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REVIEW

Silver Nanoparticles for the Therapy of Tuberculosis

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Abstract: Rapid emergence of aggressive, multidrug-resistant Mycobacteria strain represents the main cause of the current antimycobacterial-drug crisis and status of tuberculosis (TB) as a major global health problem. The relatively low-output of newly approved antibiotics contributes to the current orientation of research towards alternative antibacterial molecules such as advanced materials. Nanotechnology and nanoparticle research offers several exciting new-concepts and strategies which may prove to be valuable tools in improving the TB therapy. A new paradigm in antituberculous therapy using silver nanoparticles has the potential to overcome the medical limitations imposed in TB treatment by the drug resistance which is commonly reported for most of the current organic antibiotics. There is no doubt that AgNPs are promising future therapeutics for the medication of mycobacterial-induced diseases but the viability of this complementary strategy depends on overcoming several critical therapeutic issues as, poor delivery, variable intramacrophagic antimycobacterial efficiency, and residual toxicity. In this paper, we provide an overview of the pathology of mycobacterial-induced diseases, andhighlight the advantages and limitations of silver nanoparticles (AgNPs) in TB treatment.

Keywords: nanoparticles, antimycobacterial, *Mycobacterium*, tuberculosis, macrophage, granuloma

Introduction

The emergence of multidrug-resistance, the intercurrent immunosuppressive diseases, the relatively low-output and high costs of newly-approved antituberculous antibiotics, and the partially protective vaccines, represents the main cause of the current status of tuberculosis as a regionally re-emerging and global health problem[1–4] slowing in the same time the progress towards TB eradication. Tuberculosis infects globally more than one-third of human population,[5] and despite the lastest progress, it remains according to the latest WHO report the world's leading infectious-bacterial cause of deaths among adults, accounting only in 2018 more than 1.5 million deaths and 10 million new cases.[6,7] Moreover, in some endemic areas, TB was the first cause of hospital death.[8]

Tuberculosis (TB) is a zoonotic and anthropozoonotic disease with a complex pathogenesis, produced by bacteria from *Mycobacterium tuberculosis* complex (*MtbC*), mainly *M. tuberculosis*, and in a lesser amount by the infections with other mycobacteria such as *M. bovis, M. africanum, M. caprae, M. canetti*, and occasionally *Mycobacterium pinnipedii* or *M. microti*.[9–11] For some newly included members of *MtbC* as *M. mungi*,[12] the exact role in human tuberculosis is currently poorly understood. *MtbC* bacteria are nonmotile and non-sporulated bacilli with a distinctively thick and lipid-rich cell wall included in the Actinomycetales order. The emergence of drug-resistant strains

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of *MtbC* observed in the last decades gives rise to additional challenges to the anti-TB prevention and control efforts.[8]

Nanotechnology and nanoparticle science are emerging disciplines connecting interdisciplinary areas of research such as chemistry, physics, and medicine providing innovative approaches and new-practical solutions for several critical-issues, including bacterial-induced infectious diseases.[13,14] Metallic silver has a long history in medical applications, but its popularity markedly declined following the introduction and broad-usage of antibiotics.[15] Nowadays, in the context of continuous rise in the rate of antibiotics consumption and "antibioresistance crisis", silver in the form of AgNPs or in combination with classical antibiotics has made a remarkable comeback as a potential antibacterial molecule in the medicine and health care industry.[16,17] Intracellular survival represents peculiar pathogenic factors of Mycobacteria, and this combined with the thick, hydrophobic (waxy) bacterial cell wall rich in mycolic acid and arabinogalactan contributes to the "phagocyte sabotage", failure of the immune system to clear the septic focus, ensures the long-term persistence and furthermore, the local to systemic dissemination of infection.[18] Recent reports have shown that AgNPs have a high antimycobacterial effect in both bacterial cultures and within macrophages,[19,20] thus, the exploration of this new-concept of antimycobacterial-nanoparticles could change the current optics regarding TB-therapy.

This review explores in detail the main pathological features of mycobacteria and TB-pathogenesis, the AgNPs antibacterial mechanism of action per se and in combination with antibiotics, and not least the advantages and the limitation on using AgNP in TB therapy. Also, we up-to-date review of the main in vitro, in vivo and clinical studies assessing the antimycobacterial potential of AgNPs.

The Emergence of Drug Resistance Tuberculosis

Drug-resistant (DR) (defined as resistant to one or more antituberculosis drugs) and finally Multi-Drug-resistant tuberculosis (MDR) (defined as antibioresistace to at least rifampicin and isoniazid, the two most powerful antituberculosis drugs)[7] is the most urgent and difficult provocation in TB treatment, a major public health concern, and an important cause or global TB reemergence noticed in the last three decades.[21,22] New cases of both DR and MDR are typically expected to appear following the amplification of TB-resistance patterns through inadequate usage of antituberculosis chemotherapy, mainly the therapeutic use of ineffective-antibiotics formulations as first-line treatment and the premature stoppage of treatment and not last the inter-patient transmission of DR/MDR/XDR (Drug-/Multidrug-/Extensively drug-resistant tuberculosis) strains of TB, especially observed in areas with a high prevalence of DR/MDR-TB infections of following nosocomial transmission.[7,23,24] Infection with MDR-TB strains is associated with a high mortality rate (up to 55%, compared to 4.5−17% mortality in infections with nonresistant TB-strains). A low treatment success despite the usage of appropriate second-line treatment, and typically spans a relatively short clinical course from diagnosis to death, especially in cases with concurrent infections like HIV or reduced body mass index.[7,25-27]

The current antituberculous therapy involves the first-line treatment during a 6 to 9 months, involving four antibiotics in sequential combination (isoniazid, rifampin, pyrazinamide, and ethambutol). In case of relapse or antibioresistace, the second-line therapy-treatment (during 18–24 months) of combination therapy with second-line drugs as aminosalicylic acid, fluoroquinolones, aminoglycosides, cycloserine, linezolid, and clofazimine, which are typically more toxic, more expensive and less efficient.[28–30] In addition to poor efficiency in the case of MDR and XDR-strains of *M tuberculosis*, major adverse reactions (mainly hepatitis, gastrointestinal events) are present in more than 30% of cases following first-line therapy[31] and in 83% following the second-line antituberculous therapy.[32] Following the second-line antituberculous therapy, the adverse reactions are more severe and include mainly gastrointestinal and hepatic reactions, CNS adverse effects (including reactions raging from insomnia to psychosis and delirium), arthropathies, nephrotoxicity and electrolyte abnormalities, ototoxicity, hypothyroidism and hematological toxicity.[30,33]

The prolonged antituberculous therapy, limited antibacterial activity and intercurrent diseases are the main reason for patient noncompliance and finally, the induction of DR/MDR/XDR strains *MtbC*. The DR reaches approx. 20% among the previously treated TB patients, while the MDR tuberculosis appears in 4–10% of the same group population.[34]

The development of new antimycobacterial drugs and identification of new drug targets must take into account firstly the peculiarity of *MtbC* pathogeneses[35] and the high adaptability of this classically known as an intracellular bacterial pathogen. The most intriguing property of *MtbC*

assured by over 150 virulence factors[5,36] is the capacity of *MtbC* to survive and multiply in certain conditions inside the macrophages, monocytes and dendritic cells.[8]

Mycobacterial Infection Pathology

Mycobacteria are classified according to their pathogenesis and role in human tuberculous as *Mycobacterium tuberculosis* complex (detailed above) and non-tuberculous mycobacteria (*NTM*, previously named "atypical mycobacteria") (e.g. *M. avium, M. kansasii, M. terrae, M. abscessus*, etc).[37,38]

Non-tuberculous mycobacteria are ubiquitous, free-living, acid-fast bacteria, generally with reduced human pathogenicity (most of them are saprophytic) compared with *M. tuberculosis* complex. Even so, infections with both types of mycobacteria have several common characteristics and some NTM are used as infectious agents in experimental models of tuberculosis (e.g. *Mycobacterium marinum* in the zebrafish model of tuberculosis).[39] This material is mainly intended to review the pathogenesis of bacteria included in the *M. tuberculosis* complex with few examples of NTM when adequate.

Although a dual intracellular and extracellular-type of infectivity is described for *MtbC*, the essential mechanism of disease in TB is based on the ability of mycobacteria to inhibit within the cells of the monocyte-macrophage system (MMS) the fusion of the phagosomes (containing microbes) with.[18] Modulation of macrophage intracellular organelle compartment is essential not only for *MtbC* survival but also for its intracellular multiplication. Replication within the MMS-cells leads not only to the destruction of these cells but also of all cell populations surrounding the inflammatory focus. Within the affected organ and regional lymph nodes, this process will result is massive caseous necrosis and formation of a granulomatous reaction (caseating granuloma/tubercle)[18,40] with a typical morphology.

Virulence and Pathogenesis Factors of Mycobacterium Tuberculosis

The complex pathogenicity of *MtbC* is determined by a plethora of virulence factors and literature dedicated to these factors is vast.[5,41,42] This is particularly important in the disease process and gives TB a peculiar progression of biological events and interaction with the immune cells.

In a comprehensive review by Forrellad et al[5] the *MtbC* virulence factors were classified in nine groups based on their activity, chemical structure and bacterial location: (1) virulence factors involved in the metabolism of lipids and fatty acids, (2) bacterial-wall proteins and lipoproteins (including secretion systems cell wall), (3) proteins suppressing the antimicrobial effectors of macrophage, (4) proteases (5) protein kinases (6) proteins involved in metal transport, (7) regulator gene, (8) proteins of unknown function and (9) other virulence proteins.[5]

The main virulence factors and the mechanisms by which they enhance *MtbC* infectious capability and resistance are summarized in Table 1.

Entry into Macrophages, Monocytes, and Dendritic Cells

The route of entry into the organism of *MtbC* is most often by inhalator route; the digestive pathway and other non-respiratory route are less important for the TB transmission and are often used by other Mycobacteria of *MtbC* group (e.g. *M. mungi* is transmitted by an environmental pathway mainly through anal gland secretions and infected urine).[76]

Following the initial mechanical entrapment in the bilaminar protective mucus covering the respiratory or digestive system, mycobacteria enter in contact with the local macrophages (occasionally suspended in the respiratory mucous blanket) or, rarely, with intestinal M cells. Following mainly a specific ligand–receptor interaction with the membrane receptors (pattern recognition receptors-PPR) of macrophages, mycobacteria are engulfed by phagocytosis (Figure 1). Although macrophages are the main cells responsible for *MtbC* engulfment, all cells or the MMS, including monocytes and dendritic cells are capable of *MtbC* phagocytosis.[39,77] Other professional-phagocytic cells as neutrophils, although are capable to phagocytose and destroy *MtbC*,[78] have a less-known of the role in TB infection.

There are several phagocytic receptors (surface-expressed PPRs) that assures *MtbC* recognition and phagocytosis by macrophages/newly-recruited monocytes, such as those for: complement (CR1, CR3, and CR4), macrophage mannose receptors, CD14, surfactant protein receptors (surfactant protein A) (Sp-A), Fc (FcR) and macrophage scavenger receptors.[18,36] These receptors recognize different components of *MtbC*: lipoarabinomannan (LAM) from the bacterial cell wall is recognized by CD14 and macrophage scavenger receptors, mannose, and mannose-capped-LAM by the macrophage mannose receptor, polyanionic macromolecules by the scavenger receptors and mycolyl-arabinogalactan by the intracellular NOD2 receptors.

Table 1 A Synopsis of the m Tuberculosis Main Virulence Factor and Their Pathogenic Mechanism

Virulence Factor	Mechanism of Bacterial Virulence
• Lipoarabinomannan (LAM) and Mannose-capped-LAM	Bacterial Adherence and phagocytosis by macrophages[43]
	Inhibits phagosome maturation[44] and phagolysosomal fusion[45]
	Block transcription of IFN-g, antioxidative defense and inhibition of protein kinase C activity[46]
	DownregulateTh1 cytokine expression[47]
	Induction of IL-10 production and Inhibition of dendritic-cell maturation[48]
• Lipomannan	Induction of IL-12 production and apoptosis in macrophages[49,50]
• Cord factor (Trehalose-6,6´-dimycolate)	Inhibits acidification of phagolysosome, delayed maturation of phagosomes, phagosome-lysosome fusion[51,52]
	TB-granuloma development and maintenance(dependent mainly on TNF-α and IL6 increased production) and cachexia[53,54]
	Damage to mitochondria membranes and oxidative phosphorylation impairment[55,56]
	Induction of apoptosis and thymus atrophy[57]
• Phosphatidylinositol mannosides	Granuloma development and maintenance[58]
	Inhibition of TNF, IL-12p40 production within macrophages[59]
• Phthiocerol dimycocerosate and phenolic glycolipids	Evade recruitment of MyD88-dependent macrophage populations[60]
	Intracellular bacterial survival (bacterial protection against nitrogen intermediates species)[61]
	Bacterial Adherence and phagocytosis by macrophages[62]
	Phagosome membrane rupture followed by apoptosis[63]
• Twin-arginine transporter	Cell wall biogenesis and resistance to beta-lactam antibiotics[64,65]
• Exported repetitive protein (Erp)	Intracellular MTb growth[66]
• ESAT-6 family	T cell stimulation (gamma interferon release)[67]
	Delayed-type hypersensitivity[68]
	Downregulate ROS production and LPS-induced nuclear factor-κB activity in macrophage[69]
	Inhibit TLR2-mediated signaling in macrophage[70]
	Apoptosis of macrophage[71]
	Cytolysis of macrophages, red blood cells,[72] and pneumocytes[73] by pore formation
	Bacterial translocation from the phagolysosomes to the cytoplasm[74]
• Phenolic glycolipids	Immunosuppression (release of this pro-inflammatory mediators)[75]

Typically, the recognition and MMC-internalization of *MtbC* is mediated through the interaction of several of the PPRs listed above. The active types of PPRs influence the downstream inflammatory events and the fate of *MtbC* infection. Also, some intracellular PPRs as NOD2 (nucleotide oligomerization domain protein) are able to recognize the *MtbC* and further regulate the inflammatory process[79] (mainly mediated through the NF-kB pathway). The involvement of these receptors could also be sequential, dominating different stages of the *MtbC* infection (engulfment in the early infections vs phagocytosis in systemically disseminated TB).

Replication in Macrophages

Once internalized in macrophages (or other MMC), *MtbC* resides in a phagocytic vacuole where they are capable to delay or block the fusion of primary/early phagosomes with lysosomes (Figure 1) and thus to prevent the maturation, acidification of lysosomes, *MtbC* destruction, and activation of other antimycobacterial mechanisms.[18,83] This process is

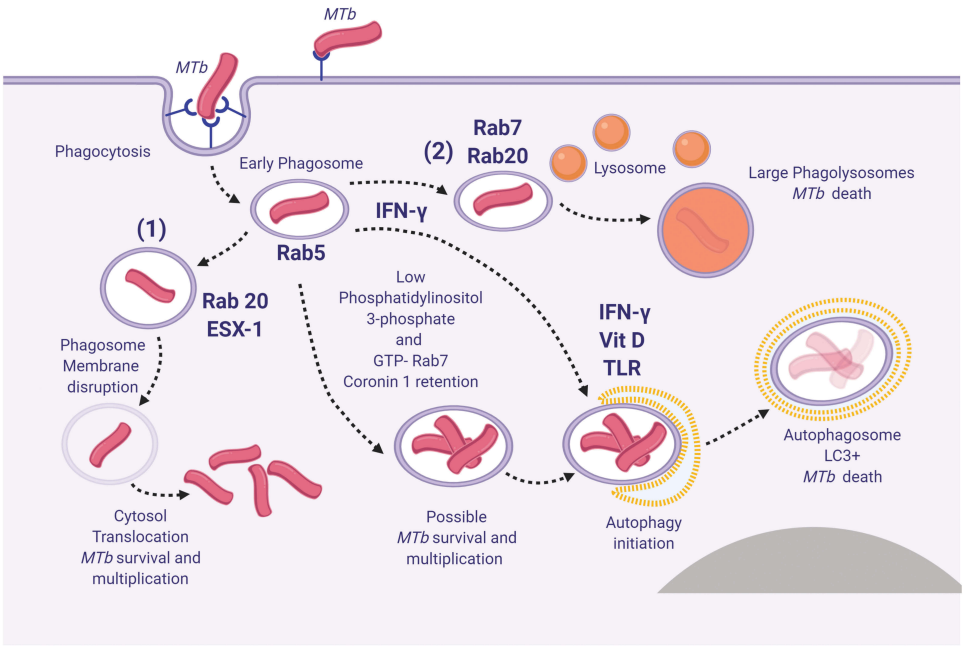

Figure 1 Spatiotemporal dynamic model of the possible fates of Mycobacterium tuberculosis (MTb) following macrophage phagocytosis (1) MTb can prevent early phagosome maturation and by the action of Rab20-trafficking, the ESX-1 will destabilize and disrupt the phagosome membrane allowing MTb direct access into the macrophage cytosol, followed in certain conditions by MTb survival and multiplication; (2) Some early phagosomes will undergo normal maturation, will fuse with the lysosomes and MTb will be killed (by reactive nitrogen intermediates, low pH, ROS, antimicrobial peptides and Fe deprivation mediated by iron scavengers, as lactoferrin, and NRAMP1);[80] occasionally MTb can survive within the mature phagolysosome; (3) Blocking of the early phagosome maturation (mainly by inhibiting PI3P generation) followed by intravesicular MTb replication; (4) Delivery of the early endosomes or early-endosomes-to autolysosomes, where typically the activity of Mtb will be suppressed. Inspired from Philips et al[81] and Schnettger et al.[82] Figure 1 was created using BioRender.
Abbreviations: NRAMP1, natural resistance-associated macrophage protein 1.

actively mediated by *MtbC* and implies a reduction of proton ATPase amount within the phagosome and inhibition of Ca^2 signals[84,85] although the exact events that lead to this effect are still controversial. Several *MtbC* pathogenic factors as sulfolipids, trehalose dimycolate, lipoarabinomannan/mannose-capped-lipoarabinomannan (MC-LAM), tryptophan aspartate coat protein (TACO) and SapM are involved in this process.[86–89] Finally, the mycobacterial phagosomes have the biochemical features of the early endosomes[90] and are a favorable milieu for *MtbC* replication and systemic (lymphatic and/or sanguine) dissemination.

Even if these mechanisms seem to robustly block the phagosome-lysosome activity, several acute-phase cytokines (as IL-1 and tumor necrosis factor-TNF) and IFNγ can stimulate the *MtbC*-infected macrophages to overcome this dysregulation of the intracellular compartments and to regain the antimycobacterial activity (essentially by changing the macrophage polarization state-discussed below).

Tuberculosis Progression: Th1 to Th2 Response Imbalance

The polarization of the immune system activity is critical in the control and evolution of the *MtbC* infections. The $CD4^+$ T lymphocytes orchestrate by the types of cytokines produced the inflammatory process (including the autoimmune processes) and are responsible for the normal multistep evolution of a typical inflammation.

In tuberculosis, initially, a T_H1 response induces a "classically" activated, M1-bactericidal macrophage (which mainly by secreting IFN-γ is able in certain limits to control the initial *MtbC* infection). Additional to T_H1, also the T_H17 cells are considered to induce a protective inflammatory response during *MtbC* infection.[91]

By the T_H2 response $CD4^+$ T secrete IL-4, IL-5, and IL-13 (promoting an "alternative" M2-activated macrophage); M2-polarised macrophages are commonly responsible for a protective effect against extracellular pathogen.[85] The T_H2 response typically is not inducing any protective activity against *MtbC* infection and replication. Moreover, T_H2 response is responsible for the development of delayed (Type IV/T cell-mediated) hypersensitivity to *MtbC* antigens (used as a diagnostic tool – intradermal reaction/tuberculin test), granuloma formation (Figure 2) and progression of clinical tuberculosis.[91–93] Although with a relative opposing effect, both T_H1/T_H2 inflammatory "phenotypes" usually coexists in *MtbC* infections. The modulation of these two

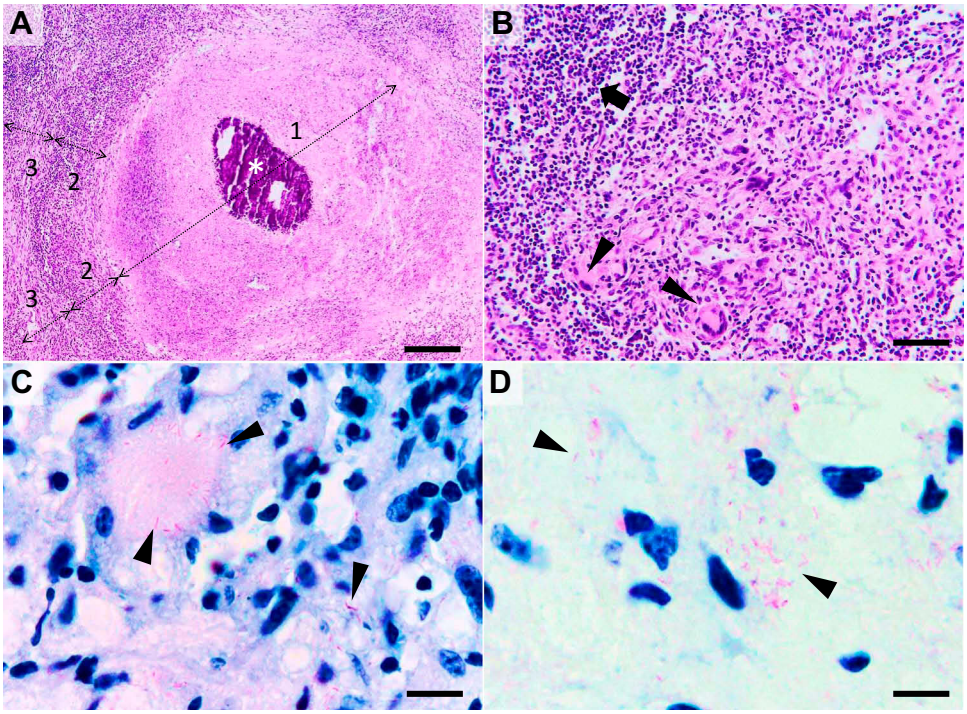

Figure 2 Histological characteristics of a tuberculous granuloma in the late caseo-calcareous stage. Image (**A**) "caseating tubercule" consisting of a large central area of caseating necrosis (zone 1) with extensive calcification (asterisk), surrounded by a reactive rim (zone 2) of lymphocytes and macrophages (including macrophage-derived epithelioid and multinucleated giant cells) and bordered by a partially formed fibrous capsule (zone 3) focally infiltrated by the above-mentioned cells; Image (**B**) detail of the leukocyte rim (zone 2), depicting several multinucleated giant cells (Langhans type) (arrowheads) admixed with fewer histiocytes, macrophages, and lymphocytes (arrow). Image (**C** and **D**) many acid-fast bacilli located intracellularly within the Langhans type multinucleate giant cells and histiocytes (image (**C**), arrowheads) and extracellularly (image (**D**), arrowheads). Image (**A** and **B**), Hematoxylin and eosin stain; Image (**C** and **D**) Ziehl–Neelsen stain for mycobacteria; ob x 4 for image (**A**) (scale bar=500 μm), x20 for image (**B**) (scale bar=100 μm), and x100 for images (**C** and **D**) (scale bar=20 μm).

components during the TB evolution is under the influence of several factors, among which individual-genetic variations ("genotype"), immune-system reactivity, microbial products (*MtbC* strain), intercurrent infections and physiological status.

Persistence of Viable Mycobacteria in Dead Cells and Necrotic Tissue

The capacity of mycobacteria to hijack the type of macrophage calls death is well known,[94] but recently a new adaptive mechanism of mycobacteria was found. Mainly, following macrophage necrosis and neutrophil necrosis, a subset of mycobacteria exploits the necrotic cell-debris as a nutrient-rich growing substrate.[95] More interestingly is the fact that tissular necrosis tends to enhance the overall mycobacterial replication.[96] In a dynamic representation of this pathogenicity, the macrophage and neutrophil necrosis represents the starting point for a vicious cycle which continues with the uptake of the Mtb-infected cell debris from the newly recruited monocytes and neutrophils, *de novo* Mtb replication, sustained infection and finally the induction of cell death.[96,97] The mycobacteria can utilize this growing niche for enhanced replication and survival, contributes to the success of mycobacteria to resist host defense and antibacterial therapy.[95,97]

Metallic Nanoparticles as Antiinfective Agents

Due to the increasing capacity of bacterial pathogens to acquire resistance to classical anti–infectious agents, nosocomial infections become a major cause of morbidity in patients of all age groups.[98] Metallic nanoparticles have unique antiviral, antibacterial, and antiparasitic properties, making them promising candidates for future applications in the treatment of infectious diseases.[99] From this class of molecules, zirconium oxide (ZrO_2NPs)[100] and Co3O4@ZrO2 (CoZ) core/shell NP[101] proved to have an antibacterial effect against both gram-negative (*E. coli* and *Pseudomonas aeruginosa*) and positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*), copper oxide nanoparticles (CuONP) have shown antifungal (*Candida albicans*) and antibacterial effect against gram-positive (*Staphylococcus aureus* and *Staphylococcus epidermidis*) and gram-negative (*E. coli* and *Proteus vulgaris*) bacteria[13,102] and iron oxide nanoparticles (FeONP)

bactericidal effect against *E. coli, Klebsiella pneumoniae*, and *Staphylococcus aureus*.[103] Gold nanoparticles (AuNP) have shown broad antibacterial effect against both gram-positive (*Staphylococcus epidermidis*) and gram-negative (*E. coli*) bacteria,[104] and following appropriate functionalization a selective antibacterial effect against methicillin-resistant *Staphylococcus aureus*.[105] Also, gold nanoparticles (AuNP) synthesized from marine seaweed *Gracilaria verrucosa* and *Gelidium pusillum* shows good biocompatibility to human embryonic kidney cells even at high concentrations of 100 and 150 μgmL^{-1}.[106,107]

Additionally to their antimicrobial properties, some form of nanoparticles posses also antiproliferative-antitumoral effect as was recently shown for magnesium oxide nanoparticles (MgONPs) synthesized from the brown algae *Sargassum wighitii*,[108] for titanium dioxide (TiO_2) nanoparticles[109,110] and for AgNPs synthesized from *Enteromorpha compressa*.[111] Also, TiO_2 nanoparticles show immunomodulatory effects,[112] having a hypothetical application in infectious diseases with a hypersensitive component (including some phases of TB).

Moreover, some nanoparticles as copper nanoparticles (CuNPs) show catalytic degradation of organic dyes with application in wastewater treatment[113] and interestingly, some nanoparticles as CuO and CuO/Cu(OH)2 show multimodal effects including in addition to antibacterial effects against *E. coli and S. aureus* also photocatalytic activity with potential application in wastewater management[114] and a dose-dependent anticancer activity against tumor rat C6 cell line.[115] A similar photocatalytic activity was shown also for zinc oxide nanoparticles synthesized from *Cyanometra ramiflora*.[116]

From the metallic nanoparticles, AgNPs are the most popular choice as anti–infectious nanoparticle-adjuvants.[17] In conjunction with appropriate-drug delivery systems as chitosan[117] AgNP per se or in combination with proanthocyanidin shown also a good in vitro antitumoral effect, against HT 29 human adenocarcinoma cells.[118,119] The antibacterial properties of AgNP, their mechanism of action and especially their antimycobacterial effects will be further detailed.

Silver Nanoparticles as an Emerging Therapeutic Approach in Mycobacterial Infections

Silver *per sei* or incorporated in different compounds has long been used empirically as antimicrobial agents and tested since the XIX century as a natural antibiotic.[15,120]

In the quest for more efficient antimycobacterial drugs that are able to overcome the "classical" issues discussed above and partially responsible for the global TB status, the antibacterial peptides and nanoparticles gained recently special attention.[19,121] Several classes of nanoparticles with intrinsic antibacterial and antibiofilm effects are proven,[122] including metallic nanoparticles (e.g copper,[123] iron,[124] gold[125] or silver-based[126]), carbon nanotubes,[127] polysaccharides as chitosan[128] and chitosan in conjunction with polycationic polymer[129] or combinations of the above-mentioned antibacterial molecules as chitosan-gold NP.[130] Among these antibacterial nanoparticles, due to their strong antibacterial activity and long-history of using silver as antiseptic, AgNPs have received most of the attention.[131,132] This new paradigm in antituberculous therapy is based on the fact that the efficiency of Ag was already proven for many classes of bacteria and their microorganisms, the long tradition in using Ag salts as disinfectants,[15] and due to the fact that unlike antibiotic drugs, most of the currently known pathogenic bacteria rarely develop resistance to metallic nanoparticles. The conditions under which this phenomenon can appear will be discussed in a separate section.

Antibacterial Effect and Mechanism of Silver Nanoparticles

Antibacterial action of AgNPs is mediated by several, generally, accepted-mechanisms (depicted in Figure 3) which in a biological context have a complementary action: 1) Direct contact with the bacteria components (biofilm and bacterial cell wall); 2) Release of bioactive ions (ex. Ag^+ ions); 3) Disruption of several metabolic pathways; 4) Generation of reactive oxygen species (ROS); 5) Genotoxicity; 6) Alteration of cell wall and cytoplasm; 7) Inhibition of bacterial DNA replication; 8) Alteration of bacterial membrane permeability and ionic change.[133–140]

These effects are mediated mainly by the primary action of the AgNP, or by the release of Ag^+ species and ROS will further disrupt the metabolic pathways and DNA. Although the main antibacterial effect of AgNPs is believed mediated by the release of bio-active Ag^+ ions,[141] more exactly, the AgNPs antibacterial mechanisms employ targeting multiple components in the bacterial cell,[142] including bacterial wall (disruption and/or increasing the membrane permeability), tRNA (transfer ribonucleic acid),

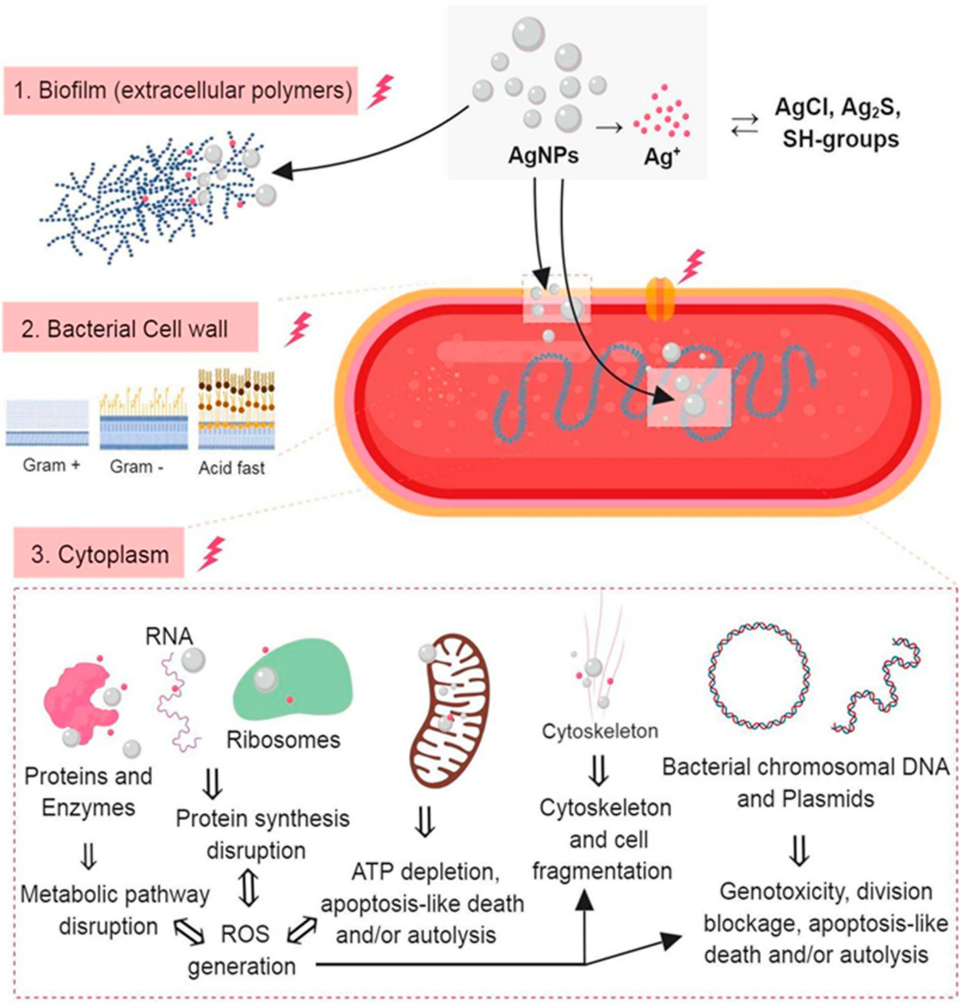

Figure 3 The three most important routes of antimicrobial action of AgNPs. 1. Accumulation and disruption of the extracellular polymers of the bacterial biofilm; silver ions (Ag^+) could also biochemically alter the biofilm overall adherence, structure, and porosity.2. AgNPs adhere to bacterial cell surface (documented for Gram-positive, negative and also for the acid-fast bacteria) resulting in microbial membrane disruption, altered transmembranar transport, cellular content leakage (mainly electrolytes dysregulation) and bacterial death (apoptosis/lysis); as for the biofilm, Ag^+ generated extracellularly contribute to the microbial cell wall disruption by biochemical alteration of the SH– groups. 3. AgNPs penetrate bacterial cell wall and access microbial cytoplasm where can interact with the organelles, cytosolic molecules (as free amino acids, peptides, and enzymes) and bacterial cytoskeleton; By direct action of AgNPs and Ag^+ results the alteration of several metabolic pathways, bacterial organelles dysfunction (mainly mitochondria), ROS generation and bacterial DNA alteration ultimately causing cell death apoptosis/lysis). Figure 3 was created using BioRender.
Abbreviations: AgNPs, silver nanoparticle; ROS, reactive oxygen species: Ag+, silver ions.

inactivating the respiratory chain (ATP depletion), enzyme and protein synthesis and DNA-binding (resulting cleavage, inhibition of replication).[138–140]

The overall bactericidal effect of AgNPs depends, in addition to the rate of Ag^+ production, also on the AgNPs size and shape, overall NP surface area, type of coating/corona, and rate of Ag^+ generation.[133–140] The difference in the efficiency of AgNPs against Gram-positive, Gram-negative or acid-fast bacteria is believed to be mainly dependent on the structural and thickness differences of their cell walls. Usually, acid-fast bacteria due to the presence of a thicker-waxy cell wall have a stronger defense-system against Ag-NPs. As in the case of the Gram-positive bacteria, this structural particularity prevents the action of Ag-NPs rendering acid-fast and gram-positive bacteria more resistance to the antimicrobial activity of Ag-NPs comparatively with Gram-positive bacteria.[143,144] For example, the Gram-positive *Bacillus subtilis*, have a cell wall of 55.4 nm, the acid-fast M tuberculosis a 20.2 nm[145] while the Gram-negative *Pseudomonas aeruginosa*, has a cell wall of only a 2.4 nm.[146] Interestingly, although there are important functional differences between the mycobacteria cell wall and gram-positive bacteria, the DNA-based molecular taxonomy of bacteria based on the high similarity to genes, groups the classical acid fast-mycobacteria as gram-positive bacteria.[147] But this overall generalization regarding the susceptibility

towards AgNPs has many exceptions, thus, AgNPS synthesized from *Bacillus brevis* has a maximum antibacterial effect against the Gram-positive, multi-drug resistant for *Staphylococcus aureus* and moderate for the Gram-negative *Salmonella typhi*.[148]

Role of Ag in Particle State (Ag^0) and Ag^+ Species in Mediating the Bactericidal Effect of Silver Nanoparticles

Ag in Particle State (Ag^0)

This is the first (direct or "primary"), of antibacterial effect of AgNPs and is considered to be due to: (1) nanoparticles damage the bacterial wall and on (2) entrance of particles into the bacterial cytosol and directly interact with the intrabacterial environment.[149–151] The adherence of the AgNPs on the bacterial surface and formation of particle agglomerates is followed by disruption of bacterial membrane integrity by induction of cell-wall pits and gaps, and alteration in membrane selectivity and permeability, including ionic transport.[151–154] This first, step is dominated by the wall changes is followed by bacterial-cytosol leakage, lost the intracellular contents and finally the collapse of the cell or apoptotic-like bacterial cell death and formation of an amorphous mass of cell debris.[149,150,155] Due to the massive loss of the bacterial content the "ghost cells" morphology is used to describe lysed bacteria following this process.[150,156]

Nanoparticles have the property to be adsorbed at the bacterial membrane mainly by electrostatic adhesion, a process mediated by surface charge of the particle- the zeta (ζ)-potential – and the outer layers of the bacterial cell wall.[151] Thus, a study designed to explore this surface-interaction between AgNP and bacteria (*Bacillus* spp), El Badawy et al found that positively charged BPEI-caped AgNPs were the most bacteriotoxic NPs, mainly due to the local agglomeration. The negatively charged citrate-caped AgNPs were the least bacteriotoxic.[151] The outer layer of bacteria (G+) is negatively charged due to the presence of carboxyl, phosphate and amino groups,[157] thus influencing the electro repulsion between bacteria and negatively charged AgNP. The highly-negatively charged bacterial wall is believed to be an important fact in explaining the superior activity of AgNP against G- compared with G+ bacteria which is frequently reported.[158]

In a similar study, positively charged AgNP by functionalization with PHMB functionalized exhibited superior antibacterial effects against *E. coli*. Also, the bactericidal activity of PHMB was enhanced by the combination with AgNPs.[159] Indeed, this hypothesis was further confirmed by Ivask et al,[160] which observed that the pathways involved in G- bacterial responses to AgNP are highly dependent on the surface characteristics of the Ag composite, including zeta (ζ)-potential.

At least partially the enhancement of the antibacterial effect of observed in AgNPs with surface-modified by surfactants (SDS) and polymers (PVP 360),[161] in addition to stabilization of particles against aggregation, can be attributed to this facilitated-adhesion to the bacterial wall.

Additionally to the wall thickness and structure, the difference in resistance of different classes of bacteria can be explained by the fact that due to the high-presence of LPS the cell wall, Gram-negative bacteria has a higher negative charge, which promotes local adhesion and membrane-clustering of particles and finally enhances the antibacterial effect of Ag-NPs.[144,162,163] Therefore, electrostatic interaction between bacterial cells (charged negatively) and AgNPs (charged positively) is critical for the antibacterial activity of NPs.[144,161,164]

Moreover, to the above-mentioned action against the bacterial wall, AgNPs have the ability to enter inside bacteria's cytosol, to form cytoplasmic precipitates and to disrupt several bacterial-physiological processes. The type of bacterial- metabolic pathways disrupted directly by the Ag in particle state is largely unknown.

Role of Ag^+ Species

The antibacterial effect of AgNP is complementary enhanced by the local elimination of Ag^+ species which have high affinity especially for thiols, selenols, organic amines and phosphates and forms strong covalent bonds.[141] The formation of this covalent bonds (e.g. silver thiolate) in which Ag act as a bridging agent linking several thiols-groups for different molecules can irreversibly alter their tridimensional structure and function[141,165] and finally will disrupt simultaneously several enzymatic pathways and constitutive cell-structure elements (DNA, cytoskeleton, plasmatic and organelle membrane, etc.). This multi-molecule disruption mediated by a broad chemical affinity and not by a targeted-element is the main cause of the complex antibacterial mechanism in comparison with classical antibiotics which typically target a narrow groups of molecules, as for example, restricted to cell membrane (beta-lactamides) or interfere with molecules synthesis and also broad spectrum of micro-organisms sensible to Ag.[15] Regarding the involvement of different metabolic pathways following the above-described mechanism,

probably one of the most important is the disruption of the ROS-regulation system (by interfering with reductase enzymes and other cofactors) and thus increasing their intracellular oxidative stress and triggering cell senescence or death. The ROS-generation as a mechanism of AgNP/Ag + action will be separately discussed.

In the AgNP/Ag+ model of action, AgNPs acts as a nanoparticulate reservoir for the continuous release of Ag+ species. The rate of release of Ag+ is dependent on many factors including NP size, surface, porosity, O_2 amount in the environment, and is mediated by release ("desorption") of chemisorbed ions from the particulate surface, oxidative dissolution (which is the main way to release Ag^+ in the aqueous environment).[166]

In a dynamic presentation of the plausible effect, the AgNP adherent on the bacterial-cell wall or entrapped inside the bacterial cytoplasm ("Trojan horse effect") will release in the adjacent environment large amounts of Ag^+ species generating a locally high concentration of antibacterial ions.[160,167]

Generation of Reactive Oxygen Species (ROS)

The generation of ROS is considered a second mechanism by which AgNPs can induce bactericidal or bacteriostatic effects. The ROS generation is due to (1) particle–cell interactions (alteration of local cell activity, e.g. inflammation-driven enhancement of oxygen respiration and oxidative/antioxidative imbalance) or due to (2) in situ production of hydroxyl radicals due to an Ag-mediated Fenton-like reaction[168,169] (acellular induction of ROS). The presence of transition metals including Fe, Cu, or Cr as synthesis contaminants enhances ROS generation via direct catalytic Haber–Weiss and Fenton-type reactions.[170] This in situ production of free radicals by AgNPs is usually enhanced by exposure to light-sources of variable wavelengths, this feature is currently explored also for photocatalytic degradation of pigments.[171,172]

The ROS generation within the activated cells is mediated by enhancement of: 1. cytoplasmic ROS (cytoROS) production by NADPH oxidase family of enzymes (e.g. endothelial, neuronal and inducible nitric oxide synthases)(eNOS, nNOS, iNOS) during inflammation; 2. Peroxisome ROS generation as a by-product of enzymatic activity (as hypoxanthine and β-oxidation, polyamine synthesis and amino acid deamination); 3. mitochondrial ROS (mitoROS) as a byproduct of metabolic-enzyme activity and mitochondrial respiration, activity upregulated, for example, by complex I NADH reductase and dehydrogenase via RET (reverse electron transfer) during inflammation. 4. lysosomal and phagolysosomal ROS mediated mainly by NADPH oxidase and myeloperoxidase produced mainly within the professional phagocytic cells (neutrophils, monocytes, and macrophages) during the intracellular destruction of microbes and removal of cell debris.[85,173] AgNPs were shown to interact with all of the above systems, including increased expression of iNOS and generation of NO,[174] impairment of mitochondrial function and ROS generation,[175,176] upregulation of peroxisome oxidative stress-related genes, such as catalase,[177] enhancement of phagolysosomal activity (reduction of lysosomes pH)[178] and macrophage and neutrophil activation and stimulation of ROS generation.[179]

Another clear advantage of using AgNPs is based on the well-known fact that NP persists much longer in the body (even years) compared with the small molecule used currently in antibacterial therapy. This would increase the long term releasing of active compounds and thus the sustained therapeutic effects.[142,180] Although AgNPs can have a direct effect on the microorganisms, the main effect is considered to be mediated through the biochemical interactions of Ag^+.[181,182]

Presence of Antibacterial – Active Products in Biosynthesized AgNPs, a Possible Source of Antibacterial Synergy?

The antibacterial effect of AgNP, especially in the green-synthesis context (plant, viral, bacterial, fungic, and algal extracts or biomimetic compounds as reducing agents),[183,184] can be, at least partially enhanced by the extra bio-active component introduced in the particle synthesis.[185,186] AgNP can be prepared using elements that possess per se an antibacterial activity. The synergistic effect between AgNP and other and bioactive phytocompounds can be expected in the green-synthesis, leading to antibacterial effects via different mechanisms as those described above.[185] Also, the concentrations of antibacterial-active compounds can be observed below the minimal dose of individual compounds. Therefore, the enhanced antimicrobial effect of NP synthesized by green-extraction which can be occasionally observed can also be determined by the presence of the bioactive molecules of the synthesis attached on the surface of nanoparticles as stabilizing agent.[186,187]

In the green-synthesis of AgNP, the biological extracts are mixed with the metal salt solutions, the bio-extract (containing starch, steroids, sapogenins, flavonoids, terpenoids, amino cellulose, etc.)[188] acts in situ as reducing agents of the silver salts (Ag^+) to form metallic silver

Ag^0, and also as capping agents to provide stability of silver nanoparticles in solution[186,189] and partially can be further be found in the structure of the AgNP as surface-stabilizing ligands.[190]

Indeed, in a recent study, Shaik et al[190] tested the efficiency of AgNP synthesized from *Origanum vulgar* against various bacteria (*Escherichia coli, Shigella sonnei, Micrococcus luteus*), and fungi (*Aspergillus flavus, Alternaria alternate, Paecilomyces variotii, Phialophora alba*). The bactericidal and antifungal efficiency was proportional to the amount of plant extract employed for the preparation of AgNPs. Similar findings of obtaining biogenic NP with broad antibacterial effect were reported by Pugazhendhi et al from AgNP synthesized from red algae *Gelidium amansii*.[191]

Also, a study which compares the antibacterial effect of AgNP produced by green synthesis (from *S. persica*) versus chemical against Gram-negative (*E. coli* and *P. aeruginosa*) and Gram-positive (*M. luteus* and *S. aureus*) bacteria shows that the green synthesized Ag-NPs exhibited slightly higher antimicrobial activity in comparison to the chemically synthesized Ag-NP.[158] The conclusion of the study was that, although *S. persica* root has antibacterial properties *per sei*, due to the small amount of active compound included in the synthesis process, the increased activity of the green-synthetized Ag-NPs was mainly due to the improved solubility of the Ag-NPs rather than the microbicidal potential of plant-derived compounds used for the synthesis of NPs.

In a study designed to assess the antibacterial effect of AgNPs from *Asparagus*spp. against 4 mycobacterium species (*M.tuberculosis, M.pheli, M.avim, and M. smegmatis*), Kote et al[185] found a direct connection between the green approach of AgNPs synthesis (mainly due to the enhanced stability) and the antimycobacterial effect. Also, some forms of biosynthesized AgNPs have multimodal action, proving simultaneous antibacterial, antimycotic and antitumoral effects as was recently shown for AgNPs produced from *Phoenix dactylifera*.[192]

In the above-mentioned studies, the enhancement of the antibacterial efficiency of AgNP produced by green synthesis is more likely mediated by the uniformity of the dispersion and a better stabilizing of molecules in aqueous solution compared with chemical synthesis.

In a study exploring comparatively the antimycobacterial effects of green-synthetized vs chemically produced AgNPs found that chemically AgNP exhibited greater efficiency in terms of mycobacterial inhibition, specificity and selectivity compared with bio-AgNPs[193]

Thus, although the presence of co-synthesis products in the green-synthesis of AgNP definitely have a role in determining and fine-tuning the biological activity of the obtained nanoparticles,[194] the exact mechanisms and the possible synergism with Ag in mediating antibacterial activity should be further explored.

Enhancement of Antibacterial Efficiency Antibiotics by AgNP

An emerging practice in antituberculous experimental therapy is to combine a metallic nanoparticle (TiNP, CuNP, AuNP, AgNP, ZnNP, etc.) with antibiotics ("nano-antimicrobials") to enhance their antimycobacterial efficiency, especially in the context of bacterial antibioresistance.[195,196] Also, antibacterial-AgNP synthesis using tetracycline as co-reducing and a stabilizing agent was described by Djafari et al.[197]

It is postulated that combining AgNPs and an antibiotic can synergistically inhibit both Gram + and Gram - multidrug-resistant bacteria.[198–200] But this synergism is observed only for certain types of antibiotics, thus Deng et al[198] showed AgNP/antibiotic synergistic growth inhibition against the multidrug-resistant bacterium Salmonella typhimurium for enoxacin, kanamycin, neomycin, and tetracycline, while ampicillin and penicillin did not show any enhancement of the antibacterial activity. Regarding the mechanisms of synergy (depicted in Figure 4), the presence of tetracycline enhances the bacterial binding of Ag, followed by an enhancement in Ag^+ release which finally leads to a high local-concentration of Ag^+ near the bacteria cell wall which leads to bacterial-growth inhibition and death.[198] Enhanced positive synergistic response against *S. aureus* and *E. coli* was observed also for AgNPs synthesized from *Argyreia nervosa* associated with seven commercial antibiotics (streptomycin, vancomycin, tetracycline, amoxicillin, gentamicin, erythromycin and ciprofloxacin).[201] Similarly, enhanced antibacterial efficiency of ceftriaxone against ceftriaxone-resistant human pathogens was reported following conjugation with biogenic AgNP.[202]

Another mechanism of AgNPs/antibiotics synergy was described by Hwang et al.[203] and is the anti-biofilm effect. This was observed following a combination of AgNPs with ampicillin, chloramphenicol, and kanamycin against various pathogenic bacteria (*Enterococcus faecium, Staphylococcus aureus, Streptococcus mutans, E. coli,*

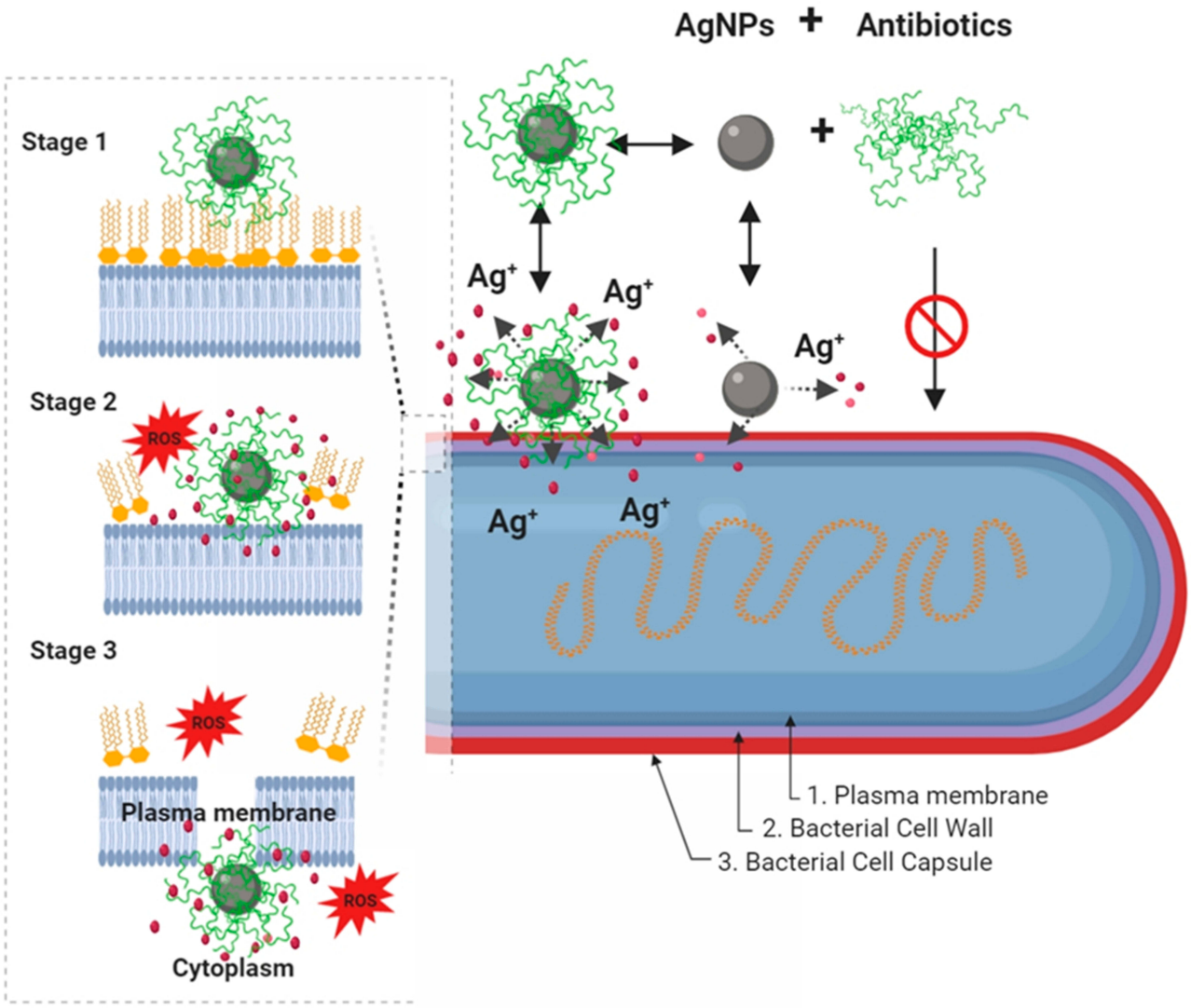

Figure 4 Schematic diagram showing, in a step by step fashion, the synergistic pathways and mechanisms of AgNP and antibiotics against multidrug-resistant bacteria (depicted in G- bacteria). Enhancement of the accumulation of the AgNPs conjugates with antibiotics within the bacterial cell membrane is associated with potentiation of Ag + release and damage of the bacterial capsule, cell wall, and plasma membrane components. In this paradigm, the pathway mediated by AgNPs is a minor antibacterial mechanism, and the activity mediated by antibiotics-only is not effective due to antibacterial resistance. In a step by step diagram of the bacterial membrane destabilization (depicted for AgNPs/nisin conjugates), the interaction between AgNP/antibiotic complexes with bacterial cell membrane (stage 1) will results in enhancement Ag+ release, in situ ROS generation, membrane-insertion of nisin (methyl)-lanthionine rings, followed by local dissolution of lipidic molecules, membrane-pore formation, and internalization of AgNPs/nisin complexes within the bacterial cytoplasm. Inspired from Deng et al[198] and Arakha et al[206] schematic concepts of AgNP/nisin and AgNPs/tetracycline complexes-mediated antibacterial activity. Figure 4 was created using BioRender.

Abbreviations: AgNPs= silver nanoparticle; ROS, reactive oxy gen species: Ag+=silver ions.

and P. aeruginosa) inhibits the formation of biofilm which is a major resistance mechanism for several types of bacteria. This antibacterial effect can be related to the high surface to volume ratio of NPs which can permit their deep infiltration into mature biofilms.[17] Recently by Farooq et -al.[204] showed an enhancement of antibiofilm efficiency of rifampicin following conjugation with silver (Rif-AgNPs) in methicillin-resistant *K pneumoniae* and *S aureus*.

Other mechanistic studies exploring the antibacterial effect of NPs, shown that AgNPs and amoxicillin, in addition to their intrinsic antibacterial activity, can form a new complex in which amoxicillin-molecules surround the AgNPs metallic core.[205]

Antimycobacterial Effect of Silver Nanoparticles

Nanotechnology brings a novel and promising therapeutic approach to improve the current antimycobacterial treatments. This include improvement of the efficiency of the currently used first-or second-line antibiotics following generation of different formulations (e.g. liposomes, solid

lipid nanoparticles, alginate nanoparticles, niosomes, dendrimers)[207] or by adding new antituberculous compounds which can synergies the classical therapy, as metallic metal-based nanoparticles (mainly silver, iron oxide, gold, copper oxide, aluminum oxide, zinc oxide, titanium dioxide, etc.).[99,187] Several of the therapeutical advantages of such nanoparticle-based therapy of tuberculosis are, among others: a) prolonged time of action, b) a high carrier ability; c) flexibility of various routes of administration, d) possibility of multiple drugs-encapsulation in the matrix, e) fewer side effects and improved compliance (especially important in prolonged anti-TB therapy).[207]

In vitro Studies

Multiple experiments carried recently determined the antimycobacterial effect of AgNP.[133,208] For example, a good activity against mycobacteria and low cytotoxicity (10 times the dose established as MIC for Mtb) on infected macrophages was recently reported by Singh et al (2016)[180] for phytogenic AgNPs.

One of the earliest reports on the antimycobacterial effect of AgNP came from Song et al.[209] who tested in vitro small, non-biogenic AgNP measuring <10 nm to several bacteria species, including beside *M. tuberculosis*, also *E. coli, S. aureus*, and *Salmonella typhi*. The antimycobacterial effect was observed at 10 ppm, and the proposed mechanism is based on the presence of AgNPs in the cytoplasm of mycobacteria and the following bacterial-metabolic disturbances.[209]

A good in vitro antimycobacterial effect, observed mainly by inhibition of the mycobacterial growth, was reported also by studies employing biogenic AgNP produced from *Plumbago auriculata*,[210] *Coriandrum sativum*,[211] *Catharanthus roseus*,[212] *Asparagus race*,[185] *Psidium guajava*,[213] *Ipomoea carnea*,[214] *Rhizopus stolonifer*,[215] and *Cucumis sativus*.[216]

In vitro inhibition of MDR and XDR strains of *M. tuberculosis* was found for physicochemically ("non-green") synthesized AgNP in doses as low 1 µg/mL.[217] Overall, no bactericidal effect was found, and although the AgNP are internalized within THP-1 macrophages, the intramacrophagic antimycobacterial effect was modest. A similar effect of multimetallic nanoparticles (MMN) including AgNP for intramacrophagic mycobacteria was reported by Ellis et al.[218] Although internalized within the phagolysosomal apparatus, the AgNP have a limited antitubercular effect for the intracellular bacteria, but increase the antitubercular effect of rifampicin. The co-administration of rifampicin led to a reduction of 68% of *M. tuberculosis* colony-forming units. Using spherical AgNP measuring 13 nm, Jafari et al.[219] observed no antibacterial effect for intramacrophagic *M. tuberculosis*, but the addition of Zn in the molecule is inducing an anti-tubercular effect. Additionally, the 5_{Ag}:5_{ZnO} report was found to have both an intracellular antibacterial effect and also no significant toxicity to normal lung (MRC-5) cell lines. By contrary, a good antitubercular effect against intramacrophagic *M. marinum* and *M. smegmatis* was observed by Mohanty et al.[19] for spherical, biogenic AgNP combined with antimicrobial peptides in doses of 0.1 and 0.5 ppm. The tested particles measured 50–100 nm and were synthesized from *Alstonia macrophylla and Trichoderma* sp. The enhanced antitubercular effect was not correlated with high levels of NO, thus the proposed antibacterial mechanism was associated with superoxide radicals formation and the activation of macrophages by cytokines. In the same study, an increased antibacterial effect against M. smegmatis was observed following the combination of NPs with the classical-antituberculosis drug rifampin.[19] Intramacrophagic killing of *M. smegmatis* internalized in RAW264.7 macrophages (in both pre/and postexposure treatments) was reported for spherical chitosan-coated AgNP (CS-AgNPs) in 3 ppm dose.[220] The bactericidal effect was time and concentration-dependent and most of the antibacterial effect was observed in the first hour of incubation. The hypothesized antibacterial mechanism was cell membrane disruption or chemical inactivation of thiol-containing molecules. Also, CS-AgNPs were noncytotoxic on RAW264.7 macrophages at the bactericidal concentration. An increased antitubercular activity was observed following the addition of gentamicin. In the same study, in addition to antimycobacterial effect, CS-AgNPs were found to be also active against other bacteria like *Staphylococcus aureus, Pseudomonas aeruginosa*, and *Salmonella typhi*.[220]

In addition to the direct antitubercular activity in doses beginning with 5 mg/l, AgNPs measuring 10–150 nm were shown to potentiation of the antibacterial effect of isoniazid, rifampicin, ethionamide, levofloxacin, ofloxacin and kanamycin against clinical isolates of *M. tuberculosis* by Kreytsberg et al.[133]

In another study employing the antimycobacterial effect of physicochemically synthesized, tetrahedral and spherical AgNP measuring 50 nm, an antibacterial effect against field isolates and standard strains of *M. tuberculosis* and *M. bovis* were reported. The minimal inhibitory concentration (MIC) was found to be 1 and 4 µg/mL for standard cultures of *M. tuberculosis* and *M. bovis*. Higher doses were needed to

inhibit the clinical isolates, being in the range of 4–32 µg/mL for *M. bovis* and 1–16 µg/mL for *M. tuberculosis*.[221]

Promising antimycobacterial results against both *M. tuberculosis* and *M. smegmatis* are also observed for AgCl-NP produced from commercial yeast extract in doses of 37 µg/mL concentration.[222] In addition to the Ag effect, in this study, the antibacterial effect could be also mediated by the activity of Cl, a potent and broad antiseptic.[223]

Using biogenic spherical (20–56 nm) AgNP synthesized from *Sesbania grandiflora*, Patel et al showed that MIC for standard cultures of M. tuberculosis is 12.5 µg/mL. This dose was half of the MIC observed for silver nitrate and approximately 30% of the MIC of Rifampicin. Also, the *Sesbania grandiflora* extract shown an antimycobacterial effect, but much higher compared with the AgNPs (100 µg/mL).[224] A similar MIC was obtained for M.tuberculosis by Punjabi et al.

An interesting strategy is combining AgNP with peptides or chitosan for antibacterial/antitumoral effect. Thus in a recent study, Abdel-Aziz et al[150] shown that spherical N,N, N-trimethyl chitosan chloride (TMC)/AgNP in a 0.98 to 125 mg/mL dose have an antibacterial effect on *M. tuberculosis* mainly by disrupting the bacterial cell wall. Also, the same nanocomposite was found to have a cytotoxic effect against A-549-lung adenocarcinoma cells in 12.3 µg/mL dose and to have reduced toxicity against normal lung cells.

Also, spherical PVP and BSA-caped AgNP measuring 5–9 nm (BSA-AgNP) and 6–45 nm (PVP-AgNP) were shown to have antibacterial effects against clinically isolated and standard *M. tuberculosis* cultures. The antibacterial effect was mediated by mycobacterial cell membrane injury, followed by bacterial lysis.[225]

Although differences regarding the sensitivity towards AgNPs were reported between different species of Mycobacteria, most of the tested materials have a simultaneous antibacterial effect against multiple species, including *M. tuberculosis, M. pheli, M. avium* and *M. smegmatis*.[185]

A synopsis of the in vitro studies using AgNPs in the treatment of mycobacteria-induced diseases, including the species and strain of mycobacteria tested, the experimental model, the type of AgNP and the main results are presented in Table 2.

In vivo Preclinical and Clinical Studies

Compared with the large number of in vitro studies exploring the potential used of AgNp as antimycobacterial drugs only a few in vivo studies were up to date carried.

In a clinical trial carried on 50 human patients with ages from 26 to 55 years suffering from with laryngeal tuberculosis, including cases with DR-tuberculosis, an AgNP aqueous suspension (Argovit-C, 10 mg/mL silver, in a concentration of 3.3%) was tested for 2 months as local therapy.[236] The AgNP group (n=30) received the treatment topically by inhalation for 2 times a day for 10 mins. The control group (n=20) received classical TB therapy. The suspension was previously characterized as containing spherical AgNP with bimodal size distribution (14.1 ± 9.9 and 50.1 ± 40.3 nm).[237]

After 60 days of therapy, the sputum was negative for *M. tuberculosis* in 93.3% of patients enrolled in the AgNP group compared with 70% of patients who received the standard anti-TB treatment. Also, the patients enrolled in the AgNP group – showed faster healing of the laryngeal TB-lesion, including ulcerations and voice function compared to standard tuberculosis drugs.[236]

An experiment designed to investigate the effect of isoniazid combined with AgNPs on MDR strains of *M. tuberculosis* was carried by Zakharov et al in 68 BALB/c inbred mice. Spherical AgNP measuring 3–60 nm were tested initially in vitro in ascending concentrations (5;25;50 µg/mL) in 651 MDR strains of *M. tuberculosis*. In the rodent study, based on the survival rates and histopathology of the lung, the combination of isoniazid and silver nanoparticles was preferable compared to the single-use of the above components.[238]

In a recently published study carried out by the same author, the effect of isoniazid combined with AgNPs was tested in 3 experimental groups of MDR-TB-infected mice: group 1 received only isoniazid (50 mg/kg); group 2 received intramuscularly AgNPs in doses of 12.5 to 125 µg/kg; group 3 received a combination of the treatments detailed for groups 1 and 2. Based on the histopathologic grading of lesions, the use of AgNPs in the treatment of TB induced by MDR strains enhances the efficiency of isoniazid.[239]

In an in vivo study carried out in 65 mice experimentally infected with MDR strains of Mycobacterium tuberculosis isolated from human patients, the efficiency of AgNP as a single therapeutic molecule or in combination with isoniazid was tested. The AgNP measured 10–150 nm. The survival rate of TB-infected animals following the combined treatment with isoniazid and AgNP was 95% and 35% in the group receiving AgNP only, compared with 100% mortality in the TB-infected control group.[133]

Table 2 A Synopsis on Studies Using AgNPs in the Treatment of Mycobacteria-Induced Diseases

	Mycobacterium Species/ Strain	Experimental Model	Nanoformulation	Tested Doses	AgNP Shape and Size Distribution	Effect on Bacteria	References
1	• *M. tuberculosis* (ATCC 25177)	Bacterial culture	TMC/AgNP*	0.98 to 125 mg/mL	Spherical 11 to17.5 nm	Inhibition of growth. Disruption of the bacterial cell wall.	Abdel-Aziz et al 2019[150]
2	• *M. tuberculosis* (H37Ra, and MDR/XDR strains)	Bacterial culture and within THP-1 macrophages	AgNP* *	1–128 μg/mL	Spherical 5.4±2.6 nm	Inhibition of growth (not bactericidal). In vitro following macrophage internalization: poor antibacterial activities.	Heidary et al 2019[217]
3	• *M. tuberculosis* (H37Rv) • *M. smegmatis* (MC2 155)	Bacterial culture	AgCl NP* (from commercial yeast)	37 μg/mL	Spherical 9 to 51 nm	Inhibition of growth.	Sivaraj et al 2019[222]
4	• *M. tuberculosis* (H37Rv) • *M. tuberculosis* (clinical isolate MDR strain) • *M. bovis* (reference strain and clinical isolate)	Bacterial culture	AgNP* (suspended in sodium citrate)	0,25 to 256μg/mL	Tetrahedral and spherical 50 nm	Inhibition of growth.	Selim et al 2018[221]
5	• *M. tuberculosis* (H37Rv)	Bacterial culture	AgNP (from *Sesbania grandiflora*)	100 μg/mL and 25 g/mL (based on MIC)	Spherical 20 to 56 nm.	Inhibition of growth.	Patel et al 2018[224]
6	• *M. tuberculosis* (H37Rv)	Bacterial culture	AgNP (from *Pseudomonas hibiscicola*)	1.25–10 mg/mL	Spherical and polygonal 10–70 nm (average 39 nm)	Inhibition of growth.	Punjabi et al 2018[226]
7	• *M. tuberculosis*	THP-1 macrophages	AgNP*, AgNP+ZnNP* and ZnNP* (embedded in PLGA polymer)	60 μg mL-1	Spherical 20 nm MMP-AgNp-1,5μm	Limited antitubercular effect (reduction with 4.5% of CFU). Increase rifampicin antitubercular potency. Global disruption to the bacterial membrane.	Ellis et al, 2018[218]
8	• *M. tuberculosis* (H37RvMTB)	THP-1 macrophages	AgNP*	1.562 ppm, 0.781 ppm, 0.390 ppm, 0.195 ppm	Spherical 13 nm	No antibacterial activities following TB phagocytosis, only after combination with ZnONP.	Jafari et al 2017[219]
9	• *M. smegmatis*	Bacterial culture	AgNP* and AgNP/VAM (conjugated with vancomycin)	Not detailed for AgNP; for AgNP-VAM inhibitory concentration was 54 μg/mL,	Spherical AgNP: 17 ± 3 nm AgNPVAM: 30 ± 3 nm	Internalization within bacteria (without specific binding of interaction). Reduction of viability and Inhibition of growth (Mild); AgNPs potentiate the effect of VAM	Sun et al 2017[227]

(*Continued*)

Table 2 (Continued).

	Mycobacterium Species/ Strain	Experimental Model	Nanoformulation	Tested Doses	AgNP Shape and Size Distribution	Effect on Bacteria	References
10	• *M. tuberculosis*	Bacterial culture	AgNP (from *Plumbago auriculata*)	0.2 to 100 μg/mL	Spherical 15–45 nm	Inhibition of growth	Jaryal et al 2017[210]
11	• *M. tuberculosis* (H73Rv)	Bacterial culture	AgNP (from *Coriandrum sativum*)	0.2μg/mL to 100	Spherical and polygonal 50–200 nm[228]	Inhibition of growth	Paarakh et al 2017[211]
12	• *M. avium subsp. paratuberculosis* (K10/GFP)	Bacterial culture	AgN* (in distilled water containing 2% fetal calf serum)	0 to 100 μg/mL	Spherical <50 nm	Inhibition of growth.	Donnellan et al 2016[229]
13	• *M. tuberculosis*	Bacterial culture	AgNP*	20 ppm and 60 ppm	Shape not specified 30–80 nm	No anti-Mtb effects	Jafari et al 2016[230]
14	• *M. tuberculosis* (MTTC300) • *M. smegmatis*	Bacterial culture	AgNP (from *Catharanthus roseus*)	5 μg/disc	Not provided (possible spherical) 38–52 nm	Inhibition of growth.	Raja et al 2016[212]
15	• *M. tuberculosis* (H37Ra) • *M. bovis* (BCG)	Bacterial culture and within THP-1 macrophages	AgNP (from *Barleria prionitis*, *Plumbago zeylanica* and *Syzygium cumini*)	0.1, 0.3, 1, 3, 10, 30, and 100 μg/mL.	Spherical and polydisperse 10–120 nm (from B *prionitis*) 60 nm (extracted from *P. zeylanica*) 9–35 nm (extracted from *S. cumini*)	Inhibition of active and dormant mycobacteria in both culture and following internalization in THP-1 macrophages	Singh et al 2016[180]
16	• *M. tuberculosis* (MTCC-300), • *M. pheli* (MTCC-1723) • *M.avium* (MTCC-1724) • *M. smegmatis* (MTCC-994)	Bacterial culture	AgNP (from *Asparagus race*)	176 mg/100 mL	Spherical and rectangular	Inhibition of growth	Kote et al 2016[185]
17	• *M. tuberculosis* (H37Ra) • *M. bovis* (BCG)	Bacterial culture	AgNp (sol A: from *Acinetobacter sp* and sol. B: from reduction of 1% trisodium citrate)	0.02–2.56 μg/mL.	Spherical (Sol A:) and Spherical-oval (Sol B) 8–12 nm (Sol A:) 1–5 nm (Sol B)	Inhibition of growth	Singh et al 2015[193]
18	• *M. tuberculosis* • *M. smegmatis* • *M. pheli*	Bacterial culture	AgNP (from *Psidium guajava*)	100–500 μL/disc	Unknown	Inhibition of growth	Kote et al 2014[213]

19	• *M. smegmatis*	Bacterial culture	AgNP (from *Ipomoea carnea*)	5 mg/mL (impregnated)	Spherical and oval 30 to 130 nm	Inhibition of growth	Daniel et al 2014[214]
20	• *M. smegmatis* (MC2155 ATCC 700084) • *M. marinum* (ATCC 927)	Bacterial culture and within RAW264.7 macrophages	AgCl NPs (sol A: from *Alstonia macrophylla* and sol B: from *Trichoderma* sp)	0.1 and 0.5 ppm	Spherical, A: 50 nm and B: 100 nm	Inhibition of growth Enhancing the destruction of Mycobacteria within macrophages (0.5pppm)	Mohanty et al 2013[19]
21	• *M. avium* • *M. smegmatis* • *M. marinum*	Bacterial culture	AgNP*	6.25, 12.5, 25, 50, and 100 μM.	Spherical 12.6 ± 5.7 nm	Inhibition of growth	Islam et al 2013[231]
22	• *M. tuberculosis* (clinical isolate)	Bacterial culture	AgNP (from *Rhizopus stolonifer*)	8 to 64 μg/mL.	Spherical 3 to 20 nm.	Inhibition of growth.	Banu et al 2013[215]
23	• *M. tuberculosis* (H37Rv and clinical isolates, including MDR and XDR strains) • *Mycobacterium spp* other than tuberculosis (no data further specified)	Bacterial culture	AgNP (from *Cucumis sativus*)	50, 31.2, 25, 15.6, 12.5, 7.8 and 6.2 g/mL	Spherical 10–20 nm	Inhibition of growth	Agarwal et al 2013[216]
24	• *M. smegmatis*	Bacterial culture and RAW264.7 macrophage culture	AgNP* (chitosan-coated: CS-AgNPs)	1,2 and 3ppm CS-AgNPs	Spherical Two size-population 55 and 278 nm	Disruption of bacterial cell wall Intramacrophagic killing of M. smegmatis (in both pre/and postexposure treatment)	Jena et al 2012[220]
25	• *M. smegmatis*	Bacterial culture	AgNP* (starch-stabilized)	0.1, 1, 2, 5, 10 μM	Spherical 20 nm	Inhibition of growth	Mohanty et al 2012[232]
26	• *M. bovis* (BCG)	Bacterial culture	AgNP* (suspended in sodium citrate)	1, 5, 10, 20 μg/mL	Spherical 20 and 30 nm	Bactericidal Activity (cell lysis)	Zhou et al 2012[233]
27	• *M. tuberculosis* (clinical isolates, including DR strains)	Bacterial culture	AgNP* (in distilled water and in combination with antibiotics)	5, 25 and 50 μg/mL	Shape not specified 10–150 nm	Inhibition of growth Potentate the effect of isoniazid, rifampicin, ethionamide, levofloxacin, ofloxacin and kanamycin	Kreytsberg et al 2011[133]
28	• *M. tuberculosis* (H37Rv and clinical isolates) • *M. xenopi*	Bacterial culture	AgNP* (BSA and PVP-capped)	1.6, 4 and 8 μg/mL	Spherical 5–9 nm (BSA nano-Ag) 6–45 nm (PVP nano-Ag)	Inhibition of growth. TB cell membrane injury; bacterial lysis	Seth et al 2011[225]

(*Continued*)

Table 2 (Continued).

	Mycobacterium Species/ Strain	Experimental Model	Nanoformulation	Tested Doses	AgNP Shape and Size Distribution	Effect on Bacteria	References
29	• *M. smegmatis* (ATCC 700084) • *M. bovis* (BCG, ATCC 35374)	Bacterial culture	AgNP*	0.22 to 25 µg/mL	Spherical and polygonal A: 20–25 nm B: 80–90 nm	Inhibition of growth	Martinez-Gutierrez et al 2010[234]
30	• *M; tuberculosis* (H37Rv)	Bacterial culture	AgNP* and AgNp/Cys (cysteine –caped AgNp)	Tested interval not provided, 6 and 10 ppm	Shape not provided AgNp 10.45 ± 0.546 AgNp/Cys 45.67 ± 0.951	Inhibition of growth	Varghese et al 2009[235]
31	• *M. tuberculosis*	Bacterial culture	AgNPs*	0.5, 1, 5, 10 and 30 ppm	Spherical (?) <10 nm	Inhibition of growth	Song at al., 2006[209]

Note: *AgNPS produced by physicochemical synthesis (non-green synthesis)
Abbreviations: DR/MDR/XDR, drug-/multi drug-/extensively drug-resistant; TMC-N, N,N-trimethyl chitosan chloride; BCG, bacillus Calmette-Guérin

The Main Limitation of the Usage of AgNP in the Treatment of Tuberculosis

Potential Toxicity of AgNPs

One of the potential drawbacks of AgNPs, as four most of the inorganic nanoparticles, is their toxicity which may limit their usage in a biological context,[240–242] but despite the extension of use in the last decades, the evidence for the toxicity of AgNPs is still unclear.[243] However, an in depth discussion regarding the toxicity of AgNP is something that goes beyond the purpose of this manuscript.

The increased production of ROS which is presented above as one of the antibacterial mechanisms of AgNP can be harmful to the normal cells if the cellular protective antioxidative mechanisms are overcome, which will trigger several detrimental biological effects like inflammation, autophagia, apoptosis, necrosis or irreversible DNA-damage followed by mutations and possible oncogenesis.[244] There are several studies in which a good antibacterial efficiency and a low-toxicity for the explored doses were observed. Thus, for AgNPs produced from *Phenerochaete chrysosporium*, a good antibacterial effect against *Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus, and Staphylococcus epidermidis* was observed but no in vitro toxic effect on mouse embryo fibroblasts for doses up to 12.5 µg/mL AgNPS.[245]

Moreover, biogenic AgNPs measuring 50–100 nm synthesized from *Alstonia macrophylla* and *Trichoderma* sp showed no cytotoxic effects on macrophages at the mycobactericidal dose (0.1 and 0.5 ppm), but the exposure to higher doses of AgNPs induced cytotoxicity and DNA-damage.[19]

Low Penetrability in Tuberculous Granulomas

A clinical limitation of the usage of AgNP in TB therapy is based on the low tissue penetrability of large molecules following a non-intravenous route of administration.[246] Also in a lack of proper functionalization following an intravenous route of administration, a low intra-lesional accumulation is predictable considering the poorly vascularization of tuberculose-lung cavities present in chronic cases of tuberculosis.[247] Additionally, the persistence of mycobacteria in the nonvascularized necrotic material within the granuloma center can assure the persistence of a reservoir of viable mycobacteria in a biological

environment largely inaccessible for large molecules and immune cells.[18]

The Immunomodulatory Effect of AgNP

Especially important in the clinical context of *MtbC* infection and macrophage polarization, Sarkar et al.[248] observed an upregulation of macrophage Hsp72 by AgNP, which is possible to be linked with further suppression of NF-κB pathway and reduction of the macrophage antimycobacterial effect. In vivo following 28 days repeated dose toxicity study in rats, AgNP in high doses induce a marked suppression of natural killer cell (NK) activities and decreased interferon-γ and interleukin (IL)-10 release in response to Concanavalin A (ConA)-mediated activation of the spleen cells.[249] Interestingly, in the same study, the lipopolysaccharide (LPS) stimulation was associated with increased IL-1β and decreased IL-6, IL-10 and TNF-α production in the spleen, proving a complex immunomodulatory effect of AgNP. The exposure of human NK cells to AgNPs also resulted in reduced viability and altered function enhancement of expression of the inhibitory receptor CD159a.[250]

The conclusion drawn by their observation brings new perspectives regarding the drug-designs which intend using AgNP in obligate intra-macrophage pathogens. But this immunomodulatory effect seems to be more complex and interferes with specific immune cell activities. Thus, although exposure of neutrophils to AgNP (50 µg/mL) was associated with reduced neutrophilic degranulation (elastase release) and oxidative burst, the overall phagocytic activity was enhanced.[251] In the same study, pre-exposure of macrophages (RAW 264.7) to AgNP, stimulates the release of inflammatory cytokine interleukin-6 as well as enhancement of phagocytic ability in response to lipopolysaccharide stimulation.[251] Also, the exposure of J774 A1 murine macrophage to AgNPs resulted in early activation of the inflammatory response by up-regulation of IL-1, IL-6, and TNF-a genes, but in a lesser amount compared with AuNPs.[252] Therefore, taking into account the divergent data regarding the immune impact of AgNPs, a better understanding of activation or suppression of immune cell pathways and functions following AgNPs needs to carry before a clinical-functional significance in the context of the complex inflammatory environment associated with mycobacterial infections to be drawn. As a solution to the above-mentioned issues in AgNP usage the utilization of a gold-silver alloy NP promise to be a viable solution in overcoming the macrophage function suppression due to the significant improvement of AgNP biocompatibility following the introduction of gold NP (AuNP) as alloy.[253] Additionally, AuNP produced from *Terminalia arjuna* show important antioxidant and anticholinesterase effects[254] which could antagonize the AsNP toxicity.

Development of Bacterial Resistance Towards Silver Nanoparticles

Although rarer and less studied compared with the classical antibioresistance, the mutation in bacteria to resist Ag is similar in certain limits to the pathway that led to chemoresistant bacterial strains.[255–258] The widespread use of Ag- and Ag-ions containing nanomaterials and nanocomposites is considered to be the main determinant in a possible bacterial selection and evolution towards a biological resistance to Ag NP and/of Ag ions.[258,259] The resistance to antibacterial Ag is reported among nosocomial infections,[260] in bacteria present within wounds, including burns,[261–264] diabetic foot ulcers,[265] dental bacteria,[266,267] or exterior natural-environments containing high amounts of Ag.[268] Occasionally, the resistance towards Ag is developed in parallel with the multidrug-resistance, as shown in *Staphylococcus aureus, klebsiella pneumoniae, Acinetobacter baumannii, and Enterococcus faecium*.[269] This cross-resistance to antibiotic and metal resistance are typically mediated when genes for different resistant phenotypes (metal/chemioresistant) are located on the same mobile genetic elements as plasmids and conserved regions of integrons.[270]

Generally observed in fast-growing bacteria, Ag-resistance was described also in Mycobacteria, as *Mycobacterium smegmatis*,[255] *M avium, M fortuitum, and M mucogenicum*.[271] Resistance to both silver nanoparticle and $AgNO_3$ was observed for saprophytic bacteria (as *Mycobacterium smegmatis*)[255] and could be proven also for the other pathogenic classes of bacteria.

As mechanism, the Ag resistance can be associated with elimination and neutralization of ionic forms of silver, as active efflux of Ag+ from bacteria (e.g by P-type ATP/SilP, membrane potential-dependent three-polypeptide cation/proton antiporter or multidrug resistance/MDR efflux pumps), increased capacity for reducing Ag+ to a neutral-oxidation state which are typically less bacteriotoxic.[272–274] Recently, in gram-negative bacteria, the resistance to AgNP was induced by overexpression of bacterial flagellum protein-flagellin, which induced aggregation of particles at the

surface of bacteria and reduction of AgNPp antibacterial effect.[192]

The difficulty in gaining such a resistance against AgNPs s is due to the fact that the antibacterial effect of nanoparticles is more complex (illustrated in Figures 2 and 3) compared with classical antibiotics.

Conclusion

Tuberculosis is still a major public health issue, but currently, nanotechnology and nanoparticle research offers several exciting concepts which may prove to be valuable tools in improving the TB-therapy, especially in the context of broad-antibioresistant stains of *MtbC*.

There is no doubt that AgNPs per sei or in conjunction with different biomolecules as peptides and chitosan have good antimycobacterial effects, but this effect is limited following macrophagic internalization of mycobacteria. A promising strategy is combining AgNPs with classical anti-TB therapeutics which synergically enhance the antimycotic activity both extra and intracellularly. Despite the encouraging in the in vitro-stage of research, still, there are few in vivo studies exploring the anti-TB potential of AgNPs. As a result of the peculiar structure and visceral distribution and of TB-induced lesions, the on-going research efforts for the synthesis of novel anti-TB nanoparticles should be focused on strategies for enhancing the local availability of antibacterial nanoparticles. Increased local availability, associated with good intra-macrophagic disponibility, a potent antimycobacterial effect, and a low-immunosuppressive and toxic effect should be the cumulative characteristics of a good nanoparticle candidate for future therapy of tuberculosis.

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Disclosure

The authors report no conflicts of interest in this work.

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