterial surface causes bacterial toxicity by binding silver ions to the proteins and DNA molecules of the cell wall as well as those inside the cell (3), leading to bacterial death. Membrane permeability might also be increased by binding silver NPs to sulfur-containing proteins, improving the infiltration of the antibiotic into the cell [171]. Another mechanism of action involved in the synergism could be ROS production (˙OH), alteration of the cell's protective function, and unwinding of DNA leading to bactericidal effects [138,172].

4.2.2 Gold NPs

Gold NPs were also widely evaluated for their impact on the antibacterial activity of antibiotics against resistant bacteria. The synergistic effects have been proven for antibiotics whose mode of action is targeted at the inhibition of cell wall synthesis, namely, amoxicillin (β-lactam) [153] and cefotaxime or ceftriaxone (carbapenems) [155] against methicillin-resistant *Staphylococcus aureus* and *Escherichia coli*, *Klebsiella pneumoniae*, and *Staphylococcus aureus*, respectively. Because gold NPs may attack bacteria in a way similar to silver NPs, the mechanism of overcoming bacterial resistance is very likely to be similar to that of silver NPs combined with β-lactam antibiotics, as described above.

On the other hand, no synergy has been observed in the case of another β-lactam antibiotic, methicillin, tested with *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* [156]. The reason why bacteria still resist methicillin after combination with gold NPs is the resistance mechanism involving a change of the target site, namely modification of transpeptidase (penicillin-binding protein).

In the case of vancomycin (glycopeptide antibiotic targeting cell wall synthesis), synergistic effects have been proven against the Gram-positive bacteria *Staphylococcus aureus*, *Enterococcus faecalis*, *Enterococcus faecium,* and *Staphylococcus epidermidis* [13,154,156]. The possible mechanism of restoring the antibacterial activity was proposed by Fayaz *et al.*, who confirmed synergistic effects of vancomycin combined with gold NPs against vancomycin-resistant *Staphylococcus aureus*. They suggest that the mechanism involves the formation of complex vancomycin-gold NPs which, instead of binding to terminal peptides, bind to the transpeptidase of glycol-peptidyl groups on the cell wall and disrupt it [13].

Roshmi *et al.* have reported that the combination of gold NPs with gentamicin [156] (an aminoglycoside antibiotic targeting protein synthesis) against *Staphylococcus epidermidis* resulted in the enhancement of antibacterial activity. Unfortunately, the authors did not describe the mechanism of resistance of *Staphylococcus epidermidis* to gentamicin, and therefore, it is hard to discuss by which mechanism gold NPs can overcome the resistance. Especially in the case of gentamicin whose antibacterial effects are resisted by four different mechanisms (reduced uptake and decreased cell permeability, alterations at the ribosomal binding sites, or production of aminoglycoside-modifying enzymes), it is extremely difficult to identify exactly the particular mechanism overcome by gold NPs. Roshmi *et al.* and Pradeepa Vidya *et al.* also reported enhancement of antibacterial activity of ciprofloxacin, levofloxacin, and rifampin (antibiotics inhibiting nucleic acid synthesis) against both Gram-positive (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*) and Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*) [155,156]. Synergies of ciprofloxacin and levofloxacin with gold NPs were confirmed against *Escherichia coli*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* and, in the case of ciprofloxacin, also against *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*. In the case of rifampin, the synergistic effect has been observed against *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*. On the other hand, synergistic effects against *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* have not been confirmed for nalidixic acid. The reason why gold NPs show or do not show synergistic effects is similar to that for silver NPs combined with quinolones. The final effect depends on the resistance mechanisms of bacterial strains. Some of them such as reduced uptake and decreased cell permeability can be overcome by disrupting the outer membrane and cell wall *via* silver NPs; others probably cannot be overcome, for example, change of the target site.

4.2.3 Titanium dioxide NPs

Roy *et al.* reported the synergistic effects of titanium dioxide combined with different classes of antibiotics against methicillin-resistant *Staphylococcus aureus* [147]. Antimicrobial activity increased in the case of antibiotics inhibiting cell wall synthesis (β-lactams, cephalosporins, glycopeptides) and protein synthesis (aminoglycosides, lincosamides, macrolides, azalides). In the case of antibiotics inhibiting nucleic acid synthesis, only a slight increase in the inhibition zone was observed and, therefore, the synergistic effect was weak [147]. The best results have been observed for penicillin, ampicillin (β-lactam), amikacin, and gentamicin (aminoglycosides). Only nalidixic acid (an antibiotic inhibiting bacterial DNA synthesis) has not shown increased antibacterial effects in combination with TiO_2 NPs. According to Muzammil *et al.*, the enhancement of antibacterial action may be explained by the interaction of titanium NPs with efflux pumps normally responsible for bacterial resistance [115].

4.3 Impact of antibiotic's mode of action and mechanism of bacterial resistance on the effectiveness of combined antibacterial treatment with nanomaterials

Pie charts in Figure 6 help to visualize that the mechanisms of action of antibiotics strongly determine the

resulting synergistic effects between different antibiotics and metal NPs. These, together with the above information, provide an interesting insight into whether an antibacterial activity can be increased or not. Enhancement of antibacterial properties of antibiotics combined with silver NPs was observed for all tested combinations with antibiotics causing cell membrane disruption (colistin). NPs enhance their antibacterial activity by disrupting the outer membrane and cell wall, which allows colistin to penetrate them and target the cytoplasmic membrane. Antibacterial effects of almost all antibiotics disrupting cell wall synthesis (amoxicillin, ampicillin, cefotaxime, ceftazidime, meropenem, imipenem, penicillin) and protein synthesis (amikacin, gentamicin, kanamycin, neomycin) could be enhanced; the few exceptions were amoxicillin, amikacin, and gentamicin since not all the combinations were evaluated by all authors in the same way. Generally, however, it may be said that bacterial resistance to antibiotics acting on cell membrane/protein/cell wall synthesis (β-lactams) could be reversed and antibiotics combined with silver NPs regain their antibacterial properties even at lower concentrations than before. Silver NPs probably interact with porin channels and peptidoglycan on the surface of the bacteria, disrupting and penetrating the cell wall, allowing the antibiotic to get inside and be effective again. In the case of β-lactam antibiotics, disruption of the cell wall and outer membrane may result in carbapenemase leaking out of the bacterial cell and decreasing its activity inside the periplasmic space, therefore reversing their mechanism of resistance. On the contrary, the antimicrobial activity of glycopeptide antibiotics (vancomycin) acting on cell wall synthesis could not be enhanced in all of the cases. Resistance mechanisms in most cases involve chemical changes of the target side (*e.g.*, D-alanyl-D-alanine to D-alanyl-D-lactate conversion) and those are difficult to overcome. However, if the mechanism of resistance is cell wall-related, NPs help antibiotics to penetrate the wall while creating pits in it [92], which allows antibiotics to get inside the bacteria and bind to its usual binding site.

The enhancement of antibacterial activity was not observed for antibiotics inhibiting folate acid synthesis (trimethoprim) and for almost all cases tested with antibiotics inhibiting nucleic acid synthesis (ciprofloxacin); however, certain differences between the obtained results were noted. Resistance to those antibiotics is mostly acquired by an irreversible chromosomal mutation that cannot be so easily reversed by the NPs.

The pie charts (Figure 6) also show results for other nanomaterials (gold NPs, TiO_2 NPs) but those materials have not been tested as extensively as silver NPs. In several cases, a single bacterial strain was tested with a certain antibiotic, so the results might be skewed. Overall, gold NPs have the ability to increase the antibacterial properties of antibiotics inhibiting protein synthesis (gentamicin), synthesis of nucleic acids (ciprofloxacin, levofloxacin, nalidixic acid, rifampicin) and cell wall synthesis (only glycopeptide antibiotics, vancomycin). However, in the case of vancomycin and rifampicin, a single bacterium tested had no effect on the enhancement of antibacterial properties. In the case of β-lactam antibiotics acting on cell wall synthesis, enhancement was observed for amoxicillin, cefotaxime, and ceftriaxone, but not for methicillin and two of the tested bacteria. Finally, the bacterial resistance of *S. aureus* was overcome by the combination of titanium dioxide NPs with all tested antibiotics acting on cell wall/protein/nucleic acid synthesis except for nalidixic acid. However, it must be stressed that only one bacterial strain was evaluated by one author.

5 Bacterial resistance to silver

It is generally known that bacteria are able to resist the antibacterial action of heavy metals by various mechanisms including efflux, extracellular barrier, reduction of metal ions, and extracellular and intracellular sequestration. The most frequent mechanism of resistance is the efflux of toxic ions outside the bacteria or forming an extracellular barrier (*e.g.* extracellular polymer substance of the biofilm), which prevents the ions from entering the cell and prevents them from the stress induced by toxic metals [173–175]. Besides that, bacteria are able to upregulate genes, which are responsible for ROS elimination, DNA damage reparation, and hydrolysis of abnormally assembled proteins, which might repair damages caused by toxic ions [176–178].

Resistance to silver and its compounds represents one of the most studied metal resistances in bacteria. Silver-resistant bacteria were first isolated in 1960 from burns treated with silver nitrate [179]. Examples of bacterial strains resistant to silver include *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Staphylococcus aureus,* and *Pseudomonas aeruginosa* [180]. Bacteria can resist silver by several mechanisms such as reduction of Ag^+ to less toxic oxidation states and decreased permeability of the cytoplasmic membrane. Nevertheless, active efflux is the most applied mechanism of how bacteria resist and eliminate the toxic effects of silver cations. The mechanism of resistance to ionic silver involves active efflux of Ag^+ from the cell by P-type

adenosine triphosphatases or chemiosmotic Ag^+/H^+ antiporters [181–185]. Simon Silver reported the resistance to silver compounds by bacterial plasmids and genes in *Salmonella* spp. strains. Silver resistance conferred by the Salmonella plasmid pMGH100 involves nine genes in three transcription units. A sensor/responder (SilRS) two-component transcriptional regulatory system governs the synthesis of a periplasmic Ag(I)-binding protein (SilE) and two efflux pumps (P-type ATPase (SilP) plus a three-protein chemiosmotic RND Ag(I)/Hþ exchange system (SilCBA)) [181].

Thanks to their ability to resist silver ions together with the constant changes of the bacterial genome and their ability to adapt to negative conditions, it is expected that bacteria may develop a resistance mechanism to AgNPs as well. Certain bacteria, at least if they have the ability, can be partially resistant to metal and metal oxide NPs by eliminating the toxic effects of metal cations or oxyanions. In this way, bacteria can eliminate one of the mechanisms of nanoparticle antibacterial activity consisting of the toxic effects of metal ions released from the NPs and, therefore, they can tolerate the toxic effect of metal NPs to a certain extent. Valentin *et al.* described resistance to both ionic silver and silver NPs in *Staphylococcus aureus*, which was associated with gene mutations involved in nucleotide synthesis, oxidative stress defense, and changes in cysteine metabolism [186]. Resistance in *Staphylococcus aureus* was also described by Elbehiry *et al.*, who has induced resistance to both silver and gold NPs, while no cross-resistance was observed [187].

Bacteria might resist NPs *via* two main approaches, they can either prevent the entrance of silver NPs or silver ions into the cell, or once it gets there reduce the amount of the antibacterial agent within the cell. For instance, *Pseudomonas putida* is able to decrease the bacterial membrane fluidity *via cis–trans* isomerization of unsaturated fatty acids [188]. However, in most cases, bacteria produce extracellular substances, which immobilize NPs and do not allow them to have contact with bacteria [189]. Yang and Alvarez described increased stimulation of biofilm development after prolonged exposure to AgNPs and upregulated quorum sensing and liposaccharide biosynthesis as the main mechanisms of resistance in *Pseudomonas aeruginosa* [190]. Khan *et al.* reported bacterial resistance in *Bacillus pumilus* and suggest that exopolysaccharide-capped AgNPs show less toxicity to various bacterial strains [191]. Protein corona formation was also reported in *Escherichia coli* after chronic exposure to NPs in continuous culture in bioreactors [192]. Besides extracellular polymeric substances other compounds can be produced by bacteria to withstand the negative effects of antimicrobials. Ellis *et al.* described the mechanism of resistance in *Pseudomonas aeruginosa* based on increased phenazine pigment production, which limits bacterial exposure to AgNPs [193]. Panácek *et al.* described bacterial resistance in *Escherichia coli* and *Pseudomonas aeruginosa,* which stems from the production of the adhesive flagellum protein flagellin. This protein triggers the aggregation and destabilization of AgNPs, which reduces their stability and therefore eliminates their antibacterial activity [48].

Once silver NPs get to the cell, the minimal inhibition concentration (MIC) has to be reached to perform their antibacterial action. Bacteria can develop resistance to AgNPs through simple genomic changes (*e.g.*, mutation of the mdtb gene) resulting in adaptation to released ions *via* an efflux system, which transports released ions through the plasma membrane to periplasm [46]. Besides, the presence of an efflux network, which works as a mechanism of resistance towards AgNPs was described also in *Bacillus subtilis* [47], *Salmonella seftenberg* [194], *Staphylococcus aureus, Klebsiella pneumoniae* [195], and *Escherichia coli* [46,192,195]. In this case, antimicrobials are pumped out of the cell and therefore MIC cannot be reached, so they cannot act as they are supposed to and perform sufficient antibacterial action.

Only Panácek *et al.* have tried to overcome the newly built mechanism of resistance. Additional stabilization *via* various surfactants and polymers was not successful, but they were able to inhibit flagellin production by the addition of pomegranate rind extract, which has suppressed the aggregation of the NPs, so NPs were able to keep their antibacterial properties [48]. As mentioned in this section, bacteria are able to build up resistance even to silver NPs, therefore, the mechanisms of resistance should be studied in more detail and new ways how to overcome them should be outlined in the near future.

6 Conclusion

The treatment of bacterial infections is no longer a simple task. Antibiotics remain the mainstay to fight bacterial infections, but due to overprescription, misuse, and overuse in animal production, episodes of resistant infections are alarmingly on the rise, and resistance of bacterial strains to antibiotics is becoming a pressing public health problem that is predicted to only worsen in the future. Many efforts have been made to overcome the emerging problem of losing the effectiveness of major antibiotics against resistant strains. Fortunately, advances in biomedical nanotechnology applications may offer a great opportunity for research in this field, open new doors, and advance the

way bacterial infections and resistant bacteria are coped with. For their small size and increased surface area, metal NPs are known to possess strong antibacterial activities, as seen from several studies. Their impact on both growth and maturation of bacterial biofilm suggests a broad spectrum of antimicrobial properties, which can be applied in the dressing of surgical tools, dental products, catheters, but also in other products as cosmetics, clothing, and food packaging [196].

Since many metallic NPs with promising antibacterial activities have not been fully investigated in combination with antibiotics against resistant strains, more studies should be conducted. To the best of our knowledge, only silver, gold, and titanium dioxide NPs have been tested in combination with antibiotics, especially against resistant strains. In many of those papers, the long-lost effectiveness of antibiotics against resistant strains was restored *via* combination with small concentrations of inorganic NPs. This finding is of great importance and could become a game-changer in combating bacterial resistance to commonly used antibiotics. However, as shown in this review, there are some combinations that do not possess those enhanced properties. As of now, no one has tried to explain the reasons why some combinations work, and others do not. The answer to this question might be found in the mechanism of action of the antibiotics or the mechanism of bacterial resistance. These have not been studied yet and might be crucial for subsequent research. Right now, it is really difficult to generalize from individual studies, mainly due to the fact that there is no standardized method for the evaluation of the synergistic effects and also because researchers perform experiments based on available NPs and bacteria, rather than targeting specific bacteria with previously described mechanisms of resistance. Without a properly characterized material (size, morphology, surface modification) and the knowledge of resistance mechanisms, correlation with basic physicochemical properties and evaluation of the synergistic effect is not possible. Therefore, standardized methods, NPs, and bacteria should be included in future studies.

The application of metal NPs in combination with antibiotics against various bacterial infections holds promise in paving the way for future therapeutics in nanomedicine. This approach may serve as an adjunct to the existing therapies and might restrain the escalating problem with resistant strains. At the same time, the possibility of acquiring bacterial resistance even to those nanomaterials needs to be studied and possible ways of preventing or overcoming it need to be described. Furthermore, the translation to clinical medicine should be preceded by a deeper knowledge of the mechanisms of bacterial resistance, mechanisms of action of NPs and by verification of combinations of certain NPs and antibiotics by *in vivo* infection models in order to better understand their pharmacokinetics and biodistribution. Finally, an important part of *in vitro* and *in vivo* testing is toxicity tests, which help us exclude combinations with extremely high toxicity.

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