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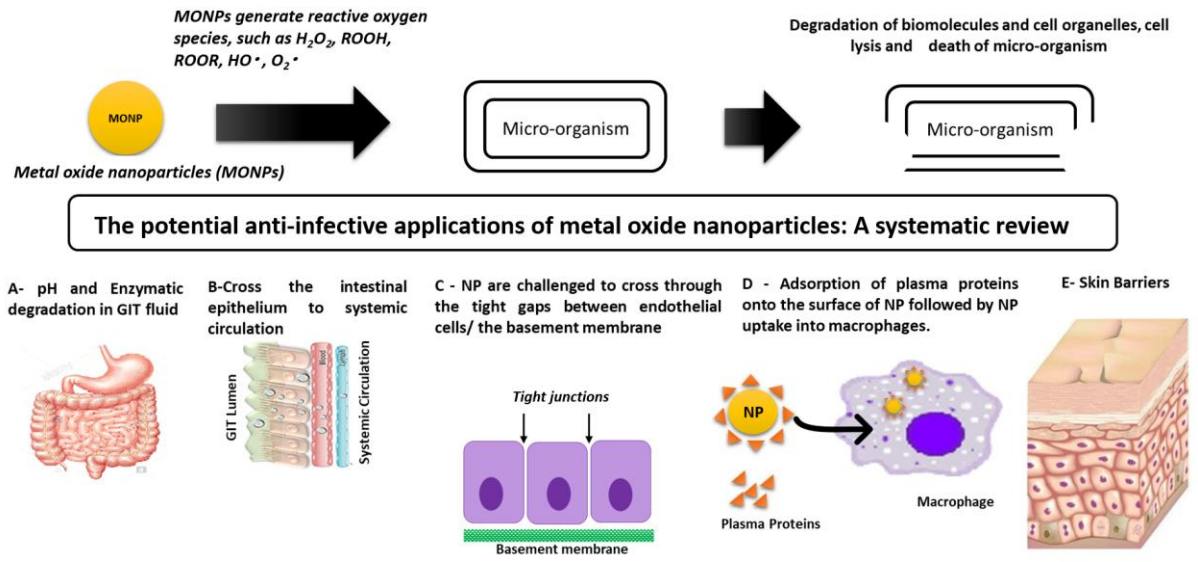
ABSTRACT

Microbial infections present a major global healthcare challenge, in large part because of the development of microbial resistance to the currently approved antimicrobial agents. This demands the development of new antimicrobial agents. Metal oxide nanoparticles (MONPs) are a class of materials that have been widely explored for diagnostic and therapeutic purposes. They are reported to have wide-ranging antimicrobial activities and to be potent against bacteria, viruses, and protozoans. The use of MONPs reduces the

possibility of resistance developing because they have multiple mechanisms of action (including via reactive oxygen species generation), simultaneously attacking many sites in the micro-organism. However, despite this there are to date no clinically approved MONPs for antimicrobial therapy. This review explores the recent literature in this area, discusses the mechansims of MONP action against micro-organisms, and considers the barriers faced to the use of MONPs in humans. These include biological challenges, of which the potential for an immune response and off-target toxicity are key. We explore the possible benefits/disbenefits of an immune response being initiated in detail, and consider the effect of production method (chemical versus green synthesis) on cytotoxicity. There are also a number of techical and manufacturing challenges, which are also discussed in depth. In the short term, there are potentially some "quick wins" from the repurposing of already-approved nanoparticle-based medicines for anti-infective applications, but a number of hurdles, both technical and biological, lie in the path to long-term clinical translation of new MNOP-based formulations.

GRAPHICAL/VISUAL ABSTRACT AND CAPTION



Metal oxide nanoparticles have efficient antimicrobial activity but there are many biological challenges restricting their application in man, as well as hurdles to scaled-up clinical manufacture.

1- INTRODUCTION

Nanomedicine is the branch of medicine that use particles sized from 1 to 1000 nm for either therapeutic or diagnostic purposes (Garnett & Kallinteri, 2006). Nanomedicine has the potential to overcome several drawbacks of conventional therapies, mainly due to the fact that the use of nanoscale particles leads to changes in physicochemical properties compared to those of the bulk. Properties such as surface charge, shape, and surface area to

volume ratio can all be varied. Nanoparticles (NPs) can be modulated to accumulate in target tissues via surface functionalization or by controlling particle size, and thus drug-loaded NPs can be used to deliver an active ingredient selectively to a particular part of the body. This permits administration of a lower dose. Targeting is also associated with reduced side effects due to the lower possibility of drug accumulation in off-target organs.

Initially, nanomedicine was developed to improve the treatment of cancer (Barenholz, 2012). More recently, researchers have developed a range of nanomedicine products for the diagnosis and treatment of myriad other diseases. However, the number of nanomedicines currently approved by the Food and Drug Administration (FDA) or in clinical trials is very small compared to the massive volume of research work published. A total of 1567 articles were identified in PubMed by searching for "nanomedicines" on 13th June 2019 (Figure 1), while only 51 nanomedicine products have been approved by the FDA and another 77 are at different stages of clinical trials (Bobo, Robinson, Islam, Thurecht, & Corrie, 2016; Ventola, 2017a). Nanomedicines approved by the FDA mainly consist of liposomes, polymers, micelles, polymer conjugated proteins, and nanocrystals. Only 12 products are metal-containing nanoparticles, such as hydroxyapatite, calcium phosphate and iron oxide (Bobo et al., 2016; Ventola, 2017b). Six iron oxide NP (IONP) products were approved for the treatment of iron deficiency, but four have been withdrawn from the market due to safety issues (this will be discussed in detail later) and only two (Ferumoxytol and Resovist) are still used (Yi-Xiang, 2015).

Despite the small number of marketed products, metal oxide NPs (MONPs) have been prepared on a very large number of occasions and found to have applications in a wide range of fields ranging from semiconductors to biomedicine (for both therapeutic or diagnostic purposes) (Bobo et al., 2016; Seabra & Durán, 2015; Ventola, 2017a). One example of biomedical applications is for antimicrobial purposes. Microbial infections comprise one of the most serious dangers to human health. They include diseases caused by bacteria, viruses, protazoans and fungi (Aderibigbe, 2017). Although there are many FDA approved antimicrobial agents, there is a need to develop new active ingredients in this field owing to the target microbes developing resistance to currently used therapeutics. There are many studies which have investigated the antimicrobial activity of MONPs: a total of 2266 articles were recognized in PubMed by searching for "metal oxide nanoparticles as antimicrobial agents" on 13th June 2019 (Figure 1). However, no MONPs have to date been FDA approved for use against infectious disease.

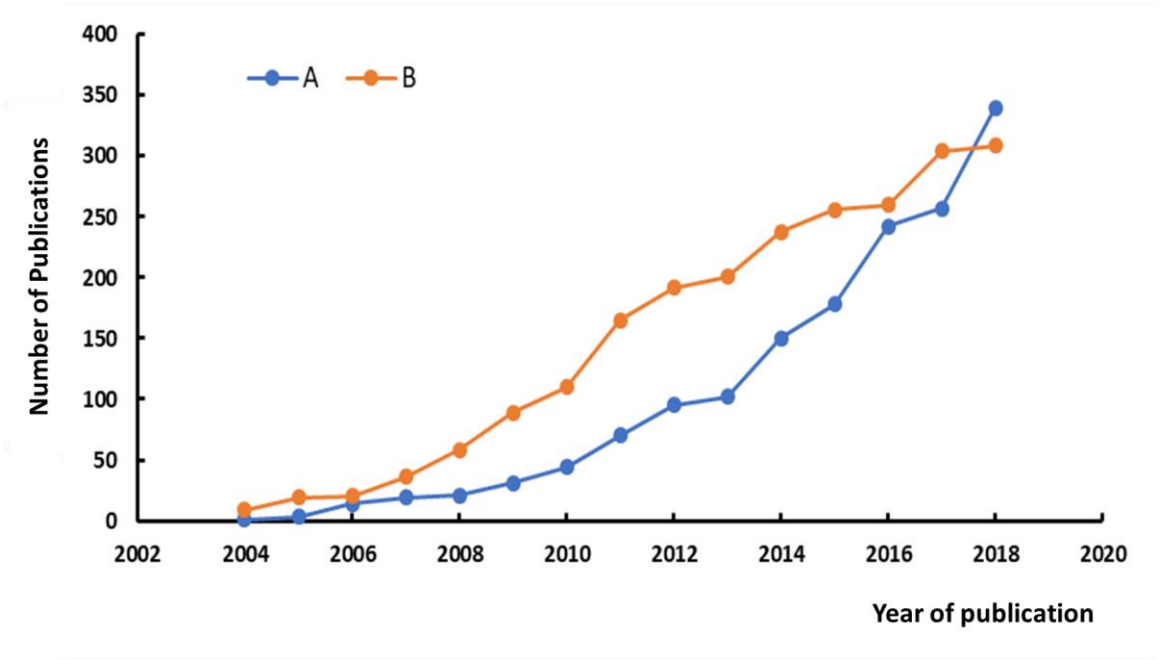


Figure 1: The number of publications (A) on nanomedicines and (B) investigating the potential applications of MONPs as antimicrobial agents published per year from 2004 to 2018 (data extracted from PubMed using the search term "nanomedicines" and "metal oxides nanoparticles as antimicrobial agents" on 13th June 2019).

2- THE NEED FOR NEW TREATMENTS

A number of factors lie behind the failure of current antimicrobial agents, and the situation is currently critical. In the case of bacterial infections, an antibiotic crisis was announced in 2013 (Fair & Tor, 2014) due to the emergence of resistant bacterial strains that cannot be treated with standard antibiotics (Fair & Tor, 2014). Several reasons lie behind the emergence of such resistance: (1) approved antibiotics only attack a single target in the bacteria (Etebu & Arikekpar, 2016) (see Figure 2); (2) over-prescription and improper use of antibiotics by the healthcare sector (Fair & Tor, 2014); and (3) the misuse of antibiotics in agriculture (e.g. in feed stock to promote animal growth (Wegener, Aarestrup, Jensen, Hammerum, & Bager, 1999) or sprayed over plants to protect them from disease and increase production (Fair & Tor, 2014)).

In the case of viral infections, again the approved antiviral therapeutics (*e.g.* direct acting antiviral agents) are designed to attack a specific target on the virus (Figure 2). Viruses are characterized by a high rate of genetic mutation, and therefore can be expected to rapidly develop resistance and cross resistance (Melikian et al., 2014; Wyles, 2013).

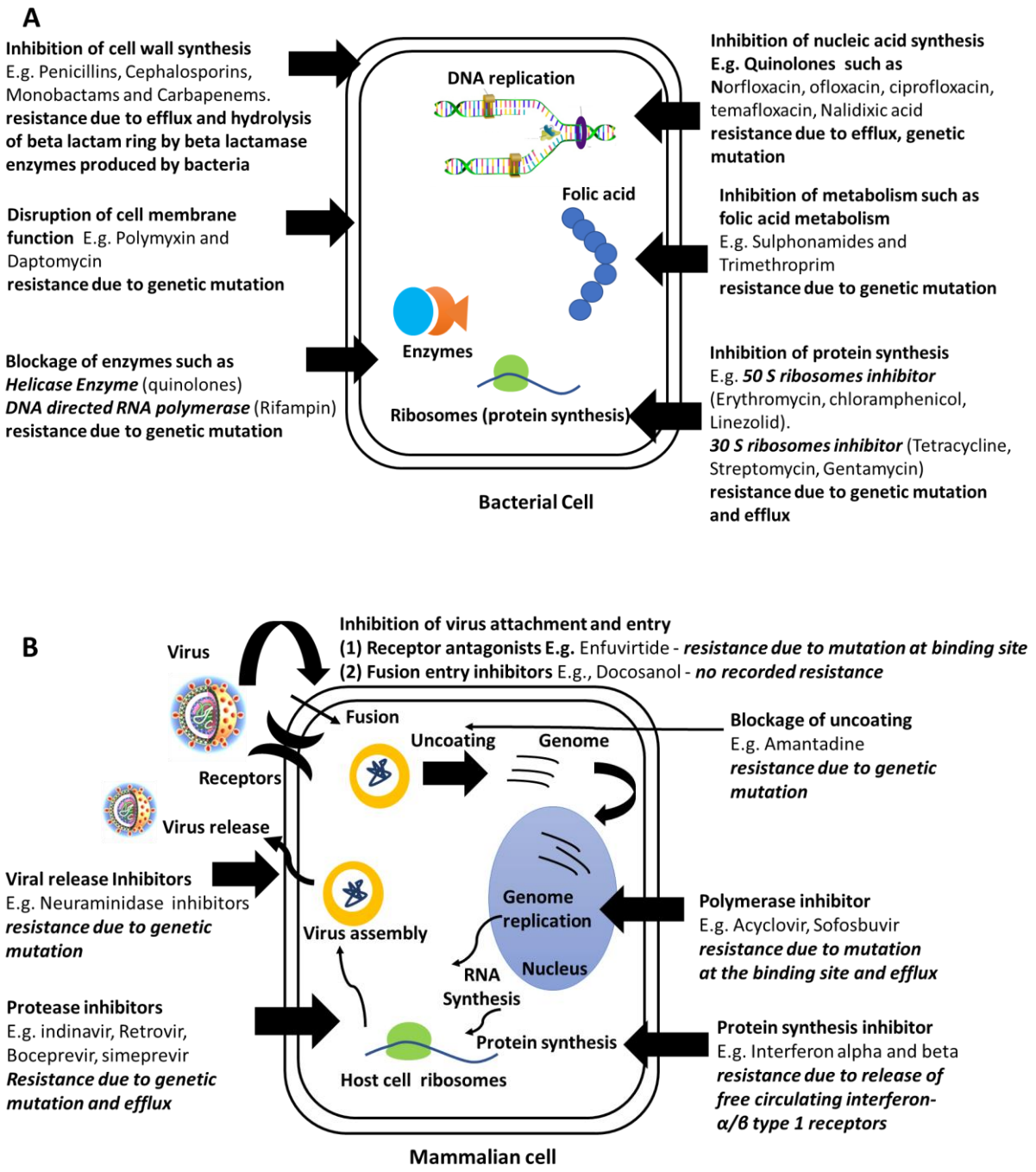


Figure 2: The mechanisms of action of current anti-infective agents, including potential resistance mechanisms against (A) antibiotics and (B) antivirals.

Additionally, the selectivity of antiviral agents makes them specific to a given virus, and they cannot generally be used for the eradication of multiple types of viruses (Martinez, Sasse, Brönstrup, Diez, & Meyerhans, 2013). This necessitates, the development of broad-spectrum antiviral agents. Given that the host cellular machinery is commonly required for viral replication and propagation, this might represent a good target for developing broad

spectrum agents, and some clinical trials of such active ingredients are underway (De Chassey, Meyniel-Schicklin, Aublin-Gex, André, & Lotteau, 2012).

Protozoan parasitic diseases mainly occur in developing countries, which has unfortunately led to their being neglected: there has been very low investment in the production of antiparasitic agents, and this has contributed to the development of resistance. Of 1300 medicinal agents developed between 1975 to 1999, only 13 were for the treatment of parasitic infections (Fairlamb, Ridley, & Vial, 2003). Unfortunately, market forces have been insufficient to drive the discovery and development of new drugs for these diseases (Pink, Hudson, Mouriès, & Bendig, 2005). This has led to new public sector and public-private partnerships, including investment by the Bill and Melinda Gates Foundation to develop new antiparasitic agents (Pink et al., 2005). One exciting development was the release of Ambisomes (amphotericin B-loaded liposomes), an antiparasitic nanomedicine with high efficacy and minimal side effects. However, its high cost ($267 per vial) is problematic, and thus it does not reach all patients (Fairlamb et al., 2003). Even in clinical trials where the medicine was supplied with a much lower price ($18 per vial) through the WHO/Gilead donation program, the estimated per-patient cost of treatment with liposomal amphotericin B was $660 (Assis et al., 2017; Bhattacharya & Ali, 2016). Therefore, the cost of antimicrobial agents is another important issue that must be restrained to allow more effective management of microbial diseases worldwide.

From the above, it can be concluded that the development of microbial resistance is the major reason for the failure of currently used anti-infective agents. Currently used active anti-infective agents target only a single aspect of microbial reproduction or survival (the selective blockage of enzymes, ribosomes, cell membrane synthesis, DNA replication, DNA gyrase, folic acid metabolism or protein synthesis (Etebu & Arikekpar, 2016). Therefore, there is ample scope for the micro-organisms to develop target-orientated resistance. For example, the antibiotic vancomycin functions by binding to the ends of glycol peptides and interfering with their cross linking to the bacterial cell wall. Bacteria such as *Enteroccoci* resist vancomycin by interfering with its binding with glycol peptides (Miller, Munita, & Arias, 2015). *Pseudomonas* biofilms can upregulate the production of efflux pumps to expel antibiotics from the bacterial cell cytosol and drive them to the extracellular milieu, leading to the biofilms becoming resistant to β-lactam antibiotics (Zhang & Mah, 2008). Degradation or hydrolysis of antibiotics by special enzymes produced by bacteria is another a self-defence mechanism which can lead to resistance (*e.g.* β-lactamase enzymes produced by *Klebsiella* for the degradation of ampicillin (Fu et al., 2007). In another example, genetic mutation of *Helicobacter pylori* causing a change in the binding site for clarithromycin is responsible for its development of resistance (Ontsira Ngoyi et al., 2015). Antiviral resistance arises for similar reasons; for instance, a mutation in the active site of thymidine kinase (the enzyme responsible for the activation of acyclovir) is largely responsible for *herpes simplex* virus becoming resistant to this drug (Morfin & Thou, 2003).

It is believed that ideally an efficient antimicrobial agent should simultaneously interact with multiple sites both on the micro-organisms and the host cell to reduce the likelihood of the micro-organism developing resistance. For example, ribavirin is a guanosine analogue that has a broad-spectrum activity against many RNA and DNA viruses. Ribavirin has several

mechanisms of action (Beaucourt & Vignuzzi, 2014) including: (1) inhibition of inosine monophosphate dehydrogenase, resulting in depletion of guanosine triphosphate; (2) interfering with mRNA-capping; (3) stimulating the host immune system to act against the invaded virus; (4) inhibiting viral RNA polymerase, and (5) enhancing virus mutagenesis due to incorporation of ribavirin triphosphate in place of guanosine triphosphate into viral RNA, resulting in viral death.

MONPs could comprise a very promising route for the development of new antimicrobial therapeutics. They have been reported to have a broad antimicrobial activity against bacteria (both Gram positive and negative), viruses, fungi, and protazoa (Aderibigbe, 2017, Raghunath & Perumal, 2017). Their efficiency stems from their mechanism of action: several are reported, but the principle mechanism involves the production of reactive oxygen species (ROS) which are potent in killing micro-organisms. Micro-organisms are not able to develop resistance to such ROS production (Raghunath & Perumal, 2017), because ROS attack multiple different sites and biomolecules in the micro-organism, resulting in their oxidation and subsequent cell death. Micro-organisms attempt to protect themselves against oxidation through the use of enzymes such as dismutase and catalase to convert ROS into water and oxygen, non-toxic by-products. However, these can be overwhelmed by the presence of very high amounts of ROS, which results in the oxidation of a range of essential molecules such as proteins, lipids, carbohydrates and DNA: this will be discussed later in more detail (Fatma Vatansever et al., 2014, Chen, Brugarolas, & He, 2011). The production of ROS by MONPs is similar to the approach employed by macrophages to eradicate microbes. Macrophages endocytose micro-organisms, trap them inside endosomes, and destroy them through the secretion of very large amounts of ROS sufficient to overcome the superoxide dismutase enzymes of the micro-organism (Slauch, 2012).

Although MONPs have been proven to have potent antimicrobial properties, they have a number of limitations that hinder their clinical application. *In vivo*, there are several challenges which they must overcome to reach their intended target. These include requirements to cross biological barriers and maintain stability in biological fluids, in addition to considerations of how the MONPs will interact with the immune system and any safety and risks associated with their long-term administration. There are also complications associated with the technical design of NPs which can selectively attack and eradicate a target micro-organism without having any hazardous effects on the patient. All of these issues will be discussed in detail below.

3- MECHANISMS OF ACTION OF METAL OXIDE NANOPARTICLES

This review will focus on the antimicrobial activity of MONPs against bacteria, viruses and parasites. A summary of the findings in the literature are given in Table 1. For instance, MONPs have been explored for their antimicrobial activities against a range of micro-organisms that are known to cause common hospital acquired infections (Khan Hassan, Baig, & Mehboob, 2017). Potent activity has been reported against a wide range of such bacteria, including *Escherichia coli*, *vancomycin resistant Enterococci*, and *methicillin-resistant Staphylococcus aureus* (Table 1). Viruses such as hepatitis B and C, influenza, human immunodeficiency viruses (HIV), rotavirus, and herpes-simplex virus that cause

around 5% of nosocomial infections (Khan Hassan et al., 2017). MONPs have demonstrated significant potential *in vitro* against hepatitis C and herpes simplex type 1 and 2 (Table 1). Parasitic infections cause a relatively small number (< 0.5%) of total nosocomial infections (Góralska & Kurnatowski, 2013), but MONPs have been shown to be effective against *Plasmodium falciparum* (malaria), helminth infections and *leishmania* species (Table 1).

Table 1: Reported antimicrobial activity of metal oxide nanoparticles

Material	***Particle Size (nm)***	***Zeta Potential (mV)****	***Test Method***	***Antimicrobial effects***	***Reference***
Antibacterial activity					
Aluminium oxide [α-Al_2O_3]	20 - 30	NA	Agar well diffusion	*Staphylococcus aureus (S. Aureus)*, *Klebsiella aerogenes, Escherichia coli (E. Coli), Pseudomonas desmolyticum*	(Prashanth et al., 2015)
Antimony trioxide (Sb_2O_3)	90 - 210	NA	Agar plate and counting of colony forming units (CFU)	*E. coli, Bacillius subtilis Streptococcus aureus*	(Baek & An, 2011)
Calcium oxide (CaO)	15 - 180	NA	Agar plate and counting of CFU	*Lactobacillus plantarum*	(Tang et al., 2013)
Calcium oxide (CaO)	16	NA	Agar well diffusion	*Staphylococcus epidermidis > Pseudomonas aeruginosa*	(Roy Arup, Gauri, Bhattacharya, & Bhattacharya, 2013)
Cadmium oxide (CdO)	60	NA	Determination of optical density; agar plate and counting of CFU	*E. coli*	(Rezaei-Zarchi et al., 2010)
Cerium oxide (CeO_2)	6 – 40	NA	Disc diffusion method	*E. coli* and *B. subtilis*	(Pelletier et al., 2010)
Chitosan based Zinc oxide NPs (ZnO)	99 - 603	-12.9 to – 35.5	Agar diffusion and micro titre methods	Antibacterial activity and biofilm inhibition activity against *Micrococcus luteus and S. aureus*	(Dhillon, Kaur, & Brar, 2014)
Chromium oxide (Cr_2O_3)	41, 65 and 79	NA	Disc diffusion	*E. coli, P. aeruginosa*	(Ananda & Gowda, 2013)
Cinnamomum verum functionalized Fe_3O_4	9.4	NA	Agar plate and counting of CFU	*S. aureus and E. coli*	(Anghel et al., 2014)

Cobalt oxide (Co_3O_4)	100 -150	NA	Broth dilution	*S. aureus and E. coli*	(Ghosh et al., 2014)
Copper oxide (CuO)	23	NA	Well diffusion	*E. coli, Enterococcus faecalis, and Klebsiella pneumenia*	(Ahamed, Alhadlaq, Khan, Karuppiah, & Al-dhabi, 2014)
Copper oxide nanorods (CuO)	Width: 60 nm, length: 8.3 μm	NA	Agar plate and counting of CFU	*E. coli*	(Pandey et al., 2014), (Khashan, Sulaiman, & Abdulameer, 2016), (Gilbertson et al., 2016), (Bondarenko, Ivask, Käkinen, & Kahru, 2012)
Copper oxide (CuO)	5 - 8	NA	Well diffusion	*K. penumeniae, Salmonella typhimurium, and Enterobacter . aerogenes*	(Kumar, Salar, & Purewal, 2014)
Copper oxide (CuO)	10 - 40	NA	Agar plate and counting of CFU	Different stains of *S. aureus*	(Hsueh, Tsai, & Lin, 2017)
Copper oxide (CuO)	20 - 27	NA	Well diffusion	*B. subtilis, S. aureus, E. coli and P. aeruginosa*	(Azam, Ahmed, Oves, Khan, & Memic, 2012)
Copper oxide (CuO)	15 – 30	NA	Determination of optical density	*E. coli and P. aeruginosa*	(Das, Nath, Phukon, & Dolui, 2013)
Copper oxide (CuO)	9.6	NA	Broth dilution	*E.coli and S. aureus*	(Jadhav, Gaikwad, Nimse, & Rajbhoj, 2011)
Copper oxide (CuO)	Nanosize: 20 – 200 nm Microsize: 200 – 2000 nm	-16.5 -28.5	Determination of optical density	*E. coli and Lactobacillus brevis*	(Kaweeteerawat et al., 2015)
Copper oxide (CuO)	< 50	NA	Determination of optical density	*P. aeruginosa*	(Guo et al., 2017)
Copper oxide (CuO and Cu_2O)	CuO = 30 Cu_2O= 40	NA	Agar plate and counting of CFU	*E. coli*	(Meghana, Kabra, Chakraborty, & Padmavathy, 2013)
Copper oxide (CuO)	20 - 95	NA	Agar plate and counting of CFU	Various nosocomial bacteria inc. *methicillin-resistant Staphylococcus*	(Ren et al., 2009)

				aureus (MSRA), S. epidermis, P. aeruginosa, and E. coli	
Copper oxide (CuO)	20 – 30	NA	Agar plate and counting of CFU	*E. coli, B. subtilis and S. aureus*	(Baek & An, 2011)
Copper oxide (CuO)	25 - 30	NA	Well diffusion	Strongly diminishes the biofilm forming uro-pathogens of *MRSA and E. coli*	(Agarwala, Choudhury, & Yadav, 2014)
Graphene oxide modified zinc oxide (ZnO) nanoparticles	170	NA	Microdilution	*E. Coli, S. typhimurium, B. subtilis, E. faecalis*	(Linlin Zhong & Yun, 2015)
Iron oxide NP (Fe_3O_4) functionalized with carvone	12	NA	Biofilm assay	Inhibited colonisation and bio-film formation of *S. aureus and E. coli*	(Holban et al., 2015)
Iron oxide (Fe_3O_4) NP impregnated polyacrylonitrile matrix	2 - 24	-34 to -20 at pH 4 to 10	Membrane agar test	*E. coli*	(Mukherjee & De, 2015)
Iron oxide (Fe_3O_4)	33 – 40	NA	Well diffusion	*E.coli, S. aureus, and Proteus vulgaris*	(Prabhu, Rao, Kumari, Kumar, & Pavani, 2015)
Iron oxide (Fe_3O_4)	10 – 120	NA	Well diffusion	*S. aureus, B. subtilis and E. coli, S. epidermidis, Bacillus Licheniformis, Brevibacillusbrevis , and Vibrio cholerae ,*	(Behera, Patra, Pramanik, Panda, & Thatoi, 2012);
Iron oxide (α-Fe_2O_3)	50 - 110	NA	Well diffusion	*S. aureus, E. coli, P. aeruginosa and Serratia marcescens*	(Ismail, Sulaiman, Abdulrahman, & Marzoog, 2015)
Iron oxide (Fe_3O_4)	10.4 – 11.4	-32.2 to + 36.3	Determination of optical density; agar plate and counting CFU	*B. subtilis and E. coli*	(Arakha et al., 2015)
Iron oxide (mixture of Fe_3O_4 and γ-Fe_2O_3)	9	-19	Determination of optical density	Concentration dependant antibacterial activity against *S. aureus*	(Tran et al., 2010)
Layered graphene sheets decorated with zinc oxide nanoparticles	30 – 40	NA	Well diffusion	*Salmonella typhi > E. coli*	(Bykkam et al., 2015)

(ZnO)					
Magnetite (Fe_3O_4)	8	NA	Determination of optical density	Concentration dependant bacteriostatic effect against *E. coli*	(Chatterjee et al., 2011)
Maghemite (Fe_2O_3)	25 – 30	NA	Well diffusion	Active against the following uro-pathogens; *MRSA, methicillin resistant Staphylococcus epidermidis (MRSE), vancomycin resistant Enterococci (VRE), Proteus mirabilis E. coli, K. penumnoiae, and P. aeruginosa*	(Agarwala et al., 2014)
Magnesium oxide, nanowire (MgO)	Width: 6 nm, length: 10 µm	NA	Well diffusion; determination of optical density	Showed a concentration dependant bacteriostatic activity against *E. coli and Bacillus species*	(Al-Hazmi et al., 2012)
Metatitinic acid (H_2TiO_3) and silicon dioxide (SiO_2) NPs incorporated into dressings	NA	NA	Disc diffusion	Inhibited growth of *E. coli, S. aureus* and *E. faecalis.*	(Krokowicz et al., 2015)
Nickel oxide (NiO)	10 – 20	NA	Agar plate and counting of CFU	Active against *E.coli, B. subtilis and S. aureus*	(Baek & An, 2011)
Nickel oxide (NiO)	20 – 30	36.8	Well diffusion	Broad spectrum antibacterial activity against gram-positive and gram-negative pathogens	(Rakshit et al., 2013)
Polyethyleneimine capped zinc oxide NPs (ZnO–PEI NP)	*3 – 7 (core) 20 (capped)*	NA	Determination of optical density	*E. coli* bearing high pathogenicity island genes	(Chakraborti et al., 2014)
Titanium dioxide	< 50	NA	Well diffusion	Active against biofilm producing	(Jesline, John, Narayanan,

(TiO_2)				*MRSA*	Vani, & Murugan, 2015)
Titanium dioxide (TiO_2)	NA	NA	Agar test and counting of CFU	Promising treatment for dental plaque	(Thomas, Raj, & Venkataramana , 2014)
Titanium dioxide (TiO_2)	23 to 134	NA		*Streptococcus mutans*	(Besinis, De Peralta, & Handy, 2014)
Titanium dioxide (TiO_2)	60	NA	Determination of optical density; agar plate method and counting CFU	*E. coli*	(Rezaei-Zarchi et al., 2010), (Alhadrami, Al, & Hazmi, 2017), (Tong, Binh, Kelly, Gaillard, & Gray, 2013)
Titanium dioxide (TiO_2)	10, 25, and 50	-33.8 to -5.48 at pH range 3.6 to 6.2	Agar plate test and counting of CFU	Size dependant activity against *E. coli*	(Lin et al., 2014)
Titanium dioxide (TiO_2)	7 - 12	NA	Quantitative assessment method as per AATCC test method 100-2004.	Size dependant antibacterial activity against *S. aureus and K. pneumonia*	(Sundaresan, Sivakumar, Vigneswaran, & Ramachandran, 2012)
Zinc oxide (ZnO)	89 - 159	*NA*	Determination of optical density	*K. pneumonia*	(Reddy, Nisha, Joice, & Shilpa, 2014)
Zinc oxide (ZnO)	12, 25, 30, 88, 142, 212, 307	NA	Determination of optical density	Size dependant growth inhibition of *S. aureus, E. coli, and B. subtilis*.	(Raghupathi, Koodali, & Manna, 2011)
Zinc oxide (ZnO)	50	NA	Agar plate and counting of CFU	Concentration dependant activity against *B. subtilis*	(Hsueh et al., 2017)
Zinc oxide (ZnO)	10 – 25	NA	Well diffusion	Active against clinical isolate of *S. aureus*	(Narasimha, Sridevi, Prasad, & Kumar, 2014)
Zinc oxide (ZnO)	30	*NA*	Agar plate and counting of CFU	*Campylobacter jejuni*	(Xie, He, Irwin, Jin, & Shi, 2011)
Zinc oxide (ZnO)	20 - 25	*NA*	Determination of optical density; agar plate and determination of zone of inhibition	*E. coli and S. aureus*	(Mirhosseini & Firouzabadi, 2013)
Zinc oxide (ZnO)	70	NA	Determination of optical density	Concentration dependant inhibitory effect of *E. coli O157:H7*	(Liu et al., 2009)

Zinc oxide (ZnO)	20 60	-21.9 -17	Agar plate and counting of CFU	Size dependant inhibitory activity against *S. aureus*	(Seil & Webster, 2012)
Zinc oxide (ZnO nano and microwires)	Microwire: Width: 200 – 500 nm, length: 2 to 4 μm Nanowire: Width: 20 – 40 nm, length: 4μm	NA	Agar plate and counting of CFU	Size and dose dependant inhibitory effect against *B. subtilis > S. aureus*	(Rago et al., 2014)
Zinc oxide (ZnO)	100 – 150	NA	Agar plate and counting of CFU	Active against *Streptococcus agalactiae* and *S. aureus*	(Huang et al., 2008)
Zinc oxide (ZnO)	50 – 70	NA	Agar plate and counting of CFU	Selective antimicrobial activity against *E. coli and B. subtilis*	(Baek & An, 2011)
Zinc oxide (ZnO)	19.8	NA	Well diffusion	Inhibited bacterial growth of *methicillin-sensitive S. aureus (MSSA), and MRSA*	(Ansari, Khan, Khan, Sultan, & Azam, 2012)
Zinc oxide (ZnO)	15, 25, and 38	NA	Agar plate and determination of zone of inhibition	Size dependant inhibitory effect against *Salmonella, Paratyphi, B. subtilis, K, pneumoniiae, S. epidermidis, E. aerogenes and MRSA*	(Palanikumar, Ramasamy, & Balachandran, 2014)
Zinc oxide (ZnO)	17 – 21	NA	Disc diffusion	Concentration dependant activity against *S. aureus, E. coli, K. pneumoniae, E. faecalis and P. aeruginosa*	(Narayanan, Wilson, Abraham, & Sevanan, 2012)
Zinc oxide (ZnO)	Nanorods: Diameter: 30 – 60 nm, length: 80 nm and widt: 50 – 200 nm, Length: 5 μm.	*NA*	Broth dilution and colony counting; agar plate; disc diffusion; microtiter plate; conductivity assay	*E. coli, Salmonella choleraesuis, P. aeruginosa, L. plantarum and Listeria monocytogenes*	(Espitia et al., 2012)

	Spherical: 37 – 47 nm. Circular: 30 – 60 nm. Acicular: 20 – 30 nm				
Zinc oxide adhered to a surface of fabric (ZnO)	I) Width, 200 – 800 nm, length: 2-4μm. II) Width 50 – 300 nm, length 1 - 2μm	NA	Agar diffusion; modified colony counting method	Bacteriostatic effect against *K. pneumoniae and S. aureus*	(Shateri-Khalilabad & Yazdanshenas, 2013)
Zinc oxide (ZnO)	20 – 217	19.7	*In vivo* rat model	Bactericidal activity against *P. aeruginosa*	(Watson et al., 2015)
Zinc oxide (ZnO)	< 100	*NA*	Well diffusion	*MRSA*	(Jesline et al., 2015)
Zinc oxide NPs (ZnO) and ultrasound application	20 and 60	-21.9 and -17	Agar plate and counting of CFU	Ultrasound stimulation led to stronger antibacterial activity against *S. aureus* than NPs alone	(Seil & Webster, 2012)
Zinc oxide nanoparticles coating a glass slide	15	NA	Agar plate and counting of CFU	Excellent antibiofilm activity against *E. coli and S. aureus*	(Applerot et al., 2010)
Zinc oxide, nanoparticle and nanorods (ZnO)	Nanoparticle: 20 nm Nanorods: width from 60 – 350 nm and length from 0.5 to 4.2μm	NA	Agar diffusion	Inhibited growth of *S. aureus*, *E. coli and Aspergillus niger*	(Jaisai, Baruah, & Dutta, 2012)
Super paramagnetic iron oxide nanoparticles (unconjugated and conjugated to zinc and iron metals)	Unconjugated: 18 nm Zinc conjugated: 20 nm Iron conjugated: 28 nm	Unconjugated: -35.5 Zinc conjugated: -40.1 Iron conjugated: -34	Bacterial biofilm and counting of CFU	Biofilm of *MRSA, E. coli, P. aeruginosa*	(Durmus, Taylor, Kummer, & Webster, 2013)
Silver and Zinc	ZnO: 50	*NA*	Agar plate and	*S. mutans*	(Kasraei, 2014)

oxide composite	Ag_2O: 20		counting of CFU		
Antibacterial activity of biogenically synthesized metal oxide NPs					
Aluminium oxide (Al_2O_3) synthesized by using leaf extracts of lemongrass	254	*+ 52.2*	Disc diffusion	*E. coli*	(Ansari et al., 2012)
Cobalt oxide (Co_3O_4) synthesized using leaf extracts of Sageretia thea	20	NA	Disc diffusion	Concentration dependant activity against *S. aureus and E. coli* Antibacterial activity increased after exposure to UV	(Khalil et al., 2017)
Copper oxide (CuO) synthesized through microbial method using *Streptomyces* species (forming CuO coated textile)	100 – 150	NA	Agar plate and determination of zone of inhibition	*E. coli, S. aureus, and A. niger* CuO nanoparticles applied to textile showed maximum zone of mycostaisis - a promising future for a textile that might decrease transmission of infectious diseases.	(Usha, Prabu, Palaniswamy, Venil, & Rajendran, 2010)
Copper oxide (CuO) synthesized using gum karya	4.8, 5.5, and 7.8	NA	Well diffusion	*E. coli and S. aureus*	(Padil & Černík, 2013)
Copper oxide (CuO) synthesized using extract of brown algae	5 – 45	NA	Disc diffusion	*Enterobacter aerogenes and S. aureus*	(Abboud et al., 2014).
Copper oxide (CuO) synthesized using Phyllanthus amarus leaf extract	20	NA	Well diffusion	*B. subtilis, S. aureus, E. coli and P. aeruginosa*	(Acharyulu et al., 2014)
Copper oxide (CuO) prepared using tea leaf and coffee powder extracts	50 – 100	NA	Disc diffusion	*Shigella dysenteriae and V. cholera*	(Sutradhar, Saha, & Maiti, 2014)
Iron oxide (Fe_3O_4) Produced using seaweed (Sargassum muticum aqueous extract)	10 – 30	NA	Disc diffusion	*E. coli, Proteus mirablis, Proteus vulgaris and S. aureus*	(Arokiyaraj et al., 2013)

Titanium dioxide (TiO_2) synthesized using the fungus Aspergillus flavus	33	NA	Well diffusion	*E. coli*	(Santhoshkumar et al., 2014)
Titanium dioxide (TiO_2) synthesized using Aeromonas hydrophila	40.5	NA	Well diffusion	*S. aureus* and *streptococcus pyogenes*	(Jayaseelan et al., 2013)
Zinc oxide (ZnO) produced using the leaf extract of Solanum nigrum	20 – 30	NA	Disc diffusion	*Salmonella paratyphi, E. coli, V. cholerae* and *S. aureus*	(Ramesh, Anbuvannan, & Viruthagiri, 2015).
Antiviral activity					
Cuprous oxide Cu_2O)	45.5 (by TEM) 92.4 (by Zeta sizer)	NA	*In vitro*, Huh 7.5.1 cells infected with HCV	Inhibited Hepatitis C virus entry (Genotype 1a, 1b and 2a) at a concentration of 2 µg/ml	(Hang et al., 2015)
Iron oxide (Fe_3O_4)	75 – 80	+ 7.25 – 7.48	In vitro, Huh7 cells infected with HCV	Induced knockdown of Hepatitis C virus genes encoding helicase and protease, essential for cirus replication	(SooRyoon Ryoo et al., 2012)
Zinc oxide, Tetrapod shape (ZnO)	Arm diameter: 200 nm to 1µm, length 5 - 30 µm	NA	*In vivo* using BALB/c mice	Interacts with Herpes simplex virus 2 inhibiting its entry into cells	(Antoine et al., 2016) (Mishra et al., 2011)
Antiparasitic activity					
Al_2O_3, CeO_2, Fe_3O_4, ZrO_2 and MgO	<50 <25 9 – 11 <100 <30	NA	*In vitro*, human blood cells infected with the parasite were treated with NPs	*Plasmodium falciparum* (malaria)	(Jacob Inbaneson & Ravikumar, 2013)
Zinc oxide (ZnO)	10 - 15	NA	*In vivo*, mice infected with parasite were treated orally with the NPs	Showed protective effect against *Eimeria Papillate* induced coccidiosis	(Dkhil, Al-Quraishy, & Wahab, 2015)
Zinc oxide (ZnO)	17	*NA*	*In vitro*, parasite in medium were treated with NPs	Helminth infection	(Khan et al., 2015b)
Zinc oxide (ZnO) and iron oxide (FeO)	20 – 30 20 – 40	NA	*In vitro,* parasites in medium were	*Helminth* infection	(Dorostkar, Ghalavand, Nazarizadeh,

			treated with NPs		Tat, & Hashemzadeh, 2017)
Titanium dioxide (TiO_2),	10 – 25	NA	*In vitro*, promastigotes in medium were treated with NPs	Promastigotes of *leishmaniasis* major	(Jebali & Kazemi, 2013) (Delavari, Dalimi, Ghaffarifar, & Sadraei, 2014)
Zinc oxide (ZnO),	10 – 30				
Magnesium oxide (MgO)	30 – 40				
Antiparasitic Activity of biogenically synthesized metal oxide NPs					
Cobalt oxide (Co_3O_4) synthesized using leaf extracts of Sageretia thea	20	NA	*In vitro*, parasites in culture media were treated with NPs	Active against *leshimaniasis*, both the axenic promastigote and amastigote cultures.	(Khalil et al., 2017)
***NA = Not available**					

3.1. Antibacterial activity

The antibacterial effects of MONPs arise from damage to cell membranes, the generation of ROS, photokilling, disturbance of metal/metal ion homeostasis, genotoxicity, and protein or enzyme damage (Figure 3) (Raghunath & Perumal, 2017). A brief description of the major mechanisms is presented below. For further detail about the mechanisms of action, readers are directed to a recent review (Raghunath & Perumal, 2017).

3.1.1. Cell wall damage

The surface charge, size of the MONPs and the nature of the bacterial cell wall (Gram negative versus Gram positive) profoundly affect the antimicrobial activity of NPs (Raghunath & Perumal, 2017). The cell walls of both Gram negative and positive bacteria have a peptidoglycan (sugar/amino acid polymer) layer, but this is thicker with Gram positive bacteria. The membrane of Gram negative bacteria is more negatively charged than Gram positive bacteria (Figure 3) (Beveridge, 1999).

Many binding forces are involved in the adhesion of NPs to the bacterial cell wall, including electrostatic, van der Waals and hydrogen bonding interactions (Parikh & Chorover, 2006). Binding is further influenced by steric effects (Neu & Marshall, 1990). The relative importance of these will depend on the net surface charge of a NP (neutral, negative or positive). It has been reported that amphiphilic molecules embedded in the walls of Gram negative (*e.g.* lipopolysaccharides, phospholipids) and Gram positive (*e.g.* teichoic acid and lipoteichoic acid) bacteria are the first molecules involved in binding with NPs (Makin & Beveridge, 1996). These amphiphilic molecules have a hydrophobic and a hydrophilic region that are able to interact with NPs approaching the bacterial cell wall. Lipopolysaccharides are reported to bind to GeO_2, α-Fe_2O_3 and α-Al_2O_3 surfaces (Parikh & Chorover, 2008).

Lipoteichoic acid is found to interact with the surface of TiO_2 NPs (Rice & Wickham, 2005; Wickham & Rice, 2008). These interaction forces facilitate the adhesion of a NP onto the cell wall of bacteria, and this is followed either by the endocytic uptake of the NP or the formation of nanoscale pores in the cell wall (Figure 3). The latter in particular permits the passage of more MONPs into the interior of the bacterium where they can interact with a range of intracellular components such as lipids, enzymes, proteins, DNA and other intracellular organelles (Raghunath & Perumal, 2017). Pores also allow leakage of intracellular components to the extracellular milieu. All these effects together result in the death of the bacterium (Raghunath & Perumal, 2017). MONPs with a positive surface charge are taken up to a greater extent than other NPs due to the prevalence of negative charges at the cell wall. This might be responsible for the selective action of some MONPs (Chung et al., 2004).

3.1.2. Production of reactive oxygen species

As noted above, ROS production is thought to be the principal mechanism underlying the antimicrobial activity of MONPs (Raghunath & Perumal, 2017). ROS comprise superoxide anions (O_2^-), hydroxyl radicals ($OH^\cdot$), hydrogen peroxide (H_2O_2) and organic hydroperoxides. ROS are normally neutralized or deactivated by the protective mechanisms present in bacterial cells, either enzymatically (catalase or superoxide dismutase) or by reducing substances such as thiol or sulphur containing compounds (*e.g.* glutathione) (Fatma Vatansever et al., 2014; Slavin, Asnis, Häfeli, & Bach, 2017a). However, these are limited in their effects and can be overwhelmed by very high ROS concentrations as discussed earlier.

MONPs dissolve and release metal ions (*e.g.* Fe^{3+}, Co^{2+}, Mn^{2+} and Cu^{2+}) both in the medium surrounding the bacteria and in the cytoplasm. Thus, after endocytic uptake of a NP into the bacteria, a certain quantity of metal ions is released into the cytoplasm. Metal ions can also easily diffuse through the cell wall of the bacterium. These two processes result in the generation of ROS inside the cell (Pereira & Oliveira, 2012). When ROS production overwhelms the cellular antioxidant defence system, oxidative stress results (Imlay, 2003; Imlay & Linn, 1988; Paravicini & Touyz, 2006; Storz & Imlay, 1999). This is associated with damage of many key biomolecules inside a micro-organism, including carbohydrates, proteins, lipids, and genetic materials (Figure 3). Oxidative stress can also lead to depletion of reduced glutathione (Jahnke et al., 2016; Madl, Plummer, Carosino, & Pinkerton, 2015), a compound which has an important role in scavenging and detoxifying ROS molecules (Ramalingam, Parandhaman, & Das, 2016). For instance, exposure of *E. coli* to ZnO and TiO_2 resulted in depletion of reduced glutathione (Ashutosh, Pandey, Singh, Shanker, & Dhawan, 2011).

The amount of ROS produced is controlled by the physicochemical properties of NPs, including their surface area, diffusibility and electrophilic nature (Raghunath & Perumal, 2017). For example, Cu_2O NPs have higher antibacterial activity than CuO NPs, indicating that the oxidation state of the metal plays a role in toxicity (Meghana et al., 2013). In this case, O_2 can oxidise Cu^+ in Cu_2O to Cu^{2+}, which can in turn react with superoxide (O_2^-), leading to sustained oxidative stress (Meghana et al., 2013). Superoxide molecules may

reduce Cu^{2+} to Cu^{+}, thereby generating H_2O_2. The latter can react with Cu^{+} to generate OH^{-} (Nieto-Juarez, Sienkiewicz, & Kohn, 2010; Slavin, Asnis, Häfeli, & Bach, 2017b). Higher concentrations of OH^{-} have been detected in cells treated with CuO NPs than those treated with Cu_2O NPs (Slavin et al., 2017b).

In addition to the production of ROS via the metal ions released from their surfaces, MONPs frequently have electron donating surfaces (Sawai et al., 1996). This endows them with the ability to generate ROS upon exposure to UV light and/or oxygen. This can lead to bacterial death *via* a process termed photokilling. Photokilling is a mechanism particularly characteristic of MONPs containing transition metals (Hongjun & Lianzhou, 2014), but ROS can be also generated from non-transition metal based materials upon exposure to light (Fatma Vatansever et al., 2014; Sawai et al., 1996). After exposure to light, the ROS produced lead to the disruption of the cell membrane, loss of permeability, damage to proteins and DNA, and damage to enzymes. Complete killing of bacteria was reported after exposure to titanium oxide (TiO_2) NP under UV light for 50 min (Tsuang et al., 2008).

3.1.3. Disturbance in metal/metal ion homeostasis

Metal ions are essential to regulate the metabolic activity of micro-organisms (Gaballa & Helmann, 1998). Excess metal ions disrupt homeostatic processes and therefore metabolic activity (Raghunath & Perumal, 2017). Excess metal ions can further bind with and cross-link genetic material either between or within DNA strands, and hence disrupt the helical nature of DNA (Raghunath & Perumal, 2017). Inside bacteria, NPs are constantly undergoing dissolution because of the electrochemical potential in solution, leading to a uniform distribution in the cell. In contrast, NPs that interact with the cell wall produce a high local concentration of ions, causing more toxicity (Hood & Skaar, 2013; Skaar & Raffatellu, 2015).

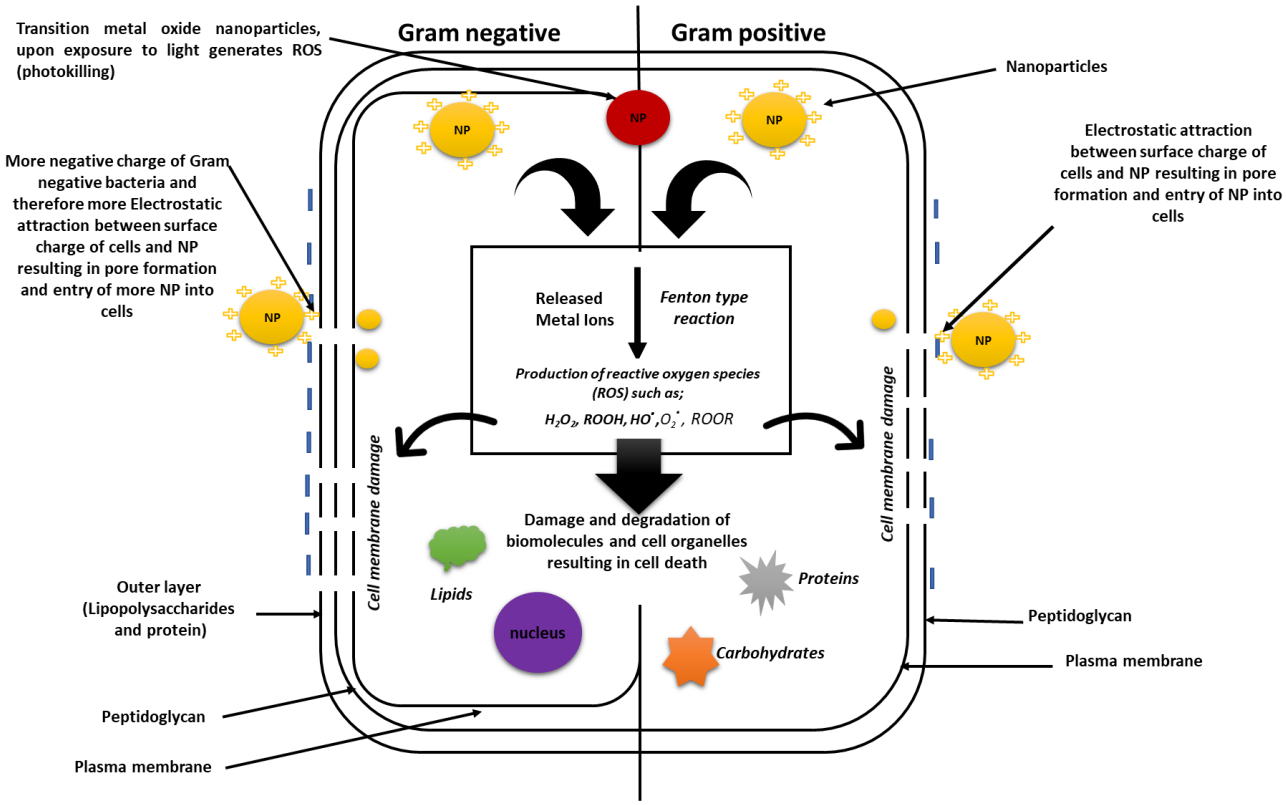


Figure 3: Mechanisms of action involved when metal oxide nanoparticles act as antibacterial agents.

3.1.4. Genotoxicity

MONPs are reported to damage both chromosomal DNA (single circular strands carrying the genetic material essential for daily metabolic activity) and plasmid (not essential for daily survival, but important at times of stress) DNA in bacteria. Such damage results in DNA oxidation and fragmentation (Giannousi, Lafazanis, Arvanitidis, Pantazaki, & Dendrinou-Samara, 2014), and has been noted with *E. coli* and *Bacillus subtillis* exposed to Cu_2O NPs (Giannousi et al., 2014).

3.2. Antiviral activity

MONPs are reported to act as antiviral agents through the attachment of the particles to the surface of the virus (Aderibigbe, 2017). This interferes with the interactions between binding sites at the exterior of the virus and specific receptors on the surface of the host cell, and therefore inhibits virus entry into the cell (Figure 4). For example, an *in vitro* study showed that Cu_2O NPs interacted with the surface of hepatitis C, inhibiting its entry into Huh7.5.1 cells and consequently inhibit viral replication (Hang et al., 2015). In addition, MONPs can be used for the delivery of a therapeutic agent (either chemically or physically attached onto the NP surface). This has been exemplified for IONPs, which were employed as targeted delivery systems carrying a DNAzyme for the treatment of hepatitis C (Ryoo, Jang, Kim, Lee, Bo, et al., 2012). *In vivo* studies on mice showed that the IONPs accumulated in the hepatocytes and macrophages in the liver, suggesting they have potential for the treatment of hepatitis C (Ryoo, Jang, Kim, Lee, Bo, et al., 2012).

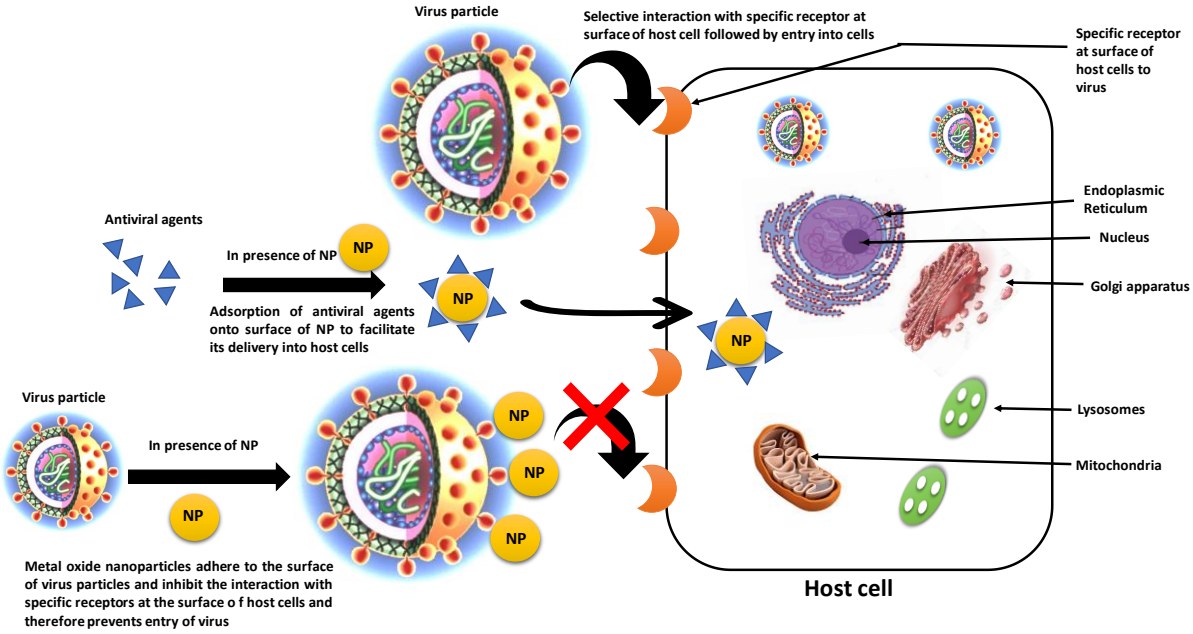


Figure 4: Mechanisms of action of metal oxide nanoparticles as antiviral agents.

3.3. Antiparasitic activity

The antiparasitic activity of MONPs involves the production of sufficiently large amounts of ROS to overcome the defence systems of the parasite. The production of ROS is initiated by metal ions released from the NPs (Aderibigbe, 2017) as previously discussed in Section 3.1.2. For instance, *in vitro* treatment of *Gigantocotyle explanatum* with different concentrations of ZnO NPs was found to kill the parasite via this route (Khan et al., 2015a). An increase in ROS has been associated with an increase of the activity of protective enzymes such as superoxide dismutase (Sawai et al., 1996), but at high concentrations of ZnO NPs (240 µg /ml), this protective system appeared to be disrupted, possibly due to the saturation of enzymes as a result of over production of hydroxyl ions and other ROS rendering the detoxification mechanism ineffective (Khan et al., 2015a).

4- TRANSLATION OF MONPS TO THE CLINIC

There are currently no MONPs formulations for antimicrobial applications in the list of FDA-approved nanomedicines (Bobo et al., 2016), despite the very significant amount of research work that has been undertaken (Table 1). To date, IONPs are the only MONPs approved by the FDA, with applications for both imaging and the treatment of iron deficiency (Anselmo & Mitragotri, 2015, 2016; Bobo et al., 2016). However, most of these IONP formulations were withdrawn and discontinued for use in the clinic (Anselmo & Mitragotri, 2015). This might be a result of several associated side-effects (*e.g.* severe lower back pain and life threatening hypersensitivity reactions) or lower imaging efficacy compared to other MRI agents (Yi-Xiang, 2015). FDA approval of these IONP formulations involved many physicochemical and biological tests at the preclinical and clinical stages. However, these did not fully guarantee biological safety and efficacy, and consequently additional tests might be required in the future. This could place obstacles to the development of MONPs for

antimicrobial activity. However, there are potentially low-hanging fruit: Ferumoxytol, which contains IONP coated with polyglucose sorbitol carboxymethylether, is an extremely safe material currently used in the clinic (Anselmo & Mitragotri, 2015; Spinowitz et al., 2008; Spinowitz et al., 2005). Given that IONPs have been shown to have antibacterial, antiviral and anti-parasite activity, the antimicrobial activity of Ferumoxytol would appear to be worthy of investigation.

The limited number of MONPs being processed into FDA approval might be a result of a lack of attention being paid to the biological and technical perspectives essential for translation to the pre-clinical and clinical stages. Key questions to be considered comprise biological challenges such as:

(1) Are the MONPs stable in the blood plasma?
(2) Can they cross key biological barriers such as the epithelium layer, and survive the gastrointestinal pH and high ionic strength of physiological fluids?
(3) Can they be selectively targeted to and taken up by an infected organ/cell?
(4) Do they initiate an immune response? If so, is this beneficial or detrimental?
(5) Can they be eliminated safely from humans after treatment?
(6) What are the potential biotoxicities associated with long-term administration?

A number of technical questions also need to be taken into account:

(1) What issues might be faced in large scale production?
(2) What physicochemical properties tests (in-process and final product) are required to allow reproducible production of MONPs?

These issues will be discussed in turn below.

4.1. Biological challenges

4.1.1. Biological barriers

There are several barriers which must be overcome by MONPs *in vivo* in order for them to have a pharmacological effect. These barriers differ according to the route of administration. This review will consider the key biological barriers encountered by NPs after application by intravenous, oral, and topical routes of administration (Figure 5).

Intravenous administration of NPs has the advantage of delivering the NP directly into the blood stream. However, many barriers must still be overcome to reach a particular desired organ. For example, plasma proteins are quickly adsorbed onto NP surfaces in the blood, forming a protein corona in a process known as opsonization (Garnett & Kallinteri, 2006). Adsorption is associated with conformational changes of the proteins, and this enhances NP recognition and elimination by macrophages of the reticuloendothelial system (RES), a part of the immune system localized in the lymph nodes, liver and spleen (Garnett & Kallinteri, 2006). Removal of NPs by the RES is very sensitive to their size. Particles greater than 200 nm are rapidly cleared by the RES of the liver and spleen (Faraji & Wipf, 2009; Kulkarni & Feng, 2013; Luís, Barros, Tsourkas, Saboury, & Cardoso, 2012). However, smaller particles are able to avoid RES uptake, and the literature reports that there is an inverse relationship

between particle size and uptake by the RES: particles of smaller sizes persist for a longer time in the systemic circulation than larger particles (Hoshyar, Gray, Han, & Bao, 2016). This has been noted for example with Au NPs (Hoshyar et al., 2016). Uptake by the RES can further be ameliorated by NP surface modification prior to administration: for instance, the attachment of polyethylene glycol (PEG) is reported to reduce protein adsorption and increase the circulation time (Stolnik, Illum, & Davis, 1995). IONPs coated with PEG accumulate to a lesser extent in the liver and spleen than their naked analogues, making them more available in the systemic circulation to be taken up by other organs (Fong-yu, Su, Yang, & Yeh, 2005).

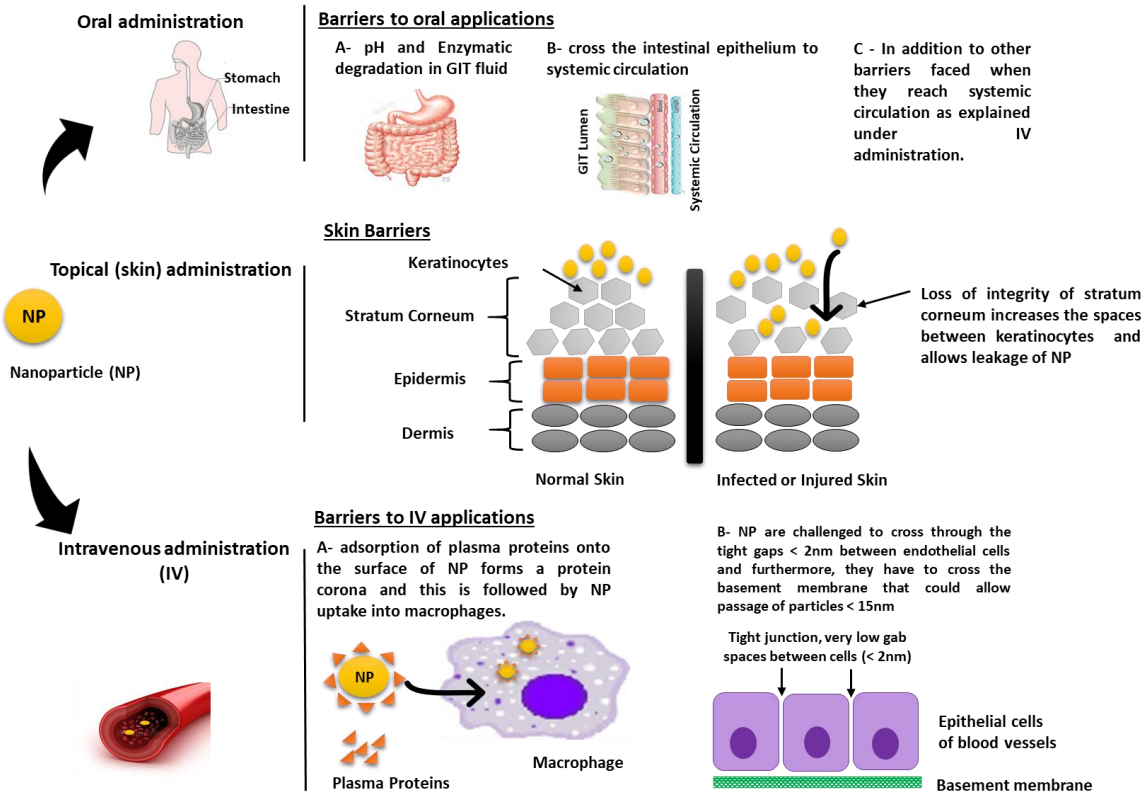


Figure 5: Challenges potentially facing NPs after oral, topical, and IV administration

The next obstacle faced by the NP will be the need to transit across the endothelium layer of the blood vessels into the extracellular fluid (Figure 5). The endothelial cells of the blood vessel membrane are tightly adhered to each other, with a gap of less than 2 nm between them (Garnett & Kallinteri, 2006). Additionally, they are supported on a basement membrane which only allows the passage of particles smaller than 15 nm. This further reduces the possibility of extravasation of NPs across the vascular endothelium (Garnett & Kallinteri, 2006). In some organs, such as the liver, the endothelium layer is more permeable and particles up to 100 nm in size can pass through (Braet et al., 2007). The spleen is also more accessible to larger particles (200 to 250 nm) (Cataldi, Vigliotti, Mosca, & Cammarota, 2017; Moghimi et al., 2017; Moghimi, Hunter, & Andresen, 2012). This means that if NPs are able to enter the systemic circulation, they are likely to accumulate in the liver and spleen. In contrast, other organs such as the brain are highly inaccessible to NPs, with very tight junctions between their endothelial cells, and thus are extremely difficult to target (Greene,

Campbell, Greene, & Campbell, 2016). However, metal-containing NPs which are able to cross the blood brain barrier (BBB) in animal models have been designed. One example of such a formulation comprises PEGylated gold NPs with particle size of 5 nm (Cheng et al., 2014). More examples of inorganic NP that are able to cross BBB can be found in a recent review by Zhou *et al.* (Zhou, Peng, Seven, & Leblanc, 2018).

If NPs manage to overcome the previous barriers to reach their target organ or tissue, they now have to cross the extracellular fluid, a jelly-like fluid filled with polysaccharides, proteins and collagen (Garnett & Kallinteri, 2006). This contains some water channels that could possibly be used for NP transportation (Garnett & Kallinteri, 2006). However, the nature of the extracellular matrix imparts a potential problem due to the possibility of protein adsorption onto the surface of the NP, leading to particle aggregation (Yue-Jian et al., 2010). Again, this might be obviated by the grafting of PEG to the particle surfaces.

After crossing the extracellular fluids, NPs are typically taken up by cells through the endocytic pathways (Porter, Moghimi, Illum, & Davis, 1992). MONPs must be able to withstand the acidic pH of the endosomes/lysosome, and/or escape into the cytoplasm of cells to exert their antimicrobial activity. However, IONPs comprise the only metal oxide system that has been extensively explored in terms of its biological fate. They were found to be significantly dissolved within the endosomes of stem cells in less than a month (Desboeufs, Michel, Pellegrino, Lalatonne, & Wilhelm, 2016). Given that a key part of the antibacterial activity of MONPs arises from metal ions being released into solution upon dissolution this may not be an issue in terms of their antimicrobial efficacy, but further investigations are required.

Oral administration of NP is likely to be favourable compared to IV administration as it is patient friendly and does not require trained medical staff or close medical observation after administration. However, orally administrated NP face additional challenges to those discussed for the intravenous route (Figure 5). These include the pH variation, potential for enzymatic degradation and high salt concentrations in the gastrointestinal tract (encouraging particle aggregation), and the need to cross its endothelium to enter the systemic circulation. Given that inorganic compounds such as MONPs are generally not endogenous, there are no mammalian enzymes which can digest them. Therefore, the digestive impact on inorganic NPs is generally not studied as an independent variable (Mccracken, Zane, Knight, Dutta, & Waldman, 2013).

The gastrointestinal fluids have a range of pH values ranging from acidic (in the stomach) to neutral and mildly alkaline pH (in the small intestine). This will affect both the surface charge and solubility of MONPs. For example, at acidic pH, cations neutralize the negative surface charge of TiO_2 NPs, resulting in particle aggregation; upon moving the NP into alkaline pH, the negative surface charge is returned and the particles disaggregate (Finnegan, 2006; Godinez & Darnault, 2010; Guiot & Spalla, 2012; Romanello & Cortalezzi, 2013). These effects can be mitigated though coating the NP; for instance, coating TiO_2 NPs with natural organic matter (phenolic and carboxylic compounds) was found to be efficient in stabilizing them against aggregation (Romanello & Cortalezzi, 2013). A number of other MONPs have also been relevealed to aggregate when suspended in simulated biological

fluids (Laijin Zhong, Yu, & Lian, 2017), with the colloidal stability found to lie in the order Fe_3O_4 < CuO < TiO_2 < CeO_2 < ZnO in all fluids tested (Laijin Zhong et al., 2017). The aggregation was found to occur because of changes in surface charges and the high salt concentrations of simulated biological fluids (Laijin Zhong et al., 2017). NP dissolution can also be a problem: γ-Fe_2O_3 NPs were reported to dissolve at the acidic pH of the stomach, relasing their metal ions into the systemic circulation (Chamorro et al., 2015).

The next expected barrier for NPs applied orally is the requirement for them to penetrate the mucus layer and the gastrointestinal epithelium layer to be available for the systemic circulation. The mucus layer is a jelly-like layer composed of water and proteins (mucin) which acts as a semipermeable barrier between the lumen and epithelium layer (Jeong et al., 2010), hindering the penetration of MONPs into the latter (Fröhlich & Roblegg, 2012). The passage of NPs through a mucus layer depends on their size and surface charge (Avdeef & Testa, 2002). Generally, neutral and positively charged NPs are able to penetrate more easily through the mucus layer (Avdeef & Testa, 2002). However, the situation is complicated and the NPs may have an influence on the composition of mucus layer: silver NPs have been reported to induce secretion of mucus of abnormal mucin composition (Jeong et al., 2010). The abnormality of mucin might be indicative of pathologic regions and requires further investigation to explore the potential toxicity of MONPs administrated orally (Jeong et al., 2010).

If NPs pass through the mucus layer, they next have to cross the epithelium of the gastrointestinal tract. The epithelial layer in the gastrointestinal tract is composed of cells linked together by intercellular junctions, restricting passage between them. All epithelia reside on a basal membrane, which separates them from the underlying connective tissue containing capillaries, lymph vessels, and lymph follicles. Therefore, MONPs have also to cross the basal membrane and the connective tissue to reach the systemic circulation (Fröhlich & Roblegg, 2012). This can be a problem: after oral administration of silver NPs into rats, a large number of NPs were detected in the connective tissue under the epithelial layer of both the small and large intestine. This was found to induce abnormal mucin composition in the intestinal mucosa (Jeong et al., 2010). Therefore, further investigation should be carried out into the pathophysiology of the gastrointestinal tract after oral administration of MONPs.

Topical application of MONPs is another route to be considered for antimicrobial therapy (Figure 5). After topical application, NPs either penetrate to deep skin layers for local effects, or permeate to the bloodstream for systemic activity (Labouta & Schneider, 2013). The biological barriers to the former, for local antimicrobial activity, are likely to be less challenging than getting the NPs into the systemic circulation. Healthy skin is divided into the epidermis and the dermis. In addition, there are two physical barriers in the epidermis: the stratum corneum (the outmost layer of the epidermis), and tight junctions (intercellular junctions that seal adjacent cells forming the stratum corneum layer) (Brandner et al., 2015; Jatana & DeLouise, 2015). Intact healthy skin does not allow permeation of NPs, but this is not the case for inflamed, injured, or infected skin (Yoshioka, Kuroda, Hirai, Tsutsumi, & Ishii, 2017). A significant amount of research has been performed to explore the penetration of NPs through healthy skin, but there is still doubt regarding the therapeutic benefit of

their topical application in humans as most studies use animal rather than human skin (Yoshioka et al., 2017), and different authors have reported strikingly different observations.

The penetration of ZnO and TiO_2 NPs through skin has been explored, but the results are contradictory. Some studies reported permeation, while others did not. For example, Tan and his colleagues observed that TiO_2 NPs of 10 to 50 nm could permeate human skin *in vivo* (Tan, Commens, Burnett, & Snitch, 1996), but in another study 20 nm TiO_2 NPs failed to penetrate human skin both *in vivo* and *ex vivo* (Pandey et al., 2014). ZnO particles < 200 nm in size (Durand, Habran, Henschel, & Amighi, 2009) and of 30 nm (Xinyu, Ishida, & Kiwada, 2007) were both found to be unable to penetrate human skin *in vitro*, and ZnO of 80 nm was unable to penetrate porcine skin *ex vivo* (Gamer, Leibold, & Ravenzwaay, 2006). In contrast, Fe_2O_3 NPs ranging from 4.6 to 10 nm could pass through incised mouse skin *in vitro* (Lee et al., 2010). The different results reported are likely due to the use of varied experimental protocols, since there is no universally agreed approach for such studies: while some authors used intact skin, others employed incised or inflamed skin; some researchers add additives to their NP formulations (*e.g.* surfactants) but others do not; and different equipment was employed for qualitative and quantitative studies. Therefore, a robust correlation between the physicochemical properties of NP (size, material, surface charge, shape) and uptake is still lacking. Further, the precise role of formulation additives (*e.g.* permeation enhancers) and the condition of the skin (healthy skin versus inflamed or infected skin) on NP penetration needs further investigation (Labouta & Schneider, 2013; Yoshioka et al., 2017).

There are a range of factors which can affect the penetration of MONPs: (1) skin factors (*e.g.* type of skin, animal or human skin, intact *vs.* incised skin, hairy skin *vs.* non-hairy skin); (2) experimental factors (*e.g.* concentration of NPs, application time, skin area, *in vivo* or *in vitro* model); (3) formulation factors (*e.g.* particle size, surface charge, material type, additives, particle stability (aggregate *vs.* individual particles) and the vehicle used to disperse the NP). All of these need to be controlled to obtain detailed insight into NP uptake. For more information, readers are directed to reviews written by Yoshioka et al. (Yoshioka et al., 2017) and Labouta and Schneider (Labouta & Schneider, 2013).

Finally, it could be expected that the use of MONPs to treat topical and local antimicrobial activity might be more applicable than trying to target the systemic circulation via the skin, because microbial infections are associated with skin inflammation and increase of the leakiness of the vascular endothelium (Bray & Geisbert, 2005). This means there is a high likelihood of NP accumulation at the site of infection. However, further studies on the pathophysiological anatomical changes accompanying the application of MONPs is still required to determine their safety.

4.1.2. Immune response

The immune system can both inhibit or potentiate the antimicrobial activity of MONPs. Hinderance of their antimicrobial activity can arise due to opsonization as previously discussed in Section 4.1.1, followed by activation of the complement pathway of innate immunity (a set of proteins which help in the recognition of foreign particles by

macrophages) (Szeto & Lavik, 2017). This in turn enhances the clearance of NPs by the RES of the liver and spleen, as has been noted for IONPs (Jain, Reddy, Morales, Leslie-Pelecky, & Labhasetwar, 2008). The activation of the complement pathway can also cause or exacerbate life-threatening hypersensitivity reactions. For example, the administration of dextran coated IONPs (*e.g.* Sinerem, Combidex, Feridex) has been associated with hypersensitivity reactions as a result of complement pathway activation. This led to their withdrawal from the market after being approved by the FDA for MRI imaging or treatment of anaemia (Banda et al., 2014; Chao et al., 2013)

On the other hand, both the innate and adaptive arms of the immune system have been reported to be activated by MONPs, which can be used as adjuvants (materials added to vaccines to boost the immune response) (Maughan, Preston, & Williams, 2014; Moreira, Neto, Kipnis, & Junqueira-kipnis, 2017). This effect of MONPs increases the possibility of invading pathogen removal. The activation of innate immunity involves immune cell recruitment and activation of antigen-presenting cells (APCs) such as monocytes, macrophages and dendritic cells. Activation of adaptive immunity involves activation of different types of T helper (Th) cells and B cells producing specific antibodies against the invaded pathogen (Mccomb, Thiriot, Krishnan, & Stark, 2013). This is summarized in Figure 6.

MONPs have been reported to modulate the immune system in a significant number of studies (Table 2) (Maughan et al., 2014). For instance, C57BL/6 mice were vaccinated with the model antigen ovalbumin (OVA) and Co_3O_4 NPs (CNP) and their efficacy compared with the Imject alum adjuvant. CNPs stimulated T helper cells with a more balanced Th1 (cellular immunity, potent against intracellular infections) to Th2 (antibody immunity, effective against extracellular pathogens) ratio than alum. Anti-OVA antibody production was less pronounced with CNP than alum, which is indicative of lower risks of allergic responses (Cho et al., 2012).

IONPs are reported to have time and dose dependent immunomodulatory effects both *in vitro* (M2 macrophage cell line) (Rojas et al., 2016) and *in vivo* (BALB/c mice) (Shen, Wang, Liao, & Jan, 2011). *In vivo* immunization of mice with IONPs coated with a surface protein from the merozoite parasite *via* intramuscular, subcutaneous or intraperitoneal routes (Pusic et al., 2013), all resulted in activation of adaptive immunity against the pathogen (*e.g.* B cell activation, with production of a significant level of anti-parasite antibodies and production of splenocyte cytokines (IL-4 and IFN-γ)). In other work (Pusic et al., 2013), bone marrow-derived dendritic cells treated with IONPs were reported to be activated. All these effects indicate that the immune response could be used to ameliorate microbial infections *via* the application of metal oxide NPs.

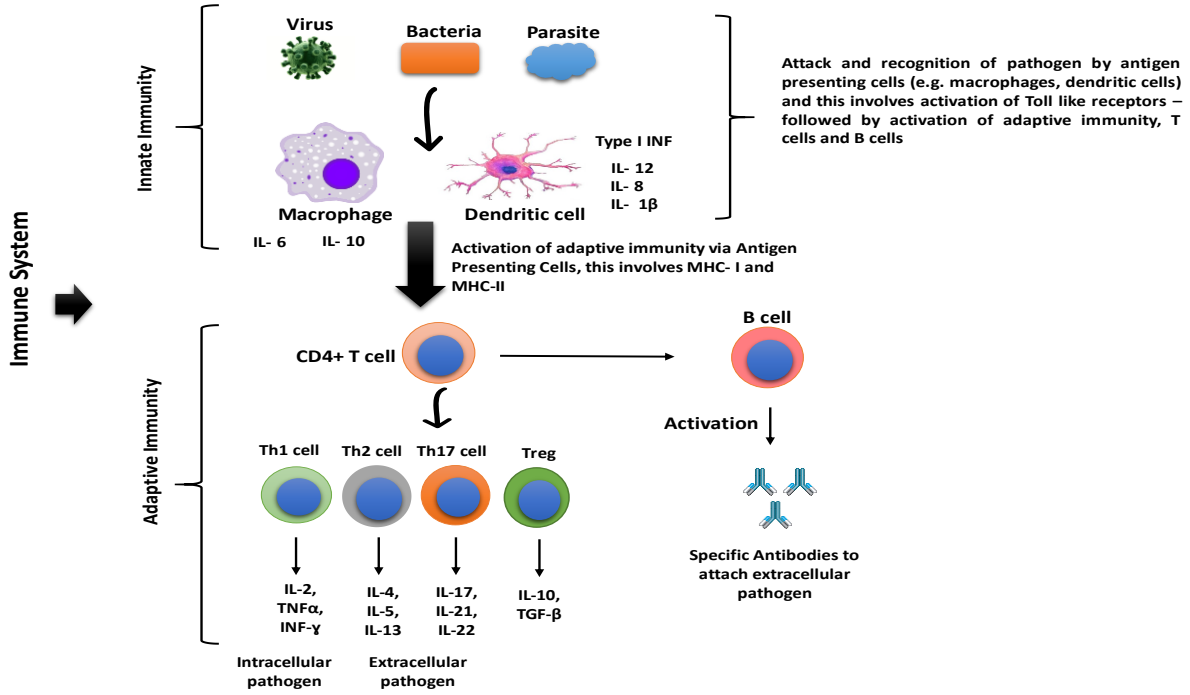


Figure 6: Immune response both innate and adaptive immunity against the invaded pathogens

Immunostimulatory effects have also been observed with TiO_2 NPs, which activated innate immunity when applied to the lungs of rats *in vivo* (Duffin, Tran, Brown, Stone, & Donaldson, 2007). TiO_2 NP are also reported to stimulate human macrophages (Lucarelli et al., 2004), and liver cells in mice after intraperitoneal injection (Cui et al., 2011). TiO_2 NPs with different physical properties (polymorphs, particle size/shape) applied to dendritic cells resulted in the activation of both innate and adaptive immunity (Schanen, Karakoti, Seal, Iii, & Warren, 2009), (Winter et al., 2011). Zirconium dioxide (ZrO_2) has also been reported to induce adaptive immunity (*e.g.* activation of T helper cells) (Hanley et al., 2009). *In vivo*, zinc oxide NPs administered with OVA generated an inflammatory response and activation of adaptive immunity in mice (Roy et al., 2014; Matsumura et al., 2010).

Such activation of the immune system by MONPs could offer an alternative route to microbial eradication. However, further studies should be performed to develop a formulation that could activate the immune system without causing hypersensitivity reactions. Further investigations are also required to explore how chemical and physical properties such as material composition, size, shape, surface charge and hydrophobicity impact the immune system, as there is limited literature regarding this. This is particularly important because it is difficult to change one parameter without affecting others: for example, it is hard to change particle size without affecting the surface charge (Labouta & Schneider, 2013; Pasquale, Preiss, Silva, & Garçon, 2015).

Table 2: Immunomodulatory effects of metal oxide nanoparticles

Name	*Study details*	*Immunomodulatory effect*	*Reference*
Co_3O_4 combined with an antigen model, OVA	*In vivo* immunization with C57Bl/6 Mice	Induces adaptive immunity *e.g.* induction of different types of T helper (Th) cells (Th1, Th2 and Th17 cells) Low level of antigen antibodies *e.g.* IgE and IgG1	(Cho et al., 2012)
IONPs (Fe_3O_4)	*In vitro* test on M2 macrophages	Time dependant immunomodulatory effects with increased production of interleukin (IL) 10	(Rojas et al., 2016)
IONPs (Fe_3O_4)	*In vivo* test on BALB/c mice	Decrease of the production of splenocyte cytokines (IL-4 and IFN-γ)	(Shen et al., 2011)
IONPs (Fe_3O_4) coated with Merozoite surface protein 1	In-vivo immunization of mice	Higher production of IL-4 compared to IFN-γ; increase production of anti-parasite antibodies	(Pusic et al., 2013)
IONPs (Fe_3O_4)	*In vitro* tests on bone marrow-derived dendritic cells (BMDCs)	Activate APCs increased IL-6, TNF-α, IFN-γ, and IL-12 production, upregulation of dendritic cells	(Pusic et al., 2013)
TiO_2	*In vivo* test on mice	Increases influx of neutrophil into lung of mice	(Duffin et al., 2007)
TiO_2	*In vitro* test on human macrophages, PMA-differentiated myelomonocytic U-937 cells	Induction of Toll-like receptors (TLRs), proteins with a major role in the immune system	(Junqueira-Kipnis, Marques Neto, & Kipnis, 2014)
TiO_2	*In vitro* tests on denderitic cells derived from human umbilical vein endothelial cells	Induces proliferation of naïve CD4+ T cells, enhance Th1 response increase IFN-γ and TNF-α production	(Schanen et al., 2009)
TiO_2	*In vitro* tests with bone marrow derived dendritic cells	Induction of surface proteins important in immunity (MHCII and CD80)	(Winter et al., 2011)
ZrO_2	*In vitro* test with human macrophages, PMA-differentiated myelomonocytic U-937 cells	Induction of TLRs	(Lucarelli et al., 2004)
ZnO	*In vitro* tests with peripheral blood mononuclear cells	Induced IFN-γ, TNF-α, and IL-12	(Hanley et al., 2009)

ZnO	*In vivo* test with BALB/c mice	Induction of TLRs	(Roy et al., 2014)
ZnO	*In vivo* imuunization test on BDA/1J mice	Increased levels of IL-4, IL-5 and IL-13 Activation of Th2 cells	(Matsumura et al., 2010)
ZnO	*In vivo* immunization with mice (BALB/c)	High levels of IgG1 and IgE Induction of IL-2, IL-4, IL-6 and IL-17 Lower levels of while IL-10 and tumour necrosis factor-α. Increased number of eosinophils and mast cells. High level of Th2 cells	(Roy et al., 2013)

4.1.3. Targeting of MONPs into infected organs

Selective delivery of NP into the site of microbial infection can be passive, depending on the properties of the blood vessels at the site of infection as previously discussed in section 4.1.1, or active (via coating the NP with specific ligands targeting receptors at the cell surface). Although active targeting strategies have been widely explored in the literature there are to date no nanomedicine products with active targeting abilities that have been approved by the FDA for the treatment of any type of disease (Bobo et al., 2016; Kamalya, Xiaoa, Pedro M. Valenciab, & Farokhzad, 2009; Ventola, 2017a). This reveals the challenges faced in targeting MONPs to infected organs while avoiding harmful effects due to accumulation in off-target organs. One example of a targeted nanoscale formulation that is being investigated is SGT-53 (SynerGene Therapeutics), which contains an anti-transferrin antibody fragment that binds with a transferring glycoprotein receptor on cancer cells (Bobo et al., 2016). This agent is in Phase 1 and 2 clinical trials for the treatment of solid tumours, glioblastoma, and metastatic pancreatic cancer (Ventola, 2017a).

4.1.4. Elimination

It is generally a prerequisite for any formulation to be eliminated from the human body after administration to avoid the hazards of long term toxicity due to accumulation in the tissues (Ionescu & Caira, 2005). MONPs are not a target for enzymatic degradation (as discussed in Section 4.1.1). This means that controlling particle size is essentially the only strategy that can be employed to enhance their elimination through the kidneys (this requires particle size < 10 nm), especially in long term administration. NPs containing essential metals such as IONPs are reported to be reused as a nutritional source by the body; this is accompanied by an increase in iron ion levels, but these return to normal levels within three weeks after intravenous administration (Jain et al., 2008). This results in lower cytotoxicity at the therapeutic dose (Bassett, Halliday, & Powell, 1986) than might be the case where non-endogenous metals are used. The biological fate of MONPs needs further study however (Desai, 2012), particularly in terms of their potential toxicological effects during short and long term administration.

4.1.5. Toxicology

MONPs can be synthesized either through traditional chemical methods or green/biogenic methods (using plant extracts or micro-organisms). The toxicological properties of some chemically synthesized MONPs were studied, and these were reported to be toxic both *in vitro* and *in vivo* due to the release of ROS and damage of intracellular components such as proteins, enzymes and DNA, and interference with the respiratory chain of the mitochondria in human cells (Seabra & Durán, 2015). It was speculated that the green synthesis of MONPs might be associated with lower toxicity to human cells due to (1) the absence of residues from toxic organic solvents and additives (*e.g.* surfactants) required for traditional chemical synthesis and (2) the possibility of the particles produced being coated with proteins or other components from the biogenic synthesis, forming a corona that might be more bio-compatible than toxic materials adsorbed in the chemical synthesis (Seabra & Durán, 2015). Table 3 summarizes the cytotoxicity of some MONPs synthesized by both chemical and biogenic methods.

The biogenic synthesis route was found to decrease the cytotoxicity of Co_3O_4 in terms of biocompatibility with human red blood cells and macrophages (Khalil et al., 2017). However, biogenically synthesized Fe_3O_4 NPs produced in *Magnetospirillum gryphiswaldense* were compared with Fe_3O_4 synthesized by a standard co-precipitation method for their cytotoxicity to L929 mouse fibroblast cells, and both showed comparable cytotoxicity (Han et al., 2007). This is contrary to another study (Yaaghoobi, Emtiazi, & Roghanian, 2012) where cell lysis of human peripheral blood cells after exposure to commercially produced Fe_3O_4 was noted, while there was no cytotoxicity or morphological changes observed with Fe_3O_4 NPs synthesized with a green method (Yaaghoobi et al., 2012).

In vivo, TiO_2 synthesized by a green method was reported to show no cytotoxic effect on Wistar rats (Wang & Fan, 2014), while chemically synthesized TiO_2 NPs were highly toxic in mice, resulting in spleen damage, immune dysfunction, alteration of gene expression, and apoptosis (Babitha & Korrapati, 2013). Other studies revealed that biogenically synthesized NPs were also associated with cytotoxicity: for example, Co_3O_4 NPs synthesized by green (Cho et al., 2012) and chemical (Papis et al., 2007; Ponti et al., 2009) methods have both been found to be cytotoxic. Therefore, the safety profile of MONPs generated biogenically is still controversial, and further investigation is necessary to understand their toxicological properties. For more information on the toxicology of MONPs, readers are directed to a recent book chapter (Saquib, Faisal, & Abdulrahman, 2018).

Table 3: Cytotoxicity studies on metal oxide nanoparticles

Nanoparticle composition	**Chemical methods**			**Biogenic methods**		
	In vitro/ In vivo test	***Toxicity***	***Reference***	***In vitro/ In vivo test***	***Toxicity***	***Reference***
Aluminium oxide (Al_2O_3)	L929 and BJ* cells	No cytotoxic effects	(Radziun et al., 2011)	No available cytotoxicity studies		
Antimony trioxides (Sb_2O_3)	Seven types of human cell lines	No cytotoxic effects	(Cooper & Harrison, 2009)	No available cytotoxic studies		

Calcium oxide (CaO)	Rats	Areas of necrosis, and haemorrhages in liver, kidney and brain	(Butt, Ejaz, Baron, Ikram, & Ali, 2015)	No available cytotoxicity studies		
Cobalt oxide (Co_3O_4)	Human lymphocytes	Morphological transformation and genotoxicity	(Papis et al., 2007); (Ponti et al., 2009)	Instilled into lung of Wistar rats	Immuno-inflammatory response associated with lung damage	(Cho et al., 2012)
Cobalt oxide (Co_3O_4)	Balb 3T3* cells	DNA damage, inflammatory responses	(Chattopadhyay et al., 2015)	Human RBCs and macrophages	Biocompatible with no marked toxicity	(Khalil et al., 2017)
Cobalt oxide (Co_3O_4)	BEAS-2B* cells	Production of ROS	(Ortega et al., 2014)	Further cytotoxicity studies are required		
Copper oxide (CuO)	A549, lung epithelial cell line	DNA damage	(Karlsson, Cronholm, Gustafsson, & Möller, 2008)	AMJ-13 and SKOV-3 cancer cell line	Cell growth arrest	(Sulaiman, Tawfeeq, & Jaaffer, 2018)
Copper oxide (CuO)	MCF-7 cells, human breast cancer cells line	Dose and time dependant autophagy	(Laha et al., 2014)	Dermal fibroblast cell line	Cell apoptosis	(Sulaiman et al., 2018)
Copper oxide (CuO)	Mice	Dose dependent apoptosis, damage to the immune system	(Siddiqui et al., 2013)	Further cytotoxicity studies are required		
Copper oxide (CuO)	Human blood lymphocytes	Decreased cell viability in a conc dependant pattern	(Assadian et al., 2017)	Further cytotoxicity studies are required		
Iron oxide (Fe_3O_4)	Human peripheral blood cells	Lysis of cells	(Yaaghoobi et al., 2012)	Human peripheral blood cells	No morphological changes	(Yaaghoobi et al., 2012)
Iron oxide (Fe_3O_4)	L929 mouse fibroblast cell line	Viability of cells was around 85%	(Han et al., 2007)	L929	Viability of cells was around 90%	(Han et al., 2007)
Titanium dioxide (TiO_2)	mice	Spleen damage, immune dysfunction, alteration of gene expression, apoptosis	(Wang & Fan, 2014)	Wistar rats	No cytotoxic effects recorded	(Babitha & Korrapati, 2013)

Zinc oxide (ZnO)	A549 cells	Decreased cell viability	(Cho et al., 2012)	*neuro2A cells	Decreased cell viability	(Darroudi et al., 2014).

*Balb 3T3 cells, immortalized cells developed from primary mouse embryonic fibroblasts; BEAS-2B cells, human airway epithelium cells; neuro2A cells, a fast-growing mouse neuroblastoma cell line; BJ cells, normal human cells, skin fibroblasts

4.2. Technical challenges

In the pharmaceutical industry, reproducible production of a dosage form is crucial. For the nanomedicine field, this requires additional tests to those needed for conventional dosage forms and involves determination of NP size, size distribution, surface charge, release of active ingredients, purity and surface functionalization (ligands for active targeting) (Desai, 2012). The stability of the nanomedicine at both the pre-clinical and clinical stages will also be vital (Desai, 2012). These properties of NPs are crucial in determining their pharmacological effects, which has led the FDA to propose a series of key tests which must be undertaken on any new nanomedicine (Table 4).

These tests are essential as they quantify the physicochemical properties of NPs, which in turn control their interactions with cells and other biological components and therefore the ultimate therapeutic outcome (Lina, Wanga, & Sridharb, 2014). For example, size regulates the circulation and navigation of nanomaterials in the bloodstream, penetration across physiological membranes, site- and cell-specific localization, and even the induction of cellular responses (Feng, 2004; Ferrari, 2008): smaller silver NPs cause greater apoptotic effects with certain cell lines (Kim et al., 2012; Sosenkova & Egorova, 2011).

The surface composition determines the surface charge and energy. The latter is relevant to the dissolution, aggregation and accumulation of nanomaterials. Surface charge affects receptor binding and physiological barrier penetration, governs NP dispersion stability or aggregation and is generally estimated in terms of the zeta potential (Lina et al., 2014). For example, the high ionic strength of physiological fluids enhances aggregate formation and therefore affects the interactions of NPs with cells. Zebrafish embryos were reported (Truong, Zaikova, Richman, Hutchison, & Tanguay, 2012) to be extremely sensitive to gold NPs under conditions of low ionic strength, in which the NPs disperse, but not at high ionic strength. The surface charge and composition of NPs further affects the composition of the protein corona formed after the introduction of NPs into a biological system, which in turn influences their interactions with cells (Huinan & Webster, 2007; Lina et al., 2014). The chemical composition of a NP has a number of effects in terms of dissolution and cellular interactions, all of which alter the viability and functionality of cells. For example, a sub-lethal pro-inflammatory response was reported with Al_2O_3 NPs in a murine macrophage cell line, while ZnO NPs induced a lethal genotoxic effect (Ralloa et al., 2015).

The shape of a nanomaterial affects cellular uptake, biocompatibility and retention in tissues and organs (George et al., 2014; Pal, Tak, & Song, 2007). It was also reported that modulation of the shape of NPs can alter their flow in the systemic circulation, adhesion

properties with cells, and circulation time (Doshi, Prabhakarpandian, Rea-Ramsey, Pant, & Shivshankar Sundaram, 2014; Geng et al., 2009).

In addition to the technical challenges encountered during the manufacture of NPs, other obstacles are faced during the evaluation of the pharmacological activity of the administrated material, since the simple pharmaco-equilibrium theory used for conventional dosage forms (measuring the drug concentration in the blood to reflect therapeutic efficacy) cannot be applied (Desai, 2012). The pharmacological activity of nanomedicines depends on (1) NP accumulation at the target site, and (2) achieving a high efficacy/risk ratio compared to conventional dosage forms (Havel et al., 2016). The measured plasma concentrations after nanomedicine application reflect the nature/number of circulating NPs and this cannot be directly correlated to pharmacological or toxicological effects.

Scaling up NP production is another technical challenge. There are four major methods used for the manufacture of MONPs: dry or wet milling, vapour, liquid and solid phase synthesis (Tsuzuki, 2009). The milling and vapour phase methods have the disadvantages of producing NPs of broad size distribution and in aggregated form, while the liquid method produces particles of narrow size distribution but with a high degree of agglomeration. The solid phase method gives a product with a uniform size, shape and low level of agglomeration (Tsuzuki, 2009). To date the vapour and liquid phase syntheses have been most widely used for the synthesis of MONPs. Suitable gas-based techniques include physical vapour deposition, chemical vapour deposition, flame pyrolysis, spray pyrolysis, laser ablation, gas condensation, and electro-explosion. Liquid approaches found to be effective are, *inter alia*, sol-gel, hydrothermal, solvothermal, sono-chemical, reverse micelle, colloidal and microwave syntheses (Tsuzuki, 2009).

Large scale production of MONPs could be performed using batch or continuous flow reactors. The latter are likely to be more applicable industrially, owing to batch-to-batch variation arising in batch processes. Continuous flow reactors can produce nanoparticles on an industrial scale with a high degree of reproducibility (Kwon et al., 2018). Two types of continuous flow systems, tubular and spinning disc reactors, have both been found to be effective for the synthesis of MONPs (Kwon et al., 2018). However, continuous flow reactors tend to result in the production of MONPs with a broader size distribution than those synthesized in batch reactors and hence require further optimization. Nevertheless, the continuous flow reactor has very high productivity (Kwon et al., 2018), and thus is expected to be the industry choice for large scale MNOP synthesis. For more detail, the readers are directed to two detailed review papers (Kwon et al., 2018; Tsuzuki, 2009).

Additionally, MONPs are known to aggregate in simulated biological fluids (Laijin Zhong et al., 2017) and further investigations are still required to stabilize them *in vivo*. Often, the integration of different components (*e.g.* polymer, drug, organic solvent, non-solvent, surfactant, etc) in the nanomedicine is necessary to achieve the desired performance. In some cases, decoration of MONPs using a ligand for targeting purposes might be required, and this imparts additional challenges such as determining a reproducible pattern of spatial orientation and distribution of the ligand molecules on the surface of the NP (Desai, 2012).

Sterilization imparts another challenge for nanomedicines production. The sterilization methods applied for conventional pharmaceutical dosage forms (autoclaving, gas sterilisation, γ-radiation *etc*) cannot be applied to nanomedicines because they cause particle aggregation and consequently affect pharmacological activity (Bozdag, Dillen, Vandervoort, & Ludwig, 2005; Kempner, 2001; Özcan, Bouchemal, Segura-Nchez, Özer, 2009; Qindeel, 2017). Instead, filtration might be a good choice for nanomedicine sterilization (Desai, 2012; Qindeel, 2017). All these technical challenges must be carefully considered by scientists developing MONPs for antimicrobial therapy. This requires additional experimental work to control the size, size distribution and surface characteristics of MONPs at both the pre-clinical and clinical stages.

Table 4: Suitable analytical techniques for determining the physicochemical properties of metal oxide NPs (Ali et al., 2016; Lina et al., 2014)

Physicochemical properties	***Technique used***	***Advantages***	***Restrictions***
Size (hydrodynamic size)	DLS	Non-destructive analysis method Rapid and reproducible measurement Measures in any liquid media, solvent of interest Hydrodynamic sizes accurately determined for monodisperse samples Modest cost of apparatus	Insensitive correlation of size fractions with a specific composition Influence of small numbers of large particles Limit in polydisperse sample measures Limited size resolution Assumption of spherical shape samples
Hydrodynamic dimension, binding kinetics	FCS	High spatial and temporal resolution Low sample consumption Specificity for fluorescent probes Method for studying chemical kinetics, molecular diffusion, concentration effect, and conformation dynamics	Limit in fluorophore species Limited applications and inaccuracy due to lack of appropriate models
Hydrodynamic size and size distribution (indirect analysis), conformation change of protein–NP conjugate, structural, chemical and electronic properties	SERS RS TERS	Enhanced spatial resolution. No need for sample preparation. Complementary data to IR No requirement for sample preparation Potential of detecting tissue abnormality Increased spatial resolution (SERS) Topological information of nanomaterials (SERS, TERS)	Limited spatial resolution (only to micrometers) Extremely small cross section Interference of fluorescence Irreproducible measurement (SERS)
Size and shape of nanomaterials	NSOM	Simultaneous fluorescence and Spectroscopy measurement Nano-scaled surface analysis at ambient conditions Assessment of chemical information and interactions at nano-scale resolution	Long scanning time Small specimen area analyzed Incident light intensity insufficient to excite weak fluorescent molecules Difficulty in imaging soft materials Analysis limited to the nanomaterial surface
Molecular weight, composition/structure, and surface properties	MS	High accuracy and precision in measurement. High sensitivity to detection (a	Expensive equipment. Lack of complete databases for the identification of molecular species.

		very small amount of sample required).	Limited application to date in studying nanomaterial bioconjugates.
Structure and conformation of bioconjugate, surface properties	IR ATR-FTIR	Fast and inexpensive measurement Minimal or no sample preparation requirement (ATR–FTIR) Improving reproducibility (ATR–FTIR) Independence of sample thickness (ATR–FTIR)	Complicated sample preparation (IR) Interference and strong absorbance of H2O (IR) Relatively low sensitivity in nanoscale analysis
Size and size distribution, shape, aggregation, and dispersion	SEM ESEM	Direct measurement of the size/size distribution and shape of nanomaterials High resolution images of biomolecules in natural state provided using ESEM	Conducting sample or coating conductive materials required Dry samples required Sample analysis in non-physiological conditions (except ESEM) Biased statistics of size distribution in heterogeneous samples Expensive equipment Cryogenic method required for most NP-bioconjugates Reduced resolution in ESEM
Shape heterogeneity, size and size, and dispersion	TEM	Direct measurement of the size/size distribution and shape of nanomaterials with higher spatial resolution than SEM Several analytical methods coupled with TEM for investigation of electronic structure and chemical composition of nanomaterials	Ultra-thin samples in required Samples in non-physiological conditions Sample damage/alternation Poor sampling Expensive equipment.
Size and size distribution, shape, structure, dispersion, and aggregation	STM	Direct measurement High spatial resolution at atomic scale	Conductive surface required Surface electronic structure and surface topography
Size and size distribution, shape, structure, sorption, dispersion, aggregation **Surface properties**	AFM	3D sample surface mapping Sub-nanoscaled topographic resolution Direct measurement of samples in dry, aqueous or ambient environment.	Overestimation of lateral dimensions Poor sampling and time consuming Analysis in general limited to the exterior of nanomaterials
Size (indirect analysis), structure **Composition** **Purity** **Conformational change**	NMR	Non-destructive/ non-invasive method Little sample preparation	Low sensitivity Time consuming Relatively large amount of sample required Only certain nuclei NMR active
Size, shape and structure for crystalline materials	XRD	Well-established technique High spatial resolution at atomic scale	Limited applications in crystalline materials Only single conformation/ binding state of sample
Size/size distribution, shape **structure**	SAXS	Non-destructive method Simplification of sample preparation	Low resolution.

		Amorphous materials and sample in solution accessible	
Surface charge and particle stability	Zeta -sizer	Simultaneous measurement of many particles (using ELS)	Electro-osmotic effect Lack of precise and repeatable measurement

Abbreviations: AFM, atomic force microscopy; ATR, attenuated total reflection; DLS, dynamic light scattering; ESEM, environmental SEM; FCS, fluorescence correlation spectroscopy; FTIR, Fourier transform infrared; IR, infrared; MS, mass spectroscopy; NM, nanomaterial; NMR, nuclear magnetic resonance; NPs, nanoparticles; NSOM, near-field scanning optical microscopy; RS, Raman scattering; SAXS, small-angle X-ray scattering; SEM, scanning electron microscopy; SERS, surface-enhanced Raman scattering; TEM, transmission electron microscopy; TERS, tip-enhanced Raman spectroscopy; XRD, X-ray diffraction.

CONCLUSION

This review explores the potential application of metal oxide nanoparticles (MONPs) for antimicrobial applications. We first consider the burden caused by microbial infections globally, and then survey the literature investigating the potential of MONPs to ameliorate these. There is extensive evidence to show that MONPs are effective in the treatment of bacterial infections, although the majority of this comes from *in vitro* studies. There is also promising evidence that MONPs will also be effective against viral and parasite-caused diseases. In large part, this efficacy is attributable to MONPs' mechanism of action involving the production of reactive oxygen species, which can circumvent the issue of antimicrobial resistance by simultaneously attacking multiple targets on a target organism.

We next consider the potential obstacles which MONP-based medicine will face *in vivo*, and how these might be overcome. Such challenges include delivering the NPs to the appropriate part of the body, the cellular response to them *in vivo*, and difficulties in large scale production and ensuring reproducibility in synthesis. The potential toxicity of the NPs to healthy cells is considered, as is the ability of MONPs to trigger an immune response *in vivo*. The latter could have both benefits and disbenefits in antimicrobial therapy, and there exists the possibility of using MONPs to stimulate the immune system to attack invading pathogens. In terms of synthesis, we evaulate routes to achieve high-throughput and high-reproducibility synthesis of MONPs, as well as the use of "green" synthetic approaches to ameliorate off-target toxicity. Finally, we discuss the technical and regulatory challenges which need to be overcome for MONP-based antimicrobial medicines to reach the clinic. Overall, it is clear that MONPs have great potential as antimicrobial agents, and there are potentially some "quick wins" from the repurposing of already-approved nanoparticle-based medicines (*e.g.* those based on iron oxide nanoparticles). There remain however a number of hurdles, both technical and biological, to the clinical translation of new MONP-based formulations. Future research needs to focus on: i) obtaining a more detailed understanding of how MONPs behave *in vivo* (in terms of their location in the body, pharmacokinetics, pharmacodynamics and toxicity); ii) new routes to high-reproducibility synthesis on the industrial scale; and, iii) developing a robust panel of quality control assays to produce systems appropriate for use in patients.

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