Chapter

In vitro Antimicrobial Activity Evaluation of Metal Oxide Nanoparticles

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Abstract

In recent years, infectious diseases, specifically those that are caused by pathogens, have seen a dramatic proliferation due to resistance to multiple antibiotics, opening the colony by opportunistic pathogens. Nanotechnology and tissue engineering have been applied in the development of new antimicrobial therapies, capable of fighting opportunistic infections. In the medical field, research on antimicrobial properties of metal oxide nanoparticles have emerged to find new antimicrobial agents as an alternative against resistant bacteria. The metal oxides, particularly those formed by transition metals are compounds with electronic properties, and most magnetic phenomena involve this type of oxides. Nanoparticles-based metal oxide properties such as shape, size, roughness, zeta potential and their large surface area, make oxides ideal candidates to interact with bacteria and able to have an antimicrobial effectiveness. The aim of this chapter is to offer an updated panorama about the relationships between the use of metal oxide nanoparticles in the medical field, with an emphasis on their role as antimicrobial agents and the properties that influence their antimicrobial response. In addition, the mechanism of nano-antimicrobial action is described and the importance of using in vitro test methods, adopted by leading international regulatory agencies, that can be used to determine the antimicrobial activity of the metal oxide nanoparticles.

Keywords: nanoparticles, metal oxide, antimicrobial, *in vitro*, methods, mechanism of action, nano-antimicrobial

1. Introduction

Infectious diseases are one of the main causes of morbidity and mortality in the world, so there is the need for research on antimicrobial agents. According to the World Health Organization (WHO), resistance to antimicrobials endangers the effectiveness of treatments for an increasing series of infections by bacteria and fungi [1]. In addition, it poses a growing threat to global public health and requires action by all sectors of government, industry, healthcare professions and society. The success of surgery and medical therapy will increasingly be compromised in the absence of effective antibiotics. On the other hand, the dissemination of multidrug-resistant infections will increase the need for laboratory tests and the use of more expensive drugs, thus increasing the cost of healthcare.

In the science of materials, there is an interest in the development of new antimicrobial therapies, capable of fighting opportunistic infections. For this reason, it is important as a first step, to take into account the different laboratory methods that can be used to evaluate antimicrobial activity *in vitro*. The aim of this methods is to detect possible drug resistance in common pathogens [2], but also which are the most appropriate assays to be used in new agents and materials what that have a potential therapy application.

Nanotechnology and tissue engineering are potential applications for the reported antimicrobial properties of nanoparticles (NPs). Some of the potential advantages of NPs, to fight against microorganisms, are that they do not generate resistance and are a safe potential antimicrobial alternative for clinical use [3–5].

However, the research in this area is needed to understand the mechanism of action of NPs and how to design better therapies. In recent years, nanoparticles have been incorporated into the medical field as an alternative to new antimicrobial agents, especially oxides based in silver (Ag), copper (Cu), and titanium (Ti) [6]. On the other hand, findings have raised concerns about their possible toxic effects in humans, triggering an interest to investigate more about the nanotoxicology and the search for new antibacterial nanomaterials with nontoxic properties for human being [7–9].

The antibacterial activity of the NPs depends of the size and shape; so it requires active research of nanometer-scale materials. Recently, basic and applied research has been done on various metal oxides with different shapes and sizes has carried out for their application in a broad scale of areas such as catalysis, in semiconductors, sensors, controlled release of drugs and as antimicrobial agents.

The physical and chemical properties of metal oxide NPs allow their interaction with biological systems, which has become of vital importance due to the increasing resistance of bacteria. Within these properties, there are shape, size, roughness, zeta potential and coatings, among others [5, 10]. The antimicrobial activity presented by the NPs of metal oxides could have a mainly therapeutic application, but it can also be extended to the food industry, to water purification and to the textile industry.

The present chapter will be focused on recent reports that explore the relationships between the use of NPs in the medical field, with an emphasis on their role as antimicrobial agents and the physicochemical properties of metal oxide NPs that influence their antimicrobial response.

Additionally, findings will address antimicrobial activity of novel metal oxides NPs based in zinc, manganese, iron and magnesium. Also, we will discuss diverse methods for the assessment of antimicrobial activity that can have uses for metal oxide NPs and which complies with quality according to official standards to evaluate antimicrobial agents and materials.

2. Antimicrobial applications of nanoparticles

The rapid emergence of resistant pathogens is occurring worldwide, endangering the efficacy of antibiotics, which have pushed medicine to evolve and save millions of lives. Resistance of the pathogens has been attributed to the overuse and misuse of antibiotic medications, as well as the lack of new strategies for antibacterial development to address the challenge [11, 12]. This challenge suggests that the focus of research on resistance pathogens must be turned to the discovery of novel strategies to fight the pathogen infections. One of the new areas that is emerging in

response for this challenging menace is the use of nanotechnology, mainly by the identification of how the manipulation of materials for the synthesis of NPs could be utilized for the therapeutic management of pathogen infections [13].

Research on the synthesis, characterization and application of NPs as an antimicrobial system is a new area of interest in the biomedical and healthcare fields due to the possible enhancement of nanoparticles within their physiochemical behavior against drug-resistant pathogens due to size effect, doping effect, could be cost-effective and they are quite stable enough for long-term storage with a prolonged shelf-life. Moreover, the NPs could be subjected to sterilization by methods of high temperature, gamma irradiation or plasma treatment without losing its properties or inactivation [14].

In reference to the biomedical field, the benefits of nanotechnology have been quite substantial, for example, there are devices with antimicrobial nanoproperties such as heart valves, catheters, and dental implants [10]. The type of nanolayers covering these kind of devices can delay or inhibits the adhesion and growth of bacteria such as *Streptococcus mutans*, *Staphylococcus epidermis*, and *Escherichia coli*. Other implantable material is bone cement based polymethyl methacrylate (PMMA) with Ag nanoparticles that have demonstrated significant reduction in the number of arthroplasty surgery-related infections, including methicillin-resistant *Staphylococcus aureus* (MRSA), *S. aureus*, *S. epidermidis*, and *Acinetobacter baumannii* infections [10, 15].

The application of nanoparticles used to fight against pathogens consist mainly of metals and metal oxides of zinc, silver, copper or titanium, because they naturally exhibit microbiocidal or microbiostatic actions and have demonstrated bactericidal activity against both Gram-positive and Gram-negative bacteria. The bactericidal application of metal NPs is based on the mechanism that affect the respiration system by photocatalytic production of reactive oxygen species (ROS) that damage cellular and viral components that ultimately leads to bacterial death, compromising the bacterial cell wall/membrane, inhibition of enzyme activity and DNA synthesis, interruption of energy transduction and the most important the pathogens do not develop resistance to metal NPs [16, 17].

Besides as metal nanoparticles could target the bacterial cell wall; there is an opportunity to dope the nanoparticles with relevant antibiotics to enhance their antibacterial action through synergy offering multiple advantages as controllable with sustained and relatively uniform distribution release in the target tissue, improving the solubility, minimized side effects and enhanced cellular internalization [14, 18, 19].

3. Metal oxide nanoparticles as antimicrobial agents

The resistance of microorganisms to the action of antimicrobial agents, especially antibiotics, is a serious public health problem, which has been a reason for the search and development of new antimicrobials through nanotechnology.

The manipulation on a nanoscale of metal oxide has provided new research in the pharmaceutical area due to the antimicrobial properties of these oxides, according to data revealed in *in vitro* studies [20, 21]. In this sense, the metal oxide NPs between 1 and 100 nm with different shapes allow their physical and chemical properties could become in some promise antimicrobial agents against infectious diseases for the recent findings about their interaction which has become of vital importance due to the increasing of infection diseases by bacterial resistance [22, 23].

3.1 Mechanisms of antimicrobial activity

There are findings about the potential mechanisms of action, where it attempts to explain the bactericidal effect of metal oxide NPs [10, 24–27]. Some of these include the action of reactive oxygen species (ROS), the electrostatic interaction, accumulation, ions delivered and contact by itself of NPs, that induce a several effects from outside and into the bacteria, and that it will be described below (**Figure 1**).

3.1.1 Formation of reactive oxygen species (ROS)

They are a group of reactive molecules produced in some metabolic processes in which oxygen participates: the superoxide anion O^{2-} which is a powerful oxidizing agent very reactive with water. Hydrogen peroxide H_2O_2 and the hydroxyl radical (•OH) which is the most reactive, since accepting one more electron, gives rise to a water molecule. Metal oxides NPs are capable of producing different reactive oxygen species may participate in different types of reactions in which they can undergo oxidation or reduction processes. ROS produce disruption of DNA, damage by oxidation of polyunsaturated fatty acids and amino acids. The alteration of the balance in the mechanisms of production and elimination of ROS, in favor of production, originates the state of oxidative stress in the bacteria cell. In the case of O_2 and H_2O_2 cause less acute stress reactions and can be neutralized by endogenous antioxidants, such as superoxide and catalase enzymes, while OH^- and O_2 can lead to acute microbial death.

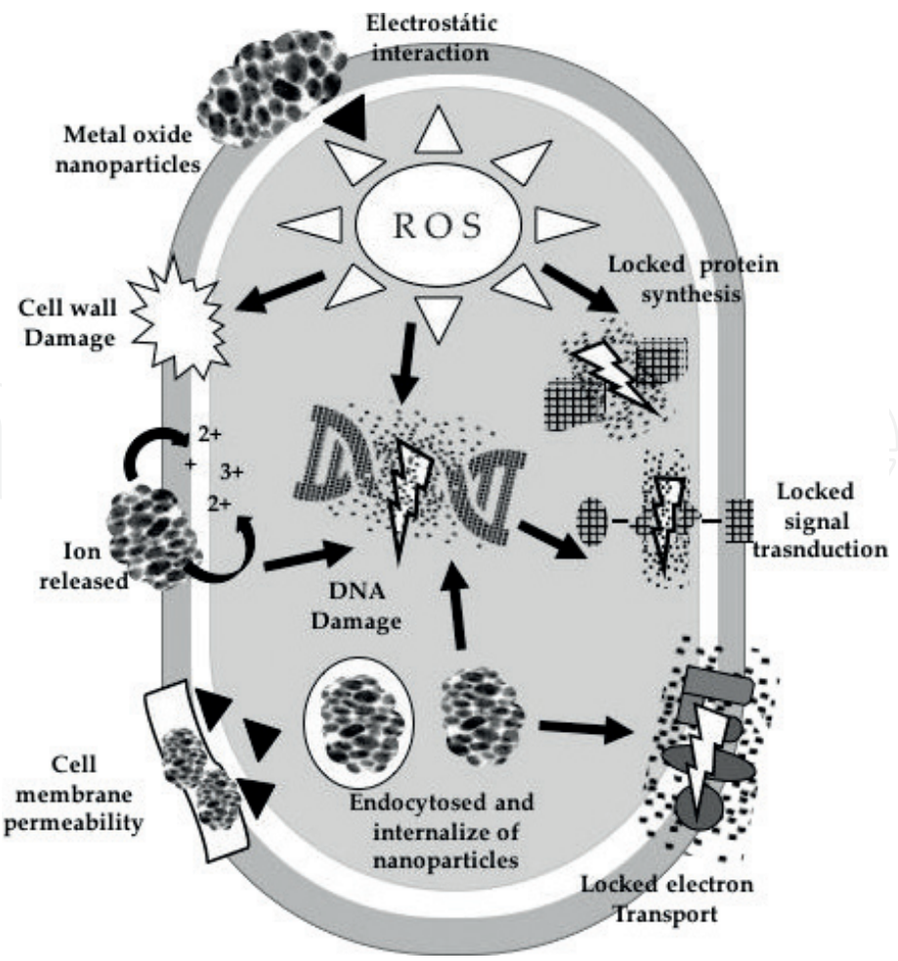

Figure 1.
Mechanisms of action of the bactericidal effect from metal oxide nanoparticles.

3.1.2 Damage to the wall-cell membrane due to electrostatic interaction and accumulation

The electronegative groups of the polysaccharides in the bacterial membrane have an attraction sites by metal cations. The difference in charge between bacterial membranes and the NPs of metal oxides leads to electrostatic attraction and thus accumulates on the bacteria surface, altering the structure and permeability of the cell membrane. Gram-negative bacteria have a higher negative charge than Gram-positive bacteria and therefore the electrostatic interaction will be stronger in Gram-negative strains. The pores of the membranes are in the order of nanometers, therefore the smaller the particle size and the greater the surface area, the greater the efficiency of the metal oxide nanoparticles. In the same way, the cations extracted from the NPs of the metal oxides and their accumulation in the cell wall, create pits in it, leading to a change in permeability due to the sustained release of lipopolysaccharides, membrane proteins and intracellular factors. In addition, this mechanism has been linked to the interruption of the replication of adenosine triphosphate (ATP) and the deoxyribonucleic acid (DNA) of the bacterium, leading to its death. One study indicates that the action of NPs depends on the components and structure of the bacterial cell. The unique components of Gram-negative bacteria, such as LPS, can prevent the adhesion of metal oxides NPs to the barrier of bacterial cells and regulate the flow of ions in and out of the bacterial cell membrane.

3.1.3 Loss of homeostasis by metal ions

The balance of metallic elements is essential for microbial survival, since it regulates metabolic functions by helping coenzymes, cofactors and catalysts. When the bacteria have an excess of metals or metal ions, there will be a disorder in the metabolic functions. Metal ions bind with DNA and alter the helical nature by cross-linking between and within the DNA strands. The metal ions neutralize the charges in LPS and increase the permeabilization of the outer membrane. The ions of metal oxides might also cause the decomposition of bacterial cells due to the diffusion of metal ions by generating large amounts of hydroxyl radicals and diffusion in bacterial cells. Other studies indicate that NPs of metal oxides slowly release metal ions through adsorption, dissolution and hydrolysis; they are toxic and abrasive to bacteria and, therefore, lyse the cells.

3.1.4 Dysfunction of proteins and enzymes

Protein dysfunction is another mode of antibacterial activity exhibited by NPs of metal oxides. The metal ions catalyze the oxidation of the side chains of amino acids resulting in carbonyls bound to proteins. The carboxylation levels within the protein molecule serve as a marker for the oxidative damage of the protein. This carboxylation of proteins will lead to the loss of catalytic activity in the case of enzymes, which finally triggers the degradation of proteins.

3.1.5 Inhibition of the transduction signal

Electrical properties of metal oxide NPs interact with nucleic acids inducing suppress of cell division by altering processes of replication of the chromosomal DNA and the plasmid in microorganism. It is known that signal transduction in bacteria is affected by NPs of metal oxide. Phosphotyrosine is an essential component of mechanism of signal transduction in bacteria. NPs dephosphorylate

the phosphotyrosine residues, which inhibits signal transduction and, ultimately, obstructs growth of bacteria.

3.2 Nanoparticle characteristics and their influence of antimicrobial activity

Other factors of the antimicrobial activity, has been sought to analyze what characteristics influence the microbial response to the action of the metal oxide nanoparticles. It is known of existing reports concerning the chemical-physics properties from the metal oxide nanoparticles, but taking into consideration factors like the shape, size, roughness, zeta potential and coatings, etc., that influence the resultant antimicrobial effectiveness [5, 10]. These results could have a mainly therapeutic application in medicine, but it can also be extended to the food industry, to water purification and to the textile industry [28].

3.2.1 Size and shape

Several reports mention that the size and shape are the most important factors to the antimicrobial activity [23, 29–31]. With respect to size there are findings where this is a crucial factor to damage the bacterial systems for many reasons. The sizes as <30 nm are factors that allows the accumulation and penetration into the bacteria causing damage and consequently leading to bacteria death (<10 nm) [23]. The same authors point out that metal oxide nanoparticles with a size greater than 10 nm promotes the permeability when coming into contact with bacteria [23]. In relation with this, the specific surface area by the nanoparticle size affects the surface to mass ratio affecting on surface reactivity. For this reason, they can also have influence in many direct mechanisms of toxicity against the bacteria and the subsequent loss of viability (**Figure 1**).

With respect to shape, it is by knowing that depending on the synthesis method, it will obtain the form of the nanoparticle [32]. Numerous studies shown various forms obtained like spherical, rod-shaped, truncated triangular, nanotubes, nanorods, nanowires, nanosphere, nanoneedles, nanorings and nanocubic [23, 33, 34]. Evidence reports that needle-shaped metal oxides nanoparticles present higher antibacterial activity than cubic shaped, based on the optical and fluorescence intensity [30].

3.2.2 Surface and zeta potential

The relation between the surface nanoparticle/nanomaterial and bacterial adhesion has not been fully studied and there are few reports about it. Some studies report that the adsorption of bacterial proteins is promoting by the surface area-to-mass ratio carry out the reduction in bacterial adhesion [35–37]. Surface nanomaterials have high degree of roughness, therefore bacteria cell membranes cannot adhere to the surface nanomaterial; so the bacteria adhesion is reduced [10, 38, 39].

The surface charge or zeta potential could be another property of the nanoparticle related with bacteria adhesion since it is important to mention that if the surfaces with negative charge are capable to decrease the interaction with bacteria charged negatively, the surface of nanomaterial with negative charge could obtain the same effect, compromising bacterial adhesion [10, 23, 40]. On the other hand, the electrostatic attraction occurs when the nanoparticles are positively charged promoting the accumulation in bacterial cell membrane, which is negatively charged and then they penetrate inside the bacteria triggering other mechanisms [23] (**Figure 1**).

3.2.3 Chemical doping

Nanoparticle chemical doping is a modification and functionalization around the surface of nanoparticles to regulate and control the interaction with bacteria and enhance their antimicrobial effect. Reports have shown this method as a factor to improve the presence of surface oxygen atoms that promote the production of reactive oxygen species (ROS) [23]. Similarly, the chemical functionalization increase of the surface-area-to-volume ratio results in increasing the antimicrobial potential activity [38]. Also, this procedure has prevented the agglomeration and the solubility in different solutions [10].

3.3 Metal oxide nanoparticles

The transition metal oxides (TMO) are compounds with unique electronic properties, most magnetic phenomena involve this type of oxides. The nanostructures formed by TMO, due to their dimensions of a few nanometers and their large surface area, are ideal candidates to interact with bacteria. It is known that NPs of the silver are excellent antimicrobial agents and they are the more studied and reported. However, *in vitro* and *in vivo* studies indicate that nanoparticles based Ag, Cu and Ti are toxic to mammalian cells derived from the skin, liver, lung, brain, vascular system, and also gives rise to a distribution in other organs, where are in accumulations [8, 9].

Therefore, is important that the different metal oxides nanoparticles will be studied and guarantee it clinical use. In particular, there are reports concerning to zinc oxide (ZnO), trimanganese tetroxide (Mn_3O_4), magnetite (Fe_3O_4) and magnesium oxide (MgO) nanoparticles that have antimicrobial properties.

3.3.1 ZnO nanoparticles

Zinc oxide is a compound with excellent antimicrobial properties. It is an n-type semiconductor with a band gap of 3.3 eV. ZnO NPs can adopt a wide variety of morphologies such as; rings, propellers, belts, wires, among others [41, 42]. The antimicrobial activity of ZnO NPs happens by different mechanisms, one of these is the ROS generation [43] inside the cell. It has been proposed that ZnO NPs can act to generate cell death, or the release of Zn^{2+} ions, whose excess generates an alteration of cellular metabolism. Some species reported as susceptible to ZnO nanoparticles are; *S aureus*, *S. epidermidis*, *Streptococcus pyogenes*, *Enterococcus faecalis* [44], *Bacillus subtilis*, *Escherichia coli* and *Klebsiella pneumonia* [41]. These bacteria can generate intra-hospital infections causing serious infectious diseases and some strains are found in water or food, so ZnO NPs can have a possible application in these areas.

3.3.2 Mn_3O_4 nanoparticles

The trimanganese tetroxide, Mn_3O_4, is a mixed oxide of manganese (Mn (II) Mn $(III)_2O_4$) is a normal spinel and crystallizes in cubic form. It occurs in nature as the hausmannite mineral. The antimicrobials properties of Mn_3O_4 NPs have been little studied. Has been reported an effect of these diseases against strains of *Vibrio cholerae*, *Shigella* sp., *Salmonella* sp., and *E. coli* [45]. The effect of the NPs of Mn_3O_4 against has been evaluated against *E. coli* and *S. aureus* through microdilution assays [46]. The results of the minimum inhibitory concentration (MICs) indicated that the bacteria *E. coli* was more sensitive to the action

of the NPs of Mn_3O_4. It was observed that the inhibitory effect proportionally increases to the concentration of Mn_3O_4 NPs, which could divide the different characteristics of the surfaces of the bacterial cells and their interaction with the NPs, therefore the mechanism of action could be focused on the bacterial wall membrane.

3.3.3 Fe_2O_3 nanoparticles

Iron oxide (III) is a very stable oxide, it crystallizes in hexagonal form and is found in nature as the mineral hematite α-Fe_2O_3. The nanostructures of this oxide take different forms as they are nanowires, nanotubes, nanospheres, etc. [47]. Although its synthesis has been widely studied, its possible antibacterial effect not. The Fe_2O_3 NPs bactericidal effect against *E. coli* and *S. aureus* has been reported, where an increase of this effect is observed, as the concentration of iron oxide NPs increases [48]. A bactericidal effect has also been seen on *P. aeruginosa* with a minimum inhibitory concentration of 0.06 mg/L [49]. Another study reports on the bactericidal activity of nanostructured hematite against a variety of Gram-positive and Gram-negative bacteria; *P. aeruginosa*, *S. aureus*, *K. pneumoniae*, *Lysinibacillus sphaericus* and *Bacillus safensis* [50]; proposing some mechanisms of action depending on the activity observed in each stage of the growth of the bacteria in question. A bactericidal effect of NPs of Fe_2O_3 against *S. epidermidis* has even been determined [51].

From its properties, its possible application in the remediation of the environment and water, as well as in the biomedical area, has been proposed, due to the different studies of cytotoxicity that have been carried out [47].

3.3.4 MgO nanoparticles

Magnesium oxide is in nature as the mineral periclase [52]. The antibacterial activity of MgO against Gram-positive and Gram-negative bacteria has been reported. It has been proposed that MgO NPs can damage the cell membrane causing the loss of intracellular contents and causing the death of bacterial cells [53]. The generation of reactive oxygen species has been attributed to the surface alkalinity of the MgO NPs [54]. The antibacterial activity of NPs of MgO against Gram-negative bacteria has been evaluated; *E. coli* and *P. aeruginosa* (500 and 1000 μg/mL) and in a Gram-positive bacterium; *S. aureus* (1000 μg/mL) [55]. The MgO NPs potentiated lipid peroxidation induced by ultrasound in the liposomal membrane. In this case the mechanism of action could be associated to the presence of defects, or to the lack of oxygen on the surface of the NP, leading to lipid peroxidation and the generation of reactive oxygen species [55]. The antibacterial effect and mechanism of action of NPs of MgO against strains of *Campylobacter jejuni*, *E. coli* and *Salmonella enteritidis* has been studied [56]. In this case, it was observed that the permeability of the bacteria's membrane, after exposure to the MgO NPs, was compromised, finding the presence of hydrogen peroxide that would subsequently cause cell death. Studies of *P. aeruginosa* and *S. aureus* versus MgO NPs showed a greater zone of inhibition in *S. aureus* than in *P. aeruginosa* [56]. Based on previous work, the authors note that the bactericidal action of MgO NPs may be due to the binding of surface oxygen to bacteria. As the surface area of the particles increases, the concentration of oxygen ions on the surface increases, which results in a more effective destruction of the cytoplasmic membrane and the cell wall of the bacteria.

4. *In vitro* methods for antimicrobial evaluation of nanoparticles based metal oxide

Bacteria exposed to antimicrobials are under selective pressure to evolve and adapt, this natural process leads to antimicrobial resistance. Human kind is facing the growing threat of rapid evolution and dissemination of bacteria resistant to multiple antibiotics. There is, therefore, an urgent need to develop new antimicrobials [57, 58].

Antimicrobial agents include disinfectants, antiseptics, and antibiotics. New agents must be exhaustively tested for efficacy and safety. Evidence-based selection of the microorganisms and the evaluation system is of paramount importance for adequate interpretation of the test results, and for extrapolating from *in vitro* to real-life scenarios.

The use of nanoparticles especially based in metal oxides emerge as new antimicrobial agents, therefore it is necessary to test the efficacy of nano-antimicrobials against representative bacterial species. One known limitation of the testing systems currently in use, is that formulations are often challenged *in vitro* with one microbial species at the time, and rarely against multi-species biofilms.

4.1 Regulatory testing

Regulatory agencies require adherence to well established evaluation systems. Regulatory tests applicable to disinfectants, antiseptics or therapeutic antimicrobials vary greatly; and could include to nanoparticles with potential use as antimicrobials.

4.1.1 Disinfectants

When evaluating chemical disinfectants, bacterial endospores are considered the microbial life-form hardest to kill, followed in descendent order by mycobacteria, bacteria in vegetative form, and viruses.

In the United States, the Food and Drug Administration (FDA) regulates chemical sterilants and high level disinfectants (HLD) that are used to reprocess medical instruments [59]. The AOAC International sporicidal and tuberculocidal tests are the accepted methods for evaluation. For a liquid chemical sterilant, the FDA standard tolerates no failures in the AOAC sporicidal test 966.04, and accepts no survivors in simulated-use testing with a challenge inoculum of six logs of spores. The FDA defines HLD as sterilants used under the same contact conditions but for only the contact time needed to reduce *Mycobacterium bovis* in 6 $\log_{10}$ in the tuberculocidal test 965.12 [60]. Moreover, to be approved, the disinfectants should be subjected to worse case scenarios, such as the presence of organic or inorganic contamination, and under simulated use conditions.

In Europe, the CEN/TC 216 technical committee produces current and future disinfectant testing standards [61]. Standard EN-14885-2006 indicates test methods to be used to substantiate claims for products intended for instrument disinfection [62], including mycobacterial/tuberculocidal (EN-14348, EN 14563), bactericidal (EN-13727, EN-14561) and fungicidal (EN-13624, EN-14562) activity tests but the terms "sterility, sterile, sterilization, sterilant" fall outside the scope of CEN/TC 216.

In the US, high, intermediate and low level disinfectants, are regulated by the Environment Protection Agency (except HLD intended to reprocess medical instruments, which fall under FDA's jurisdiction). Intermediate level

disinfectants must be tuberculocidal. Products effective only against vegetative bacteria and viruses are regarded as low level disinfectants [63].

Special testing procedures may be applicable to some pathogens of epidemiological interest, such as *Clostridium difficile* [64]. Disinfectants are intended for use on inanimate surfaces. In general, their high concentration precludes their use on living tissues.

4.1.2 Antiseptics

Antiseptics are antimicrobials intended for use on skin and mucous membranes. The same as with low level disinfectants, antiseptics are tested against *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* in vegetative form. In the US, antiseptics are regulated by the FDA.

4.2 Antimicrobial activity tests

A relevant test microorganism is chosen: preferably a strain from the American Type Culture Collection (ATCC) or a similar repository. Although, wild-type bacteria from clinical samples also have been used. All necessary controls must be included to assess test reliability and reproducibility. Also, it is important to differentiate between kill and inhibition of growth.

The antimicrobial capability of nanoparticles has been explored by this techniques due studies have suggested that NPs are excellent microbicidal activity [16, 65]. The *in vitro* tests described below are the ones that the most have been used and the regulatory agencies recommend to determine antimicrobial activity of chemical formulations and can be used in the studies of nano-antimicrobials. The use of such tests depends on the objectives and the type of information it want to obtain.

A first approach if nanoparticle has antimicrobial activity is to conduct an antimicrobial activity test, such as a disc diffusion test.

4.2.1 Disk-diffusion method

Mueller-Hinton agar (pH 7.2–7.4) is the culture medium of choice. To standardize disc diffusion, the agar is poured into either Petri dish to only 4 mm in depth, as indicated in the Clinical and Laboratory Standards Institute method [66].

The bacteria are suspended to a 0.5 McFarland turbidity standard equivalent to 150 × 106 cfu/mL. From this suspension, 100 μL are uniformly spread onto the agar. Filter-paper discs 6 mm diameter, containing the test nano-antimicrobial, will be placed over the seeded agar (alternatively, a 50–100 μL well, punched into the agar, will contain the test antimicrobial). After overnight incubation at 37°C, the plates will be examined to assess inhibition rings around the disc.

The size of the nanoparticle, its rate of diffusion, the agar's porosity, and possible charge interactions between the antimicrobial and the agar may affect diffusion and the final size of the inhibition zone. In theory, the highest concentrations will be near the antimicrobial-containing disc and will be diluted away from the center (**Figure 2**).

4.2.2 Agar dilution method

This method is the gold standard for assessing the minimal inhibitory concentration (MIC) [67]. In this method, the melted agar is mixed to contain serial dilutions of the nano-antimicrobial. The resulting antimicrobial containing medium is plated into Petri dishes. An aliquot containing 10^4 cfu of the test microorganism

is placed onto the agar's surface, and incubated overnight. Then, the plates will be examined for growth to determine the last effective concentration to inhibit growth (**Figure 3**).

4.2.3 Broth dilution method

This method is often used because is more versatile and less laborious than the agar dilution method. Its microtiter plate version (broth microdilution), allows for testing more microorganisms against diverse concentrations of nano-antimicrobials, and can be automated.

Test tubes or wells in a microtiter plate, are prepared with bacteriological broth containing serial dilutions of the test nano-antimicrobial, and seeded with

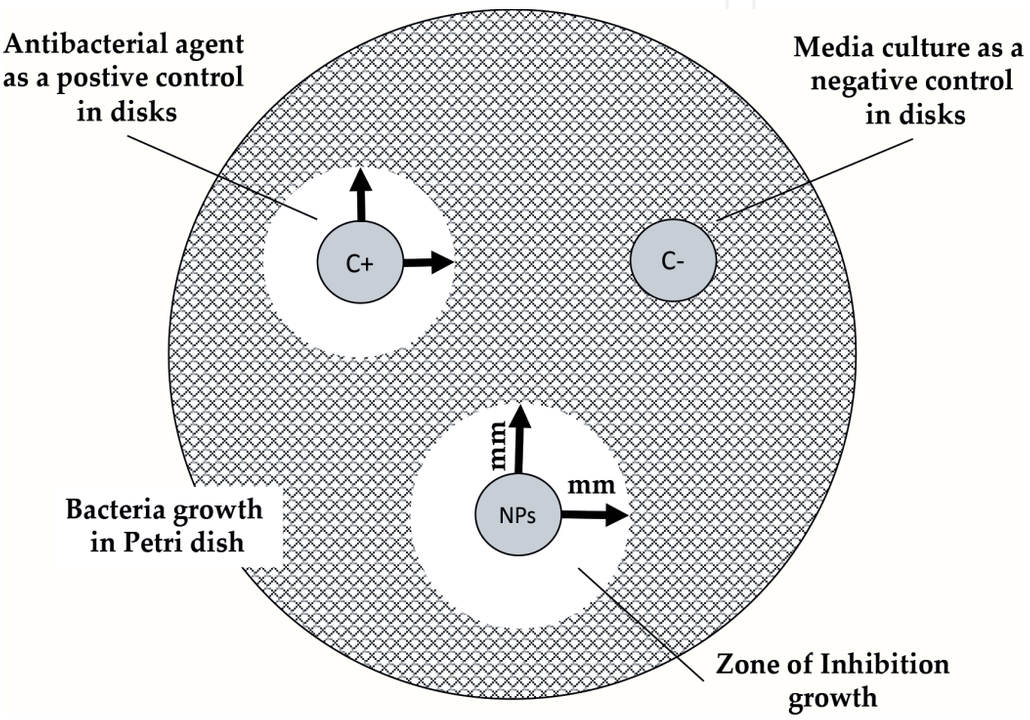

Figure 2.
Disk-diffusion method with NPs.

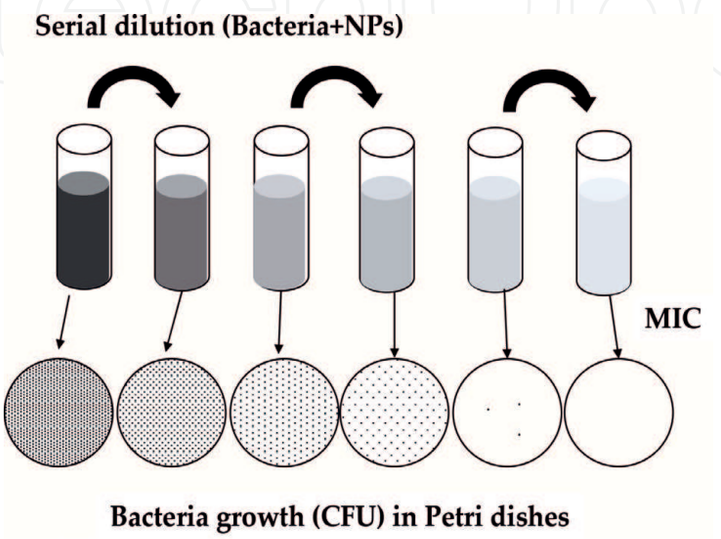

Figure 3.
Agar dilution method with NPs.

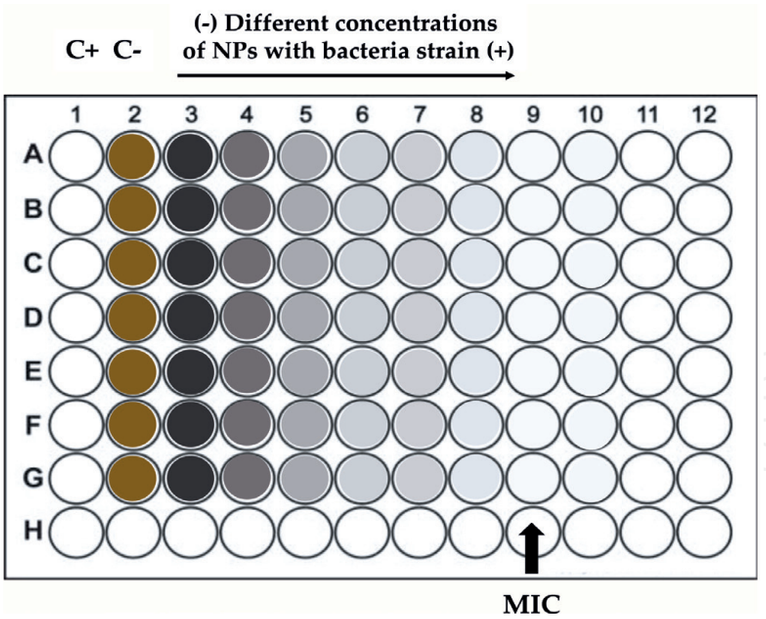

Figure 4.
Broth dilution method with NPs.

bacteria. After, overnight incubation, the tubes or wells are inspected for growth. The lowest concentration of nanoparticles that results in no-growth is the MIC [68] (**Figure 4**).

4.2.4 Time-kill method

After adding the test formulation to a broth culture, antimicrobial activity can be assessed *in vitro* by collecting sequential samples to count survivors. Time-kill allows the assessment of *in-vitro* synergy or antagonism between nano-antimicrobials.

For the time-kill experiments, Mueller Hinton broth is prepared with serial dilutions of the test antimicrobial, alone or in combination. The nano-antimicrobial concentrations may span a range above and below the formulation's MIC, previously obtained from agar dilution tests. Broths are then inoculated with 106 cfu/mL and incubated overnight at 37°C. From time 0 when bacteria are first exposed to the test antimicrobial, samples are obtained at 30 min intervals for up to 6 h. The samples are then plated on nutrient agar. After incubation overnight at 37°C, survivor counts are plotted to obtain a 'time-kill curve' [69].

5. Conclusions

Taking into account the future development and applications of the metal oxides nanoparticles in medicine, a constant search as emergent antimicrobial agents is required, due to the increase of diseases caused by microorganisms resistant to the action of antimicrobial agents as antibiotics.

Implementation of the metal oxides nanoparticles as an alternative to combat bacterial resistance due to increased findings in the mechanisms by which they act, have been the key to a better understanding and approach about the effect and kinetics that metal oxides nanoparticles have on microbial strains.

For this reason it is necessary to establish guidelines and quality standards to research nano-antimicrobials given the fact that there are many alternative methods *in vitro* testing to achieve this objective, but some of them present

limitations, while searching for new methods could be able to present specific results that allow us to compare them with *in vivo* testing.

Acknowledgements

This work was financially supported by Facultad de Odontología, División de Estudios de Posgrado e Investigación and Instituto de Ciencias Aplicadas y Tecnología in Universidad Nacional Autónoma de México.

Conflict of interest

The authors declare no conflicts of interest.

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References

[1] World Health Organization. Antimicrobial resistance. 2018. Available from: http://www.who.int/mediacentre/factsheets/fs194/en/ [Accessed: October 15, 2017]

[2] Reller LB et al. Antimicrobial susceptibility testing: A review of general principles and contemporary practices. Clinical Infectious Diseases. 2009;**49**(11):1749-1755. DOI: 10.1086/647952

[3] Jan T et al. Synthesis, physical properties and antibacterial activity of metal oxides nanostructures. Materials Science in Semiconductor Processing. 2014;**21**:154-160. DOI: 10.1016/j.mssp.2014.01.006

[4] Kalyani RL, Venkatraju J, Kollu P, Rao NH, Pammi SVN. Low temperature synthesis of various transition metal oxides and their antibacterial activity against multidrug resistance bacterial pathogens. Korean Journal of Chemical Engineering. 2015;**32**(5):911-916. DOI: 10.1007/s11814-014-0262-5

[5] Mamonova I et al. Biological activity of metal nanoparticles and their oxides and their effect on bacterial cells. Nanotechnologies in Russia. 2015;**10**(1-2):128-134. DOI: 10.1134/S1995078015010139

[6] Ling D, Hyeon T. Chemical design of biocompatible iron oxide nanoparticles for medical applications. Small. 2013;**9**(9-10):1450-1466. DOI: 10.1002/smll.201202111

[7] Karlsson HL et al. Copper oxide nanoparticles are highly toxic: A comparison between metal oxide nanoparticles and carbon nanotubes. Chemical Research in Toxicology. 2008;**21**(9):1726-1732. DOI: 10.1021/tx800064j

[8] Jeng HA, Swanson J. Toxicity of metal oxide nanoparticles in mammalian cells. Journal of Environmental Science and Health Part A. 2006;**41**(12):2699-2711. DOI: 10.1080/10934520600966177

[9] Adamcakova-Dodd A, Thorne PS. Grassian VH: In Vivo Toxicity Studies of Metal and Metal Oxide Nanoparticles. In Handbook of Systems Toxicology. Edited by: Daniel A Cascinao, Saura C Sahu. Chichester, UK: John Wiley & Sons, Ltd; 2011:803-834. DOI: 10.1002/9780470744307.gat244

[10] Wang L, Hu C, Shao L. The antimicrobial activity of nanoparticles: Present situation and prospects for the future. International Journal of Nanomedicine. 2017;**12**:1227. DOI: 10.2147/IJN.S121956

[11] Livermore DM. The 2018 Garrod lecture: Preparing for the black swans of resistance. The Journal of Antimicrobial Chemotherapy. 2018;**73**(11):2907-2915. DOI: 10.1093/jac/dky265

[12] Ventola CL. The antibiotic resistance crisis: Part 1: Causes and threats. Pharmacy and Therapeutics. 2015;**40**(4):277-283. PMID: 25859123

[13] Aslam B et al. Antibiotic resistance: A rundown of a global crisis. Infection and Drug Resistance. 2018;**11**:1645-1658. DOI: 10.2147/IDR.S173867

[14] Huh AJ, Kwon YJ. Nanoantibiotics: A new paradigm for treating infectious diseases using nanomaterials in the antibiotics resistant era. Journal of Controlled Release. 2011;**156**(2):128-145. DOI: 10.1016/j.jconrel.2011.07.002

[15] Prokopovich P et al. Potent antimicrobial activity of bone cement encapsulating silver nanoparticles capped with oleic acid. Journal of Biomedical Materials Research. Part B, Applied Biomaterials. 2015;**103**(2):273-281. DOI: 10.1002/jbm.b.33196

[16] Seil JT, Webster TJ. Antimicrobial applications of nanotechnology: Methods and literature. International Journal of Nanomedicine. 2012;**7**: 2767-2781. DOI: 10.2147/IJN.S24805

[17] Díez-Pascual AM. Antibacterial activity of nanomaterials. (Basel). 2018;**8**(6):1-6. DOI: 10.3390/nano8060359

[18] Gao W et al. Nanoparticle-based local antimicrobial drug delivery. Advanced Drug Delivery Reviews. 2018;**127**:46-57. DOI: 10.1016/j.addr.2017.09.015

[19] Qidwai A et al. Advances in biogenic nanoparticles and the mechanisms of antimicrobial effects. Indian Journal of Pharmaceutical Sciences. 2018;**80**(4):592-603. DOI: 10.4172/pharmaceutical-sciences.1000398

[20] Dizaj SM et al. Antimicrobial activity of the metals and metal oxide nanoparticles. Materials Science and Engineering: C. 2014;**44**:278-284. DOI: 10.1016/j.msec.2014.08.031

[21] Hoseinzadeh E et al. Sensitivity coefficient and death kinetics of *Escherichia coli* and *Staphylococcus aureus* to zinc oxide and copper oxide nanoparticles. Journal of Isfahan Medical School. 2012;**30**:200

[22] Hajipour MJ et al. Antibacterial properties of nanoparticles. Trends in Biotechnology. 2012;**30**(10):499-511. DOI: 10.1016/j.tibtech.2012.06.004

[23] Hoseinzadeh E et al. A review on nano-antimicrobials: Metal nanoparticles, methods and mechanisms. Current Drug Metabolism. 2017;**18**(2):120-128. DOI: 10.2174/1389200217666161201111146

[24] Hemeg HA. Nanomaterials for alternative antibacterial therapy. International Journal of Nanomedicine. 2017;**12**:8211. DOI: 10.2147/IJN.S132163

[25] Lemire JA, Harrison JJ, Turner RJ. Antimicrobial activity of metals: Mechanisms, molecular targets and applications. Nature Reviews Microbiology. 2013;**11**(6):371. DOI: 10.1038/nrmicro3028

[26] Raghunath A, Perumal E. Metal oxide nanoparticles as antimicrobial agents: A promise for the future. International Journal of Antimicrobial Agents. 2017;**49**(2):137-152. DOI: 10.1016/j.ijantimicag.2016.11.011

[27] Stankic S et al. Pure and multi metal oxide nanoparticles: Synthesis, antibacterial and cytotoxic properties. Journal of Nanobiotechnology. 2016;**14**(1):73. DOI: 10.1186/s12951-016-0225-6

[28] Vázquez Olmos AR, Vega Jiménez AL, Paz Díaz B. Mecanosíntesis y efecto antimicrobiano de óxidos metálicos nanoestructurados. Mundo Nano. Revista Interdisciplinaria en Nanociencia y Nanotecnología. 2018;**11**(21):29-44. DOI: 10.22201/ceiich.24485691e.2018.21.62545

[29] Azam A et al. Antimicrobial activity of metal oxide nanoparticles against Gram-positive and Gram-negative bacteria: A comparative study. International Journal of Nanomedicine. 2012;**7**:6003. DOI: 10.2147/IJN.S35347

[30] Kim DH et al. Effect of the size and shape of silver nanoparticles on bacterial growth and metabolism by monitoring optical density and fluorescence intensity. Biotechnology and Bioprocess Engineering. 2017;**22**(2):210-217. DOI: 10.1007/s12257-016-0641-3

[31] Gao M et al. Controlled synthesis of Ag nanoparticles with different morphologies and their antibacterial

properties. Materials Science and Engineering: C. 2013;**33**(1):397-404. DOI: 10.1016/j.msec.2012.09.005

[32] Somorjai GA, Park JY. Colloid science of metal nanoparticle catalysts in 2D and 3D structures. Challenges of nucleation, growth, composition, particle shape, size control and their influence on activity and selectivity. Topics in Catalysis. 2008;**49**(3-4):126-135. DOI: 10.1007/s11244-008-9077-0

[33] Simon-Deckers A et al. Size-, composition-and shape-dependent toxicological impact of metal oxide nanoparticles and carbon nanotubes toward bacteria. Environmental Science & Technology. 2009;**43**(21):8423-8429. DOI: 10.1021/es9016975

[34] Stoimenov PK et al. Metal oxide nanoparticles as bactericidal agents. Langmuir. 2002;**18**(17):6679-6686. DOI: 10.1021/la0202374

[35] Sukhorukova I et al. Toward bioactive yet antibacterial surfaces. Colloids and Surfaces B: Biointerfaces. 2015;**135**:158-165. DOI: 10.1016/j.colsurfb.2015.06.059

[36] Song Y, Chen L. Effect of net surface charge on physical properties of the cellulose nanoparticles and their efficacy for oral protein delivery. Carbohydrate Polymers. 2015;**121**:10-17. DOI: 10.1016/j.carbpol.2014.12.019

[37] Young J-j et al. Positively and negatively surface-charged chondroitin sulfate-trimethylchitosan nanoparticles as protein carriers. Carbohydrate Polymers. 2016;**137**:532-540. DOI: 10.1016/j.carbpol.2015.10.095

[38] Ben-Sasson M et al. Surface functionalization of thin-film composite membranes with copper nanoparticles for antimicrobial surface properties. Environmental Science and Technology. 2013;**48**(1):384-393. DOI: 10.1021/es404232s

[39] Anselme K et al. The interaction of cells and bacteria with surfaces structured at the nanometre scale. Acta Biomaterialia. 2010;**6**(10):3824-3846. DOI: 10.1016/j.actbio.2010.04.001

[40] Pan X et al. Investigation of antibacterial activity and related mechanism of a series of nano-$Mg(OH)_2$. ACS Applied Materials and Interfaces. 2013;**5**(3):1137-1142. DOI: 10.1021/am302910q

[41] Król A et al. Zinc oxide nanoparticles: Synthesis, antiseptic activity and toxicity mechanism. Advances in colloid and interface science. 2017;(249):37-52. DOI: 10.1016/j. cis.2017.07.033

[42] Wang ZL. Nanostructures of zinc oxide. Materials Today. 2004;7(6):26-33. DOI: 10.1016/S1369-7021(04)00286-X

[43] Kaftelen H et al. EPR and photoluminescence spectroscopy studies on the defect structure of ZnO nanocrystals. Physical Review B. 2012;**86**(1):014113-9. DOI: 10.1103/PhysRevB.86.014113

[44] Santhoshkumar J, Kumar SV, Rajeshkumar S. Synthesis of zinc oxide nanoparticles using plant leaf extract against urinary tract infection pathogen. Resource-Efficient Technologies. 2017;**3**(4):459-465. DOI: 10.1016/j.reffit.2017.05.001

[45] Chowdhury A-N et al. Oxidative and antibacterial activity of Mn_3O_4. Journal of Hazardous Materials. 2009;**172**(2):1229-1235. DOI: 10.1016/j.jhazmat.2009.07.129

[46] Azhir E et al. Preparation, characterization and antibacterial activity of manganese oxide

nanoparticles. Physical Chemistry Research. 2015;**3**(3):197-204. DOI: 10.22036/pcr.2015.9329

[47] Tadic M et al. Synthesis of core-shell hematite (α-Fe_2O_3) nanoplates: Quantitative analysis of the particle structure and shape, high coercivity and low cytotoxicity. Applied Surface Science. 2017;**403**:628-634. DOI: 10.1016/j.apsusc.2017.01.115

[48] Rufus A, Sreeju N, Philip D. Synthesis of biogenic hematite (α-Fe_2O_3) nanoparticles for antibacterial and nanofluid applications. RSC Advances. 2016;**6**(96):94206-94217. DOI: 10.1039/C6RA20240C

[49] Irshad R et al. Antibacterial activity of biochemically capped iron oxide nanoparticles: A view towards green chemistry. Journal of Photochemistry and Photobiology B: Biology. 2017;**170**:241-246. DOI: 10.1016/j.jphotobiol.2017.04.020

[50] Muthukumar H et al. Iron oxide nano-material: Physicochemical traits and in vitro antibacterial propensity against multidrug resistant bacteria. Journal of Industrial and Engineering Chemistry. 2017;**45**:121-130. DOI: 10.1016/j.jiec.2016.09.014

[51] Groiss S et al. Structural characterization, antibacterial and catalytic effect of iron oxide nanoparticles synthesised using the leaf extract of *Cynometra ramiflora*. Journal of Molecular Structure. 2017;**1128**:572-578. DOI: 10.1016/j.molstruc.2016.09.031

[52] Rankin DW. CRC Handbook of Chemistry and Physics. In: David R, editor. Lide. Boca Raton: CRC (Taylor and Francis Group); 2009. DOI: 10.1021/ja069813z

[53] Jin T, He Y. Antibacterial activities of magnesium oxide (MgO) nanoparticles against foodborne pathogens. Journal of Nanoparticle Research. 2011;**13**(12):6877-6885. DOI: 10.1007/s11051-011-0595-5

[54] Yamamoto O et al. Antibacterial characteristics of $CaCO_3$–MgO composites. Materials Science and Engineering: B. 2010;**173**(1):208-212. DOI: 10.1016/j.mseb.2009.12.007

[55] Krishnamoorthy K et al. Antibacterial activity of MgO nanoparticles based on lipid peroxidation by oxygen vacancy. Journal of Nanoparticle Research. 2012;**14**(9):1063. DOI: 10.1007/s11051-012-1063-6

[56] Bindhu M et al. Structural, morphological and optical properties of MgO nanoparticles for antibacterial applications. Materials Letters. 2016;**166**:19-22. DOI: 10.1016/j.matlet.2015.12.020

[57] Brooks BD, Brooks AE. Therapeutic strategies to combat antibiotic resistance. Advanced Drug Delivery Reviews. 2014;**78**:14-27. DOI: 10.1016/j.addr.2014.10.027

[58] Gottlieb S. FDA's Strategic Approach for Combating Antimicrobial Resistance. September 14, 2018. Washington, DC. Available from: https://www.fda.gov/NewsEvents/Speeches/ucm620495.htm

[59] US Food and D. Administration, Guidance for Industry and FDA Reviewers: Content and Format of Premarket Notification [510 (k)] Submissions for Liquid Chemical Sterilants/High Level Disinfectants. Rockville, MD: US Department of Health and Human Services, Food and Drug Administration; 2000. pp. 8-9. Available from: www.fda.gov/medicaldevices/deviceregulationandguidance/

guidancedocuments/ucm073773.htm [Accessed: November 17, 2018]

[60] AOAC International. Official Methods of Analysis of the Official Analytical Chemists. Arlington, VA: Ed: Kenneth Helrich, AOAC Inc.; 1990. pp. 133-146 (chapter 6)

[61] Holah JT. CEN/TC 216: Its role in producing current and future European disinfectant testing standards. International Biodeterioration and Biodegradation. 2003;**51**:239-243. Available from: https://standards.cen.eu/dyn/www/f?p=204:7:0::::FSP_ORG_ID:6197&cs=1C21F982635037747B259BDC2783DB513

[62] Comité Européen de Normalisation. EN 14885:2006 Chemical disinfectants and antiseptics—Application of European Standards for chemical disinfectants and antiseptics. Brussels, Belgium. 2015

[63] US Environmental Protection Agency. Product Performance Test Guidelines OCSPP 810.2000: General Considerations for Testing Public Health Antimicrobial Pesticides. Guidance for Efficacy Testing. EPA 712-C-17-002. Washington, D.C. February; 2018

[64] US Environmental Protection Agency. EPA MLB SOP-MB-31: Procedure for the OECD Quantitative Method for Testing Antimicrobial Products against Spores of Clostridium difficile (ATCC 43598) on Inanimate, Hard, Non-porous Surfaces. Washington, D.C. December; 2017

[65] Hoseinzadeh E, Makhdoumi P, Taha P, Hossini H, Pirsaheb M, Omid Rastegar S, et al. A review of available techniques for determination of nano-antimicrobials activity. Toxin Reviews. 2017;**36**(**1**):18-32. DOI: 10.1080/15569543.2016.1237527

[66] CLSI. Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard-Eleventh Edition. Clinical and Laboratory Standards Institute document M02-A11. Wayne, PA; 2012

[67] Baker CN et al. Comparison of the E test to agar dilution, broth microdilution, and agar diffusion susceptibility testing techniques by using a special challenge set of bacteria. Journal of Clinical Microbiology. 1991;**29**(3):533-538. PMC269813/

[68] Wiegand I, Hilpert K, Hancock RE. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nature Protocols. 2008;**3**(2):163-175. DOI: 10.1038/nprot.2007.521

[69] MacGowan A et al. A new time-kill method of assessing the relative efficacy of antimicrobial agents alone and in combination developed using a representative β-lactam, aminoglycoside and fluoroquinolone. Journal of Antimicrobial Chemotherapy. 1996;**38**(2):193-203. DOI: 10.1093/jac/38.2.193