

Potential antibacterial mechanism of silver nanoparticles and the optimization of orthopedic implants by advanced modification technologies

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Abstract: Infection, as a common postoperative complication of orthopedic surgery, is the main reason leading to implant failure. Silver nanoparticles (AgNPs) are considered as a promising antibacterial agent and always used to modify orthopedic implants to prevent infection. To optimize the implants in a reasonable manner, it is critical for us to know the specific antibacterial mechanism, which is still unclear. In this review, we analyzed the potential antibacterial mechanisms of AgNPs, and the influences of AgNPs on osteogenic-related cells, including cellular adhesion, proliferation, and differentiation, were also discussed. In addition, methods to enhance biocompatibility of AgNPs as well as advanced implants modifications technologies were also summarized.

Keywords: antibacterial mechanism, biocompatibility, osteogenic-related cells, orthopedic implants, silver nanoparticles, surface modification

Introduction

Implant-associated infection as a common postoperative complication of orthopedic surgery often results in patient suffering, financial burden, and even fatalities.^{1,2} Application of antibiotics is the most common approach to treat infection. However, the rise of resistant organisms has made routine antibiotic prophylaxis ineffective. What is worse, bacterium can rapidly form biofilm on the implant surface, making antibiotics unable to penetrate and develop antibacterial function.³ Thus, it is urgent to find an antibacterial agent that can kill drug-resistant bacteria and modify the prosthesis to prevent biofilm formation.

Silver (Ag) has been demonstrated to possess effective antibacterial effect and has been vastly used in medicine.⁴ Moreover, Ag can be manufactured into silver nanoparticles (AgNPs) through nanotechnology to have improved physical, chemical, and biological properties.⁵⁻⁷ Currently, application of AgNPs on orthopedic implant modification to prevent implant-associated infection has drawn more attention.^{8,9} In the field of orthopedic investigation, AgNP-coated external fixation pins, proximal femur or tibia mega-prostheses, and AgNPs containing bone cement have been innovated, and they show an infection inhibition trend.¹⁰⁻¹² Since AgNP-coated orthopedic implants represent a promising approach to prevent infections, the specific antibacterial mechanism of AgNPs and its effect on osteogenic-related cells need to be understood.¹³

Many research studies have investigated the antimicrobial activity of AgNPs, but the possible antibiotic mechanisms and potential hazard are still unclear.^{14,15} Castiglioni et al concluded that AgNPs cause cytotoxicity in various cell lines in a

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dose-dependent manner.¹⁶ However, the effect of AgNPs on osteoblast and osteoclast, which are responsible for bone formation and resorption respectively, is not included. In addition, optimal dosage of AgNPs on osteogenic-related cells is still controversial. Hence, the effect of AgNPs on osteogenic-related cells needs to be considered in order to screen the optimal concentration. Although Prabhu et al summed up the potential toxicity of AgNPs, they did not propose how to reduce the toxicity effect.¹⁷ Biocompatibility is a precondition for the application in medicine. Aiming to enhance the biocompatibility of AgNPs, biosynthesis process can be applied to change the morphology and surface characteristics of AgNPs.¹⁸ Furthermore, recent studies have proven that surface modifications with biomolecules, polymers, or metal ions are effective strategies that can also fulfill this mission.^{19–21}

In this review, we discuss the antibacterial mechanisms and antibiofilm activity of AgNPs, which are the fundamental of implants application. The disputed cellular effects of AgNPs on osteogenesis-related cells are discussed, and some advice for implants designed are provided. Methods such as biosynthesis, adjustments of physical properties, and combining with biomolecules to enhance the compatibility of AgNPs are highlighted. Finally, different modification methods of AgNP introduction into orthopedic implant are also summarized.

Antibacterial mechanisms and antibiofilm activity of AgNPs

As described above, AgNPs can destroy multiple drug-resistant strains and prevent biofilm formation, indicating significant potential in antibacterial application.²² Although the antibacterial mechanisms of AgNPs have been discussed extensively, the exact effect of AgNPs on bacteria is still undefined. To our knowledge, two antibacterial mechanisms are widely accepted, namely contact killing and ion-mediated killing. In this section, specific antibacterial mechanisms and antibiofilm activity were described in detail.

Antibacterial mechanisms through direct contact with microorganisms

AgNPs have more outstanding physiochemical and biological properties beyond bulk silver. It has been reported that AgNPs can anchor to the bacterial cell wall and consequently infiltrate it. This action will cause physical changes in the bacterial membrane, like the membrane damage, which can lead to cellular contents leakage and bacterial death (Figure 1A).^{23,24} It was also demonstrated that the antibacterial effect of AgNPs on Gram-negative bacteria was stronger than Gram-positive bacteria. This phenomenon

can be explained by the existing difference in the cell wall thickness between Gram-positive bacteria (30 nm) and Gram-negative bacteria (3–4 nm), which are mainly composed of peptidoglycan.²⁵ In addition, it has been proved that the cellular membrane of bacteria has a negative charge due to the presence of carboxyl, phosphate, and amino groups.²⁶ The positive charge confers electrostatic attraction between AgNPs and negatively charge cell membrane of the microorganisms, thereby facilitates AgNP attachment onto cell membranes.²⁷ Hence, enhanced antibacterial effects can be obtained by altering the surface charge of AgNPs to achieve stronger attractive force.²⁸

After adhesion to the bacterial wall, AgNPs can also penetrate the membrane and enter the bacteria. There is a size-dependent antibacterial effect, namely smaller nanoparticles has a large surface area in contact with the bacterial cells and can reach the cytoplasm more often than larger nanoparticles.²⁴ When AgNPs penetrate inside the microbial cell, it may interact with cellular structures and biomolecules such as proteins, lipids, and DNA. Interaction between AgNPs and cellular structures or biomolecules will lead to bacterial dysfunction and finally death. In particular, AgNP interaction with ribosomes lead to their denaturation causing inhibition of translation and protein synthesis (Figure 1B). It is also speculated that AgNPs interact effectively with the carboxyl and thiol groups of β -galactosidase, inhibit intracellular biological functions, and lead cell death.²⁹

Furthermore, the antibacterial mechanism of AgNPs is also due to their ability of producing high levels of reactive oxygen species (ROS) and free radical species such as hydrogen peroxide, superoxide anion, hydroxyl radical, hypochlorous acid, and singlet oxygen.^{30–32} Under normal circumstances, ROS generated in cells is limited and can be eliminated by antioxidant systems.³³ AgNPs exert antibacterial effect through inactivation of respiratory chain dehydrogenases and eventual excess ROS generation, which inhibited respiration and growth of cells.^{34,35} AgNPs can downregulate the expression of antioxidant enzyme such as glutathione (GSH), superoxide dismutase, and catalase, which can accelerate the accumulation of ROS.²² Increased ROS lead to an apoptosis-like response, lipid peroxidation, depletion of GSH, and DNA damage.^{36,37} In addition, the antibacterial activity of AgNPs was also influenced by adenosine triphosphate (ATP)-associated metabolism and ROS.³⁸

Antibacterial mechanisms mediated by the release of silver ions

There are several evidences suggesting that the released silver ions (Ag^+) from AgNPs are important in the antibacterial

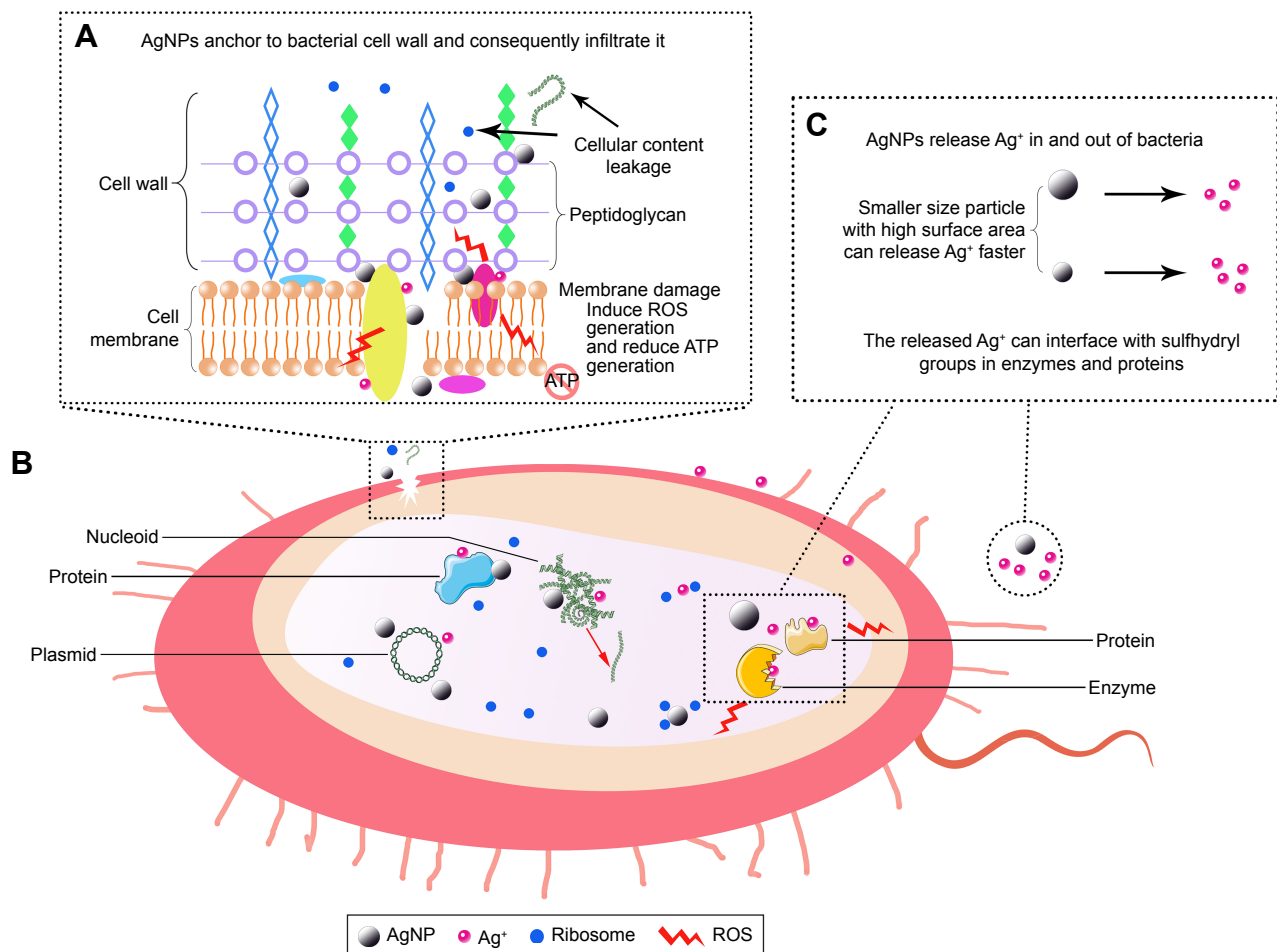


Figure 1 Antibacterial mechanisms of AgNPs. **(A)** The local enlarged picture shows that AgNPs can anchor to the bacterial cell wall and consequently infiltrate it. This action can lead to membrane damage and cellular content leakage. Furthermore, AgNPs or Ag⁺ can bind to the protein present in the cell membrane, which are involved in transmembrane ATP generation. **(B)** AgNPs can penetrate inside to microbial cell, and then AgNPs and the released Ag⁺ can interact with cellular structures and biomolecules such as proteins, enzymes, lipids, and DNA. The increased ROS lead to an apoptosis-like response, lipid peroxidation, and DNA damage. **(C)** AgNPs can sustainably release Ag⁺ in and out of bacteria, and Ag⁺ can interact with proteins and enzymes.

Abbreviations: AgNP, silver nanoparticle; ROS, reactive oxygen species.

activity.^{7,39–41} One of the important parameters of AgNPs against microbes is the surface area of the nanomaterials. AgNPs can sustainably release Ag⁺ in and out of bacteria. The highest concentration of released Ag⁺ was observed in the case of AgNPs with the highest surface area. The lowest concentration of Ag⁺ released was noted for AgNPs with the lowest surface area, resulting in weak antimicrobial property.⁴² The mechanism of the antimicrobial action of Ag⁺ is closely associated with its interface with sulfhydryl groups in enzymes and proteins (Figure 1C). For instance, Ag⁺ can bind to proteins that are present in the cell membrane to form stable bonds resulting in protein deactivation. The proteins are involved in transmembrane ATP generation and mediate ion transport across cell membranes.⁴³ Besides, micromolar ranks of Ag⁺ have been described to uncouple respiratory electron transport from oxidative phosphorylation and limit respiratory chain enzymes or obstruct with

the membrane penetrability to protons and phosphate.^{44,45} In addition, it has been found that Ag⁺ can form complex with nucleic acids, where it preferentially interact with the nucleosides. Ag⁺ intercalates between the purine and pyrimidine base pairs, disrupts the H-bonds between base pairs of the anti-parallel DNA strands, which prevent cell division and reproduction eventually.^{46,47}

Ag⁺ as a heavy metal ion can cause the increase of cellular oxidative stress in microbes, which is another antibacterial mechanism. Long et al⁴⁸ prepared four different AgNPs by coating with different ligand and investigated the mechanism of AgNP-dependent antibacterial activity. The released Ag⁺ from AgNPs was suggested to interact with respiratory chain proteins on the membrane, interrupt intracellular O₂ reduction, and induce ROS production.⁴⁸ The thioredoxin (Trx) system, which is composed of nicotinamide adenine dinucleotide phosphate, thioredoxin reductase (TrxR), and Trx,

is one of the major disulfide reductase systems used by bacteria against oxidative stress.⁴⁹ It was demonstrated that Ag⁺ binds to the active sites of *Staphylococcus aureus* TrxR and Trx and leads to oligomerization and functional disruption of TrxR as well as Trx. Ag⁺ also depleted intracellular thiol levels in *S. aureus*, disrupting bacterial thiol-redox homeostasis, and the increased ROS induced bacteria death finally.⁵⁰ Although AgNPs can kill bacteria through the two different action modes mentioned above, the antibacterial mechanism is usually considered as the synergistic effect generated from AgNPs and Ag⁺.³²

With the wide application of AgNPs, researchers start to worry about the potential development of bacterial resistance to AgNPs. In a recent report, it was found that AgNPs enhanced bacterial resistance to antibiotics by promoting stress tolerance through induction of intracellular ROS.⁵¹ In addition, Gram-negative bacteria *Escherichia coli* 013, *Pseudomonas aeruginosa* CCM 3955, and *E. coli* CCM 3954 can develop resistance to AgNPs after repeated exposure. This resistance was due to the production of flagellin, an adhesive protein of the bacterial flagellum, which caused the aggregation of AgNPs and thereby eliminated their antibacterial effect.⁵² Indeed, bacterial resistance exists, and the mechanism is the aggregation of AgNPs. However, AgNPs are always incorporated into the implant surface in a dispersed state. Thus, further studies are needed to verify whether bacterial resistance develop in AgNP-coated implant surface.

Antibiofilm activity of AgNPs

Biofilms are communities of microorganisms attached to a solid surface. Once the biofilm is formed on the implant surface, it protects microorganisms from antibiotic treatment and causes serious consequences.^{53,54} The antibiofilm activity of AgNPs has been demonstrated in a number of studies. One pioneering study was performed to analyze the interactions of AgNPs with *Pseudomonas putida* biofilms. The results suggested that biofilms are impacted by the treatment with AgNPs.⁵⁴ Du et al⁵⁵ synthesized AgNPs by using benzoin gum extract and tested their antibiofilm effect by using *E. coli*. The AgNPs exhibited the antibiofilm activity at concentrations up to 10 µg/mL.⁵⁵ AgNPs were fabricated in situ and immobilized on the titanium surface. This modified surface can reduce bacterial biofilm formation in vitro by inhibiting bacterial adhesion and *icaAD* transcription. In addition, the antibiofilm activity of the immobilized AgNPs is independent of silver release, and AgNPs can defend several cycles of bacterial exposure in vitro and reduce implant-associated

periprosthetic infection in vivo.⁵⁶ In another study, AgNPs were incorporated into porous titanium implants in the grown oxide layer and to create a micro-/nanoporous structure on the surface of the implants. Antimicrobial assays showed strong antimicrobial activity against methicillin-resistant *S. aureus* including released activity, surface antimicrobial activity, and prevention of biofilm formation.⁵⁷ These evidences showed that implant can be endowed with antibiofilm activity with AgNP incorporation.

Cellular effects of AgNPs on osteogenesis-related cells

Biocompatibility of AgNPs on osteogenesis-related cells, especially osteoblast, osteoclast, and mesenchymal stem cells (MSCs) should be concerned due to their key roles in bone regeneration.^{58,59} In this section, we discuss the influence of AgNPs on the abovementioned cell activity, adhesion, proliferation, and differentiation.

Effects of AgNPs on osteoblast and osteoclast

Bone metabolism is a critical factor during implants relative to bone integration, in which the osteoblast and osteoclast are responsible for bone formation and absorption during the integration, respectively.⁵⁹ AgNPs could be uptake into osteoblasts and could cause the first manifestation of cell injury through generation excessive nitric oxide, that is, swelling of the endoplasmic reticulum.⁶⁰ AgNPs were reported showing a cytotoxicity effect on osteoblasts in a dose-dependent manner and impaired cell viability at a concentration of 10 µg/g of AgNPs.⁶¹ In addition, higher cytotoxicity concentrations of AgNPs were observed from other studies, which was 25 µg/mL and 50 µM, respectively.^{16,62} However, from the results mentioned above, the proper concentration of AgNPs was concluded to be 10 µg/mL aiming for medical application, possessing effective antibacterial and good biocompatibility simultaneously. Furthermore, size-dependent cytotoxicity effect was also observed. When several cell lines were treated with three different characteristic sized AgNPs, the smaller particles exhibited stronger cytotoxic effects on osteoblast, which is due to the size and surface area discrepancy release of Ag⁺ from AgNPs.^{63,64}

Despite the side effect, AgNPs were demonstrated to possess the capacity of enhancing mineralization and alkaline phosphatase (ALP) expression in MC3T3-E1 cells at a concentration of 20 µg/mL. The underlying mechanisms were the miRNA regulation of expression of mothers against

decapentaplegic (Smad) transcription factor 1 and 5, and Runt-related transcription factor 2 (Runx2), which were related to osteogenesis.⁶⁵ Furthermore, some results indicated that the incorporation of AgNPs into biomaterials might lead to decreased cytotoxicity by reducing the cellular uptake of AgNPs.⁶⁶ In addition, cell spreading is suggested to be beneficial to osteoblast differentiation and also results in better cell–cell communication, which is reported being critical to coordinate cell behavior.⁶⁷ When AgNPs were incorporated into TiO₂ nanotube and cultured with MC3T3-E1 cells, some favorable effects on promoting cell spreading were observed from cell morphology assay after culturing for 3 days.⁶⁸ Another study indicated the same trend, and no significant cytotoxicity was observed when the osteoblast-like MG63 and MC3T3 cells were exposed to AgNPs, and the MG63 cell even promoted cellular proliferation.⁶⁹ Furthermore, nanostructure properties of implant surface were enhanced by the incorporation of AgNPs, which is a benefit for promoting osteogenesis with increased cell attachment, viability, and osteogenic gene expression (ALP, Runx2, and OCN).⁷⁰

Cellular effects of AgNPs on viability and differentiation of MSCs

MSCs act as multipotent precursors of various cells, which can differentiate into osteoblasts, adipocytes, and chondrocytes by a proper induction condition. Due to the key role of MSCs in bone regeneration, some researchers reported different effects of AgNPs on MSCs.

Similar to the effect on osteoblast and osteoclast, AgNPs exert cytotoxic effects on MSCs in a dose- and time-dependent manner. Greulich et al⁷¹ investigated the biocompatibility of 100 nm AgNPs in human mesenchymal stem cells (hMSCs). The cytokine level of interleukin-8 (IL-8) was significantly higher than that of IL-6 and VEGF at concentrations of

5 µg/mL and above.⁷¹ In addition, AgNPs can be absorbed into cells and then induce DNA damage, cell death, and functional impairment of MSCs. Distribution analysis showed that AgNPs were mainly located in the cytoplasm and the nucleus. Cytotoxic and genotoxic effects were positively correlated with dose. Interestingly, the migration ability of hMSCs was not impaired at subtoxic concentrations.⁷² In terms of MSC proliferation, some studies have demonstrated that AgNPs can promote cell proliferation at nontoxic concentration. Jung et al⁷³ proved that AgNPs increased cell proliferation at subtoxic concentrations and decreased cell proliferation at concentrations above 10 µg/mL. In addition, hypoxia-inducible factor-1 α acting as a transcription factor in regulating metabolism, development, proliferation, and pathology under hypoxic conditions plays a key factor in AgNPs-mediated cell proliferation in hMSCs.⁷³

In the aspect of differentiation, Sengstock et al observed that AgNPs attenuate the adipogenic and osteogenic differentiation of hMSCs even at nontoxic concentrations, whereas chondrogenic was unaffected.⁷⁴ Conversely, Samberg et al exposed human adipose-derived stem cells in both undifferentiated and differentiated states to various concentrations from 0.1 to 100 µg/mL with 10 or 20 nm AgNPs, and no significant effect was observed on cell differentiation.⁷⁵ Qin et al⁷⁶ revealed that 4 µg/mL of AgNPs was safe to urine-derived stem cells (USCs). At this concentration, AgNPs can promote osteogenic differentiation of USCs, induce actin polymerization, increase cytoskeletal tension, and activate RhoA.⁷⁶ In another study, AgNPs promoted MSC differentiation even at a much higher concentration (4–20 µM). Furthermore, AgNPs were also considered to be able to promote the formation of fracture callus and induce early closure of the fracture gap in vivo (Figure 2). The mechanisms may be via multiple routes: 1) chemoattraction

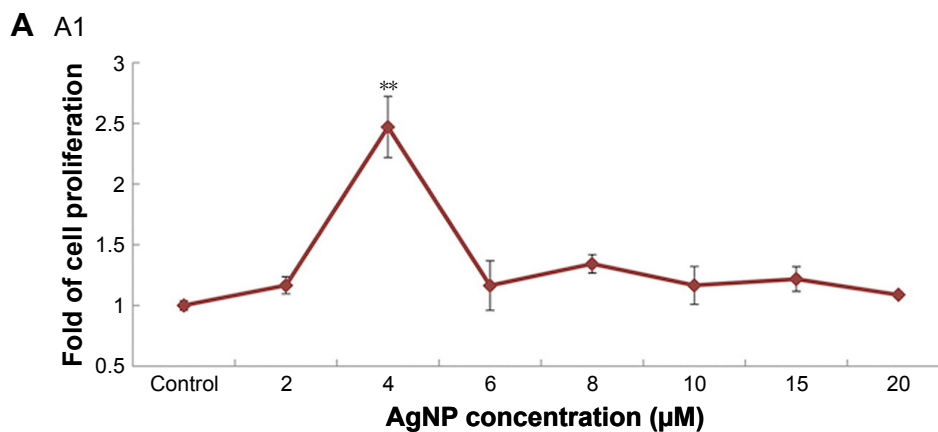


Figure 2 (Continued)

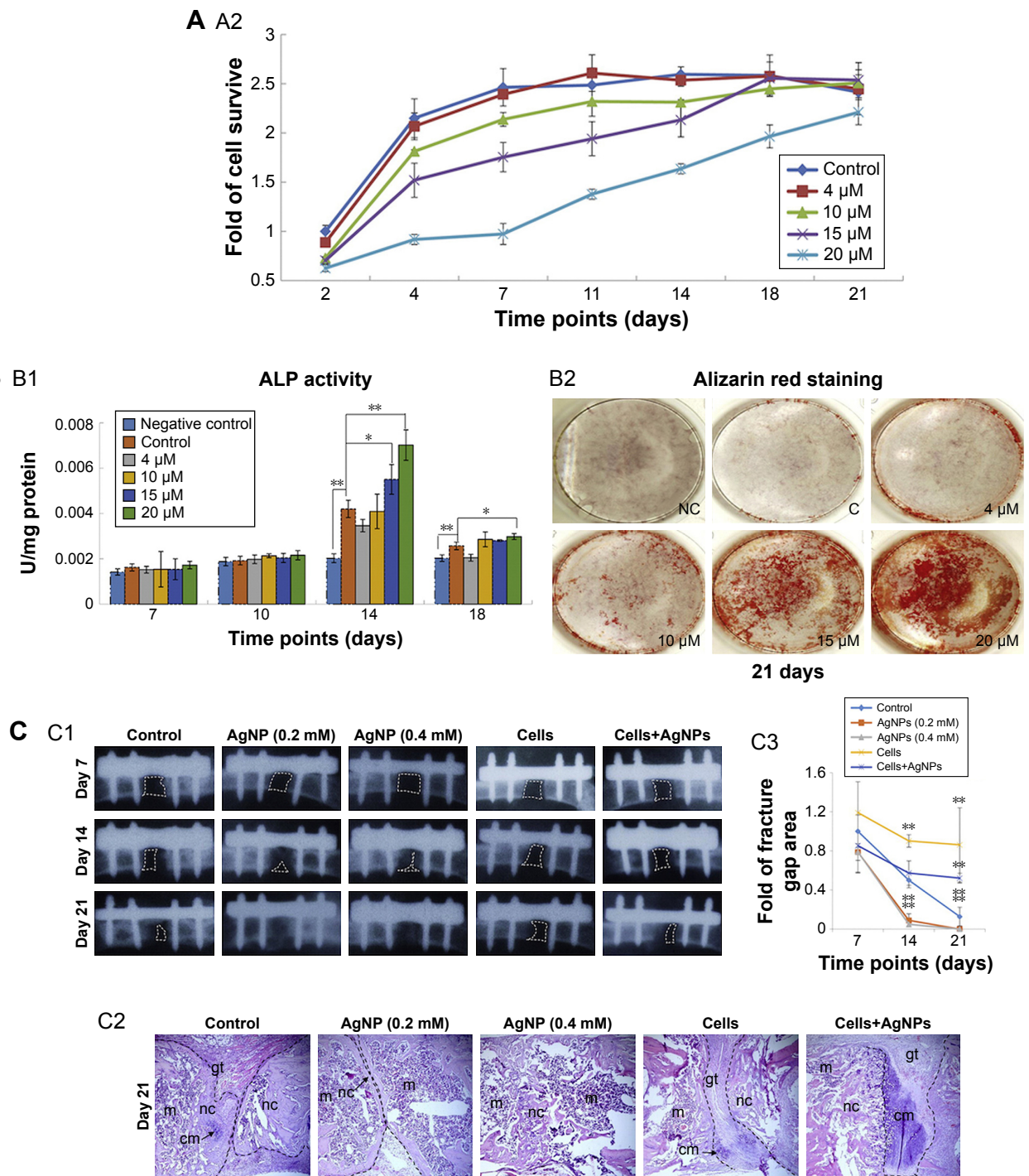


Figure 2 (A) AgNPs increase MSC proliferation and do not reduce cell viability at low concentration. (A1) Cells were cultured with different concentrations of AgNPs for 2 days to test cell proliferation. (A2) Cell viability assay was performed to test the effect of AgNPs on MSC. **(B)** ALP activity (B1) and Alizarin red staining (B2) indicated that AgNPs promote osteogenic differentiation of MSC in vitro. **(C)** AgNPs promote fracture healing in vivo. Plain X-ray radiograph of the fracture sites; broken line demarcates the unfilled fracture gap (C1). Hematoxylin and eosin staining of the middle section of the fracture site of each treatment group was shown. Broken lines indicate the two ends of the fracture femoral bone (C2). The areas of the fracture gap of each treatment groups at different postoperative days were quantified and shown in (C3). **Note:** Reprinted from *Nanomedicine* 11(8), Zhang R, Lee P, Lui V C, et al, Silver nanoparticles promote osteogenesis of mesenchymal stem cells and improve bone fracture healing in osteogenesis mechanism mouse model, 1949–1959, Copyright (2015) with permission from Elsevier.⁷⁷ * $P < 0.05$, ** $P < 0.01$. **Abbreviations:** AgNP, silver nanoparticle; ALP, alkaline phosphatase; MSC, mesenchymal stem cell; m, marrow; nc, new callus; gt, granulation tissue; cm, cartilage matrix.

of MSCs and fibroblasts to migrate to the fracture site; 2) induction of the proliferation of MSCs; 3) induction of osteogenic differentiation of MSCs via induction/activation of TGF- β /BMP signaling in MSCs.⁷⁷

As discussed above, these findings suggest that AgNPs could be used as a biocompatible agent within a particular dosage window. Maybe AgNPs $\leq 10 \mu\text{g/mL}$ is a safe dose window for osteogenesis-related cells. The cellular effects

of AgNPs on osteogenesis-related cells with respect to size of particles, exposure doses, type of cell line, major outcome of each study, and their mechanisms were summarized in Table 1.

Methods to improve biocompatibility of AgNPs

Due to great application of AgNPs, their biocompatibility should be paid attention for medical use. In this part, we focus three methods to enhance the biocompatibility of AgNPs, namely biosynthesis process, adjustment of physical properties, and biomolecule combination.

Biosynthesis to improve biocompatibility

The traditional chemical and physical methods are widely used for AgNP synthesis. However, these methods always have associated risks, such as chemical precursor contamination, solvent toxicity, and hazardous byproduct formation, which make alternative synthetic methods imperative.⁷⁸ At present, bio-enthused synthesis of nanoparticles provides advantages over chemical and physical methods as it is environment-friendly, no need to use high-pressure, high temperature, and no toxic chemicals are needed in biological method.⁷⁹

There are many resources such as bacteria, yeast, fungi, and various parts of plants can be used in the nanoparticle synthesis. Plant extracts frequently offer good manipulation and control over crystal growth and stabilization.⁸⁰ The major advantage of using plant extracts for biosynthesis is that they are easily available, safe, and nontoxic. In most cases, there are various metabolites that can be used to reduce Ag⁺.¹⁷ Through adjustment reaction parameters, AgNPs can be achieved with better yield, controlled size, shape, greater particle stability, more biocompatibility, scalability, and applicability.^{78,81} For example, AgNPs can be green synthesized by using *Artemisia tournefortiana* Rchb ethanol extract; these AgNPs with spherical shape showed powerful antibacterial ability.⁸² In addition, *Mangifera indica* inflorescence aqueous extract was used to reduce AgNO₃ to produce green AgNPs. These particles with an average diameter of 40 nm showed powerful killing effect for Gram-negative (*Klebsiella pneumoniae*, *P. aeruginosa*, and *E. coli*) and Gram-positive (*Staphylococcus mutans* and *S. aureus*) strains. Importantly, AgNPs exhibited no significant toxic effect on HeLa cell line below 25 µg/mL (Figure 3).⁸³ Kasithevar et al synthesized AgNPs by using aqueous leaf extract of *Alysicarpus monilifer*; these green synthesized AgNPs were biocompatible with normal Vero cell line and have high antibacterial activity.⁸⁴ In conclusion, biosynthesis approach is a promising

method to produce AgNPs with both significant antibacterial effect and biocompatibility.

Adjustment of physical properties

The fundamental characteristics of metallic nanoparticles strongly depend on their shapes, sizes, configurations, crystallinity, and structure whether they are in solid form or in hollow geometries. Thus, controlling such parameters can achieve the desired properties of the nanoparticles.¹⁸ In this section, we discuss the role of different sizes and shapes of AgNPs, which are the two main properties in biomedical application.

Many studies have demonstrated that smaller AgNPs showed better antimicrobial activity. For instance, mono-dispersed AgNPs with sizes of 25, 35, 45, 60, and 70 nm were obtained, and cell viability test was performed by using human lung fibroblast. The smaller AgNPs can cause severe cell apoptosis and necrosis and generation of high levels of ROS.⁸⁵ Similar results were observed in other in vitro studies. Compared with larger AgNPs, increased apoptosis, activation of cytokines/chemokines IL-8, IL-1β, tumor necrosis factor, macrophage inhibitory protein, and ROS production were observed in macrophages treated with smaller AgNPs.⁸⁶

The shape of AgNPs can impact the degree of particle toxicity. In order to comprehensively investigate these facts, spherical, rod-shaped, truncated triangular AgNPs, and their skin penetration capabilities were studied. From the results, it was found that triangular AgNPs could be an ideal candidate for topical applications which can reduce systemic toxicity, compared to the rod-shaped and spherical AgNPs.⁸⁷ In another study, spherical, rectangular, penta, and hexagonal AgNPs of different dimensions were biosynthesized, and the results showed that the spherical AgNPs possessed more antimicrobial effect than other shaped AgNPs.⁸⁸ The electron charge transfer properties are also affected by different types of AgNPs. Regardless of the size required, narrow size distributions and more “spherical” shapes can improve sample quality.⁸⁹ In a word, the above studies have highlighted the role of size and shape in potentiating AgNP-based cellular effect.

Combination with biomolecules

Surface modification of nanomaterials is essential where the surface layer facilitates the reduction of surface energy. It can also provide a protective coating that prevents nanoparticles from agglomeration, thus increasing their long-term stability.⁹⁰ Many organic molecules can be used for surface functionalization. Examples include small molecules like lipids, vitamins, peptides, and sugar and larger ones such as

Table 1 Different size, dose of AgNPs, and their effect on osteogenesis-related cells and corresponding mechanisms

Size of AgNPs (nm)	AgNP exposure dosage ($\mu\text{g/mL}$)	Cell type	Cellular effects	Mechanisms	Reference
30	5, 10, 25	Saos-2 and hBMSCs	Half maximal inhibitory concentration around 25 $\mu\text{g/mL}$ and no influence on differentiation	Upregulate hsp70 expression to adjust	16
18.3 \pm 2.6	30, 60	Human fetal osteoblast cells	Induce cell death	Excess NO generation	60
\leq 10	1–30	hMSCs and osteoblasts	Impairment of cell viability at a concentration of 10 $\mu\text{g/g}$	Excess ROS generation	61
6	10, 25, 50	Osteoblastic cells	Show cytotoxicity above 50 μM	–	62
50, 3,000, 30,000	62.5–256	Osteoblasts and osteoclasts	MIC: OB viability: 128 $\mu\text{g/mL}$; OB differentiation: 64 $\mu\text{g/mL}$; OC viability: 256 $\mu\text{g/mL}$; OC differentiation: 128 $\mu\text{g/mL}$	Size-dependent release of Ag ⁺	63
10, 50, 100	10, 20, 40, 80, 160	MC3T3-E1	Stimulated apoptosis	Excess ROS generation	64
23.0 \pm 2.0	20	MC3T3-E1	Enhance mineralization	Upregulate Smad1/5 and Runx2 expression	65
5–8	–	MG-63 osteoblastic cells	Promote osteoblast proliferation	Micro-galvanic effect between the AgNPs and titanium matrix	66
–	–	MC3T3-E1	Promoting cell spreading	–	68
4, 5–25	–	MG63 and MC3T3-E1	Mo significant cytotoxicity and even good cytocompatibility	Mammalian and bacterial cells interact differently with the same materials	69
100	50, 5, 4, 3.5, 3, 2.5, 1, 0.5	hMSCs	Cytotoxicity occurred at concentrations above 5 $\mu\text{g/mL}$	–	71
46 \pm 21	10, 1, 0.1	hMSCs	Cytotoxic at concentrations of 10 $\mu\text{g/mL}$, DNA damage at 0.1 $\mu\text{g/mL}$	–	72
9.5 \pm 1.7	15, 10, 7.5, 5, 2.5, 1	hMSCs	Promote cell proliferation and IL-8 expression below 10 $\mu\text{g/mL}$	HIF-1 α is a key factor	73
80	10, 5, 2.5	hBMSCs	Impair the adipogenic and osteogenic differentiation, chondrogenic differentiation was unaffected	–	74
10, 20	0.1, 1, 10, 50, 100	hASCs	Do not influence differentiation, but a minimal decrease in viability	–	75
19.4 \pm 5.2	0.1, 0.5, 1, 2, 4, 8, 16, 32, 64	USCs	Promote osteogenic differentiation at 4 $\mu\text{g/mL}$	Activate RhoA and increase cytoskeletal tension	76
5–15	4, 10, 15, 20	mMSCs	Promote cell proliferation and osteogenic differentiation promote the formation of fracture callus, and induce early closure of the fracture gap	Induce cells to migrate to the fracture site and proliferation; activate TGF- β /BMP signaling	77

Abbreviations: AgNP, silver nanoparticle; hBMSC, human bone marrow stromal cell; hMSC, human mesenchymal stem cell; hASC, human adipose-derived stem cell; USC, urine-derived stem cell; IL, interleukin; NO, nitric oxide; ROS, reactive oxygen species; HIF-1 α , hypoxia-inducible factor-1 α ; MIC, minimum inhibitory concentration; OB, osteoblast; OC, osteoclast.

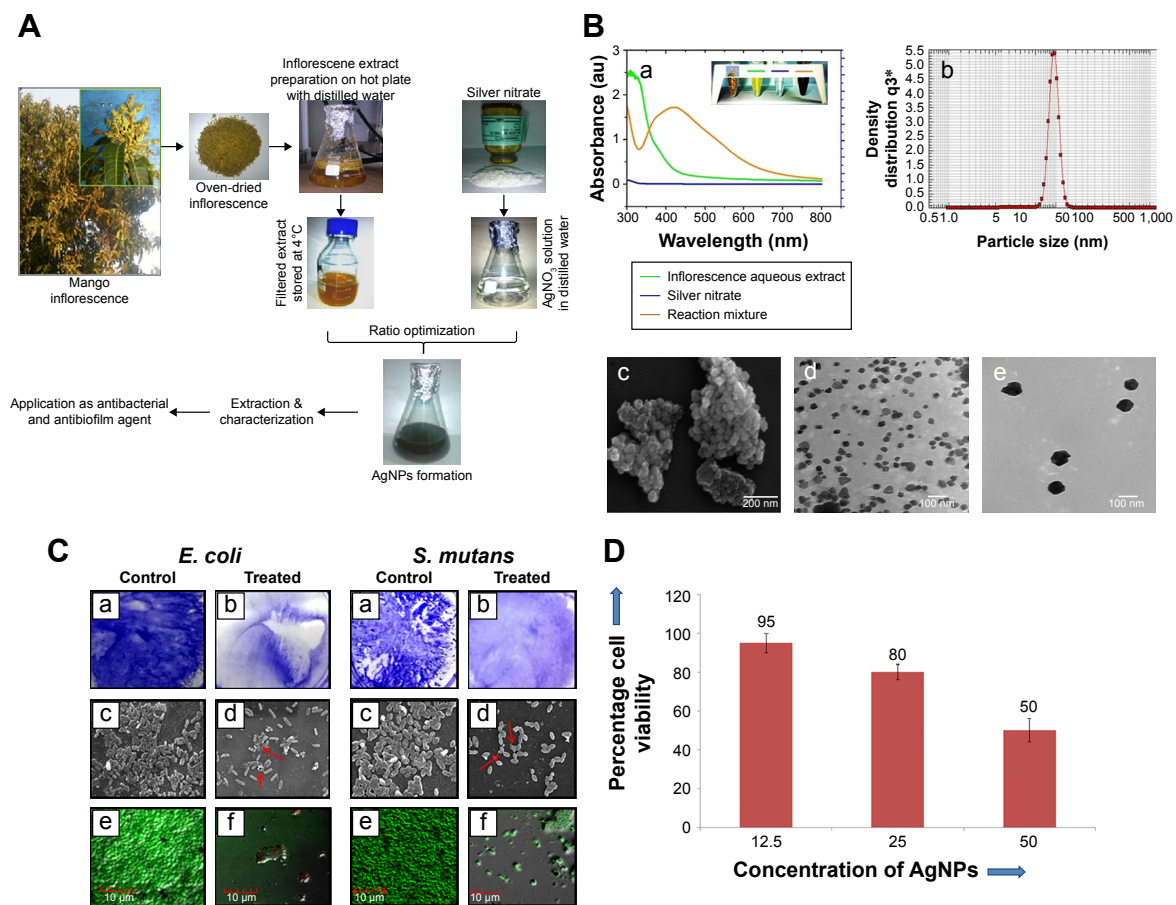


Figure 3 (A) Graphical presentation of green silver nanoparticles biosynthesis. (B) Characterization of green AgNPs: (a) ultraviolet-visible spectra indicated the synthesis of AgNPs were extremely stable. (b) Nanophox particles size analyzer graph showed the size of the particle was ranging between 30 and 70 nm with an average particle size of 40 nm. (c) Field-emission scanning electron microscope image. (d and e) Transmission electron microscope images. * $P < 0.05$. (C) Visualization of the biofilm inhibition: (a and b) images display control and treated biofilm stained by crystal violet. (c and d) images show the difference in biofilm after treatment as visualized by the SEM. (e and f) are confocal laser scanning microscope images of the control and treated biofilms. Red arrows indicate the damage in the bacterial cells due to action of AgNPs. (D) Cell line (HeLa) toxicity assessment of the AgNPs.

Note: Reproduced from Qayyum S, Oves M, Khan AU. Obliteration of bacterial growth and biofilm through ROS generation by facily synthesized green silver nanoparticles. *PLoS One*. 2017;12(8):e0181363.⁹³

Abbreviations: AgNP, silver nanoparticle; SEM, scanning electron microscope.

natural polymers including proteins, enzymes, DNA, and RNA.⁹¹ In this section, we discuss several biomolecules especially chitosan (CS), which is commonly used in surface modification.

CS is a linear polysaccharide, which has randomly distributed β -(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit).⁹² It is a highly biocompatible biodegradable material and shows antibacterial properties against pathogenic bacteria, with potential application as an antimicrobial agent.⁹³ These properties make CS suitable for surface modification of AgNPs. The hydrogels based on CS and modified with AgNPs exhibit toxicity in relation to *S. aureus* and did not exert any negative impact on the cells of the dermis.⁹⁴ AgNPs embedded in CS are capable to interact with amine and hydroxyl groups of the CS molecule, subsequently form complexes and produce

stable nanostructured film. From the results, the CS/nAg nanocomposites showed enhanced antibacterial and biocompatibility properties.⁹³ Low-molecular-weight CS (LMWC) has better water solubility and biological activity than higher degree of polymerization of CS. Compared with AgNPs coated with high-molecular-weight CS, AgNPs coated with LMWC were more effective against methicillin-resistant *S. aureus* and showed better biocompatibility and lower body absorption characteristics.⁹⁵ In another study, an agarose composite embedded with CS-coated AgNPs was synthesized. This scaffold showed good swelling ratio, excellent hemocompatibility, and appreciable antibacterial activity. Moreover, it also showed good biocompatibility to several cell lines and benefits for cell sustained growth.²⁰ These studies suggest that CS is a good material for surface modification of AgNPs.

Except CS, there are many other biomolecules that can be used in combination with AgNPs for surface functionalization. Polyethylene glycol (PEG) can reduce osponization process in which nanoparticles are directed to liver through macrophages.²¹ In addition, Kwon et al demonstrated that polyvinylpyrrolidone was a more suitable surfactant than PEG for AgNP capping, which could decrease aggregation and reduce cytotoxicity.⁹⁶ Furthermore, silk fibroin (SF) as a natural polymer shows the ability of reduction and osteogenic regeneration. It can reduce Ag^+ to Ag^0 by the Tyr residues with strong electron-donating property and also exposes to disperse and stabilize the produced AgNPs to maintain the stability of the SF-AgNPs composite solution. The SF-AgNPs composite showed good biocompatibility and improved osteogenic differentiation by decreasing the Ag^+ release from AgNPs (Figure 4).⁹⁷ Advantages of combination with biomolecules is that the surface chemistry properties of AgNPs can be easily controlled to realize multifunctionalization. Many biomolecules can meet our requirements to enhance the biocompatibility of AgNPs for medical use, which is worthy to investigate in vivo reaction in future study.

Modification of orthopedic implants by using AgNPs

Over the past decades, metallic implants have been widely used in orthopedic fields. However, it is biologically inert and cannot induce new bone regeneration or inhibit bacterial activity. AgNPs as an antibacterial agent are usually used to modify orthopedic implant surface to obtain antibacterial and potential osteointegration ability. In this section, we discuss various surface modification techniques for AgNP coating onto implants surface (Figure 5), as well as their effects on antibacterial and bone formation.

Plasma immersion ion implantation (PIII)

PIII is proven to be economical and effective and have already been widely applied in the modification of semi-conductors and biomedical industry. The process involved accelerating positive ion incorporation vertically into the negative potential surfaces in the electric field.⁹⁸ It can deposit different ions on many biomaterials, including metal, ceramics, and polymers.⁹⁹ Moreover, PIII can efficiently control the distribution and concentration of incorporated element in material by regulating the modification parameters.¹⁰⁰

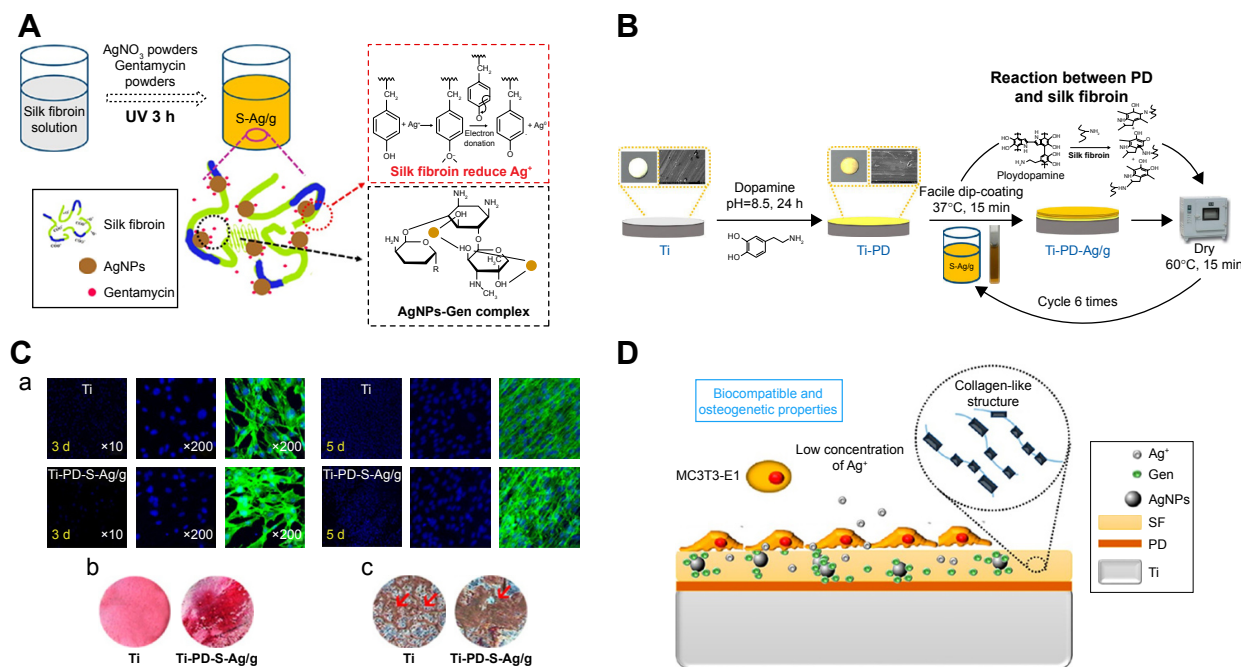


Figure 4 (A) Schematic of in situ preparation of SF/AgNPs/Gen composite solution and the possible reduction mechanism of Ag^+ by SF as well as the interaction between AgNPs and gentamicin. (B) Illustrative diagram of fabrication process for PD-S-Ag/g coatings on titanium substrate, scheme is not in real scale. (C) SF-coated AgNPs shows good biocompatibility and improved osteogenic differentiation. (a) Fluorescent images for 3 and 5 days with actin stained with FITC (green) and nuclei stained with DAPI (blue). (b) Collagen secretion on different specimens for 28 days. (c) Calcium deposition (red arrow) on different specimens for 28 days. (D) Mechanism on the AgNPs/Gen-contained SF-based coatings.

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Abbreviations: AgNP, silver nanoparticle; PD, poly-dopamine; SF, silk fibroin.

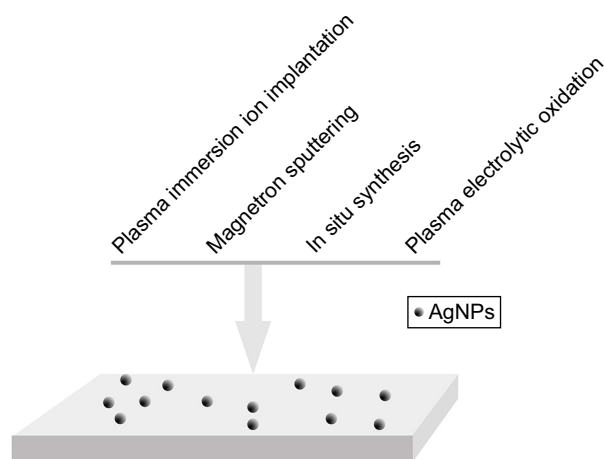


Figure 5 Strategies and main techniques for the introduction of AgNPs onto implants surface.

Abbreviation: AgNP, silver nanoparticle.

AgNPs can be incorporated into the near-surface of implant by PIII process. This technique significantly improved the stability of surface coating with a negligible amount of Ag^+ release. The immobilized AgNP implant surface not only inhibited bacterial adhesion and biofilm formation in vitro but also reduced implant-associated infection in vivo. While, the biocompatibility of titanium implants was also enhanced due to the change of implant surface physical topography.^{101,102} In addition, Ag-PIII-treated titanium accelerated the osteointegration of titanium implant by initiating the ERK1/2 signal.¹⁰² In another study, similar results have been observed by activating the integrin- α 5-orchestrated MAPK/ERK signal cascade.¹⁰³ Moreover, by increasing the PIII process time (1, 2, and 3 h), the diameter of AgNPs becomes larger, thus increasing implant surface roughness. The concentration of AgNPs can be effectively controlled within the safe range by changing the fabrication time.¹⁰⁴ Furthermore, two or more different metallic ions can be introduced on the implant surface through PIII process to obtain more biological functions.^{105,106} For instance, Zn and Ag were simultaneously and sequentially implanted into titanium by PIII. The advantages of Zn and Ag are preserved, the Zn/Ag microgalvanic couple formed on Zn/Ag dual-ion coimplanted titanium shows the best osseointegration as well as good antibacterial properties.¹⁰⁶

Magnetron sputtering

Magnetron sputtering constitutes one of the physical vapor deposition techniques that have been widely used in applications involving the metal-mechanic industry with great success. The sputtering technique has advantages of coating

thinly, thickness uniformity, and high bonding strength to metal. Many stable bioactive coating can be deposited on various implant surface uniformly through magnetron sputtering.^{107,108} Magnetron sputtering requires simple equipment and thus is easy to control. In addition, it has low substrate temperature, high film forming rate, and strong film adhesion.¹⁰⁹

AgNPs coating on M2 tool steel and Si substrate by the sputtering technique, the antibacterial efficiency, and tribological properties of the substrate were significantly enhanced.^{110,111} By controlling magnetron sputtering time, coating morphological shapes could be controlled, as could the quantity of deposited structures.¹¹² In addition, the stable coating on the implant surface can achieve long-term antibacterial effect through magnetron sputtering technique. Furthermore, AgNPs incorporated on the implant surface by sputtering can increase water contact angle and improve hydrophobicity of the substrate surface.¹¹³ It was found that with a decreased sputtering time, surface roughness and wettability were higher. An increasing roughness value is well known to be associated with increased osteoblast adhesion. However, it is also beneficial to bacterial adhesion.¹¹⁴ In Liu et al study,¹¹⁵ the homogeneous AgNP coating was uniformly distributed on the surface of the polyetheretherketone (PEEK) samples via magnetron sputtering. The AgNP-coated samples had a significant increase in surface roughness ($P < 0.05$) as the thickness of their AgNP coating increased. Their antibacterial rates were above 99%, indicating that the AgNP-coated PEEK implant materials have strong antibacterial and bactericidal effects against *S. mutans* and *S. aureus* in the oral cavity.¹¹⁵

In situ synthesis

In situ synthesis is a method through chemical reaction on the implant surface to incorporate different nanoparticles. This technology is simple, economical, and available for surface modification. However, adhesion between nanoparticles and substrate surface is not tight.

AgNPs have been obtained on different TiO_2 substrates, through reduction in AgNO_3 solutions. The amount of deposited nanoparticles depends on the concentration of the silver nitrate solution and reactive time.^{116–118} There is no doubt that the released Ag^+ showed powerful antibacterial effect, but the key point is how to prolong the release time. To enhance the adhesion on the substrate and prolong antibacterial time, an initial layer on Ti surfaces using phase-transited lysozyme was established. Then, AgNPs were synthesized on CS and hyaluronic acid layer, and multilayer coatings were prepared on Ti surfaces via a layer-by-layer self-assembly technique.

This system showed obvious antibacterial effect even in 14 days.⁷ In another study, a mussel-inspired self-polymerized polydopamine anchor was employed, and the released time was up to 28 days.¹¹⁹ A titanate nanowire film was produced on Ti substrate by an alkali hydrothermal reaction and subsequently doped by AgNPs through an ultraviolet light chemical reduction. The release of Ag⁺ was detrimental for the growth, proliferation, and differentiation of MC3T3. However, the additional CS will alleviate this cytotoxicity effect.¹²⁰ During bacterial infection, pH level around the peri-implant surface decreases as low as pH 5.5.¹²¹ Dong et al¹²² construct a pH-dependent AgNP releasing titanium implant to control peri-implant infection. Once the pH was doped to 5.5, the robust released AgNPs from implant system which efficiently controlled bacterial growth. Moreover, this system was biocompatible and showed osteoinductive properties.¹²² In situ synthesis is a simple method, but the key point is how to design a safe and suitable system to achieve long-term antibacterial effect.

Plasma electrolytic oxidation (PEO)

PEO also known as micro-arc oxidation is a method that develops ceramic-like surfaces on the implant surface. The oxidation layer can also offer a wide variety of mechanical, biomedical, tribological, and antibacterial properties through the incorporation of several ions and particles.^{123,124} Nowadays, this method is widely used in the modification of implants to prepare bioactive coatings.

PEO can dope the surface of the implants with fully dispersed and firmly attached AgNPs all within the span of a few minutes. In addition, factors such as voltage and time were not affected significantly due to the addition of AgNPs in electrolyte.¹²⁵ AgNPs were immobilized in the oxide layer on titanium implant to create a micro-/nanoporous structure through PEO technique. The coating showed strong antimicrobial activity without any signs of cytotoxicity, because AgNPs are entrapped in an in-depth growing oxide layer which fully immobilizes them and prevents them from freely circulating through the blood stem.⁵⁷ Furthermore, the AgNPs were found to be very stable in biological fluids with material loss, as a result of dissolution, to be <0.07% for the silver nanocoatings after 24 h in a modified Krebs-Ringer bicarbonate buffer, which shows a stable coating with a significant effect for preventing bacterial growth.¹²⁶

Three-dimensional printing (3DP) silver-containing scaffolds

Additive manufacturing technology, also called 3DP, has emerged recently. It can precisely fabricate scaffolds with

defined shape, size, porosity, and pore size distribution, which is beneficial for bone growth.^{127,128} In addition, more and more complex orthopedic diseases can be solved based on this technique.¹²⁹ In this section, we discuss the silver-containing 3D scaffolds in orthopedic field.

Many materials can be used to produce scaffold via this technology, such as metal, polymer, and inorganic materials. 3D scaffolds with a porosity structure is suitable for loading ions, nanoparticles, and biomolecules to achieve multifunctional application.¹³⁰ In Correia et al study,¹³¹ the tricalcium phosphate (TCP)/sodium alginate (SA) scaffolds were produced by 3DP. Subsequently, AgNPs were incorporated into scaffolds through two different methods, direct incorporation and physical adsorption. Both of them present appropriate mechanical properties, biocompatibility, and bactericidal activity, which is suitable for being used in bone tissue regeneration (Figure 6).¹³¹ Similarly, graphene oxide and AgNPs nanocomposites were successfully modified on the β -TCP scaffolds by a simple soaking method to achieve biofunctions with antibacterial and osteogenic activity.¹³² However, through soaking method, AgNP burst was released from the 3D scaffolds in short time, and this manner easily causes cytotoxicity. To control the release, a mixture of nanocomposites such as bioceramic and polyvinyl alcohol in different compartment can achieve different release kinetics.¹³³ Notably, a growing number of researchers focus on the bio-printing technology, which is a bio-printer that is used to dispense “bioinks,” consisting of cells, scaffolds, and biomolecules, in a spatially controlled manner.¹³⁴ Biological structures fabricated via bio-printing can encapsulate cells and bioactive agents directly, which include various biomimetic tissues such as the blood vessels, liver, heart, and tumors.¹³⁵ Furthermore, 3DP technology is a continuous development, and it will play a bigger role in regenerative medicine.

Conclusion

Although orthopedic implants have made huge improvement for functional reconstruction of patients with bone fractures or defects, however, implant failure and revision surgeries are needed once the infection occurs. AgNPs with strong antibacterial efficacy are being used extensively in implant surface modification to prevent implant-related infection. The potential antibacterial mechanisms of the AgNPs were summarized, and their roles in optimization of orthopedic implants to provide some advice for implant designing were highlighted in this review. Future directions should focus on stability and long-term AgNP release, exploration of suitable size, shape, as well as the novel method of surface modification, such as 3DP technology.

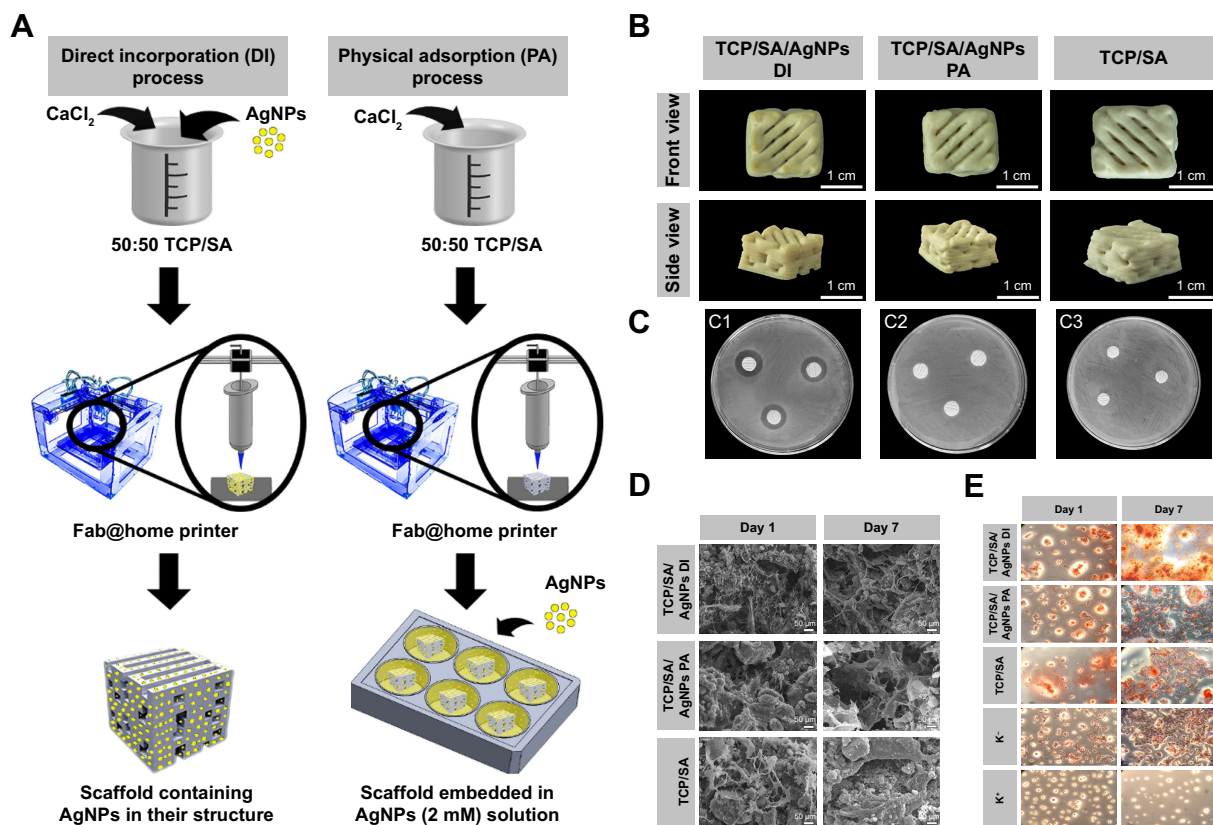


Figure 6 (A) Schematic representation of scaffold production by using the DI and PA processes. (B) Macroscopic images of the scaffolds produced by DI, PA, and without AgNPs (TCP/SA) (front and side view). (C) Characterization of the bactericidal activity of the produced scaffolds, DI process (C1), PA process (C2), TCP/SA scaffold (C3). (D) SEM images of osteoblast morphology in the presence of the scaffolds. (E) Qualitative evaluation of mineralization on the 3D scaffolds by human osteoblast through alizarin red staining.

Note: Reprinted from *Int J Biol Macromol*, 93, Correia T R, Figueira D R, De Sa K D, et al, 3D Printed scaffolds with bactericidal activity aimed for bone tissue regeneration, 1432–1445, Copyright (2016) with permission from Elsevier.¹³¹

Abbreviations: AgNP, silver nanoparticle; TCP, tricalcium phosphate; SA, sodium alginate; SEM, scanning electron microscope; DI, direct incorporation; PA, physical adsorption.

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Disclosure

The authors report no conflicts of interest in this work.

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