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Review Article

Nanoparticulate Antibiotic Systems as Antibacterial Agents and Antibiotic Delivery Platforms to Fight Infections

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Today's human society, product of decades of progress in all fields of knowledge, would have been unimaginable without the discovery of antibiotics and more generally of antimicrobials. However, from the beginning, the scientific community was aware that microorganisms through various strategies were able to hinder and render vain antibiotic action. Common examples are the phenomena of persistence, tolerance, and resistance, up to the creation of the feared bacterial biofilms. Antibiotics are a precious but equally labile resource that must be preserved but at the same time reinforced to safeguard their effectiveness. Nanoparticulate systems such as nanobactericides, with their inherent antibacterial activity, and nanocarriers, which operate as drug delivery systems for conventional antibiotics, are innovative therapies made available by nanotechnology. Inorganic nanoparticles are effective both as nanobactericides (AgNPs, ZnONPs, and TiO_2NPs) and as nanocarriers (AgNPs, AuNPs, ZnONPs, and TiO_2NPs) against sensitive and multi-drug-resistant bacterial strains. Liposomes are among the most studied and flexible antibiotic delivery platforms: conventional liposomes allow passive targeting at the mononuclear phagocytic system (MPS); "stealth" liposomes prevent macrophage uptake so as to eradicate infections in tissues and organs outside MPS; thanks to their positive charge, cationic liposomes interact preferentially with bacterial and biofilm surfaces, acting as innate antibacterials as well as drug delivery systems (DDS); fusogenic liposomes have fluid bilayers that promote fusion with microbial membranes; and finally, ligand-targeted liposomes provide active targeting at infection sites. Dendrimers are among the most recent and attractive nanoparticulate systems, thanks to their multibranched nanoarchitecture, which equipped them with multiple active sites for loading antibiotics and also interacting with bacteria. Finally, nanoantibiotics represent a new hopeful generation of antibiotic candidates capable of increasing or even restoring the clinical efficacy of "old" antibiotics rendered useless by the resistance phenomena.

1. Introduction

Although antimicrobial therapy is considered a milestone in medicine and incontestably antimicrobial agents have transformed human health by saving millions of lives, the scientific world and beyond has become aware that antimicrobial resistance is a global health emergency and a huge challenge for the successful therapy of many common infections. According to the World Health Organization (WHO), antimicrobial resistance (AMR) is the ability of a microorganism (like bacteria, viruses, and some parasites) to stop an antimicrobial (such as antibiotics, antivirals, and antimalarials) from working against it [1]. As a result, standard treatments become ineffective and infections persist and may spread to others. Antibiotic resistance is a narrower term, since it refers to resistance to drugs that treat infections caused by bacteria. The WHO reports that antimicrobial/antibiotic resistance concerns many pathogens and it is spread in every region of the world [2]; this has been endorsed by Hendriksen et al. [3] who have used metagenomic analysis of untreated sewage from 79 sites around the world to obtain representative data on AMR. According to the review on antimicrobial

resistance, chaired by the economist Jim O'Neill, up to 50,000 lives are lost each year to antibiotic-resistant infections in Europe and the USA alone, with an economic loss of between 20 and 35 billion dollars [4]. In the same analysis, it was estimated that AMR would cause 10 million death in 2050, i.e., 2 million more than cancer. AMR is a multisectorial task; in fact, it has medical but also economic, political, ecological, and sociological dimensions, which is why we need a multifaceted approach to manage and control it through monitoring, surveillance of practice and use, educational initiatives, and policy, but most of all through the development of innovative therapies.

2. Antibiotic Resistance

Undeniably, antibiotics have improved the quality of life and the life expectancy of humankind not only because of their direct activity against infectious disease pathogens but also because they made possible modern medical procedures. What we call the pre-antibiotic era ended with the accidental discovery of the first antibiotic in 1928 by Sir Alexander Fleming [5], and only after ten years from that event, Florey and Chain isolated the active molecule, which was named penicillin; commercial production began thereafter [6].

Notwithstanding the antibiotic efficacy in treating bacterial infections, it was soon clear which bacteria became resistant to them, and Fleming himself in his 1945 Nobel Prize lecture warned of the risks of antibiotic resistance: "The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant" (Figure 1) [7].

The complete lack of awareness of end users gave rise to misuse and mismanagement of antibiotics, and this contributed with the passage of time to select resistant bacteria. Resistance genes can be the result of spontaneous DNA mutations that can be transferred to progeny by vertical gene transfer, or of the acquisition of foreign DNA-like plasmids, transposons, and bacteriophages by horizontal gene transfer. This kind of resistance just described, to which Fleming referred, is defined as acquired resistance, and it represents the selective pressure achievement. In addition to acquired resistance, there is another type of resistance termed intrinsic resistance, where genetic traits are independent of previous antibiotic exposure [8] and confer to all members of a particular bacterial genus the innate ability to resist the action of an antibiotic as a consequence of the bacteria's structural or functional characteristics.

Resistance is inevitable and relentless, and the development of new molecules or chemicals utilized from those already known is certainly a commitment not to be taken lightly. In any case, the introduction of new compounds would not be enough; as a matter of fact, the history of antibiotics has taught us that generally after a short time from the placing on the market of a certain molecule, the resistance in its comparisons do not take long to appear. For this reason, pharmaceutical companies do not receive appropriate incentives to develop new antibiotics [9]. The main challenge regarding antibiotic resistance is multidrug resistance, i.e., the simultaneous development of resistance to several antibiotic classes which can occur when a bacterial strain has several different resistance genes, each providing resistance to a particular antibiotic, or when a single resistance mechanism gives resistance to more than one antibiotic. Bacteria of this type are called "*super bugs*" because they cause infections which cannot be eradicated with conventional antibiotics. Centres for Disease Control and Prevention have just published the 2019 Antibiotic Resistance Threats in the United States, and according to this, more than 2.8 million antibiotic-resistant infections happen in the U.S. each year, which cause the death of more than 35,000 people [10]. Even in Europe, the data is extremely alarming; an ECDC (European Centre for Disease Prevention and Control) study assesses that about 33,000 people die each year owing to bacteria-resistant infection [11]. These alarming data confirm that in reality, we never won the war against microorganisms; we only postponed it and that antibiotic resistance is a global health emergency that should not be underestimated if we want to avoid the threat of a post-antibiotic era. Therefore, it is imperative to do something as soon as possible.

3. Common Mechanisms of Antibiotic Resistance

Among the innate or acquired mechanisms through which microorganisms can avoid the lethal action of antibiotics, there are several alternatives (Figure 2).

3.1. Decreased Uptake of the Antibiotic from Bacterial Cell. The external membrane of Gram-negative bacteria is a semipermeable barrier that prevents the entry of large polar molecules into the cell. Small polar molecules, including many antibiotics, can enter the cell through protein channels, the porins. Bacteria can lose or mutate these channels and therefore slow down the rate of entry of the drug into the cell or prevent it from completely entering, reducing its concentration at the destination site. For example, this is what happens in Gram-negative bacteria that become resistant to β-lactams, tetracyclines, and chloramphenicol [12].

3.2. Increased Efflux of the Antibiotic from Bacterial Cell. Efflux pumps are protein transporters located in the cytoplasmic membrane of all cell types. They are active transporters; therefore, they require a chemical energy source to perform their function. Microorganisms can hyperexpress efflux pumps and then expel antibiotics. The increased efflux is a typical resistance mechanism against macrolides, tetracyclines, fluoroquinolones, and chloramphenicol [13].

3.3. Inactivation of the Antibiotic. The destruction/inactivation of the antibiotic is a common resistance mechanism. Bacterial resistance against aminoglycosides, β-lactams, macrolides, and chloramphenicol is usually due to the production of an enzyme capable of inactivating them:

(i) β-Lactamase (penicillinase) inactivates the β-lactam ring of penicillins and cephalosporins by hydrolysis

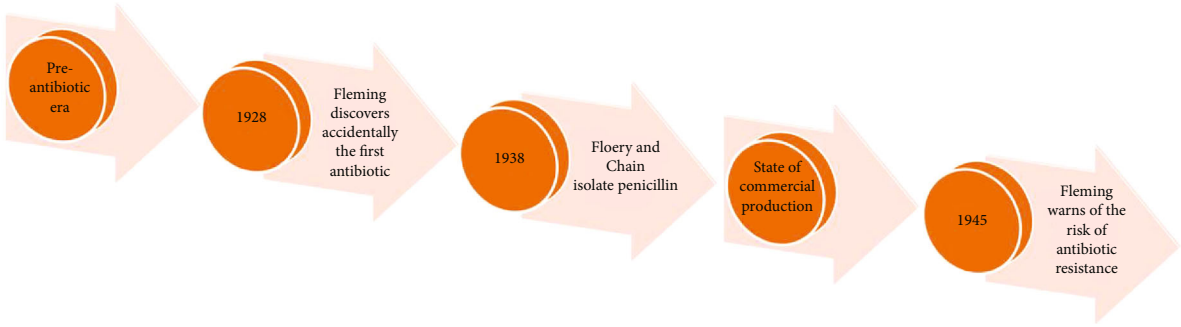

Figure 1: History of antibiotics (timeline).

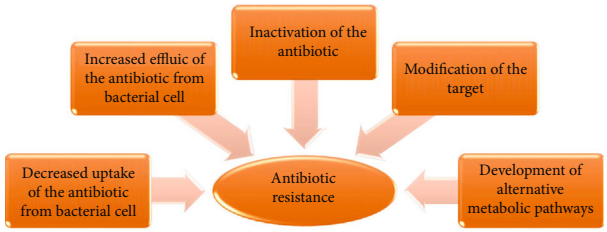

Figure 2: Mechanism of antibiotic resistance.

(ii) Acetyltransferase, adenyltransferase, and phosphotransferase can inactivate aminoglycosides

(iii) Esterase hydrolyzes the lactone ring of macrolides

3.4. Modification of the Target. The mutations occur in the coding genes for antibiotic target structures that can cause changes in the amino acid composition and/or conformation of the target protein. Such changes can lead to a lower affinity of the drug for its target or a prodrug for the enzyme that converts the prodrug to an active drug. These alterations may be due to the mutation of the natural target (e.g., resistance to fluoroquinolones), the modification of the target (e.g., ribosomal protection to macrolides and tetracyclines), or the acquisition of a resistant form of the native sensitive target such as staphylococcal resistance to methicillin, which is caused by the production of a particular Penicillin-Binding protein (PBP2′, PBP2a, or MecA) that binds with poor affinity to all β-lactams [14, 15].

3.5. Development of Alternative Metabolic Pathways. The extreme cases of resistance involve complex metabolic adaptation mechanisms and other changes such as those for vancomycin-resistant Enterococci and methicillin-resistant Staphylococci. The mechanism of resistance to vancomycin is linked to a metabolic remodelling of the cell envelope. In normal bacteria, the peptidoglycan peptide ends with the D-Ala-D-Ala residues to which vancomycin binds with high affinity, thus blocking the wall synthesis process. Resistant bacteria are able to synthesize peptidoglycan with a modified chemical structure whose terminal residues are D-Ala-D-lactate (and other similar structures such as D-Ala-D-Met and D-Ala-D-Phe). Vancomycin binds to the D-Ala-D-Ala dipeptide with an affinity of about 1000 times greater than when it binds to D-Ala-D-lactate. The consequence of the lower affinity towards the modified target is that the antibiotic is not able to inhibit the growth of the microorganism that synthesizes the modified peptidoglycan. Another example of metabolic pathway alteration is the resistance against the sulphonamides: *S. aureus* and *N. meningitidis* increased synthesis of *para*-aminobenzoic acid (PABA), which is the competitive molecule of these drugs. The greater the quantities of PABA produced, the lower the binding of sulphonamides to dihydropteroate synthase.

4. Tolerance and Persistence

Antibiotic resistance is considered the most common trick put in place by bacteria to survive and even proliferate in the presence of antibiotics, and as we know, it is based on genetic traits inheritable through vertical or horizontal gene transfer; however, it is not the only one. Unlike antibiotic resistance, tolerance and persistence are linked to epigenetic traits; therefore, they are not inheritable and can be promoted by stressful conditions such as the presence of an antibacterial agent. The tolerance phenotype allows bacterial populations to stop replication and other main metabolic reactions without going into a latent state of life. In practice, it is as if the bactericidal drug becomes bacteriostatic. An alternative to tolerance is that most of the bacterial cells can trigger apoptosis, but a very small number of bacteria survive in a dormant state and are not metabolically active; these are called persister cells [16]. In this latent state of life, the normal target of antibacterial drugs is present but is simply not active. An important contribution to the survival of the persisters is the creation of a biofilm that protects them from the action of both antibacterials (even at high concentrations) and immune system cells. The persisters can resume growth and replicate when environmental conditions return favourable. *In vivo*, this phenomenon is responsible for relapses [17, 18].

5. Biofilm

The bacterial biofilm represents an ecological niche with a complex structure within which microorganisms are trapped and at the same time protected by a self-produced matrix called an extracellular polymeric substance (EPS). More than

95% of the EPS is represented by H_2O [19, 20], while the rest is mainly a mixture of proteins and polysaccharides, but also of DNA and cellular residues.

The organization of microorganisms in biofilms allows the maintenance of a stable synergistic collaboration between cells in order to establish a fortified microbiome, which is closely associated with various forms of bacterial resistance, and allows evading the antibacterial action of chemotherapy and also the host's immune system causing chronic infection disease [21]. Combating bacterial biofilm is one of the major global medical challenges; one of the main reasons is the marked tendency of bacteria to adhere to both human and medical implant surfaces. For the latter case, there is a pressing need to do research on antibiofilm materials. These can be achieved by loading them with antibacterial substances or coating them with antiadhesive/antibacterial immobilizing agents; however, in this case there is the disadvantage that the antibacterial compounds can spread to the neighbouring tissues by promoting antibiotic resistance. To avoid this, the most promising approach is to design antibiofilm materials coated with nanostructures on the surface [22].

6. Nanoantibiotics: Nanobactericides and Nanocarriers

Bacteria put in place various mechanisms to circumvent the action of antibiotics thanks to their continuous ability to evolve and adapt to different environments, which is why the development of antibiotic-resistant bacterial strains is a certain and inescapable calamity towards which we have the moral duty to do something as soon as possible. Implementation of international plans to regulate their use, information campaigns to increase the level of awareness by the final users, and obviously development of new antibiotic molecules are reasonable preventive measures; however, they are not sufficient. It is time to turn the situation around and innovate our antibiotic arsenal for overcoming the problem of drug resistance; this can be achieved by developing new types of antibacterial agents or by giving new life, or rather a new appearance, to conventional antibiotics. To innovate the way to fight infections, it is necessary to know the limits that need to be surmounted.

The failure of many conventional antibiotic therapies is often related to the unfavourable pharmacokinetic characteristics of therapeutic agents, among which include low bioavailability, poor ability or inability to cross biological barriers, short half-life, and low chemical-physical stability. Added to these is the poor compliance of patients. Therapeutic regimens are established based on the type of bactericidal activity of the antibiotic molecule, which generally follows three main models [23, 24]:

(1) Concentration-dependent bactericidal activity with moderate-prolonged persistent effects

(2) Time-dependent bactericidal activity without persistent effects

(3) Time-dependent bactericidal activity associated with prolonged persistent effects

The study of the pharmacokinetics-pharmacodynamics of antibiotics and of the so-called pharmacokinetic/pharmacodynamic indices (PK/PD indices) [23] is a rather hot topic as wrong therapeutic regimens not only do not allow us to achieve the desired clinical result but it also promotes the development of resistance, because what fails to kill bacteria fortifies them. By making the best use of the characteristics of the therapeutic agent, therapeutic regimes can be established to prevent the development of bacterial resistance [25]. This arduous work is very challenging to implement, as it should be done specifically for each antibiotic agent. A huge help can be provided by drug delivery systems that allow delivering antibiotics at the right concentrations, for the right period of time, and also at the right place, in the case of drug targeting.

Nanotechnology is an innovative field that can offer the opportunity of treating infections in a pioneering way through nanoparticles. The term nanotechnology refers to the manipulation of matter at nanoscale level (≈1-100 nm), and it was first employed in 1974 by the Japanese scientist, Norio Taniguchi [26]. Many studies confirmed the intrinsic antimicrobial activity of various types of organic and inorganic nanoparticles (NPs), and indeed nanoparticles own newsworthy properties with respect to bulk material, which are extremely valuable for antimicrobial activity: small size and high surface area-to-volume ratio. The nanometered and controllable size permits NPs to interact with bacteria and cross more easily both bacterial envelopes and host's cell membranes, so as to interfere with essential microbial metabolic pathways [27] and to allow eradication of intracellular infections as well. On the contrary, current antibiotics generally do not reach high intracellular concentrations because of transport scarcity, and the situation worsens in the presence of resistance mechanisms such as decreased uptake and increased efflux. The high surface area-to-volume ratio is responsible for NPs' high reactivity: as the surface area-to-volume ratio increases, so does the percentage of atoms at the surface and surface forces become more dominant. Also, the shape is a critical feature, since a correlation between morphology and the activity of nanoparticles has been verified: a rod-like shape is more effective than a spherical shape to eradicate biofilm [28, 29].

A further very attractive trait of nanoparticulate systems is also the possibility of functionalizing the surfaces specifically linking chemical functional groups to have, for example, a targeted delivery and a specific charge, in order to enhance antibiotic activity [30].

Nanoparticles can show antimicrobial/antibacterial activity by themselves or can act as drug delivery systems for conventional antibiotics; in both cases, they are known as "nanoantibiotics." Typically, but not exclusively, inorganic nanoparticles show intrinsic antibiotic activity and fight against bacteria using multiple mechanisms of action; therefore, they are denominated "nanobactericides," while the "nanocarriers" are nanoparticle-based delivery systems proposed to transport old antibiotics, among these there

are liposomes, the first nanotechnology to be used for this purpose [31], dendrimers, polymeric nanoparticles, and also metallic nanoparticles. Antibiotics may be adsorbed, dissolved, encapsulated, or entrapped into nanocarriers in order to improve their pharmacokinetic/pharmacodynamic properties.

In this regard, nanocarriers (Figure 3) confer several advantages including improvement in biodistribution; increase in bioavailability and drug solubility; reduction of dosage; prolonged systemic circulation; drug cotransport; and sustained, controlled, and targeted delivery of drugs. Definitely, nanocarriers allow a reduction of side effects and an increase in therapeutic efficacy or restoration of clinical efficacy by overcoming the mechanisms of antibiotic resistance; not the least, NPs can allow a drug-modified release in order to achieve the just mentioned clinical objectives. By virtue of their extraordinary potential as possible new generation antibiotics, nanoparticles have attracted the attention of the scientific world, which is therefore investigating the various facets of their antibacterial activity both as antimicrobial nanomaterials and as carriers of old antibiotics to better understand advantages and limitations.

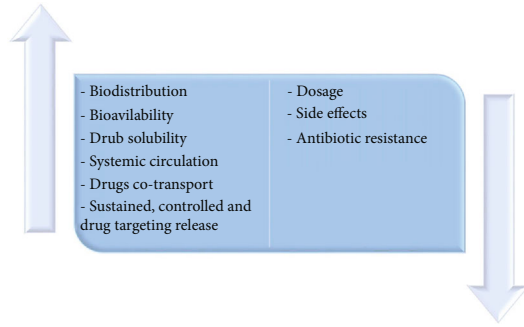

Figure 3: Advantages of nanocarriers.

7. Inorganic Nanoparticles: Metallic and Metal Oxide Nanoparticles

7.1. Inorganic Nanoparticles as Nanobactericides and Nanocarriers. History teaches us that even before the advent of the antibiotic era, various types of inorganic substances were well known for their properties of killing or inhibiting bacterial growth. These compounds include heavy metals such as silver, gold, titanium, zinc, iron, and copper. Hence, metal- and metal oxide-based nanoparticles are often named "nanobactericides" by virtue of their inherent antibacterial activity also and above all due to the nanometric size [32] and the ability to interface with bacterial surfaces both through weak and nonspecific interactions such as hydrophobic interactions [33], electrostatic attraction [34], Van der Waals forces [35], and through specific receptor-ligand bonds [36]. NPs penetrate bacterial envelopes damaging bacterial cell membrane structure through different mechanisms [37] increasing membrane permeability leading to concentration of NPs inside the membrane and cellular uptake [38]; once inside, they deeply alter the microbial pathways [27] interfering with the essential cellular components such as enzymatic proteins, DNA, ribosomes, and lysosomes: the consequences for the bacterial cell are disastrous. The damage to the membrane prevents permeability regulation, which results in electrolyte imbalance but also causes blocking of electron transport and therefore of oxidative phosphorylation; even only this type of damage can already lead to cell death. The NP-protein interaction results in their denaturation which causes enzymatic inactivation and other protein deactivation and therefore alteration of all those processes in which they are involved. NPs also establish contact with DNA bases inducing DNA damage, inhibiting DNA replication, and modifying gene expression levels [39]; the latter activity is implicated in the prevention of biofilm formation [40]. Ultimately, the NPs cause bacterial cell death through multiple and nonspecific mechanisms. Thus, on one side NPs reduce the probability of the development of resistance because it is extremely unlikely that multiple mutations may occur simultaneously in the same bacterium [41]. On the other side, it brings out the problems of the toxicity and safety of inorganic nanoparticles in the perspective of a clinical translation. Therefore, the scientific community is investigating, especially by *in vitro* but also *in vivo* studies, the toxicity mechanisms and trying to understand how the nanoparticle characteristics, such as type of metal or metal oxide nanomaterial, size, shape, and surface along with dosages and routes of administration, can influence toxicity towards eukaryotic cells, tissues, and organs [42]. For example, size is very important, according to Dos Santos et al.; the greater the size of AgNPs, the greater the risk of adverse effects [43]. Certainly, physicochemical characteristics are an important starting point to hypothesize toxicity, but the type of route of administration and accumulation sites contribute to its severity [44]. Deep knowledge of their potential toxicity and impact on human health is essential before these nanomaterials can be used in biomedical applications, that is why it is imperative to carry out more detailed *in vivo* studies performed on animal models in order to establish the NPs' concentrations that guarantee the therapeutic effect with the least adverse effects. In this regard, it can certainly be useful to engineer the nanoparticle systems to gain a targeted delivery in order to confine the therapeutic/toxic action to the infection site.

7.2. Inorganic Nanoparticle Antibacterial Mechanisms. Multiple NP antibacterial mechanisms are frequently classified into three categories as shown in Figure 4 [30, 41, 45].

7.2.1. Oxidative Stress. The nanoparticle antimicrobial activity is mainly due to the production of reactive oxygen species that cause lethal oxidative damage. Under usual circumstances, aerobic metabolism is responsible for the formation of reactive oxygen species such as the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical ($^{\bullet}OH$), and singlet oxygen (O_2). In prokaryotic cells, as well as in eukaryotic cells, ROS are normally reduced by cell antioxidant machinery. However, when their production exceeds their shutdown, cellular redox homeostasis is lost and this leads to oxidative stress: ROS are thus unimpeded to oxidize biomolecules including proteins, DNA bases, and lipids [46].

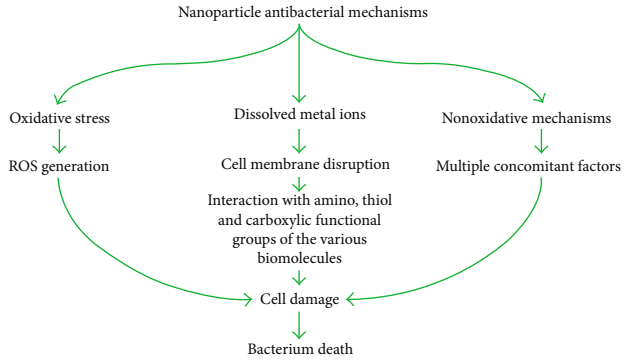

Figure 4: Multiple nanoparticle antibacterial mechanisms.

The structure of DNA and proteins is altered, while lipid peroxidation damages the cell membrane compromising its permeability and obviously also oxidative phosphorylation. The final result is severe cell damage leading to bacterium death [47]. It has been shown that metal nanoparticles can generate the four ROS mentioned above. Precisely, different NPs yield different ROS; thus, MgO and CaO give rise to O_2^-, while ZnO H_2O_2 and $^{\bullet}OH$ arise through different mechanisms. Nano-TiO_2 and ZnO are phototoxic because under natural sunlight they produce reactive oxygen species [48] through a photocatalytic process; moreover, for the ZnONPs, ROS production in the dark was also demonstrated, albeit in small quantities [49]. Ultrasound stimulation can enhance nanoparticle antibacterial activity promoting ROS production: in the presence of ultrasound, ZnO-NPs produce greater quantities of hydrogen peroxide [50].

7.2.2. Dissolved Metal Ions. Inorganic nanoparticles can release metal ions in solution, which are adsorbed through the bacterial membrane disrupting it [41]. Inside a microbial cell, metal ions interact with amino, thiol, and carboxylic functional groups of the various biomolecules inducing various damages [30]. In the case of AgNPs, it has been proven that the antimicrobial action is mainly due to Ag^+ release [51]. Silver ions interact with sulfhydryl groups in enzymes preventing catalytic activity, with DNA inhibiting their replication; in addition, they hinder cell wall synthesis in Gram-positive bacteria [41]. It has also been found that the palladium nanolayers are microbicides because they release palladium ions [52]. In contrast, the participation of metal ions in the metal oxide nanoparticle antibacterial mechanism is limited [30].

7.2.3. Nonoxidative Mechanisms. Leung et al. have minutely investigated the antibacterial mechanisms of three different MgO nanoparticle samples, which are ecofriendly and economical materials, against *E. coli* under UV light, natural light, or in the dark. The data collected showed that two of the MgO samples did not yield ROS at all, and only one type generated a small amount; indeed, membrane lipopolysaccharide and phosphatidylethanolamine were not oxidized, and expression of ROS-associated genes was also not increased. Moreover, MgONPs were not located in the cytosol, and there were not no considerable quantities of Mg ions in solution; nevertheless, fundamental bacterial metabolic processes were affected. According to the results obtained, MgO nanoparticle toxicity is not related to the induction of oxidative stress, whereas membrane damage is probably the result of multiple concomitant factors including the nano-MgO direct contact; the effect on pH; and albeit not in large quantities, the release of Mg^{2+} ions [41, 45, 53].

In the wake of MgONPs, other types of NPs have been shown to act as antibacterial agents through different nonoxidative mechanisms among which the interaction of NPs with the cell wall is one of the most crucial. The antibacterial action due to direct nanomechanical contact between the bacterial envelope and the nanoparticle has also been confirmed for AgNPs. In this regard, Pallavicini et al. have obtained antibacterial surfaces both against planktonic bacteria and against biofilm grafting monolayers of silver nanoparticles on bulk surfaces [54]. The bacteria have a multilayered structure that protects them from the external environment and allows them to maintain their shape, electrolytic homeostasis, and gradient of nutrients, and the last electron transport chain operates in the bacterial cell membrane, which also has a fundamental role in cell communication and apoptosis [30]. Gram staining with crystal violet allows distinguishing and classifying bacteria according to the different structures and components of bacterial envelopes, which automatically determines a different NP absorption pathway. Gram-positive bacteria have a thick peptidoglycan surrounding a cellular membrane and teichoic acids. These are copolymers of glycerol phosphate or ribitol phosphate and carbohydrates linked via phosphodiester bonds, which are anchored to the lipid membrane (lipoteichoic acids (LTAs)) or covalently bound to peptidoglycan (wall teichoic acids (WTA)) and impart a negative charge to the surface. The negatively charged phosphate group is homogeneously distributed along the cell wall and attracts NPs preventing their aggregation.

Gram-negative bacteria possess a thin layer of peptidoglycan between two membranes, an inner cytoplasmatic membrane and an outer membrane. A major component of LPS is the outer membrane, a large molecule consisting of a lipid and a polysaccharide, which preserves its integrity and stability, and it is the only constituent to confer a negative charge that attracts NPs.

Based on structural differences listed, the Gram-positive bacteria cell wall is porous and easily penetrated by nanoparticles, which are attracted by the high negative surface charge; on the contrary, the Gram-negative cell wall, especially because of the outer membrane, represents a barrier to NP penetration. The obvious consequence, confirmed by several studies, is that NPs have a greater antibacterial activity towards Gram-positive bacteria than towards Gram-negative ones [30, 55].

Furthermore, in order to kill bacteria, nanoparticles must be adsorbed on the bacterial surface thanks to the establishment of electrostatic interactions; therefore, positively charged NPs such as AgNPs, TiO_2NPs [56], ZnONPs, and AuNPs [57] have the advantage of being attracted to the high negative surface charge of Gram-positive bacteria. Once adsorbed on the surface, the NPs act by oxidative and

nonoxidative mechanisms that damage the constituents of the cell wall by altering the cell shape and the membrane permeability, thus dissipating the electrochemical gradient and finally triggering cell death [41].

Precisely because of their intrinsic antimicrobial activities, the use of inorganic nanoparticles in combination with antibiotics or as antibiotic carriers has several advantages in addition to those previously anticipated and essentially common to all drug delivery systems. Many works highlight the presence of a synergistic effect in the concomitant use of drugs and metallic nanoparticles, which allows improving the therapeutic agent efficacy: antibiotic MIC values against examined pathogens are reduced [58], in this way low concentrations guarantee therapeutic performance at low toxicities, and bactericidal activity against resistant strains is restored [59].

7.3. Types of Inorganic Nanoparticles. The following paragraphs analyze the antibacterial properties of the main metallic nanoparticles studied against sensitive and multidrug-resistant pathogenic bacteria, both alone in the guise of nanobactericides and in combination with the most common antibiotics.

7.3.1. Silver Nanoparticles. Silver nanoparticles have had a huge impact in the biomedical field since various properties make them extremely interesting, such as the antibacterial, antifungal, antiviral, anti-inflammatory, antiangiogenic, and antitumor activities, and not least as that of a drug carrier [60]. The antimicrobial effects of silver compounds have always been well known since ancient times, but after the introduction of antibiotics in therapy, silver preparations have taken on a marginal role; however, the development of bacterial resistance has rekindled the spotlight on their potential clinical use. AgNPs are broad-spectrum antibacterials; in fact, they act against Gram-negative and Gram-positive bacteria, including antibiotic-resistant strains and bacterial communities of biofilms, but the effect is more intense against Gram-negative ones since the Gram-positive thick peptidoglycan layer prevents NPs from reaching the cytoplasm. As proof of this, Amato et al. have evaluated the MIC values of glutathione-coated AgNPs (GSH-AgNPs) on *S. aureus* and *E. coli* detecting a large gap between the two types of bacteria: 180 μg/mL for *S. aureus* and 15 μg/mL for *E. coli*. Also for *E. coli*, the MIC values for colloidal and ionic silver were very similar, while for *S. aureus*, the NP MIC was 1 order of magnitude greater than the Ag^+ MIC [61]. These results suggested a different bacterium-NP interaction depending on the different bacterial structure. Taglietti et al. have confirmed all this through TEM characterization of bacteria cultures exposed to GSH-AgNPs and to completely ionized silver. From the images obtained, the authors came to the conclusion that the effects GSH-AgNPs are greater against *E. coli* compared to those against *S. aureus* as, in contrast to the Gram-positive bacteria, Gram-negative ones have a thin bacterial envelope that allows nanoparticles to easily penetrate the cytoplasm and reach their targets. [62]. In addition, while Mie et al. was investigating the activity of green AgNPs against 8 microorganisms of which 4 were Gram-positive and 4 were Gram-negative, they found a greater activity towards Gram-negative ones while analyzing the diameters of the diffusion disks. AgNPs at a concentration of 100 μg/mL resulted in an average inhibition zone diameter of 8.0 mm for *P. mirabilis*, *P. aeruginosa*, and *S. marcescens*, and 7.5 mm for *S. typhi*. For all Gram-positive bacteria tested (*S. epidermidis*, MRSA, *B. subtilis*, and *S. faecalis*), the average diameter of the inhibition zone was 6.0 mm [63]. The activity of AgNPs against resistant strains is well known; in fact, in addition to that just mentioned against MRSA, Lara et al. have also detected bactericidal action against multi-drug-resistant *P. aeruginosa*, ampicillin-resistant *E. coli*, and erythromycin-resistant *S. pyogenes* [64]. All the authors agree that their effectiveness is strictly connected to certain features such as the form, whether metallic or ionic, the shape, the size, and obviously the concentration. Metallic Ag is weakly antibacterial, while Ag^+ is responsible for most of the antibacterial activities. A smaller size involves a greater surface area through which nanoparticles can interact with microbial surfaces and can release Ag^+ ions [18]. AgNPs with a triangular or truncated triangular shape have greater efficacy thanks to a high surface density of atoms [18, 65]. As for the concentration, this depends on the size in a directly proportional way: the smaller the size, the lower the concentration necessary to obtain a certain antibacterial effect [66].

The AgNP-antibiotic effects are attributable to various activities including the release of Ag^+ ions, the oxidative stress, and also the nonoxidative mechanisms. Hence, the species responsible for the various damages are the AgNPs per se, the Ag^+ ions, and the ROS generated. AgNPs adhere to bacterial surfaces, thanks to the electrostatic attraction and interaction with thiol groups of cell wall proteins, and they accumulate inside cellular envelopes causing alteration of charge; therefore, they have potential for the modification of the phospholipid bilayer and impaired cell transport. The cell wall becomes more permeable, the bacterial cell becomes round, and the morphology of the membrane is compromised by the formation of many "pits" and gaps that lead to cytoplasmic leakage and most likely to cell death [60, 67–69]. Once inside the cell, silver nanoparticles are also responsible for the increase in the quantity of ROS, which causes direct lipoperoxidation-mediated damage to the cell membrane and hyperoxidation of DNA and proteins [68, 70, 71].

Most of the mechanisms of action listed above are attributable to silver ions released by AgNPs:

(1) Electrostatic interaction with LPS which involves permeability alteration, membrane hole creation, and proton gradient dissipation

(2) Inhibition of cytochromes and therefore of the electron transport chain

(3) Interaction with sulfhydryl groups of biomolecules altering their activity

(4) Obstructing the synthesis of the cell wall of Gram-positive bacteria

(5) Interaction with microbial DNA with blockade of replication

(6) 30S ribosomal subunit denaturation and consequent blocking of protein synthesis

(7) Generation of reactive oxygen species [18, 41, 68, 70, 72]

As metallic nanoparticles, AgNPs have plasmonic photothermal properties, and their size and shape are directly correlated with their plasmonic properties, particularly with localized surface plasmon resonance (LSPR). A specific incident excitation wavelength allows conversion of light to thermal energy in a controllable way. The plasmonic photothermal properties of AgNPs can be used to create "on demand" antibacterial silver nanoparticles. D'Agostino et al. grafted a layer of triangular silver nanoplates having specific LSPR features on bulk glass surfaces. When the surface is irradiated with a laser of the right wavelength, in addition to the "classic" long-term antibacterial effect due to the release of Ag^+ ions, the nanoparticles show a laser-switchable photothermal action which allows an increase in antibacterial activity as needed [73]. Silver has an active role also in wound healing; in fact, AgNPs stimulate fibroblast proliferation and their differentiation into myofibroblasts, promoting wound contraction, and also the proliferation and relocation of keratinocytes [74]. However, if the antibacterial activity is based on a highly sustained Ag^+ release, wound healing ability requires low concentrations of silver ions. In this regard, Pallavicini et al. have synthesized AgNPs coated with pectin (p-AgNPs) which, thanks to the low Ag^+ release with time, have shown to promote the proliferation of fibroblasts but at the same time their antibacterial and antibiofilm activity is preserved thanks to the pectin coating [54]. Ultimately AgNPs induce microbial cell death by multiple mechanisms against which microorganisms rarely manage, probably only after prolonged treatments, to raise a resistance, which generally consists in decreased uptake and increased outflow [18]. Numerous *in vitro* studies have shown that AgNPs are not only effective broad-spectrum nanobactericides, but they can also exhibit synergistic antibacterial activity in combination with various classes of antibiotics and can also act as efficient antibiotic carriers capable of improving their performance and efficacy both against sensitive and resistant bacteria. The scientific literature is full of interesting and profitable examples of AgNP-antibiotic combinations. In one of the first studies, MIC values of AgNPs and amoxicillin, alone and in combination, against *E. coli* were determined. The values for AgNPs and amoxicillin alone were 40 μg/mL and 0.525 mg/mL, respectively, while AgNP-amoxicillin combination values were significantly reduced to 5 μg/mL and 0.150 mg/mL, respectively. The authors put forward several hypotheses to explain the mechanisms behind this synergistic effect against both sensitive and resistant strains:

(a) In the case of resistance versus one of the two agents, it is presumable that the antibacterial activity depends on the component towards which bacteria are sensitive

(b) In the case where strains are sensitive to both association components, the synergistic effect may be due to the formation of AgNP-amoxicillin complexes where each nanoparticle is surrounded by many amoxicillin molecules thanks to the chelating reactions between the hydroxyl and amido groups of amoxicillin and nanosilver. Later, Durán et al. pointed out the presence of another more important binding between amoxicillin and silver nanoparticles, the sulfur bridge [75]. When these chelated complexes contact the bacterial surfaces, they guarantee achievement of high concentrations of antibiotics

(c) Amoxicillin is a hydrophilic molecule, while AgNPs have hydrophobic characteristics; therefore, AgNPs as carriers facilitate the transport of amoxicillin through lipid bacterial envelopes [76]

Shahverdi et al. evaluated the antibacterial activities of 14 different antibiotics (penicillin G, amoxicillin, carbenicillin, cephalexin, cefixime, gentamicin, amikacin, erythromycin, tetracycline, cotrimoxazole, clindamycin, nitrofurantoin, nalidixic acid, and vancomycin) through the disk diffusion method in the presence of AgNPs (10 μg per disk, with an average size of 22.5 nm) against *E. coli* and *S. aureus*. In the presence of AgNPs, an increase in zone inhibition sizes was noted in *S. aureus* more than in *E. coli*, only for the antibiotics penicillin G, amoxicillin, erythromycin, clindamycin, and vancomycin. The highest increases were in the case of vancomycin, amoxicillin, and penicillin G. Penicillin G and erythromycin associated with silver nanoparticles had the greatest antibiotic activity against *E. coli*; in both cases, the zone of inhibition of the antibiotic alone was 8.0 mm, while with AgNPs + antibiotic it became 12.0 mm. The AgNP combinations with penicillin G, vancomycin, and amoxicillin were the most effective against *S. aureus*. Pen G alone did not inhibit *S. aureus*, while in association with AgNPs an area of inhibition of 12 mm was observed. Vancomycin likewise had no activity against *S. aureus*, whereas with AgNPs there were areas of inhibition of 13 mm. The amoxicillin-AgNP association showed the greatest increase in the area of inhibition from 7.5 for amoxicillin alone to 14.0 mm [58]. Fayaz et al. studied the antibacterial activity of ampicillin, erythromycin, kanamycin, and chloramphenicol in the presence of biogenic AgNPs (size range: 5-40 nm) against four microorganisms: against *S. aureus* and *Micrococcus luteus* (Gram-positive) and against *S. typhi* and *E. coli* (Gram-negative) [77]. Various combinations were tested using the disk diffusion method (10 μg of AgNPs per disk). In all cases, a significant increase in antibacterial activity was noticed which, unlike what was found by Shahaverdi et al., was greater against Gram-negative bacteria than Gram-positive, most likely because weakly charged AgNPs are attracted to Gram-negative bacteria negative LPS, while Gram-positive bacteria have a more rigid and cross-linked cell wall and are therefore more difficult to penetrate [58]. For all microorganisms, the association which showed the greatest synergistic effect was that between AgNPs and ampicillin. The greatest percentage increase of the zone of inhibition of this association (81.82%) was

detected against *S. typhi* (ampicillin alone, 11 mm; AgNPs + ampicillin, 20 mm), followed by the increase against *E. coli* which was 75.00% (ampicillin alone, 12 mm; AgNPs + ampicillin, 21 mm), while against the Gram-positive *S. aureus* and *M. luteus* the percentages are lower, respectively 72.73% (ampicillin alone, 11 mm; AgNPs + ampicillin, 19 mm) and 70.00% (ampicillin alone, 10 mm; AgNPs + ampicillin, 17 mm). Ampicillin molecules were linked to each other by weak bindings and interacted with the surrounding "nanosilver core," thus forming AgNP-ampicillin complexes. In contact with the bacterial cell wall, complexed ampicillin inhibits the synthesis of peptidoglycan, favouring the penetration of the complex, which, once inside the cytoplasm, reacts with DNA hindering its unwinding [77]. The AgNP-ampicillin complex was also effective against ampicillin-resistant and β-lactamase producers *Enterobacter* sp., *P. aeruginosa*, *K. pneumoniae*, and *E. coli* [78]. Ampicillin, gentamicin, kanamycin, streptomycin, and vancomycin were combined with biogenic AgNPs (15 μL per disk) synthesized from *Phoma glomerata*. Combined antibacterial effects were evaluated by the disk diffusion method against *S. aureus*, *E. coli*, and *P. aeruginosa*. All antibiotics in association with AgNPs show greater antibacterial activity than antibiotic molecules alone. The activity was increased especially against Gram-positive bacteria, except for streptomycin. The biggest synergistic effects were noticed for the AgNP-vancomycin and AgNP-ampicillin complexes, perhaps because in both cases antibiotics inhibit cell wall synthesis by facilitating the entry of AgNPs into the bacterium [79]. AgNPs functionalized with ampicillin by the latter's thioether moiety have been shown to be more effective compared to AgNPs alone against both pathogenic and nonpathogenic strains of *E. coli* and *P. aeruginosa*. In particular, silver nanoparticles alone had an MBC of 4 μg/mL, while AgNPs functionalized with ampicillin (AgNP-AMP) exhibited an MBC of 1 μg/mL against all *E. coli* strains and the ampicillin-resistant *P. aeruginosa* [80].

Silver nanoparticles obtained by the reduction of aqueous Ag^+ ions using *Dioscorea bulbifera* tuber extract have been combined with piperacillin, erythromycin, chloramphenicol, vancomycin, and streptomycin. The efficacy of the AgNP-antibiotic complexes has been tested on both Gram-positive and Gram-negative bacteria. Inhibition zone diameters of the complexes were increased compared to those of the antibiotics alone, demonstrating that all the antibiotics examined acted synergistically with the AgNPs. It was reported that the greatest increase (11.8-fold) came from AgNP-streptomycin against *E. coli*, followed by AgNP-chloramphenicol (4.9-fold) and AgNP-vancomycin (4.2-fold) against *P. aeruginosa* and AgNP-piperacillin (3.6-fold) and AgNP-erythromycin (3.0-fold) against multi-drug-resistant *Acinetobacter baumannii* [81]. Naqvi et al. used *Aspergillus flavus* for a biologically and more ecofriendly synthesis of AgNPs which they subsequently combined with five conventional antibiotics. The antibacterial activities of both antibiotics combined with AgNPs and antibiotics alone were evaluated against multi-drug-resistant strains including *E. coli*, *P. aeruginosa*, *E. faecalis*, *M. luteus*, *A. baumannii*, *K. pneumoniae*, and *Bacillus* spp. The average values of the zones of inhibition obtained using the Kirby-Bauer disk diffusion method are in descending order as follows: ciprofloxacin + AgNPs (23 mm) > imipenem + AgNPs (21 mm) > gentamycin + AgNPs (19 mm) > vancomycin + AgNPs (16 mm) > AgNPs (15 mm) > imipenem (14 mm) > trimethoprim + AgNPs (14 mm) > ciprofloxacin (13 mm) > gentamycin (11 mm) > vancomycin (4 mm) > trimethoprim (0 mm). The average increase in diameters was 2.8-fold [82]. Synergistic effects have also been demonstrated between AgNPs and doxycycline since the effectiveness of the complex against *K. pneumoniae* was greater than that of the two components alone [83]. All reviewed papers that study the antimicrobial potential of AgNPs are *in vitro* studies, as although these nanoparticles, as well as other metal nanoparticles, have an indisputable antibiotic activity that makes them potential new generation antibiotic candidates, they also give serious toxicity problems, which prevent their use as systemic antimicrobials. The biocidal activity and consequently the therapeutic efficacy are closely related to the physicochemical characteristics of the particles, as well as the toxicity phenomena [43]. Establishing qualitatively and quantitatively the features that guarantee the biocidal action and that simultaneously limit the adverse effects is indispensable to ensure that the progress in nanotechnology can be truly spent in pharmacological approaches. At present, many *in vitro* and *in vivo* toxicity studies regarding AgNPs have been performed, one of which has evaluated a 28-day systemic toxicity effect of 20–100 nm sized AgNPs on rats using intravenous administration. Both the sizes of AgNPs examined caused a delay in the growth of rats, and a severe increase in spleen size and weight due to an increase in the number of both T and B cells was also found. Moreover, accumulations of AgNPs in the spleen, liver, and lymph nodes have been noted from a histopathological evaluation [84]. AgNPs are also neurotoxic, and they are in fact able to cross the blood-brain barrier [85] and accumulate in the brain following ingestion and inhalation [85]. AgNPs cause various damages at the cellular level including damage to the cell membrane that causes cell leakage, DNA damage, damage to the mitochondrial membrane, and ROS production which in turn contributes to the aforementioned damage and ultimately triggers apoptosis [43]. For all these reasons, the antibacterial potential of AgNPs cannot be exploited to cure a whole-body infection but remains confined to antibacterial surface treatment. Indeed, medical applications of AgNPs include medical coating such as materials used in surgery and for the impregnation of surgical masks, wound management, and antimicrobial formulations for topical use [86].

7.3.2. Gold Nanoparticles. Gold nanoparticles can have different sizes and shapes like nanospheres, nanorods, nanoprisms, and nanocages, and as with AgNPs, these factors influence their properties, among which the most visible one is the colour. So metallic gold is gold in colour, while colloidal gold has different colours which are the result of the absorbance phenomena dependent on specific LSPR bands and scattering; thus, nanospheres are ruby red, nanorods are blue or black, and so on [87].

AuNPs are useful in different biomedical fields such as biosensors, genomics, photothermolysis of cancer cells, and

in the fight against microbial infections. They have a 360-degree activity since they constitute an emerging platform for bacterial detection [88], as well as being nanobactericides and ideal nanocarriers of antibiotics [89]. Really, there are conflicting opinions on the antibacterial activity of gold nanoparticles. Most scientists are convinced that AuNPs alone do not possess intrinsic antibacterial activity. Therefore, they cannot be considered nanobactericides [18, 90]. Others have found weak bactericidal activity only at high concentrations probably because the agent is a residue from chemical synthesis [91, 92]. Cui et al. have found that AuNPs have powerful bactericidal activity against a multi-drug-resistant strain of *E. coli* [93]. They investigated the probable molecular mechanisms of action by transcriptomic and proteomic analysis and the data obtained showed the following:

(1) Downregulation of 43 genes encoding two subunits of ribosome and of the ribosomal protein S10 that binds tRNA

(2) Downregulation of some subunits of the F-type ATP synthase, also induced by norfloxacin and salicylite [94]

(3) Downregulation of a type of peroxidase (AhpC), which scavenges low-level H_2O_2

Therefore, the gold NPs act by

(1) Inhibiting the binding of the tRNA to the ribosome and therefore the protein synthesis

(2) Decreasing the activity of the F-type ATP synthase, which involves alteration of the electrochemical gradient and membrane potential and the decrease of ATP levels, which lead to a general decline of cellular metabolism

(3) Decreasing the ability of low-level H_2O_2 scavenging, but which in any case does not involve oxidative stress, in fact, unlike the other metallic nanoparticles, AuNPs are not responsible for the genesis of ROS [93]

Hence, unlike most metallic nanoparticles, AuNPs have a ROS-independent bactericidal mechanism, which most likely guarantees low levels of toxicity towards mammalian cells. Notwithstanding the use in the various epochs of gold as a therapeutic agent, both as bulk gold and at nanoscale level, has had ups and downs, so much so that their use as antibacterial agents is still controversial, AuNPs are universally believed to be ideal nanocarriers of various molecules including vaccines, antimicrobial peptides and conventional antibiotics. In particular, in the latter case there are various *in vitro* demonstrations in the literature of increasing antibiotic activity efficacy and avoidance of resistance mechanisms. The first to use this interesting delivery platform were Gu et al. in 2003 who synthesized vancomycin- (Van-) capped Au nanoparticles (Au@Van) finding the increase in activity compared to free vancomycin and the ability to kill VRE and *E. coli* strains. Surprisingly for *E. faecium*, *E. faecalis*, and *E. coli* strains, the MIC values decreased from >128 μg/mL to 2 μg/mL [95]. AuNPs have been synthesized and conjugated in a one-step process to avoid interference from functionalizing agents, with ampicillin, streptomycin, and kanamycin. AuNP-antibiotics along with their corresponding free antibiotics were tested against *E. coli* DH5α, *M. luteus*, and *S. aureus*, and the minimal inhibitory concentration of each antibiotic compared to their AuNP-conjugated form in each bacterial strain was determined. MIC values were significantly reduced only in the case of aminoglycosides. For free streptomycin, values range from 14.0 for *E. coli* to 22.0 for *M. luteus*. For AuNP-streptomycin, they become 7.0 and 17.0, respectively. For kanamycin, the MIC values of the free antibiotics are 30.0 for *E. coli*, 32.5 for *M. luteus*, and 9.0 for *S. aureus*; when the antibiotic is conjugated, they become 12.0, 23.0, and 5.8, respectively. The major change in MIC is that of AuNP-kanamycin, which differs by 60% compared to the free antibiotic [96]. The different behaviour of AuNP-Amp was subsequently charged with a rapid precipitation of ampicillin from suspension [97]. So much so that, Chamundeeswari et al. synthesized chitosan-capped gold nanoparticles coupled with ampicillin and noted that the complex with a 50% reduction in the dosage of ampicillin has a bactericidal activity 2 times greater than the antibiotic alone. In fact, MIC values were 2-fold decreased when compared with free ampicillin: they were found to be 27.4 μg/mL for *E. coli* and 20.6 μg/mL for *S. aureus* and *K. mobilis* [98]. Amino-substitutes of pyrimidines, which in themselves have no antibiotic activity, when combined with the surface of gold nanoparticles produce a complex that shows multiple bactericidal mechanisms: it destroys the bacterial membrane because it sequesters bivalent ions such as Ca^{2+} and Mg^{2+}, causing loss of cytoplasmic material; interacts with the DNA; and inhibits protein synthesis [99]. Fayaz et al. have used the nonpathogenic fungus *Trichoderma viride* to synthesize biological gold nanoparticles on whose surface are bound vancomycin molecules. They investigated the antibacterial activity of vancomycin and vancomycin-bound gold nanoparticles against test strains (*E. coli* ATCC 8739 175 40, *S. aureus* ATCC6538, and vancomycin-resistant *S. aureus*) using the liquid broth dilution method. These AuNPs were found effective since MIC values of vancomycin alone against *E. coli*, *S. aureus*, and VRSA were 175, 2, and 50 (μg/mL) respectively, while MIC values of vancomycin-bound gold nanoparticles were 40, 1.5, and 8 (μg/mL). The authors suggested the nonspecific binding with transpeptidase as a probable mechanism of action [100]. Vidya et al. prepared AuNPs functionalized with two fluoroquinolones, levofloxacin and ciprofloxacin, and two cephalosporins, cefotaxime and ceftriaxone. The MIC and MBC values against MDR *E. coli*, *S. aureus*, and *K. pneumoniae* were reduced compared to free antibiotics, and *E. coli* was the most susceptible of all. In particular, MIC values of free levofloxacin, cefotaxime, ceftriaxone, and ciprofloxacin were all >10 μg/mL for all strains tested; contrarily, MIC values of the gold nanoparticles functionalized with the same antibiotics were significantly lower. The lowest MIC values for *K. pneumoniae* were those of

ceftriaxone-AuNPs and ciprofloxacin-AuNPs (both 0.281 μg/mL), for *S. aureus* levofloxacin-AuNPs were the most effective (0.562 μg/mL), while for *E. coli* the lowest MIC was that of ciprofloxacin-AuNPs (0.140 μg/mL) [101]. AuNPs conjugated with cefotaxime have been tested on *E. coli* and *K. pneumoniae* CTX-M-15 positive (the most common extended spectrum β-lactamase in the Enterobacteriaceae family) strains that are positive and therefore completely resistant to cefotaxime. The cefotaxime delivery on AuNPs has restored its bactericidal activity. MIC of cefotaxime-conjugated AuNPs was found as 1,009 and 2,018 μg/mL against the study strains of *E. coli* and *K. pneumoniae*, respectively, whereas MBC were 2,018 and 4,037 μg/mL for the same, respectively [102]. For the first time, Haddada et al. reestablished susceptibility to doxycycline by conjugating it to PEGylated-gold nanoparticles. The resulting complex increased the penetration and antibacterial activity of the antibiotic on its own, proving to be active against a broad spectrum of human pathogens. The authors tested them against two *S. aureus* strains (ATCC 25923, ATCC 700699), *E. faecalis*, and two *E. faecium* strains (BM4147, ATCC 19434T). MIC values of doxycycline alone against the listed strains were respectively 32, >32, >32, 32, and 32 mg/L, whereas MIC values of doxycycline-conjugated PEGylated-gold nanoparticles were found to be respectively 1, 2, 2, 2, and 2 mg/L [103]. According to Lee and Lee, AuNPs work in synergy with cefotaxime and ciprofloxacin against *Salmonella*. Gold nanoparticles alter calcium homeostasis, while antibiotics are implicated in the increase of ROS and together cause apoptosis-like death [104].

Chavan et al. used ampicillin as a reducing and capping agent to obtain ampicillin-coated gold nanoparticles. Amp-AuNPs accumulate on the bacterial surface and cause pores to form at the membrane level through which they enter the cell. Amp-AuNPs have proven effective against ampicillin-resistant *E. coli*, and since they have good adhesive properties, they can interfere with the formation of biofilm [105].

7.4. Types of Metal Oxide Nanoparticles

7.4.1. Zinc Oxide Nanoparticles. According to both the U.S. Food and Drug Administration (FDA) and the European Food Safety Agency (EFSA), zinc oxide is a safe substance. Therefore, it is used in the food field (not only as a food additive but also as a material for food packaging to preserve colour and ensure microbiological safety [106–108]) and in cosmetics (for its antibacterial and deodorant properties and as a sunscreen such as TiO_2). Among the metal oxide nanoparticles, ZnONPs have attracted attention thanks to their optical, physical, and antimicrobial properties. Indeed, ZnONPs are extremely interesting nanomaterials for various industrial applications such as in optical communications and in the manufacturing of rubber, paint, coating, lubricants, and cement, to name a few [109], but also for biological applications especially in anticancer and antibacterial fields [110]. It is legitimate to think that if zinc oxide has good antibacterial properties, nanosize-ZnO, with its large surface area-to-volume ratio, is a better antibacterial compared to its bulk counterpart. In fact, ZnONPs are effective against a large number of both sensitive and resistant human pathogens, and also have great stability, good toxicological profile, and low cost. Particularly, studies have confirmed that ZnONPs have a marked selective toxicity towards various Gram-positive and Gram-negative bacterial species, while they are biocompatible with human cell lines [111]. Due to their intrinsic antibacterial activity, ZnONPs alone can be considered nanobactericides, but since their synergistic action with some classes of antibiotics has been demonstrated, they can be used also as drug carriers to enhance conventional antibiotic activity and overcome resistance mechanisms [112]. ZnONPs have multiple bactericidal mechanisms:

(1) Intracellular ROS generation

(2) Release of Zn^{2+} ions

(3) Mechanical damage to the cell wall resulting from adhesion to the bacterial surface

The antibacterial activity of ZnONPs is positively influenced by various elements including particle size and concentration, surface characteristics, morphology, and exposure to UV light [112]. The smaller the nanoparticle size, the greater the surface area available to contact bacteria and the easier the permeation through the bacterial envelopes. In addition, the dissolution of the Zn^{2+} ions and the generation of H_2O_2 are surface-area-dependent events [113, 114]. Ultimately, a smaller size enhances ZnONPs' antibacterial efficiency. In this regard, Raghupathi et al., by examining the antibacterial properties of zinc oxide nanoparticles against both Gram-positive and Gram-negative microorganisms, found that the viability of the bacterial cell significantly decreased with a decrease in particle size from 212 nm to 12 nm. ZnONPs larger than 100 nm showed bacteriostatic activity against MRSA, while ZnONPs smaller than 12 nm had bactericidal activity [114]. ZnO surface modification can be obtained through thermal annealing, which involves an increase in the quantity of O_2 absorbed on the surface and therefore a greater production of ROS or by using coating agents that induce greater Zn^{2+} release or improving ROS genesis [112]. Toxicity, the number of active facets (high density corresponds to high antibacterial activity), and the internalization mechanism depend on the shape of ZnONPs. Spherical ZnONPs penetrate with greater difficulty than rods and wires [115]. Talebian et al. found that flower-shaped ZnONPs are more active against *S. aureus* and *E. coli* than rod- and spherical-shaped ones [116].

ROS production is considered the main antibacterial mechanism, and at the same time, the major cause of ZnONP nanotoxicity [112, 113]. Among metal oxides, ZnO is the one with the highest photocatalytic efficiency. When ZnO absorbs UV light, a photoinduced oxidative process is triggered through which ROS, mostly hydrogen peroxide and superoxide ions, are produced [113]. The conditions in which ROS genesis occurs are still controversial. According to some authors, this is an event limited exclusively to exposure to light, while other studies report the presence of ROS also in the dark [117, 118]. Ann et al. compared the

bactericidal activity of two forms of ZnO, rod and plate, against *E. coli* and *S. aureus*, in the presence and absence of UV-A illumination. It was found that exposure to light for 20 minutes significantly increases bacterial inhibition regardless of morphology [119]. Therefore, although production in the dark of reactive oxygen species by ZnONPs is not to be excluded, photocatalytic production involves a high amount of ROS and therefore an increase in bactericidal activity [112].

The release of the Zn^{2+} ions in solution has been proposed as another likely mechanism of action. Zn^{2+} ions inhibit active transport and amino acid metabolism and cause enzyme disruption. Although Zn^{2+} release is commonly accepted, its contribution to bactericidal activity is still controversial, in fact ZnONPs are stable and insoluble in H_2O; therefore, the distribution of ions in the medium is limited [120]. Nonetheless, Pasquet et al. demonstrated that antimicrobial activity of the ZnO nanoparticles on *E. coli*, *S. aureus*, *P. aeruginosa*, *Candida*, and *A. brasiliensis* depends significantly on the Zn^{2+} ions, and their release is influenced not only by nanoparticle properties such as size, surface characteristics, porosity, and morphology but also by the characteristics of the medium in which the dissolution process takes place, like pH and UV illumination [121]. ZnONPs dissolve rapidly producing Zn^{2+} ions only in acidic conditions (pH 4.5), while they remain intact at neutral or biological pH, hence they can presumably have an antibacterial effect at the lysosomal level [122]. Zn^{2+} is less bactericidal and toxic when compared with Ag^{+}, which is certainly the most potent bactericidal activity among metal ions but also extremely cytotoxic. For these reasons, many researchers aim to associate Ag^{+} together with other metal ions such as Zn^{2+} in order to obtain a synergistic antibacterial effect together with a lower toxicity. An interesting and recent work by Fan et al. compares the antibacterial activity of Ag^{+} with that of the Zn^{2+} ions. These ionic species are derived from two solutions, i.e., silver nitrate solution ($AgNO_3$) and zinc nitrate hexahydrate solution ($Zn(NO_3)_2 \cdot 6H_2O$), respectively, and not from dissolution processes of nanoparticle systems; notwithstanding, it might seem a topic far from the intention of this review, but what emerges helps to analyze more critically the bactericidal action of Zn^{2+}. The purpose of Fan et al.'s work was to find the most powerful Ag^{+}–Zn^{2+} atomic ratios against *E. faecalis* biofilm on dentin (the best ratios were 1 : 9 and 1 : 12). The authors determined the minimum inhibitory concentration (MIC) and bactericidal concentration (MBC) of Ag^{+}, Zn^{2+}, and Ag^{+}–Zn^{2+} at different atomic ratios by a serial microdilution assay. From the results obtained, the MIC and MBC values for Ag^{+} were 6.4×10^{-2} μg/mL and 12.8×10^{-2} μg/mL, respectively, while the MIC and MBC values of Zn^{2+} were undetectable for its limited antibacterial ability. These results confirm the strong antibacterial activity of silver and highlight that the bactericidal ability of Zn^{2+} is much weaker than Ag^{+}, generally being inhibitive rather than bactericidal to *E. faecalis*. The authors also reported that when Zn^{2+} was mixed with Ag^{+}, the antibacterial effects would be significantly improved compared to Ag^{+} alone. Even if the pattern and mechanism behind this synergistic activity is still unclear, the authors speculate that Zn^{2+} depolarizes the membrane potential; this does not in itself cause the rupture of the bacterial cell membrane and the leakage of cytoplasmic material but can certainly favour the accumulation of Ag^{+} ions and therefore their antibacterial action [123]. Considering the very weak antibacterial activity of the Zn^{2+} ions and bearing in mind that the release of Zn^{2+} ions from ZnONPs is ultimately very scarce, it can be concluded that this really does not significantly influence the antibacterial activity of the ZnONPs. The third bactericidal activity proposed is the result of the attack of ZnONPs on the bacterial cell wall by electrostatic forces, since ZnONPs are positively charged in water [124, 125]. The interaction causes disorganization of the envelope, destruction of the membrane, and outflow of cytoplasmic material [126, 127]. Defects in the particle surface make it much more abrasive than it already is by increasing this antibacterial mechanism [113]. Various studies show synergistic antibacterial activity between ZnONPs and some conventional antibiotics; in fact, different antibiotics showed different activities in the presence of nanosized ZnO. Thati et al. treated the *S. aureus* clinical isolate simultaneously to a subinhibitory concentration (100 μg/disk) of zinc nanoparticles and 25 different antibiotics. The disk diffusion method was used to assay the possible synergy between ZnONPs and antibiotics positioned in the same plate. An increase in the inhibition zones was observed in all candidate antibiotics, but the significant enhancement of antibiotic activity was found in the ZnONP associations with β-lactams (penicillin and amoxicillin/clavulanic acid, 10 mm), aminoglycosides (amikacin, 10 mm), and cephalosporins (cefalexin, cefotaxime, and ceftazidime, 8 mm) [128]. Conversely, the efficacy of Banoee et al.'s ZnONPs (20-45 nm sized at 500, 1000, and 2000 μg/disk concentration) combined with 14 different antibiotics was verified against *S. aureus* and *E. coli*. In the presence of ZnONPs, the antibacterial activity of amoxicillin, penicillin G, and nitrofurantoin against *S. aureus* decreased; only for the ZnONP-ciprofloxacin combination was an increase of 22% and 27% observed in the zones of inhibition against *E. coli* and *S. aureus*, respectively. The reasons probably lie in the interaction between ZnONPs and the two proteins that regulate the outflow and permeation of fluoroquinolones, respectively: NorA and Omf. Thus, ZnONPs decrease the outflow of ciprofloxacin from *S. aureus* because they interfere with NorA while increasing its permeation because they interact with the membrane protein Omf [59].

Norfloxacin, ofloxacin, and cephalexin were conjugated to ZnONPs, and the antibacterial activities of the three different concentrations of the nanodrug conjugates (10, 50, and 100 μg) were tested against *S. aureus*, *E. coli*, and *P. aeruginosa* by well diffusion assay and biofilm inhibition study and compared to that of identical concentrations of free zinc oxide nanoparticles against the same bacterial strains. The antibacterial activity of free ZnONPs has been confirmed since they inhibited all the tested bacteria: in *S. aureus*, the zones of inhibition were found to be 16, 18, and 19 mm at 10, 50, and 100 μg concentrations, while in *E. coli* and *P. aeruginosa*, the zones of inhibition were found to be 14, 30, and 31 mm and 19, 21, and 23 mm, respectively. Concerning the ZnONP-antibiotic conjugates, an increase in the zone of

inhibition and inhibition of biofilm in a dose-dependent manner was observed for all antibiotics against all three pathogens; this clearly reveals the synergistic activity of antibiotics with ZnONPs. By way of example, in *S. aureus*, the zones of inhibition of ZnO nanoparticles with ofloxacin (at 10, 50, and 100 μg concentrations) were 14, 25, and 31 mm. Those of ZnO nanoparticles with norfloxacin were 31, 44, and 50 mm, while those of ZnO nanoparticles with cephalexin were 29, 37, and 38 mm. Norfloxacin and cephalexin with ZnONPs also revealed similar antibacterial activity against all the tested bacteria [129].

Iram et al. tested the bactericidal activity of different antibiotic-nanoparticle combinations against vancomycin-resistant enterococci (VRE). Ciprofloxacin, erythromycin, methicillin, and vancomycin were conjugated with three types of metal oxide nanoparticles, CaO, MgO, and ZnO. The ZnO-antibiotic combinations were found to have the lowest MIC values and were therefore the most effective, proving that the ZnONPs allow restoring the susceptibility of VRE strains. In particular, ZnONPs at concentrations 0.625 mM, 1 mM, 1.25 mM, 1.5 mM, 2 mM, and 2.5 mM have been shown to be efficient in reducing the MICs of the antibiotics. ZnO nanoparticles at concentrations of 1 mM (< 50 nm), 1.5 mM (<100 nm), and 2.5 mM (<5 μm) effectively reduced the MIC range of ciprofloxacin from 16-256 μg/mL to 2-16 μg/mL, 4-32 μg/mL, and 4-256 μg/mL respectively. ZnO nanoparticles at concentrations of 1 mM (50 nm), 1.25 mM (100 nm), and 2.5 mM (5 μm) allowed the reduction of the MIC range of erythromycin from 1024-2048 μg/mL to 128-512 μg/mL. Methicillin in combination with ZnO particles at concentrations of 1.25 mM (50 nm) and 2 mM (100 nm) showed reduction of MIC from 32-256 μg/mL to 8-64 μg/mL, while ZnONPs at 2.25 mM (5 μm) resulted in a reduction to 8-128 μg/mL. Vancomycin combined with ZnONPs at concentrations of 0.625 mM (<50 nm), 1 mM (< 100 nm), and 1.25 mM (<5 μm) showed reduction of MICs against VRE from 256-512 μg/mL to 16-32 μg/mL with < 50 nm ZnO and to 32-64 μg/mL with <100 nm and <5 μm ZnO particles [130].

7.4.2. Titanium Dioxide Nanoparticles. TiO_2NPs have exceptional properties such as biological and chemical inertness, chemical and mechanical stability, corrosion resistance, hydrophilicity, and low production cost, which make them expendable in different industrial fields. Pigments and paints represent the main applications. In particular, titanium dioxide is the most widely used white pigment and an opacifier not only in the paint industry but also in cosmetics, medicines, and food. Other applications include paper and plastics followed by glass, metal patinas, catalysts, and electric conductors [131]. In addition to all these attractive features and uses, in 1972 Kenichi Honda, an associate professor, and Akira Fujishima, his graduate student, discovered the photocatalytic properties of TiO_2 since they found the photoelectrolysis of water took place on a TiO_2 electrode [132]. Thirteen years after the discovery of the "Honda-Fujishima effect," some researchers began to employ this ability to produce a photochemical sterilization system based on titanium dioxide powder [133, 134], and thereafter, a series of works followed in which nano-TiO_2 was used for the disinfection of surfaces, water, and wastewater [135–137]. In effect, like ZnO, TiO_2 is a metal oxide semiconductor photocatalyst whose antibacterial activity primarily depends on the photocatalytic production of ROS [138], and TiO_2NPs have the same photocatalytic characteristics as the bulk TiO_2 combined with the well-known and advantageous features of nanoparticles [139]. TiO_2NPs have broad-spectrum antimicrobial activity; in fact, they are active against both Gram-positive and Gram-negative bacteria and fungi, and in addition, they are ecofriendly [140]. For these reasons, TiO_2NPs are useful and effective not only for both the disinfection and sanitation processes of water and surfaces but also as nanoantibiotics and drug delivery systems of conventional antibiotics, as shown by the data in the literature.

Kühn et al. suggested the use of titanium dioxide-coated surfaces in all places where regular disinfection is required, like clinical and laboratory environments, to prevent contamination. Under UV-A rays and in the presence of water and oxygen, TiO_2 generates highly reactive OH-radicals that kill bacteria. The effectiveness of this disinfection procedure has been established taking into account the destruction of germs such as *E. coli*, *S. aureus*, *E. faecium*, *P. aeruginosa*, and fungus *C. albicans*. The authors found that TiO_2NPs have the greatest antimicrobial efficiency against *E. coli*, followed by *P. aeruginosa*, *S. aureus*, *E. faecium*, and finally, *C. albicans* [135]. Gelover et al. have shown the greater efficacy of photocatalysis with sol-gel-immobilized TiO_2 films over glass cylinders in inactivating both the total and faecal coliforms naturally present in spring water compared to solar disinfection (SODIS) [136]. As already reported for the other nanosized structures, the antibacterial activity of TiO_2 is closely related to nanoparticle properties such as size, morphology, and crystal nature, and also above all, because TiO_2 photocatalytic efficiency depends on them [141]. In this regard, TiO_2 nanotubes, thanks to their hollowed shape, gain a large surface area through which ROS can be generated; therefore, they possess a high antimicrobial activity [139]. TiO_2 in anatase cystalline form has a band gap energy of 3.2 eV that allows electron-hole pair production with the use of UV-A light or radiation with a shorter wavelength. The photogenerated holes and electrons are responsible for TiO_2 photocatalytic antimicrobial activity as they react with water and oxygen to produce ROS [142], mainly hydroxyl radical and superoxide radical anion, but also hydrogen peroxide and singlet oxygen [143]. The high production of reactive oxygen species not balanced by their elimination causes the oxidative phenomena affecting various bacterial cellular constituents. At the cell wall level, lipids undergo peroxidation, porins and outer-membrane proteins are damaged [143], and polysaccharide chains are cleaved [144]. All this makes the cell membrane more easily accessible. ROS also oxidize membrane phospholipids causing increased fluidity, loss of cellular components, a rapid initial leakage of K^+ followed by a slower release of RNA and proteins, cell lysis, and finally probable microrganism mineralisation [142, 145]. Microscopy studies on *P. aeruginosa* treated with TiO_2NPs also highlighted the loss of cytoplasmic material: "bubble-like protuberances which expelled cellular material"

[146]. Obviously, the damage to the membrane also involves inhibition of the respiratory chain.

E. coli cells treated with photoactivated TiO_2NPs were visualized by SEM, and some constituents of the cell membrane were analyzed [147]. The following morphological changes have been highlighted: increase in cell volume and development of honeycomb structure. The authors suggest that the molecular basis of shape and structural changes is to be found in the decomposition and peroxidation of cell membrane fatty acids [147].

Kubacka et al. treated *P. aeruginosa* cells with TiO_2 + UV and analyzed proteomic and gene expression changes. Expression of genes coding for proteins and enzymes critical for the membrane and the bacterial cell wall has been altered: most of the enzymes participating in lipid metabolism were overexpressed while proteins coding genes involved in pilus biosynthesis and murein and lipopolysaccharide metabolisms were downregulated. Presumably, to compensate for the initial attack on the cell wall, bacterial cells tried to fortify their "second defense barrier," the membrane. Genes related to stress, detoxification, and DNA repair mechanisms were found expressed at high levels, while, as regard the respiratory chain, the expression levels of ubiquinol-dependent cytochrome oxidases were increased but "a strong decrease of the coenzyme-independent respiratory chains" was noted. TiO_2-UV treatment also decreased the ability of *P. aeruginosa* cells to assimilate and transport inorganic phosphate: 7 genes linked to iron homeostasis and Pi assimilation, including Pho regulon which regulates biofilm synthesis and pathogenicity [148], were expressed at low levels [140].

Carré et al. studied the effects of TiO_2NPs, under UV-A irradiation and in the dark, on the viability of *E. coli* cells and on lipids and microbial proteins. After application of TiO_2 and UV-A light, the following were observed:

(1) A significant reduction in cell viability by counting on agar plate

(2) Lipoperoxidation caused by 50% of the superoxide anion radical

(3) Alteration of the protein spots identified through 2DE-gels

The disappearance of a protein spot can be linked to various reasons such as oxidation, denaturation, and cutting but it is not automatically an indication of a protein failure. The proteins involved are varied in function and location: porins, chaperone proteins, enzymes, and proteins involved in the transport and metabolism of various substrates. The proteomic profiles obtained were somewhat interesting since, of the 22 spots in the control, 14 and 22 spots were missing after treatment with TiO_2 0.1 g/L + UV-A and TiO_2 0.4 g/L + UV-A, respectively, for 30 min. While surprisingly, 7 and 19 spots disappeared after treatment with TiO_2 only at a concentration of 0.1 g/L and 0.4 g/L, respectively. All this indicates that TiO_2NPs show both irradiation-dependent and irradiation-independent antibacterial activities [117, 143]. Li et al. tested the bactericidal activity of hybrid Ag-TiO_2 nanoparticles in both light and dark conditions. In the dark, hybrid nanoparticles have greater antibacterial activity than AgNPs and TiO_2NPs; this demonstrates the presence of a synergistic antibacterial mechanism independent of titanium dioxide photocatalytic production of ROS. Also under UV light, hybrid Ag-TiO_2 nanoparticles presented stronger bactericidal activity than UV alone, AgNPs + UV, or TiO_2 NPs + UV [149].

Roy et al. were the first to test the interaction of TiO_2NPs with antibiotic molecules in order to restore their effectiveness against multi-drug-resistant bacteria. The fruitfulness of the TiO_2NP-antibiotic interaction without any irradiation was detected through the disk diffusion method: in all cases, there was an increase in zone inhibition sizes. The highest increase in area by the antibacterial activities were observed for penicillin and amikacin (10 mm) followed by ampicillin and gentamycin (in each 9 mm), oxacillin, cloxacillin (8 mm), amoxycillin, cephalexin, cefotaxime, ceftazidime, vancomycin, streptomycin (in each 7 mm), erythromycin, clindamycin (6 mm), and tetracyclin (5 mm). A moderate increase was noted for ciprofloxacin, rifampicin, sulphazidime, and cotrimoxazole (4 mm). Finally, chloramphenicol (3 mm) followed by norfloxacin and clarithromycin (2 mm) demonstrated the lowest increase. The results obtained showed a synergism of action between TiO_2NPs and all the antibiotics tested against MRSA; in particular, the synergy is especially high with penicillins, cephalosporins, and aminoglycosides; therefore, the combination of the latter antibiotics with TiO_2NPs can represent a promising therapeutic approach against multi-drug-resistant bacterial strains [150]. Recently Ullah et al. isolated MRSA strains and assessed their susceptibility/resistance against some antibiotics, TiO_2 nanoparticles, and their combinations. All strains were found to be susceptible to vancomycin, quinupristin/-dalfopristin, and teicoplanin, while they were highly resistant to erythromycin, penicillin, and tetracycline. TiO_2NPs alone showed the greatest efficacy at a concentration of 2 mM for 12 hours of incubation while TiO_2NPs 3 mM in combination with erythomycin increased the antibacterial activity of the antibiotic since the MIC decreased from the 0.25-1024 mg/L to 2-16 mg/L range [151].

8. Liposomes for Antibiotic Delivery

Liposomes are semipermeable spherical vesicles made up of amphiphilic lipids (phosholipids) arranged to form one or more *lamaellea* or concentric bilayers separated by aqueous compartments surrounding an entrapped central aqueous volume. Hydrophilic portions of phospholipids are in contact with the internal or external aqueous environments while hydrophobic moieties are in contact with each other. Their sizes are in a wide range between 20 nm and several micrometers, so liposomes are considered both nanoparticle and microparticle systems. Since then, liposomes have become the most widely studied and universally used drug carrier systems for both topical and systemic administrations; not only that, that they can be applied in cosmetics as penetration enhancers, adjuvants in vaccination, and signal enhancers/carriers in medical diagnosis. As carriers, they are ideal both for hydrophobic molecules, which can be

loaded inside lipid bilayers, and for hydrophilic compounds that are located in the central aqueous core or in the aqueous compartments located between *lamellae*. Based on size and bilayer number, liposomes can be classified into multilamellar vesicles or MLVs (>0.5 μm), oligolamellar vesicles or OLVs (0.1-1 μm), large unilamellar vesicles or LUVs (>0.1 μm), and small unilamellar vesicles or SUVs (< 0.1 μm). Each type of vesicle has a typical aqueous volume/-lipid ratio value; the higher the ratio, as in the case of LUVs, the greater the ability to carry hydrophilic drugs, while vesicles with low aqueous volume/lipid ratio such as MLVs and SUVs are optimal for carrying hydrophobic drugs like many antibiotics [152]. There are several advantages that have made liposomes extremely popular as drug delivery systems; first of all, they are nontoxic and biodegradable as their composition is similar to that of biological membranes being made up of natural or synthetic lipids. Secondly, the liposomal system is exceptionally ductile since size, lipidic composition, surface charge (zeta potential), and surface modifications can be finely modulated to obtain enhanced circulation half-life, controllable release kinetics, and targeted drug delivery. Liposomes are masking systems or, as Bangham used to call them, "trojan horses" which allow safeguarding incorporated active principles from any degradation. Another very interesting feature is their fusogenic membrane property; in fact, their lipid bilayer structure allows them to merge with ease with all biological membranes, including bacterial ones [31, 153]. Thanks to this property, in case of intracellular infections, liposomes permit the achievement of effective intracellular concentrations overcoming one of the major limitations of some antibiotic agents and thus preventing antibiotic resistance onset. For all these reasons, liposomes are the most successful nanocarriers and the most patented and approved drug delivery vehicles for clinical use [154]. Drugs indicated for the treatment of various diseases can find benefit from being transported by liposomes; surely among these there are anticancer, antifungal, antiviral, and antibiotics agents, due to their low therapeutic index, severe side effects, and resistance mechanisms.

After Gregoriadis first encapsulated an antibiotic in liposomes, thousands of similar works have followed one another. Engineering liposomes in order to obtain specific pharmacokinetic and pharmacodynamic properties guarantees the achievement of therapeutic success; for this reason, research on liposome technology has generated a great variety of liposomal formulation for different purposes. As for antibiotics, liposomes are the means to broaden the spectrum of antibacterial action in order to circumvent resistance, to defeat forms of resistance such as biofilms, to improve drug concentration at the infection site reducing toxicity and enhancing efficacy, and to deliver multiple active molecules that fight synergistically to eradicate infections. To achieve these goals, different types of liposomes have been created.

9. Conventional Liposomes

Also called "first-generation liposomes," these were the first to be used for pharmaceutical purposes [155], and they can be neutral if they only consist of neutral phospholipids and cholesterol in varying quantities, negatively charged if acidic phospholipids (phosphatidic acid) are included in the lipidic composition, or positively charged if they contain stearylamine. Conventional liposomes protect encapsulated molecules from degradation and ensure passive targeting towards tissues or organs that have a discontinuous endothelium (liver, spleen, and bone marrow). In fact, after intravenous administration, these liposomes are recognized and quickly removed from systemic circulation by the reticuloendothelial system (opsonization). Therefore, they are ideal for carrying antimicrobial drugs to treat infectious diseases involving phagocytes [155], but when it is necessary to fight infections localized elsewhere, they have a limited and disadvantageous use. The binding of opsonins to liposomes is size-dependent, and mononuclear phagocytic system (MPS) uptake is greater for larger liposomes; therefore, SUVs have a half-life longer than that of MLVs [156]. Neutral and negatively charged liposomes are removed from the circulation more slowly than positively charged ones, hence the latter are toxic [157–159]. Neutral, negative, and positive conventional liposomes were mainly used in earlier works or whenever the intent was to achieve antibiotic active concentrations at the MPS level. Increasing intracellular residence of antibiotic agents is not only fundamental for intracellular infections but also in the case of extracellular infectious agents such as *S. aureus*. In fact, because these are phagocytized by immune cells as a defense, it is essential to increase their intraphagocytic concentration to kill the pathogen. So, if free dyhydrostreptomycin (DHS) is not capable to enter macrophages, the one carried by liposomes can penetrate phagocytic vacuoles containing *S. aureus* killing it [160].

A few years later, Desiderio and Campbell attempted to achieve the same goal by encapsulating an aqueous solution of cephalotin in MLVs. The encapsulated cephalotin was superior to the free one in killing intraphagocytic *S. typhimurium* as the liposomal vehicle allowed achievement of high concentrations in infected murine macrophages and killing 60% of the microorganisms after 60 min of incubation [161]. Streptomycin and chloramphenicol, transported by neutral or anionic large unilamellar liposomes, showed an antibacterial intracellular activity increased by more than 10-fold compared to respective free drug activity against *E. coli* located within mouse macrophages [162]. The broad-spectrum antibiotic piperacillin is readily inactivated by staphylococcal β-lactamases. By encapsulating it in large unilamellar liposomes prepared with phosphatidylcholine and cholesterol (1 : 1), Nacucchio et al. protected antibiotics from hydrolysis by restoring their bactericidal efficacy against β-lactamase-producing *S. aureus* [163].

Liposomal vancomycin and teicoplanin have an intracellular antimicrobial effect significantly greater than corresponding free forms against MRSA present in human macrophages [164]. According to Pumerantz et al., only vancomycin carried by conventional liposomes is able to kill MRSA persisting inside the alveolar macrophages because, compared to free vancomycin and that encapsulated in stealth liposomes, it is the only one that can reach sufficient intracellular concentrations [165].

10. Long Circulating Liposomes

Steric stabilization of liposomes increases their longevity in biological fluids preventing macrophage uptake [166]; this is achieved by adding to conventional lipids hydrophilic molecules that mimic erythrocyte membranes such as monosialoganglioside (GM1) [167, 168] or hydrophilic polymer such as PEG. These types of liposomes are called "second-generation liposomes" or long circulating liposomes or stealth liposomes (PEG coated) because steric hindrance does not allow recognition and uptake by the mononuclear phagocyte system, so they can accumulate in other tissues or organs outside MPS and their circulation times are extended [155, 168, 169]. Bakker-Woudenberg et al. investigated whether encapsulation of ciprofloxacin in PEG-coated liposomes could increase its therapeutic potential in treatment of *Klebsiella pneumoniae* pneumonia in rats. Liposomal formulation was found to have a greater therapeutic efficacy than free ciprofloxacin because it gave rise a decreased clearance and elevated and prolonged ciprofloxacin concentrations in the blood and tissues [169]. One year later, the same authors tested the aforementioned liposomal system to improve the efficacy of ciprofloxacin in two rat models of *Pseudomonas aeruginosa* pneumonia, both acute and chronic. Long circulating liposomes of ciprofloxacin guaranteed prolonged concentrations of drug in the blood, which protected against early-stage septicemia of acute infection, while in chronic infection, they allowed the elimination of more than 99% of bacteria in lungs [169].

Vancomycin standard formulations fail to reach high effective concentrations to kill MRSA-infected alveolar macrophages because they have poor intracellular and lung tissue penetration. Muppidi et al. have shown that encapsulation within liposomes increases intracellular efficacy of vancomycin and specifically that vancomycin transported by PEGylated liposomes compared to that encapsulated in non-PEGylated liposomes has prolonged blood circulation time and it accumulates more at target site, i.e., in lungs, and less in kidneys; therefore, it is more effective and less toxic [170].

11. Cationic Liposomes

Cationic liposomes consist of lipids with a positive residual charge such as 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), oleic acid (OA), stearylamine (SA), dioctadecyldimethylammonium bromide (DODAB), dioctadecyldimethylammonium chloride (DODAC), and dimethylaminoethane carbamoyl cholesterol (DC-chol). The presence of a positive charge provides specific electrostatic interaction with negatively charged molecules such as DNA; therefore, cationic liposomes are one of the most commonly used nonviral gene delivery vectors [171], and also with bacterial cell wall and biofilms, both negatively charged [172–175]. Moreover, molecules like DODAB and DODAC are quaternary ammonium compounds that form stable vesicles in aqueous solutions with antimicrobial properties since they cause flocculation and death of bacteria [176, 177]. Unlike harmless neutral conventional liposomes charged only with antibiotic drugs, cationic vesicles alone are bactericides. On one hand, this entails the disadvantage of cytotoxicity towards mammalian cells, while on the other hand, it can suggest a probable synergistic action between the cationic bilayer antimicrobial lipids and antibiotics encapsulated inside [176]. This encouraged the application of cationic liposomes as drug-releasing systems in treating bacterial infections involving natural surfaces such as skin and teeth or biomedical devices. Sanderson and Jones have encapsulated vancomycin and gentamicin in cationic liposomes (DODAB) and investigated their antibacterial potential against biofilms of the skin-associated bacteria *Staphylococcus epidermidis*. Liposomal vancomycin was more active than free vancomycin and liposomal gentamicin, in the latter case probably due to gentamicin slow passage through the membrane. Based on the results, the authors suggested topical or intravenous administration of liposomal vancomycin to treat bacterial infections within and outside human body, including biomedical devices [178]. Vancomycin encapsulated within SA and DODAB was found to be more effective to inhibit bacterial growth from *S. aureus* biofilms than an equal amount of free vancomycin [172]. Similarly, the effectiveness of penicillin G encapsulated in cationic liposomes containing 22 mole percentage of DC-chol on pen-G-sensitive *S. aureus* biofilm was tested. Despite the lower concentration and shorter exposure time, liposomal benzyl penicillin was able to reduce the biofilm formation rate by 4 times compared to the nonencapsulated one [179].

Drulis-Kawa et al. tested *in vitro* thirteen different liposomal lipid compositions, eight cationic, two neutral, and three anionic, containing meropenem and gentamicin against *P. aeruginosa*, *K. pneumoniae*, *E. coli*, and *S. aureus*. Regardless of the antibiotic agent transported, cationic liposomes were more effective than neutral and anionic ones both against Gram-negative and Gram-positive bacteria. In particular, two cationic formulations led to a greater decrease in MIC, PC/DOPE/DOTAP 3 : 4 : 3, which also contained the fusogenic lipid DOPE (see Section 12), and PC/Chol/DOTAP 3 : 4 : 3 [180]. As regard antibiotics already in possession of a broad and excellent antimicrobial activity such as cefepime, cationic liposomes have not increased efficacy but in any case carriers have been found able to guarantee protection from degradation, decrease of adverse effects, and better encapsulation-efficiency percentage (EE%) compared to conventional ones [181]. Thanks to their ability to degrade peptidoglycan, bacteriophage endolysins have an antimicrobial potential, which is interesting only for Gram-positive bacteria. Bai et al. have encapsulated the phage-derived endolysin BSP16Lys in cationic liposomes (hexadecylamine as cationic lipid) with the hope of extending its spectrum of action to Gram-negative. The formulation, tested against *E. coli* and *S. typhimurium*, has proven effective in reducing cell viability [182].

12. Fusogenic Liposomes

Fusogenic liposomes (Fluidosomes®) have soft lipid bilayers thanks to the presence of lipids such as dioleoylphosphatidylethanolamine (DOPE) and cholesterolhemisuccinate (CHEMS) which increase their fluidity to promote

fusion with biological membranes and in particular with microbial ones [183, 184] even and especially if these latter, due to the resistance mechanisms, constitute a significant barrier to permeability. In this way, in addition to protecting drugs from enzymatic inactivation, they also allow to surmount resistance mechanisms [185]. Moreover, fluid liposomal drug formulations improve antibiofilm activity of various antibiotics [186]. Ultimately, fusogenic liposomes, together with the already discussed cationic liposomes, thanks to their ability to facilitate interaction between liposomes and bacteria microorganisms and their biofilms, are considered an optimal delivery system for antibiotic agents [187].

Gram-negative bacteria are intrinsically resistant to glycopeptide antibiotics such as vancomycin, as these are unable to cross their outer membrane. With the aim of widening vancomycin antibiotic spectrum also to Gram-negative bacteria and allowing drug accumulation in the periplasmic space, Nicolosi et al. prepared fusogenic and pH-sensitive SUVs consisting of a lipid mixture 4 : 2 : 4 of DOPE/DPPC/-CHEMS and loaded with vancomycin. Their antibiotic activity was tested on clinical isolates of *E. coli* and *A. baumannii* and compared to that of conventional liposomes loaded with vancomycin and free vancomycin. Neither free vancomycin nor vancomycin encapsulated in nonfusogenic liposomes demonstrated bactericidal activity against the two tested Gram-negative strains, while vancomycin loaded in fusogenic liposomes showed a MIC value of 6 mg/L both against both tested strains. This is an extremely interesting data, especially considering that *A. baumannii* is sensitive to a few antibiotic molecules [184].

Fusidic acid is a lipophilic bacteriostatic agent, which due to its structure has difficulty passing through the bacterial membrane; this translates into an activity limited to Gram-positive bacteria only. Fusidic acid resistant strains that demonstrate a reduction in cell membrane permeability are very common. Thus, in order to facilitate its penetration through the bacterial cell membrane both in resistant and nonresistant strains, fusidic acid was loaded into fusogenic small unilamellar liposomes and its activity was tested *in vitro* against 25 wild strains of Gram-positive and Gram-negative bacteria including *S. aureus*, *S. epidermidis*, *K. pneumoniae*, *A. baumannii*, *E. coli*, and *P. aeruginosa*. Liposomal fusidic acid, compared to the free one whose efficacy has been confirmed only and exclusively on Gram-positive strains, exhibited augmented growth inhibitory activity against Gram-positive strains and an additional action against Gram-negative bacteria. With regard to Gram-positives, the lowest MIC is that against *S. epidermidis* which passes from 0.25 μg/mL (free fusidic acid) to 0.15 μg/mL, while for Gram-negative the lowest value is that against *A. baumannii*, 37.5 μg/mL [183].

13. Liposomal Codelivery

It is well known that combined and simultaneous transport of multiple active ingredients has enormous advantages, first of all, because if two molecules show a synergistic mechanism of action this involves a reduction in dosages, an increase in efficacy, and a decrease in toxic effects. This principle can also be translated to antibiotic agent codelivery or to combined transport of an antibiotic and other molecules or substances with known antimicrobial activities. In this case, the combination and synergy between different mechanisms of action allow fighting infections that are difficult to eradicate with monotherapy alone and circumvent resistance mechanisms. For these purposes, several liposomal formulations have been conceived. Cyclic lipopeptide daptomycin has a renowned potent antibiotic activity against Gram-positive bacteria; daptomycin-resistant strains are rare, but among these, there is certainly MRSA. PEGylated liposomes loaded with daptomycin alone, with clarithromycin alone, or with a mixture of daptomycin and clarithromycin with an optimized ratio of 1 : 32, were tested on MRSA infected-mice. In coencapsulated PEG-liposomes clarithromycin presence allowed use of only 1 : 30 of the dose of daptomycin present in liposomes with only daptomycin. The cotransport of daptomycin with clarithromycin was found to be a more effective liposomal delivery system against MRSA and less toxic than liposomes containing individual antibiotics giving that the amount of daptomycin used was one-thirtieth [188]. Colistin (polymyxin E) is a last-line antibiotic therapy for the infections caused by MDR Gram-negative pathogens including *P. aeruginosa* due to the rapid onset of resistant strains and i.v. dose-dependent nephrotoxicity. Ciprofloxacin is a first-line therapeutic agent but nonetheless not free from resistance phenomena. Colistin-ciprofloxacin combination, the first nebulized and the second administered orally, has proven to be an effective therapeutic approach against multi-drug-resistant (MDR) *P.aeruginosa* responsible for pneumonia associated with cystic fibrosis [189]. Colistin and ciprofloxacin show a synergistic action as colistin interacts with outer membrane lipids increasing ciprofloxacin membrane permeation. To fully utilize their synergism of action against *P. aeruginosa* pulmonary infections, Wang et al. made anionic liposomes containing colistin and ciprofloxacin for pulmonary codelivery and analyzed cytotoxicity *in vitro* in the A549 human lung epithelial cells and antimicrobial activity against clinical isolate resistant strains to both drugs. The results showed that this new liposomal formulation was safe to be used for pulmonary delivery and preserved drug synergism [190].

Bacterial biofilms are of extreme concern because they are fortified bacterial communities invisible to the immune system and resistant to antibiotics. To effectively treat bacterial biofilm infections, Hou et al. designed a lysozyme-associated liposomal gentamicin consisting of negatively charged liposomes loaded with gentamicin and stabilized with cationic lysozyme. The authors tested this new formulation against *P. aeruginosa* and *S. aureus* biofilms and compared the effects obtained on the biomass and living cells of the biofilm with those of gentamicin and lysozyme alone. The new LLG formulation was more effective in destroying and preventing the formation of biofilms of both Gram-positive and Gram-negative bacteria [191].

Some metals known for their antimicrobial characteristics including gallium, bismuth, and silver, have also been coencapsulated in liposomal transport systems together with other antibiotic substances. Liposomal gentamicin

formulation with gallium metal (Lipo-Ga-GEN) was created and tested against *P. aeruginosa* by Halwani et al. Gentamicin delivered inside Lipo-Ga-GEN in low quantities (0.94 mg/L) is more effective against the highly resistant strain of *P. aeruginosa* (PA-48913) (MIC value of 2 mg/L) in reducing quorum-sensing molecules and eradicating the biofilm, compared to free gentamicin (MIC value of 256 mg/L) [192].

Tobramycin and bismuth-ethanedithiol coencapsulated in liposomal formulation (LipoBiEDT-TOB) lowered the levels of *P. aeruginosa* quorum-sensing signal molecules in a consistent way than respective free forms, preventing more biofilm formation. Even in the case of preconstituted biofilms, the efficacy of the liposomal formulation is greater than tobramycin and bismuth alone, as liposomes penetrated and killed bacteria inside the biofilms [193]. The same formulation of LipoBiEDT-TOB was administered to rats chronically infected with *P. aeruginosa* in reducing the production of quorum-sensing molecules and virulence factors; therefore, the authors have suggested it as a probable treatment for the management of chronic pulmonary infection in cystic fibrosis patients [194]. The antimicrobial essential oil of *Melaleuca alternifolia* (tea tree oil) [195] and silver ions were coencapsulated at subminimal lethal concentrations in a controlled release liposomal carrier and added to suspension cultures of *P. aeruginosa*, *S. aureus*, and *C. albicans*. The formulation augmented antimicrobial efficacy of the individual agents despite the low concentrations of the two components. It guaranteed a controlled release of antimicrobials, low toxicity, and optimal therapeutic performance [196].

14. Ligand-Targeted Liposomes

With the liposomal technologies described so far, i.e., by varying liposome size, membrane fluidity, surface charge, and greater or lesser hydrophobicity, a nonspecific passive target recognition mechanism is achieved. Bacterial infection triggers the inflammatory process that causes a vascular permeability increase through its mediators, and dysfunction in lymphatic drainage. Both involve passive targeting at the infection sites. To obtain active targeting, the specificity deriving from molecular recognition between a liposomal surface ligand and its receptor is required [197]; for this purpose, the liposome surface is empowered with different types of molecules including mono- and oligosaccharides, proteins, antibodies, and fragments of antibodies. Antibodies, which recognize surface antigenic determinants of *Streptococcus oralis*, responsible for dental plaque, have been conjugated to liposomes by binding to DOPE. The immunoliposome antioralis was developed; despite having lower affinity for *S. oralis* than free antibodies, it showed greater affinity and therefore greater antimicrobial activity than unmodified liposomes [198].

As known, vancomycin glycopeptide is poorly absorbed after oral administration. Intestinal cells contain folic acid receptors; therefore, to increase vancomycin oral uptake, Anderson et al. trapped the glycopeptide inside folic acid-coated anionic liposomes (folic acid-poly(ethylene oxide)-cholesterol). The formulation was tested *in vitro* in a Caco-2 cell model and *in vivo* in male Sprague-Dawley rats. Vancomycin Caco-2 cell uptake was increased by 5.7-fold, while the relative bioavailability of vancomycin carried by uncoated and folic acid-coated liposomes was augmented respectively by 3.9-fold and 12.5-fold compared to free vancomycin [199]. Mannose-modified liposomes recognize mannose receptors highly expressed by macrophages and dendritic cells, so ciprofloxacin transported inside them can be conducted towards alveolar macrophages and is therefore more effective than that encapsulated in conventional liposomes [200]. Bardonnet et al. developed liposomes in which were present tetraethylene glycol oside, ligands for *H. pylori* adhesins, to transport antibiotics against *Helicobacter pylori*. The glycosylated vesicles were stable at acidic pH while still guaranteeing pH 4 in the internal aqueous compartment [201]. Thanks to the mucoadhesive properties of chitosan, chitosan-coated liposomes encapsulated with rifampicin have been successfully used as a carrier for the delivery of rifampicin to lungs by nebulisation [202].

S-thanatin is a synthetic antimicrobial peptide analogous to thanatin, which unlike this has a threonine instead of a serine in position 15 and a broader-spectrum activity [203]. Since membrane phospholipid represents target for *S*-thanatin [203], Fan et al. wanted to employ its bacterial affinity to develop a liposomal carrier with specific bacterial targeting by binding Ts to the liposomal surface through a PEG-linker (Ts-LPS). Due to the fact that Ts is positively charged, it is electrostatically attracted to negatively charged bacterial surfaces and intercalates at the cell membrane level promoting its fusion with liposomal bilayers. The authors encapsulated levofloxacin inside Ts-LPs and tested the activity against 17 multi-drug-resistant clinically isolated *K. pneumoniae* strains. MIC values were found 2-16 times lower than that of free levofloxacin. According to the authors, a possible explanation is as follows: Ts guarantees the selective bacterial targeting as it anchors the bacterial envelopes interacting with the negatively charged components; it favours the fusion of liposome with the bacterial cell thus compromising the bacterial membrane integrity, the electron transport chain, and the pump outflow. All of these have two important consequences: an increase in uptake and a decrease in outflow of levofloxacin [204]. Lectins are carbohydrate-binding proteins involved in cellular/molecular recognition processes and in attachment and binding of bacteria and virus sugars. Therefore, lectins conjugated to the surface of liposomal carriers can specifically localize the release of encapsulated drugs. Cationic liposomes (stearylamine) loaded with metronidazole and coated with concanavaline-A lectin were tested *in vitro* against *Streptococcus mutans* biofilm. The liposomal formulation, stable under various pH conditions, showed nearly 100% bacterial growth inhibition. According to the authors, conc-A allows liposomal targeting transport and specific release of metronidazole to the surface "glycocalyx" of bacterial biofilm [205]. Wheat germ agglutinin (WGA) is a lectin and agglutinin naturally produced from wheat to protect itself from pathogens such as insects, fungi, and bacteria. Thanks to its bioadhesive properties, WGA acts as a targeting ligand for liposomes enhancing their binding to oral cells. Wijetunge et al. prepared WGA liposomes encapsulating amoxicillin and assessed their therapeutic potential

against *Streptococcus mutans*, often responsible for oral ulcerative lesions, in an oral epithelial–bacterial coculture system. WGA liposomes have shown *in vitro* a sustained release of amoxicillin, a 48 h stay in oral cells, and a significant reduction in oral cell damage [206].

15. pH-Responsive Liposomes

The great versatility of liposomal carriers allows incorporating into them stimuli-responsive biomaterials which as a consequence to changes in the microenvironment such as pH, temperature, light, and redox potential, and induces liposomal bilayer destabilization by triggering specific release of active pharmaceutical ingredients [207]. Therefore, stimuli-responsive liposomes allow for programmable release of a drug in response to exogenous or endogenous stimuli. pH changes in healthy tissues are associated with the onset of various pathological processes, first of all cancer but also diabetes, Huntington's disease, and bacterial infections [208]. Since infection sites and cellular endosomal compartments, as well as the tumor microenvironment, have in common an acidic pH, pH-sensitive drug delivery systems allow for a site-specific release of the drug that, as regard antibiotic drugs this means reaching higher concentrations at the infection site, higher intracellular/endosomal concentrations necessary to eradicate intracellular persistent infections, greater antibacterial efficacy, and lesser appearance of resistance mechanisms. For these reasons, pH-responsive liposomes are the most used stimuli-responsive liposomes in targeting antibiotics.

The release of the transported drug depends on the pH of the microenvironment surrounding liposome and on the pKa values of the bilayer constituent molecules, because protonation/deprotonation rates of the functional groups vary resulting in morphological modifications and changes in permeability of the liposomal membrane [207]. Zwitterionic lipids such as phosphatidylcholine and phosphatidylethanolamine and their derivatives, and amino-acid-based lipids with primary amines have been used to develop pH-sensitive liposomes [188, 209]. Since zwitterionic lipids possess both acidic and basic functional groups, in physiological conditions they are negatively charged (pH > pI), while in an acidic environment they have a positive charge (pH < pI) which favours their interaction with bacteria [210].

Despite the advantages that a pH-sensitive release can confer to antibiotic drugs, most pH-responsive liposomal systems have been developed for the active targeting of anticancer drugs, and only in recent times has this new programmable release modality catch on also in the bacterial infection field.

The aminoglycoside gentamycin is an antibiotic with a broad-spectrum antibacterial activity but which due to its hydrophilicity scarcely crosses the cell membranes and is therefore not able to reach lethal intracellular concentrations; this represents an obvious disadvantage in fighting infections caused by intracellular pathogens. To facilitate the intracellular transport of gentamycin, Cordeiro et al. [211] encapsulated it in a pH-sensitive fusogenic liposome carrier previously studied which allowed *in vitro* the fusion of the vescicle in dependence on the pH thanks to the presence of DOPE and the pH-sensitive lipid *N*-succinyl DOPE [212]. The authors characterized its efficacy *in vivo*, and the results showed that the liposomal encapsulation of gentamycin increased plasma concentrations of the antibiotic, precluded drug accumulation in the kidneys (toxicity site), and redirected the antibiotic to the liver and spleen, and that finally, thanks to its sensitivity to pH, the formulation enhanced the intracellular delivery of the drug increasing its antibacterial activity by 104 times [211]. Recently Jadhav et al. developed new pH-sensitive lipids consisting of three hydrocarbon tails (C18), a carboxylic group, and a secondary amino group for the transport of vancomycin. The presence of a secondary amine, more basic than primary amine usually used in pH-sensitive lipids, guarantees easier protonation in acid medium and therefore an immediate responsiveness of the carrier. The acidic pH induces structural modifications of the lipids and deformation of this new pH-sensitive liposomal formulation allowing an increased and prolonged release of vancomycin. The antibacterial efficacy of the carrier was tested *in vitro* e *in vivo*. MIC values found *in vitro* against *S. aureus* and MRSA at pH 6.5 were lower than MICs at pH 7.4; subsequently, these findings were also confirmed *in vivo* against MRSA in a mouse skin infection model [209].

Omolo et al. have reported in a recent work the creation of a pH-sensitive liposome with "On" and "Off" switches thanks to the synthesis and insertion in the bilayers of a biosafe quaternary lipid (oleic-acid-derived quaternary lipid (QL)) and oleic acid (OA). Based on the MD simulations performed, QL and OA constitute a supramolecular complex in the liposomal membrane that acquires "Off" conformation at basic pH and has an "On"' configuration at acid pH, allowing the pH-sensitive release of the drug. The change in pH conditions and deprotonation/protonation states influencing the two lipids interactions are as follows: at basic pH, OA is deprotonated and therefore negatively charged and interacts with quaternary nitrogen of QL ("Off" position), while at acidic pH (positively charged liposome), OA is in neutral form while QL remains positive; this entails a slight repulsion between the two components of the complex which thus undergoes a rearrangement to the "On" position. The authors encapsulated vancomycin in the OA-QL liposomes and first studied the *in vitro* drug release, then determined the MIC values against both MSSA and MRSA and finally tested the antibacterial activity *in vivo* on a mouse skin infection model. *In vitro* studies have shown that the structural and charge liposomal changes occurring at acidic pH act synergistically as the former leads to an increase in the release at the infection site and the latter guarantees a positive charge that favours interaction with the negatively charged bacterial surfaces. All this increases the specificity of targeting. Compared to free vancomycin, the MIC values at pH 7.4 were 4-fold lower both against *S. aureus* and MRSA, while at pH 6 the decrease was more consistent, respectively, of 8- and 16-fold. The OA-QL liposomes exhibited their efficacy in eliminating MRSA infections even in animal models, since in the untreated group skin samples, there were signs of inflammation and abscesses while the groups treated with OA-QL had none [208].

16. Dendrimers

The dendrimers are symmetric multibranched macromolecules consisting of a central nucleus from which branching units originate all around and terminate with superficial functional groups. Precisely, the term dendrimer does not refer to a molecule itself but to a defined topological pattern and to the ordered and hyperamified nanoarchitecture that recalls that of a tree; in fact, dendrimer derives from the Greek word "dendron" which means tree, but also that of neuronal dendrites [213]. Therefore, theoretically, dendrimers can be constituted by any repetitive building block molecule increasing exponentially the number of possible dendrimer molecules that can be developed.

Dendrimeric molecules are obtained by means of repeated condensations of the same unit through two types of synthetic approaches: divergent, from the core to the periphery, or convergent, from the periphery to the nucleus, up to a size between 2 and 5 nm [214]. Thus, a globular structure composed of three architectural regions is carried out: the central nucleus or multifunctional core, the branches called "dendron" attached to the core where each ramification represents a generation (G-1, G-2, and G-3), and finally the peripheral active sites [214]. The research field of dendrimers is a hastily growing area since these arborescent nanostructures have attracted attention thanks to a series of characteristics and unique properties that make them expendable in different application fields including drug delivery and antimicrobial applications. Their nanometric dimensions, in addition to the presence of a large number of cavities and branches where active sites are widely present, guarantee a high surface area-to-volume ratio which is ideal for loading large quantities of antibiotic agents and greatly promotes interactions with microorganisms and not least interesting, molecules with antibiotic activity can act as a constitutive unit of the dendrimer [215]. For this reason, dendrimers represent new excellent candidates both as nanobactericides, considering that some of them have proven intrinsic antibacterial activities including cationic dendrimers, and also as nanocarriers capable of boosting therapeutic activity of old antibiotic molecules. Nanomolecules with positively charged surfaces are known to have antimicrobial potential because they can establish contact with negatively charged bacterial envelopes through electrostatic interactions. This general principle, already evaluated for the other nanoparticles mentioned above in this review, also can be extended to dendrimers. Biocidal dendrimers are therefore positively charged and contain amine or tetraalkyl ammonium groups as functional groups [214, 216], while anionic dendrimers show no efficacy [217]. PAMAM dendrimers are active both against sensitive and resistant bacteria [218] and following electrostatic interaction with bacterial surfaces cause membrane disruption and loss of cytoplasmic material. Unfortunately, parallel to the favourable microbicide activity, biocide dendrimers show toxicity towards mammalian cells. To overcome this obstacle while preserving therapeutic efficacy, Jevprasesphanth et al. modified the amino groups of cationic PAMAM dendrimers with PEG chains [219]. Functionalizing dendrimers with quaternary ammonium salts (QAC) increases their antimicrobial activity compared to the same nonassociated salts because it allows the achievement of high surface concentrations [220]. Silver complexes of PAMAM dendrimers and silver-PAMAM dendrimer nanocomposites have shown considerable antimicrobial activity *in vitro* against *S. aureus*, *P. aeruginosa*, and *E. coli* [221]. Dendrimers made of polyglycerol, functionalized with chitosan, and complexed with boron were highly viscous, biocompatible, and effective bactericides against *S. aureus*, hence they have been studied as antimicrobial coating [222]. The production of an antimicrobial peptide in dendrimeric form, in addition to increasing its stability to peptidases and proteases, enhances antimicrobial activity compared to the monomeric form [223]. Amphiphilic dendritic dipeptides self-assemble in solution and act as pore-forming proteins positioning in the membranes altering permeability [224].

The antimicrobial peptide dendrimer G3KL composed only of natural lysine and leucine residues alternating in the branches, was found to be mildly toxic to human red blood cells and effective against multi-drug-resistant *P. aeruginosa* and *A. baumannii* [225, 226]. Antimicrobial dendrimeric peptides have also been shown to be effective in preventing the formation of bacterial biofilms, hindering their development and destroying also mature biofilms [227]. Multivalent fucosyl-peptide dendrimers have been found effective in inhibiting *P. aeruginosa* LecB protein preventing bacterium attachment on the tissue surfaces and the biofilm formation [228]. Reymond et al. also investigated whether biofilm formation could be inhibited by blocking the action of LecA and LecB lectins from *P. aeruginosa*. The four glycopeptide dendrimers synthesized with high affinity to the lectins, two each, were efficient in blocking *P. aeruginosa* biofilm formation and also in inducing dispersal biofilm *in vitro* [229]. The presence of the pharmacophore dipeptide tryptophan and arginine within antimicrobial dendrimeric peptides enhances antibacterial activity and membrane selectivity as tryptophan guarantees lipophilicity and arginine the positive charge [230]. Bahar et al. developed the arginine-tryptophan-arginine 2D-24 dendrimeric peptide that has proven effective against *P. aeruginosa* normal planktonic and persister cells and also against *P. aeruginosa* biofilm cells, as it is able to penetrate the biofilm matrix [231].

The high surface area of the dendrimers guarantees a high number of interaction/reaction sites; the multivalent surfaces are not only related to the innate antimicrobial activity of some of them as already seen, but also make dendrimers an ideal platform for antibiotic loading and delivery among other things with the advantage of carrying both hydrophobic and hydrophilic drugs, the first ones inside internal cavities and the last ones on the surfaces [215]. Dendrimer multivalence combined with their arborescent topological configuration confers a huge advantage in the fight against bacterial infections as the enormous surface area ensures a large number of active sites available for antibiotic molecule conjugation and accessible surfaces for interaction with the bacteria. Encouraging contact between high concentrations of antibiotics and bacteria contributes to discouraging the onset of antibiotic resistance. Different dendrimers

have been used as drug delivery systems (DDSDDS), but certainly the most studied are PAMAM dendrimers.

Sulfamethoxazole (SMZ) loaded on PAMAM dendrimers, unlike pure SMZ, is soluble in water, shows prolonged release, and has bactericidal activity from 4- to 8-fold greater against *E. coli* [232]. PAMAM dendrimers have also proven ideal and biocompatible carriers of water insoluble quinolone antimicrobials such as nadifloxacin and prulifloxacin, considering that they exhibited increased solubility and antimicrobial activities [233]. Vancomycin conjugated to PAMAM dendrimers active sites manifested an increased bond avidity towards two cell wall models, one susceptible, (D)-Ala-(D)-Ala, and the other one resistant, (D)-Ala-(D)-Lac, to vancomycin. Choi et al. exploited these high-avidity multivalent vancomycin dendrimer-based nanosystems as ligands for bacteria targeting of iron oxide nanoparticles to achieve rapid uptake by bacterial cells [234].

Erythromycin macrolide is slightly soluble in water, but when it is transported by both hydrosoluble cationic (PAMAM-NH2) and anionic (PAMAM-OH) dendrimers, its solubility increases by 8- and 7-fold respectively. Regarding the antibacterial activity against *S. aureus*, erythromycin conjugated to PAMAM dendrimers has a slightly positive effect compared to the free antibiotic; the MBC values were found lower than 2-fold in the case of anionic and 4-fold dendrimers in case of cationic dendrimers, thus confirming the innate antibacterial activity of the latter [235].

Like PAMAM, poly(propylene imine) dendrimers (PPI) have also proved to be optimal DDS in boosting the therapeutic power of antibiotic agents. Coadministration of low doses of nadifloxacin and PPI-G4 dendrimers is more effective against *E. coli*, *P. aeruginosa*, and *P. hauseri* than higher doses of the antibiotic alone [236]. The PAMAM dendrimer G-4 has been successfully employed as intracellular drug delivery vehicles of azithromycin in both *Chlamydia*-infected HEp-2 cells and chlamydial inclusions [237]. Hydroxypyridinones (HPs) are a family of N-heterocyclic metal chelators with proven antimicrobial activities [238]. Zou et al. developed hydroxypyridinone hexadentate-based dendrimers and tested their antimicrobial activity alone and in combination with norfloxacin against *S. aureus* and *E. coli*.

The results obtained confirmed the biostatic activity of the HPs both against Gram-positive and Gram-positive bacteria and in addition highlight a strong synergistic bactericidal effect between norfloxacin and HPs. Evidently, iron deprivation makes bacteria more sensitive to the antibiotic [239]. Like other multifunctional nanoparticles, dendrimers can also act as targeted drug delivery of drugs, making antibiotic treatments more localized, and therefore more effective, and safer. In *Chlamydia*-infected tissues, there is an overexpression of folate receptors so folate-functionalized dendrimers are efficient DDS for antibiotics and anti-inflammatory drugs to attenuate infection and associated inflammation caused by *Chlamydia* [240]. The need for antibiotic targeting is even more acute when the infections to be defeated are located in poorly perfused tissues such as wounds. Wong et al. developed a light-controlled dendrimeric nanocarrier with cell wall targeting for specific delivery of ciprofloxacin to Gram-negative bacteria for wound treatments. In particular, the authors attached photocaged ciprofloxacin to an LPS-targeted PAMAM (G5) dendrimer as it is conjugated to ligands of the outer membrane. Such a dendrimer simultaneously allows a localized and stimulus-controlled release of the payload at the Gram-negative cell wall, which can be used in light-based therapies to treat infected wounds [241]. Many works on dendrimers have confirmed on one hand the cationic dendrimers' possession of innate antimicrobial activity but at the same time of toxicity towards mammalian cells and on the other hand declared that anionic dendrimers are not intrinsic antimicrobials and are not very toxic. Wrońska et al. recently evaluated the antibacterial activity of levofloxacin coadministered with a maltose-modified third-generation PPI dendrimer (cationic) and with an anionic phosphorus dendrimer AN-G4. The antimicrobial activity has been tested against *P. hauseri*, *E. coli*, and *S. aureus*. Both cationic and anionic dendrimers have shown a synergistic effect with levofloxacin with the advantage of making effective lower doses of antibiotics and using anionic dendrimers instead of cationic ones so as to reduce toxicity, environmental pollution, and why not, the appearance of antibiotic resistance [242]. Another fluoroquinolone, ciprofloxacin, also showed synergistic effects with dendrimers. Svenningsen et al. conjugated it to a G0 DAB-core PAMAM dendrimer and tested the complexes against four different clinically relevant bacterial strains, some of which are antibiotic-resistant bacterial strains: *Enterococcus faecalis* vancomycin-resistant, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Salmonella enterica*. The MIC values of the conjugated dendrimers and the unconjugated control dendrimer were obtained by microbroth dilution assay. Control nonconjugate dendrimer lacks antibacterial activity because in place of amino terminal groups it had acetyl groups (MIC > 256 mg/L), while ciprofloxacin-dendrimer conjugates demonstrated an increased antimicrobial activity against all four bacterial strains examined (MIC values between 0.25 and 0.5 mg/L). The authors explained the complete lack of antibacterial activity of the nonconjugated dendrimer as well as the increased activity of the conjugates with the presence of a synergy related to the action mechanism of ciprofloxacin. Fluoroquinolone noncovalently binds topoisomerases (mainly topoisomerase II in Gram-negative and topoisomerase IV in Gram-positive) stabilizing them by preventing the development of DNA;. PAMAM dendrimers on the other hand are renowned for promoting gene transfection; therefore, it is likely that PAMAM dendrimers enhance DNA binding by promoting the activity of ciprofloxacin [243].

17. Conclusions

This review was aimed at providing a detailed and comprehensive knowledge of the recent achievements in the field of nanoparticulate antibiotic systems analyzing the research papers available. The final reference list of 245 articles was selected based on keywords shown in Figure 5.

Among all the major countries are the United States, China, India, Italy, United Kingdom, and Canada as shown in Figure 6.

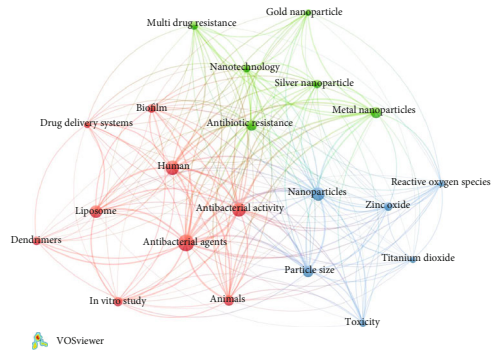

Figure 5: Representation of keywords.

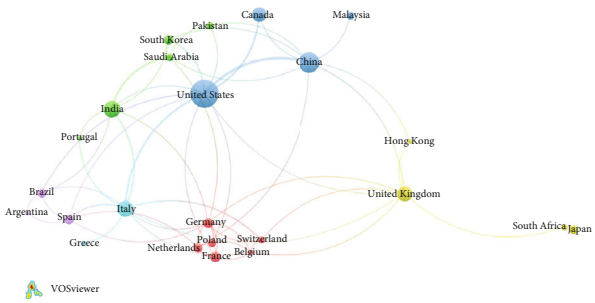

Figure 6: Representation of the distribution of authors' country of origin.

In addition to representing the predominant life form on Earth, bacteria are among the most resilient organisms thanks to their continuous ability to evolve and adapt to different environmental contexts. An appropriate and effective antibacterial therapy cannot disregard the latter fundamental notion. For centuries, humankind has lived at the mercy of continuous epidemics, which, in addition to causing the death of thousands of human lives, have hindered human progress in various fields, until Fleming's serendipitous discovery of penicillin turned the tables in our favour. That was the turning point in a battle against bacterial infections, but as we all know, it did not allow us to win the war. With the introduction of antibiotics in therapy, we basically triggered the start of a natural selection process so as with the passage of time, bacteria have learned to survive by adapting to our "magic bullets." Antibiotic resistance and more generally antimicrobial resistance, is a worldwide problem due to both the epidemiological and economic impact that requires a multitasking approach to be managed. According to estimates by the British Government, in 2050 AMR could cause the death of 10 million people per year, to which must be added the clinical and financial burden that could lead to the collapse of worldwide health systems. To avoid this apocalyptic scenario, it is our duty to reinvent the "old" antibiotics in the light of the most innovative strategies in order to make them effective as long as possible. Since most bacteria have an average diameter between 0.2 and 2 μm, nanotechnology represents a pioneering research field that allows us to develop nanoparticulate systems that not only can represent valid antibiotic delivery systems but which, when also equipped with innate antibacterial activities, can also act as an antibacterial alone or in synergy with the loaded drug. Scientific literature is full of demonstrations of the enormous therapeutic potential of "nanoantibiotics" which, thanks to their nanometric dimensions, the high surface/volume ratio and the possibility of a targeted delivery, have the ability to reach infection sites, anchor themselves to the bacterial cell wall and, if they have appropriate size, to penetrate through it. Nanobactericides are all those nanoparticle systems that show innate antibacterial properties, such as the nanoparticles of some metals and metal oxides [244, 245], but also the cationic liposomes and dendrimers including the cationic and PAMAM ones. They act through multiples and nonspecific antibacterial mechanisms; this on the one hand certainly does not cancel out but reduces the probability that resistance may arise against them but on the other hand makes them unspecific bactericidal agents and therefore often toxic to mammalian cells. Reasonably, this disadvantage can only be mitigated by thinking in terms of increasing concentration on the active site through active targeting. The aforementioned nanobactericides, as well as other nanoparticle systems without intrinsic antibacterial activity such as the majority of liposomes and dendrimers, have proved to be excellent drug delivery systems able to give a new life to the "old" antibiotics. Indeed these nanoparticulate delivery platforms improve drug pharmacokinetic/pharmacodynamic characteristics allowing them to overcome the various limitations related to the drugs and the pathogens and last, but not least, they permit multiple antibiotic molecule codelivery. All that disadvantage the therapeutic failure and therefore the development of resistance phenomena. Moreover, nanobactericides used by nanocarriers often have the advantage of acting synergistically further enhancing therapeutic efficacy. In conclusion, the field of nanotechnology provides a plethora of promising and forward-looking strategies for boosting conventional antibiotic therapies and countering the onset of the huge burden of multidrug resistance.

Data Availability

The data used to support the findings of this study are available from the corresponding authors upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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