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REVIEW

Enhancing ZnO-NP Antibacterial and Osteogenesis Properties in Orthopedic Applications: A Review

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Abstract: Prosthesis-associated infections and aseptic loosening are major causes of implant failure. There is an urgent need to improve the antibacterial ability and osseointegration of orthopedic implants. Zinc oxide nanoparticles (ZnO-NPs) are a common type of zinc-containing metal oxide nanoparticles that have been widely studied in many fields, such as food packaging, pollution treatment, and biomedicine. The ZnO-NPs have low toxicity and good biological functions, as well as antibacterial, anticancer, and osteogenic capabilities. Furthermore, ZnO-NPs can be easily obtained through various methods. Among them, green preparation methods can improve the bioactivity of ZnO-NPs and strengthen their potential application in the biological field. This review discusses the antibacterial abilities of ZnO-NPs, including mechanisms and influencing factors. The toxicity and shortcomings of anticancer applications are summarized. Furthermore, osteogenic mechanisms and synergy with other materials are introduced. Green preparation methods are also briefly reviewed.

Keywords: antibacterial property, composite material, orthopedic implant, osteogenic activity, zinc oxide nanoparticles

Introduction

Pathological conditions of bones, like osteoporosis or cancer, often cause structural changes such as trabecular bone reduction and cortical bone thinning, which increase the likelihood of fractures and can lead to other bone defects. In addition to diseases, trauma is the main cause of bone defects.[1–4] Although intrinsic self-healing abilities may naturally repair minor defects, therapeutic interventions are often necessary for defects larger than 6 mm.[5]

At present, autografting is considered the gold standard for treating critical bone defects.[6] Although autografts have good histocompatibility and are non-immunogenic, the process of implantation may further harm the patient and could cause chronic pain, bleeding, and other complications.[7,8] Another common treatment is the allografting of bone tissue transplanted from a donor. However, compared with autografting, it may lead to immune rejection and even serious infection.[9] Artificial bone replacement is a widely accepted clinical treatment that has been used to treat bone defects for decades. However, prostheses can pose challenges such as infection and aseptic loosening.[10,11] Fortunately, their biological performance can be improved by proper modifications, including the introduction of nanoparticles (NPs).

NPs are usually defined as objects with a dimension of 1–100 nm.[12] These nano-scaled materials may possess different physical and chemical properties compared with

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the bulk ones due to their high surface area to volume ratio.[13,14] NPs are widely applied in industry, cosmetics, and food products.[15,16] As a trace element, zinc participates in various metabolic processes. A substantial proportion of zinc (~30%) is stored in bone, and the pathological reduction of zinc levels impairs bone growth.[17–19] Zinc plays an important regulatory role in bone formation. In addition to being a component of inorganic minerals, it also activates proteins involved in bone homeostasis.[20] Zinc oxide NPs (ZnO-NPs) are a common type of zinc-containing nanoparticles that have gained increasing attention in biological research because of their low toxicity, biological compatibility, bioactivity, and chemical stability.[21] ZnO-NPs can accelerate bone growth and mineralization.[22] In addition, they possess selective toxicity toward bacteria and normal cells.[23,24] These biological properties imbue ZnO-NPs with considerable potential in orthopedic applications. In fact, studies have confirmed that implants of various materials, like metals and polymers decorated with ZnO-NPs via doping or coating, show better antibacterial and osteogenic abilities.[25,26]

To our knowledge, this is the first article focused on summarizing the recent progresses of ZnO-NPs in orthopedic applications. The first part is a brief review on the green preparation methods. Then, The mechanisms, influencing factors, and recent progress in antibacterial applications are discussed (Figure 1). The third section discusses the mechanisms and reducing methods of toxicity and proposes a hypothesis on the limitations of ZnO-NPs in anticancer applications. The final section highlights the osteogenic properties of ZnO-NPs. This article reviews research studies on the antibacterial, toxic, anticancer, and osteogenic properties of ZnO-NPs in orthopedics over the last 5 years. This review also sheds light on the ability of ZnO-NPs to exert both antibacterial and osteogenic effects, and their considerable potential in synergies with other co-decorated materials to enhance the biological activity of implants.

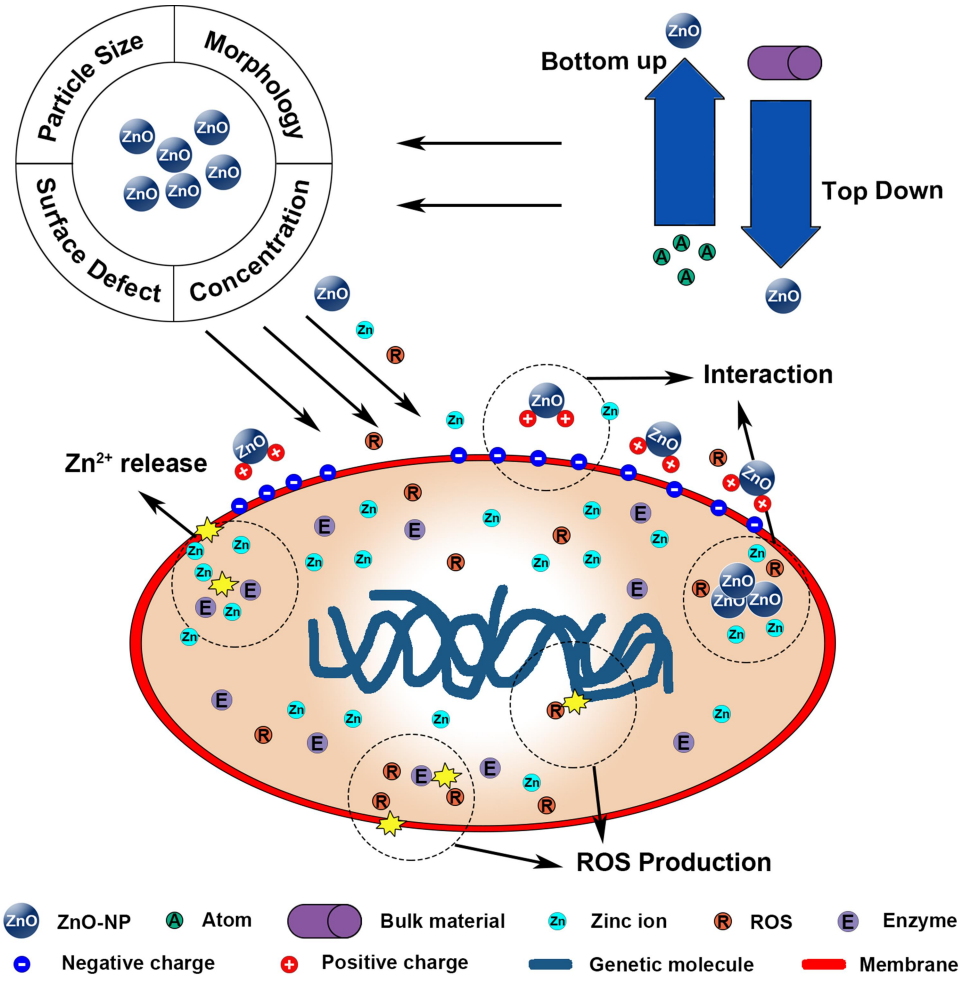

Figure 1 Schematic of ZnO-NP preparation strategies, antibacterial mechanisms, and influencing factors.
Abbreviations: ROS, reactive oxide species; ZnO-NPs, zinc oxide nanoparticles.

Green Fabrication Process of ZnO-NPs

There are Two strategies adopted for NPs preparation: top-down and bottom-up.[27,28] The top-down strategy refers to breaking bulk materials into nano-scale particles, but it produces imperfect surface structures.[29] In contrast, the bottom-up strategy refers to the self-assembly process in which atoms gathered into a nucleus and finally aggregate into NPs.[30] As the most widely used preparation strategy, Products obtained through bottom-up strategies usually have homogeneous chemical NPs compositions.[31] ZnO-NPs can be prepared by physical, chemical, and biological methods. Physical methods often follow the top-down strategy and include: grinding, milling, thermal evaporation, pulsed laser deposition, and others.[32,33] Although large quantities of ZnO-NPs can be produced in a short period of time, problems like high energy consumption, uneven size distribution, and low product nanometer rate still need to be solved.[30,34] Chemical methods like electrochemistry, chemical reduction, and photochemical reduction techniques usually follow a bottom-up strategy.[33] NPs produced via chemical methods are homogeneous in size.[35] However, toxic chemical reagents used during synthesis often remain on NP surface, which limits their use in medical applications.[23,36,37]

ZnO-NP biosynthesis is mostly categorized as wet chemical synthesis involving plants or microorganisms. Biosynthesis is environmentally friendly and safe, and the obtained ZnO-NPs are usually more biocompatible since they were functionalized by biochemicals.[32,38] Customized sizes and structures can be obtained by modulating extract parameters like the original source (different plants or microorganisms), pH, and concentration.[32,39,40] ZnO-NPs can be obtained through plant extracts using milder solvents such as water and ethanol, and the reducing and capping agents in extracts like phenols and flavones can stabilize the NPs through electrostatic, steric, hydration, and van der Waals forces.[41–44] The preparation process of plant extract-assisted biosynthesis is relatively simple and can be done in three steps (Figure 2). Generally, the first step is plant extract preparation. Next, zinc salts are added to plant extracts as precursors. During this stage, metal ions are reduced into NPs and then stabilized by reducing and capping agents. In the final step, ZnO-NPs are obtained after other synthesis processes like high temperature annealing.[39,45] Wang et al[45] successfully obtained spherical ZnO-NPs with a diameter of 20 nm, using the extract of *Artemisia annua*. Although NP sizes were relatively homogeneous, the product showed a specific aggregation phenomenon, which could lead to toxicity. Hu et al[46] obtained spherical ZnO-NPs with a diameter of 8 nm from the *Cucurbita pepo* leaf

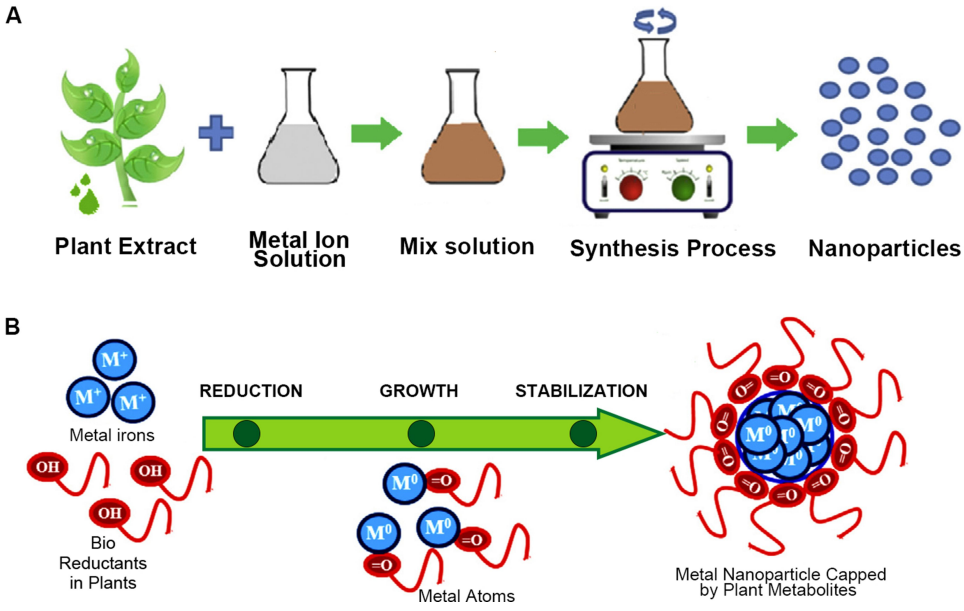

Figure 2 Schematic diagram of ZnO-NP preparation from plant extracts.
Notes: (**A**) green preparation process through plant extract utilization. (**B**) Mechanisms of green preparation through plant extracts utilization. Reprinted from Applied Materials Today, Volume 5, Shamaila S, Sajjad AKL, Ryma N-u-A, et al, Advancements in nanoparticle fabrication by hazard free eco-friendly green routes, Page: 150–199, Copyright (2016) with permission from Elsevier.
Abbreviation: ZnO-NPs, zinc oxide nanoparticles.

extract using a classic mixing-stirring-annealing process. Interestingly, they claimed no aggregation occurred. This discrepancy may be explained by the different extracts and capping agents with diverse stabilizing effects.

Microorganisms including bacteria, fungi, and algae can also participate in ZnO-NP biosynthesis,[47,48] as these microorganisms have a self-defense mechanism for heavy metal ion exposure.[49] The preparation process can be divided into microorganism cultivation, biochemical activities of microorganisms, intra- and extracellular transport of metal ions, intracellular nucleation, and finally NP formation.[35,49] Shamsuzzaman et al[50] used a *Candida albicans* suspension as a reducing and capping agent, and successfully prepared quasi-spherical ZnO-NPs with a diameter of ~15–20 nm. Sanaeimehr et al[51] also obtained ZnO-NPs with good anticancer effects using *Sargassum muticum* extract.

It is worth noting that biosynthetic methods are not perfect. The extract components may vary and some even remain unknown, which means that further purification of functional biochemicals is needed.[52] In conclusion, Future explorations of biosynthesis should be focused on details of the mechanisms and purification of functional biochemicals from plants or microorganisms. Successful large-scale biosynthesis of custom-structured ZnO-NPs will enhance their application in biological fields.

Antibacterial Properties of ZnO-NPs

Antibacterial Mechanism and Influencing Factors

Prosthesis-associated infections are a major cause of implantation failure and can occur due to improper surgical performance or post-operation contamination from surrounding tissues.[53] Revision of the prosthesis increases the financial burden of patients and also causes secondary injury.[54] There are five stages in the development of prosthesis-associated infection: 1) reversible attachment; 2) irreversible attachment; 3) microcolony formation; 4) proliferation and maturation; and 5) matrix detachment.[55] During the proliferation and maturation stage, colonies begin to form biofilms. These protective extracellular matrixes greatly enhance the resistance of bacteria against the immune system and bactericides.[56–59] It is therefore essential to inhibit bacterial attachment and growth at the early stage, which could be achieved using implants with antibacterial properties.

ZnO-NPs possess good antibacterial properties; however, the specific mechanism is not clear. Antibacterial properties come from three aspects: the interaction between NPs and bacteria; the release of zinc ions (Zn^{2+}); and the generation of reactive oxide species (ROS).[24,60] Bacteria possess negative charges on their surfaces because of negatively charged cell wall components like teichoic and lipoteichoic acid.[61] Conversely, ZnO-NPs are positively charged in water suspensions.[62]

Electrostatic attraction between bacteria and ZnO-NPs leads to the accumulation of NPs on the bacterial surface, which changes the zeta potential of the bacteria and destroys the potassium channels on cell membranes, eventually leading to lipid peroxidation and increased permeability.[63,64] This membrane dysfunction also causes enhancing internalization, which finally leads to excessive intracellular ZnO-NP accumulation and altered metabolism.[65] Zn^{2+} also plays an important role in antibacterial properties; they can combine with functional proteins, thereby changing cell membrane permeability. As the intracellular Zn^{2+} concentration increases, interaction with the thiol group of the enzyme is strengthened, thus affecting various bacterial enzymatic reactions, weakening glycolysis, and inducing cell death.[66,67] ROS are highly reactive oxygen-containing chemical species that are involved in maintaining cellular homeostasis under normal conditions. However, high ROS levels may induce oxidative stress and toxicity.[68,69] ZnO-NPs can produce ROS when subjected to light or ultraviolet irradiation because of the electron-hole pair activation, but active redox cycling on NP surfaces will still produce ROS in aqueous solutions even in darkness. Excessive ROS will directly damage bacterial lipids, proteins, and DNA, ultimately killing the bacteria.[70–72]

Many factors can affect ZnO-NP antibacterial performance. A smaller size corresponds to a larger specific surface area and smaller volume, thus enhancing Zn^{2+} dissolution, ROS generation, and abilities to adsorb and penetrate cell membranes.[65,73-76] Mahamuni et al[77] obtained ZnO-NPs with a diameter range of 15–100 nm through polyol synthesis. ZnO-NPs with the smallest diameter of 15 nm showed the best antibacterial effect on *Staphylococcus aureus*. Similarly, Wang et al[78] found that bacteria are more sensitive to small-sized particles, by comparing the antibacterial properties of ZnO-NPs with diameters of 15, 50, and 90 nm.

Surface defects on particles also play an important role in mediating antibacterial properties. Physical defects like

irregular protrusions and sharp edges can mechanically damage bacteria, and surface electronic states caused by chemical defects may enhance ROS production.[79] In addition, antibacterial properties can be manipulated by different morphologies such as nanocombs, nanorods, nanobelts, nanowires, nanosheets, nanoflowers, and snowflakes, and such structures can be achieved by changing the preparation method.[80–83] Compared with spherical particles, rod-shaped particles are more likely to penetrate the cell membrane, and thus have stronger bactericidal properties.[84] Talebian et al[85] compared the same volumes of ZnO-NPs in flower, rod, and ball structures and showed that flower-shaped particles had the highest specific surface area with more Zn^{2+} release and the strongest antibacterial properties. Various unique structures can confer many possibilities for orthopedic applications of ZnO-NPs. However, the mechanisms of ZnO-NP morphology on biological properties are not yet well understood. Therefore, more studies are needed particularly focused on specific mechanisms and optimal structures. Besides the intrinsic properties of ZnO-NPs, external environmental factors also affect antibacterial properties. Li et al[86] examined the toxicity of ZnO-NPs toward *S. aureus* in ultrapure water, NaCl solution, minimal Davis medium (MD), and Luria–Bertani (LB) medium. They found that the solubility of ZnO-NPs in various solutions were as follows: LB, 68 mg/L; MD, 38 mg/L; NaCl, 14 mg/L; and ultrapure water, 6.9 mg/L. However, the strongest antibacterial properties were observed in ultrapure water. The difference in solubility was due to the different components of each solution. Chloride ions can form complexes with zinc ions in NaCl solution, and phosphates in other solutions can form $Zn_3(PO_4)_2$ with zinc ions to accelerate dissolution. However, these reactions reduce the concentration of zinc ions, thereby reducing the antibacterial ability.

The species and metabolism of bacteria can also influence antibacterial behaviors. Gram-negative bacteria are more resistant to ZnO-NPs because lipopolysaccharides on the cell wall prevent ZnO-NP adhesion and internalization.[87] Antimicrobial tests show that the minimum inhibitory concentrations (MICs) of Gram-positive compared with Gram-negative bacteria are 50%–85% lower.[88] Elizabeth et al[89] observed an increased Zn^{2+} release in the presence of bacteria. This is because bacteria lower the surrounding pH during the metabolic process, thereby accelerating dissolution.[90]

Antibacterial Properties of ZnO-NP-Modified Implants

ZnO-NPs modifications imbue implants with good antibacterial properties. Elizabeth et al[89] covered titania nanotubes and titania nanoleaf with ZnO-NPs through precipitation. Compared with pure nano-patterned materials, the antibacterial properties of modified samples were significantly improved. Artifon et al[91] mixed polyetherimide (PEI) with different concentrations of ZnO-NPs and obtained a series of ZnO/PEI scaffolds by electrospinning. The antibacterial effect increased with higher ZnO-NP content. In addition to direct toxicity toward bacteria, ZnO-NP-modified implants can also regulate the immune system and enhance antibacterial properties. Wang et al[92] incorporated ZnO-NPs on the surface of a titanium substrate through magnetron sputtering to form a nano-coating. The modified samples exhibited good antibacterial properties during in vitro experiments. Interestingly, the authors reported even better antibacterial effects with in vivo experiments. Subsequently, they cultured macrophages with modified samples in vitro and found increased secretion of pro-inflammatory factors and enhanced phagocytosis ability against bacteria, indicating that immune system regulation played a considerable role in in vivo antibacterial ability.

In order to obtain materials with good antibacterial and osteogenic effects, ZnO-NPs are typically used as antibacterial agents in combination with osteogenic materials. The most common co-modified material is hydroxyapatite (HA).[93–95] Maimaiti et al[26] produced coatings of nano-HA (nHA) and ZnO-NPs and employed polypyrrole as a dual regulator using the pulse electrochemical deposition method. This coating exhibited good antibacterial properties while retaining mineralization functionality. Shitole et al[96] obtained a series of multi-concentration gradient poly-epsiloncaprolactone (PCL)/nHA/ZnO scaffold through electrospinning by mixing PCL and nHA with ZnO-NPs in various concentrations. Although the antibacterial activity increased with higher concentration, MG-63 cell adhesion decreased on scaffolds with 15 wt% and 30 wt% ZnO-NPs, which indicated a cytotoxic effect. The final recommended concentration of ZnO-NPs was 10 wt%, which reduced the adhesion rates of *S. aureus* and *E. coli* by 80.8% and 82.1%, respectively, compared to the control group.

To enhance the antibacterial effect of ZnO-NPs, antibiotics and other antibacterial NPs can be given in conjunction with ZnO-NPs to facilitate antibacterial synergy. Banerjee et al[97] prepared ZnO-NPs by precipitation,

subsequently modified them with pancreatin to obtain ZnO-NPs-PK and studied their ability to inhibit MRSA (methicillin-resistant *S. aureus*). They found that the MIC to inhibit 90% of MRSA isolates was 50% lower for ZnO-NPs-PK compared with bare ZnO-NPs. Furthermore, combining ZnONPs-PK with vancomycin at a concentration of 1/4 MIC almost completely inhibited MRSA growth. This phenomenon suggests that MRSA treated with low-concentration ZnO-NPs are more susceptive to certain antibiotics. However, because the antibacterial mechanism of ZnO-NPs is not fully understood, combining them with antibiotics and ZnO-NPs may not achieve a stable antibacterial effect. Alves et al[98] pre-treated MRSA with low-concentration ZnO-NPs, which made MRSA develop a slight tolerance to the main antibacterial mechanism of ZnO-NPs. Subsequently, the antibacterial ability of antibiotics with different mechanisms against ZnO-NP-tolerant MRSA were tested in sub-concentrations. The authors claimed that the inhibitory effects of vancomycin and rifampicin were not obvious, indicating that the main antibacterial mechanism of ZnO-NPs in this experiment were similar to those antibiotics, which inflict RNA damage and affect cell wall synthesis. This also means that without clarifying the antibacterial effect of ZnO-NPs, the combined application with various antibiotics will have low efficiency and uncertainty and may even trigger toxicity. Combining Ag nanoparticles (AgNPs) and ZnO-NPs while modifying implants has been drawing increasing attention. AgNPs are the most widely studied antibacterial agents, with broad-spectrum antibacterial effects and no known resistant strains.[99] The combined application with AgNPs enhances antibacterial ability and reduces the toxicity associated with a high concentration of a single material.[100–102] Zhang et al[100] mixed different proportions of AgNPs and ZnO-NPs (the total amount of these two NP concentration was10 wt%) with HA powder (90 wt%) and coated it on the surface of the titanium sheet using laser cladding. The antibacterial activity against *S. aureus* of coatings with only ZnO-NPs was relatively low, only 35.9%. Although the antibacterial rate of coatings with 10 wt% AgNPs reached 93%, it caused cytotoxicity in normal cells. Thus, the Ag7ZnO3HA coating (Ag/ZnO/HA = 7:3:90 wt%) was found to be the best biological coating. The antibacterial rate of *S. aureus* reached 85.8%, and the coating retained good biocompatibility and osteogenic effects (Figure 3).

ZnO-NPs have shown great potential for antimicrobial modification of implants, but challenges still exist. The underlying antibacterial mechanisms remain to be clarified, and the effects of combined application with antibiotics and the ability to regulate the immune system require further research.

Toxicity and Anticancer Properties

Toxicity Mechanisms and Reducing Methods

Low toxicity is a prerequisite for orthopedic implantation to minimize harm to the patient. The toxicity mechanisms of ZnO-NPs are quite similar to antibacterial mechanisms, that is, interactions between particles and cells, Zn^{2+} release, and ROS generation. Among these, it is generally believed that ROS generation is the main cause of eukaryotic cell death.[103–106] ZnO-NPs internalized by cells may enter the mitochondria and generate excessive intracellular ROS through depolarization of mitochondrial membrane and impairing the electron transport chain. These ROS may cause oxidative stress and directly destroy the membranes of the mitochondria and other organelles to induce apoptosis.[107]

The US Food and Drug Administration listed ZnO-NPs as "generally regarded as safe" (GRAS) substances (21CFR182.8991). However comparing with magnesium oxide nanoparticles (MgO-NPs), which are also classified as GRAS substances (21CFR184.1431) and widely studied in the orthopedic field, ZnO-NPs show more biotoxicity.[108,109] Ivask et al[110] tested the toxicity of MgO-NPs and ZnO-NPs against human alveolar epithelial A549 cells, human epithelial colorectal Caco-2 cells, and the murine fibroblast cell line, Balb/c 3T3. The MgO-NPs showed non-toxicity at concentrations less than 100 μg/mL, while ZnO-NPs showed toxicity to all three cell types at concentrations higher than 30.2 μg/mL.[110] Therefore, ZnO-NP applications need to be within an appropriate range of concentration.

Studies have shown that their toxicity is dose dependent. Sudhakaran et al[111] obtained spherical ZnO-NPs with diameters of ~43 nm with a wet chemical method. Subsequently, ZnO-NPs were administered to healthy adult Wistar rats through intravenous and intraperitoneal routes (10 mg/kg). Although the rats had no obvious toxicity on gross examination, histological damage was found in the liver. NPs also can affect embryonic development. Yan et al[112] injected ZnO-NPs into chicken embryos at a daily dose of 5 μg for 9–12 days and observed embryonic craniofacial defects. Suriyaprabha

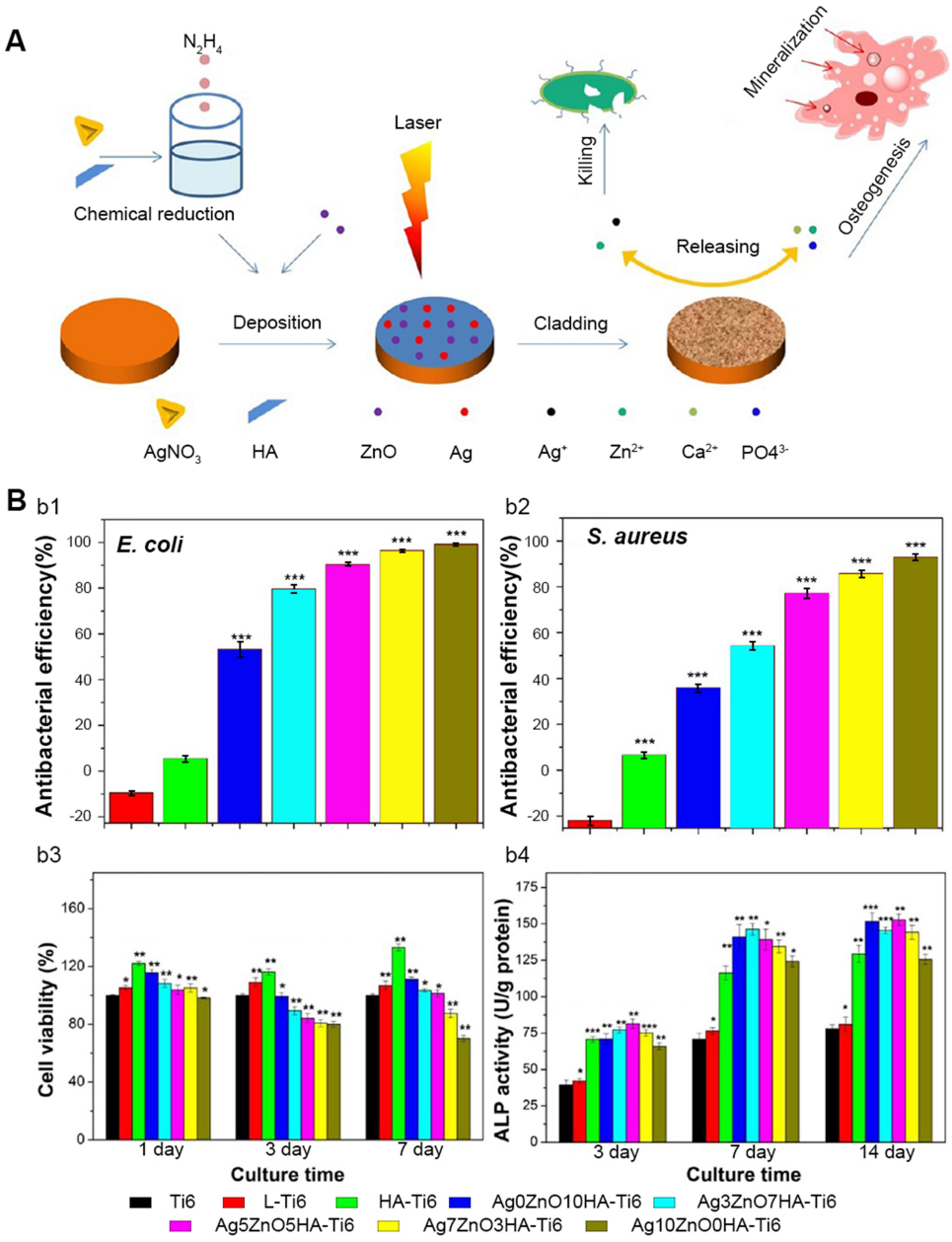

Figure 3 Combined application of ZnO-NPs and silver enhances antibacterial properties while reducing toxicity.

Notes: (**A**) Schematic of the Ag/ZnO/HA composite coating fabrication process and the antibacterial and osteogenesis processes. (**B**) Biological functions of Ag/ZnO/HA composite coating: (b1) antibacterial efficiency of the samples against *E. coli*; (b2) antibacterial efficiency of the samples against *S. aureus*; (b3) MTT assays of cell viability after culturing for 1, 3, and 7 days; (b4) osteogenic effects evaluated with ALP activity assays for 3, 7, and 14 days. The Data are presented as mean ± SD (n = 3): *P < 0.05, **P < 0.01, and ***P < 0.001 (*t*-test). Reprinted with permission from Zhang Y, Liu X, Li Zet al Nano Ag/ZnO-Incorporated Hydroxyapatite Composite Coatings: Highly Effective Infection Prevention and Excellent Osteointegration. ACS Appl Mater Interfaces. 2018;10(1):1266–1277. Copyright (2018) American Chemical Society.

Abbreviations: ALP, alkaline phosphatase; *E. coli, Escherichia coli; S. aureus, Staphylococcus aureus*; ZnO-NPs, zinc oxide nanoparticles.

et al[113] treated zebrafish embryos with different ZnO-NP concentrations and reported that only those >90 μg/mL induced abnormal embryo development. ZnO-NPs can also damage lung and reproductive functions.[114,115] However, since factors like size, temperature, solvent properties, pH, and others affect ZnO-NPs toxicity, it is difficult to determine a safe concentration range. Fortunately, they are unlikely to damage organs and embryos in orthopedic applications since ZnO-NPs are incorporated or coated on the implant. However, the implants can be toxic to the peri-prothesis tissues.

Even distribution is essential when decorating implants with ZnO-NPs. Aggregation during modification may lead to high local ZnO-NP concentrations, resulting in toxicity.[116] Abdulkareem et al[117] coated the surface of a titanium (Ti) substrate with ZnO-NPs and HA, to create

a HA/ZnO nanocomposite coated layer. The coating showed excellent antibacterial properties, but NP aggregation led to a reduction in bioactivity. Fortunately, this disadvantage can be avoided by using suitable preparation methods. Maimaiti et al[26] produced an HA coating doped with ZnO-NPs by pulse electrochemical deposition. The particles were distributed uniformly, and the coating showed no toxicity while exhibiting good antibacterial and osteogenesis properties.

Besides inhibiting aggregation, decorating with materials that may reduce Zn^{2+} release and ROS levels is also an option. Li et al[118] used atomic layer deposition to deposit the ZnO seed layer on a Ti substrate surface (Ti-ZnOs), and then the ZnO nano-rod arrays were grown with a hydrothermal method (Ti-ZnO). Subsequently, polydopamine (PDA) coating was applied (Ti-ZnO/PDA), and arginine-glycine-aspartic acid-cysteine (RGDC) peptides were immobilized to obtain the final sample (Ti-ZnO /PDA/RGDC). Ti-ZnO/PDA/RGDC exhibit good antibacterial and osteogenesis properties and less toxicity. This reduction in toxicity can be attributed to the reducibility of PDA, which consumes the ROS generated by ZnO-NPs. In addition, the covalent bonds between Zn^{2+} and PDA can reduce Zn^{2+} release, thereby further lowering toxicity and enhancing biocompatibility (Figure 4). Although the toxicity of ZnO-NPs is relatively low compared with other NPs, attention still needs to be paid to their concentration and distribution in orthopedic applications.[119] Suitable implant preparation methods are needed to ensure homogeneous particle distribution, and further investigation is required to determine the mechanism of toxicity and a safe concentration range.

Introducing less-toxic materials can also reduce the toxicity of ZnO-NPs. Vidic et al[120] obtained ZnO/MgO mixed NPs through the combustion of zinc-magnesium alloys, and then tested the antibacterial and cytotoxic properties of ZnO-NPs, MgO-NPs, and ZnO/MgO mixed NPs against *E. coli, Bacillus subtilis*, and HeLa cells. Compared with MgO-NPs, ZnO-NPs exhibited better antibacterial properties against both bacterial species and stronger toxicity against HeLa cells. The antibacterial properties of the mixed NPs were better than those of MgO-NPs, while their cytotoxicity was lower than that of pure ZnO-NPs.[120] This combination successfully reduced toxicity, while maintaining antibacterial properties. However, reports on the co-application of MgO-NPs and ZnO-NPs in orthopedics are few. In fact, MgO-NPs can both reduce the toxicity of ZnO-NPs and promote bone repair. The combination of ZnO-NPs and MgO-NPs may present favorable potential for orthopedic applications and requires further studies.

Limitations of Anticancer Effects in Orthopedic Applications

The anti-cancer properties of ZnO-NPs are a research hot spot aimed at improved bio-applications. The mechanism is the same as for toxicity. Many studies have reported ZnO-NP anti-cancer effects on multiple cancer cell lines, including bladder, breast, lung, and others.[121–124] However, very few reports have focused on ZnO-NP treatment for bone tumors. After tumor resection, bone defects need to be filled with implants that can assist in the elimination of remaining cancer cells and enhance bone healing.[125]

Highly proliferative cells are more sensitive to ZnO-NPs and the strong toxicity of ZnO-NPs against rapidly proliferating cells may delay the healing process.[126] Taccola et al[127] examined the response of bone marrow mesenchymal stem cells (BMSCs) and differentiated osteoblasts to ZnO-NPs. At a concentration of 20 μg/mL, BMSCs with strong proliferating potential are significantly inhibited, while ZnO-NPs have little effect on osteoblasts with lower proliferation abilities. Kim et al[128] suggested that ZnO-NPs participate in mitochondrial dysfunction and lead to BMSC apoptosis. These findings suggest that ZnO-NPs with anti-cancer effects may damage normal cell proliferation and may not suitable for treating bone cancer.

Osteogenic and Chondrogenic Abilities of ZnO-NPs

Osteogenic Mechanism of ZnO-NPs

The osteogenic properties of ZnO-NPs are due to Zn^{2+} release. As an essential trace element, Zn^{2+} participates in various enzyme catalytic activation reactions.[22] Furthermore, Zn^{2+} can promote bone growth, mineralization, and formation.[129,130] ZnO-NPs produce Zn-OH groups when in contact with water molecules that act as apatite nuclei and accelerate mineralization.[131] In addition, Zn^{2+} can activate the mitogen-activated protein kinase pathway, which promotes expression of the osteocalcin gene region. Moreover, cells exposed to excessive Zn^{2+} may up-regulate the expression of Zn^{2+} transporters such as ZIP1, and its overexpression can enhance Runx2 expression, thus enhancing bone formation.[132,133]

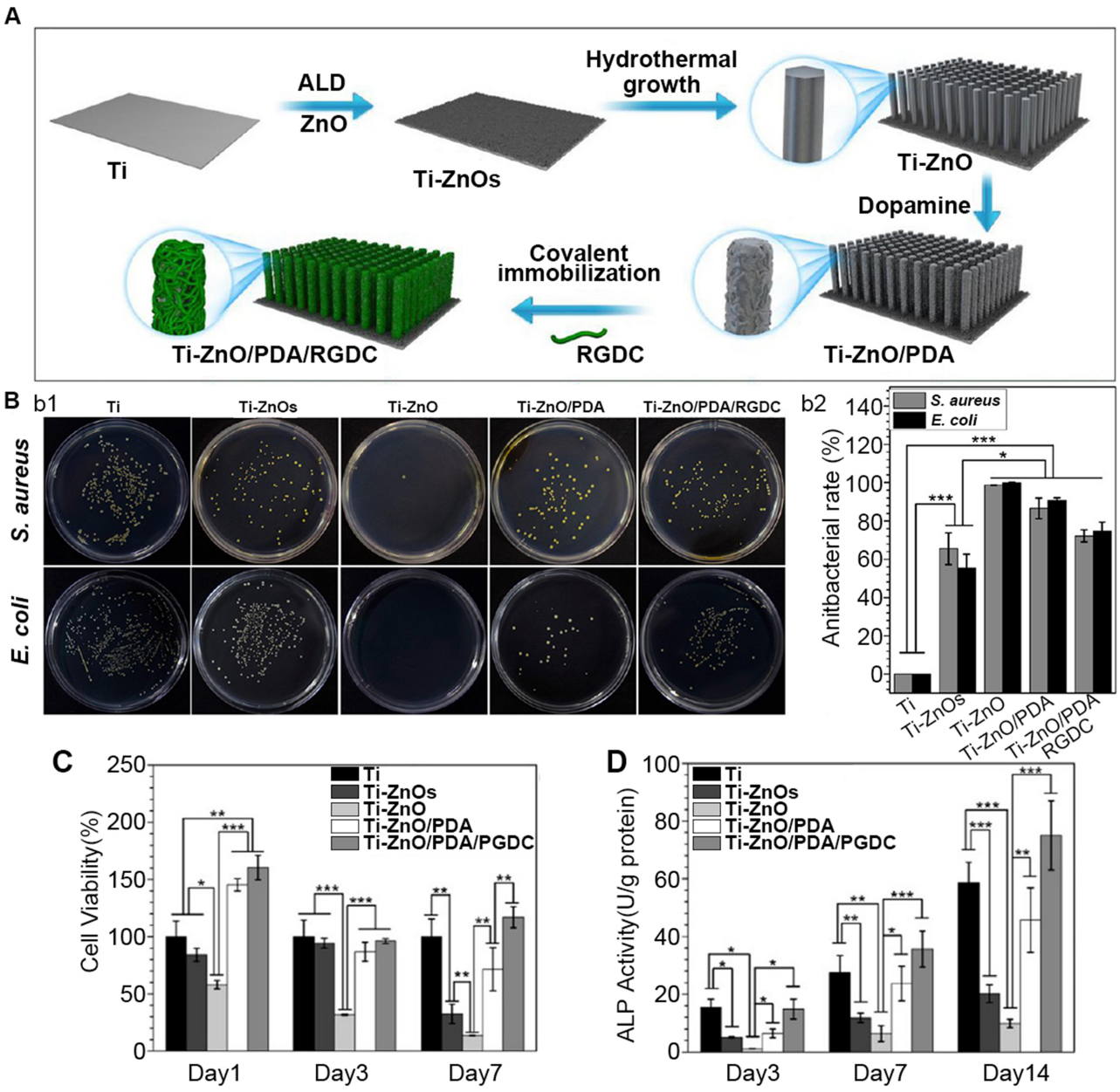

Figure 4 DOPA coatings reduce ZnO-NPs toxicity.

Notes: (**A**) Schematic of the ZnO/PDA/RGDC NR array fabrication process. (**B**) Antibacterial properties of different samples against *S. aureus* and *E. coli*: (b1) re-cultivated bacterial colonies on agar culture plates after dissociation from various surfaces, *S. aureus* and *E. coli* seeded at a concentration of 10^7 CFU/mL, (b2) antibacterial rate of different samples against *S. aureus* and *E. coli*. (**C**) MTT cell viability results after culturing in the presence of different samples after 1, 3, and 7 days. (**D**) ALP activities of osteoblasts cultured in the presence of different samples after 1, 3, and 7 days. The data are presented as mean ± standard deviation: *P < 0.05, **P < 0.05, and ***P < 0.001 (*t*-test). Reprinted with permission from Li J, Tan L, Liu XM et al Balancing bacteria-osteoblast competition through selective physical puncture and biofunctionalization of ZnO/polydopamine/arginine-glycine-aspartic acid-cysteine nanorods. ACS nano. 2017;11(11):11,250–11,263. Copyright (2017) American chemical society.

Abbreviations: ALP, alkaline phosphatase; DOPA, dopamine; NR, nanorod.

Yamaguchi et al[134] demonstrated that 10–250 μmol/L Zn^{2+} inhibited osteoclast differentiation. They pointed out that Zn^{2+} suppresses nuclear factor-κB signaling by reducing levels of tumor necrosis factor-α in vivo. This inhibitory effect decreases osteoclast generation and enhances the proliferation of osteoblasts, with a net effect of greatly enhanced osteogenesis. Park et al[135] studied the upstream signaling pathway of RUNX2 and found that Zn^{2+} can activate protein kinase A signaling, by up-regulating cyclic adenosine monophosphate (cAMP), and thus enhancing nuclear translocation of phosphorylated cAMP response element-binding protein and upregulated RUNX2 expression.

ZnO-NPs can improve the activity and differentiation of mesenchymal stem cells (MSCs) and osteoblasts on the

implant surface. These properties are ascribed to the larger modified implant surface area, which may possess more active sites that tend to absorb more proteins.[136] Furthermore, studies have shown that physical properties of the scaffold are very important and can affect bone regeneration.[137–140] These physical properties can also be optimized by ZnO-NPs modifications. Sahmani et al[93] mixed different proportions of ZnO-NPs with HA, then sintered the mixed materials, and coated the resulting scaffolds with gelatin-ibuprofen. They found that the higher the ZnO-NP weight fraction, the higher the rate of degradation. Li et al[141] proposed that doping ZnO-NPs into beta-phase poly (vinylidene fluoride) can produce a scaffold with a bone-like Young's modulus, which can promote bone repair in vivo. However, several studies reported that doping ZnO-NPs would increase the water contact angle and reduce the material's hydrophilicity, impairing osteoblast adhesion.[96,142]

Osteogenic Properties of ZnO-NP-Modified Implants

Wang et al[45] found that ZnO-NP osteogenic ability is concentration dependent (Figure 5). They reported that the expressions of alkaline phosphatase and collagen significantly increased in MG63 cells cultured with 10 μg/mL ZnO-NPs compared to 5 μg/mL. They proposed that more ZnO-NPs produce a better osteogenic effect within the non-toxic concentration range. Shen et al[143] applied a ZnO coating onto a micrometer-scale patterned Ti substrate using a hydrothermal method. The ZnO-NP-containing samples promoted osteoblast proliferation and differentiation. Furthermore, quantitative tartrate-resistant acid phosphatase (TRAP) activity analysis of RAW264.7 cells was performed, and the ZnO-NPs modified samples had the lowest TRAP activity, indicating the inhibition of osteoclast differentiation.[143] The results of subsequent animal experiments corroborated the in vitro results that ZnO-NP modification could efficiently promote new bone tissue formation after implantation for 4 and 12 weeks.

The combination of HA and ZnO-NPs is very common in orthopedic experiments and can promote osteogenesis via multiple mechanisms. Shitole et al[96] incorporated nano-HA and ZnO-NPs into the PCL scaffold, which greatly improved its osteogenic effect. Maimaiti et al[26] proposed that the HA/Zn coating achieved better osteogenic performance for bone formation compared to the pure HA coating. Gnaneshwar et al[144] firstly doped ZnO-NPs into HA particles to obtain ZnO/HA particles, and then incorporated them to poly (L-lactic acid)-co-PCL and silk fibroin nanofibrous scaffolds. They found that scaffolds doped with ZnO/HA particles possessed stronger osteogenesis effects than scaffolds doped with the same quantities of ZnO-NPs and nHA. This result could guide the development of new co-modification strategies with various NPs in the future.

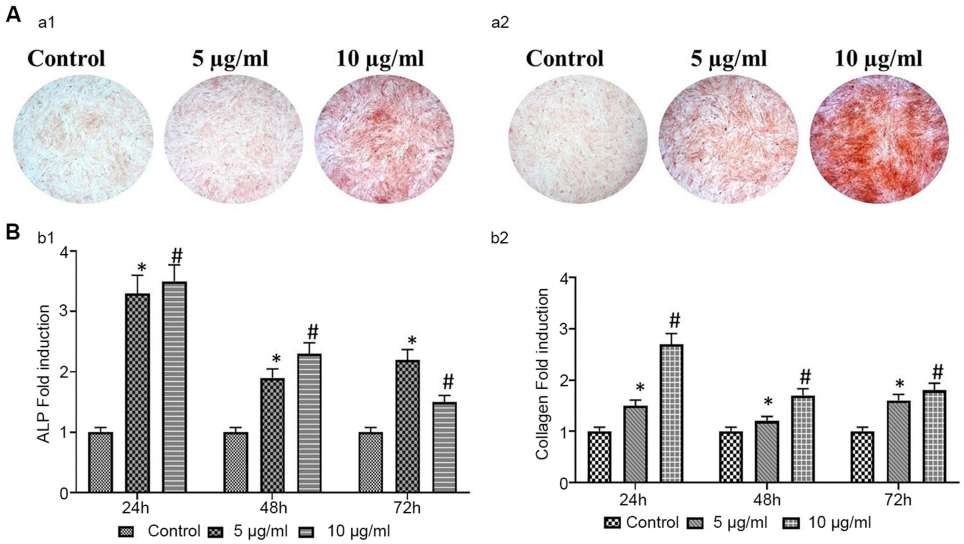

Figure 5 Effect of ZnO-NP concentration on osteogenesis activity.

Notes: (**A**) Alizarin red stain for calcium mineralization in osteoblasts treated with ZnO-NPs (0, 5, and 10 μg/mL) for: (a1) 5 and (a2) 7 days. (**B**) mRNA expression in osteoblasts treated with ZnO-NPs (0, 5, and 10 μg/mL) for 24, 48, and 72 h; (b1) ALP; (b2) collagen. Data are presented as mean ± SEM values from three experiments: *P < 0.05, #P < 0.01 compared with 0 μg/mL-treated osteoblasts. Reprinted from Journal of Photochemistry and Photobiology B: Biology, Volume 202, Wang D, Cui L, Chang X, Guan D. Biosynthesis and characterization of zinc oxide nanoparticles from Artemisia annua and investigate their effect on proliferation, osteogenic differentiation and mineralization in human osteoblast-like MG-63 Cells, Page: 111,652. Copyright (2020) with permission from Elsevier. **Abbreviations:** ALP, alkaline phosphatase; ZnO-NPs, zinc oxide nanoparticles.

AgNPs, As a strong antibiotic, also have the ability to promote bone formation.[99] Interestingly, AgNPs and ZnNPs may act synergistically to promote osteogenesis.[100,101] In the study of Deng et al,[101] AgNPs and ZnNPs were uniformly doped on the surface of sulfonated polyetheretherketone (PEEK), using a layer-by-layer technique. Compared to PEEK doped with pure silver or ZnO-NPs, the hydrophilicity of ZnO/Ag/PEEK was reduced, cell adhesion was impaired in the early stage, and the number of MG-63 cells on ZnO/Ag/PEEK were the highest on day 5. Reverse transcription polymerase chain reaction of cells co-cultured with the sample on day 14, showed significantly higher expression of osteogenic genes with ZnO/Ag/PEEK compared with Ag/PEEK and ZnO/PEEK (Figure 6). Although the synergistic mechanism of AgNPs and ZnO-NPs is not clear, their combined application has considerable potential for osteogenesis.

Interestingly, ZnO-NPs can promote cartilage formation at lower concentrations. Khader et al[25] prepared ZnO/PCL scaffolds with a concentration gradient of 1–10 wt% ZnO-NPs with an electrospinning technique. They found that under concentrations of 1–2.5wt%, the expression of cartilage markers such as SOX-9 and collagen type II were significantly increased in MSCs. Moreover, the scaffold with 10 wt% ZnO-NPs promoted osteogenic properties rather than chondrogenic differentiation, which is consistent with the findings of a previous study.[145]

Mirza et al[145] studied the chondrogenic effects of 1% ZnO-NPs decorated with poly (octanediol citrate) polymer, and found that they improved chondrocyte viability, upregulated the expression of cartilage matrix-specific genes (*COL2A1* and *ACAN*), and inhibited the expression of the matrix degradation gene (*MMP-13*).This cartilage-promoting ability could be attributed to Zn^{2+} release. Some

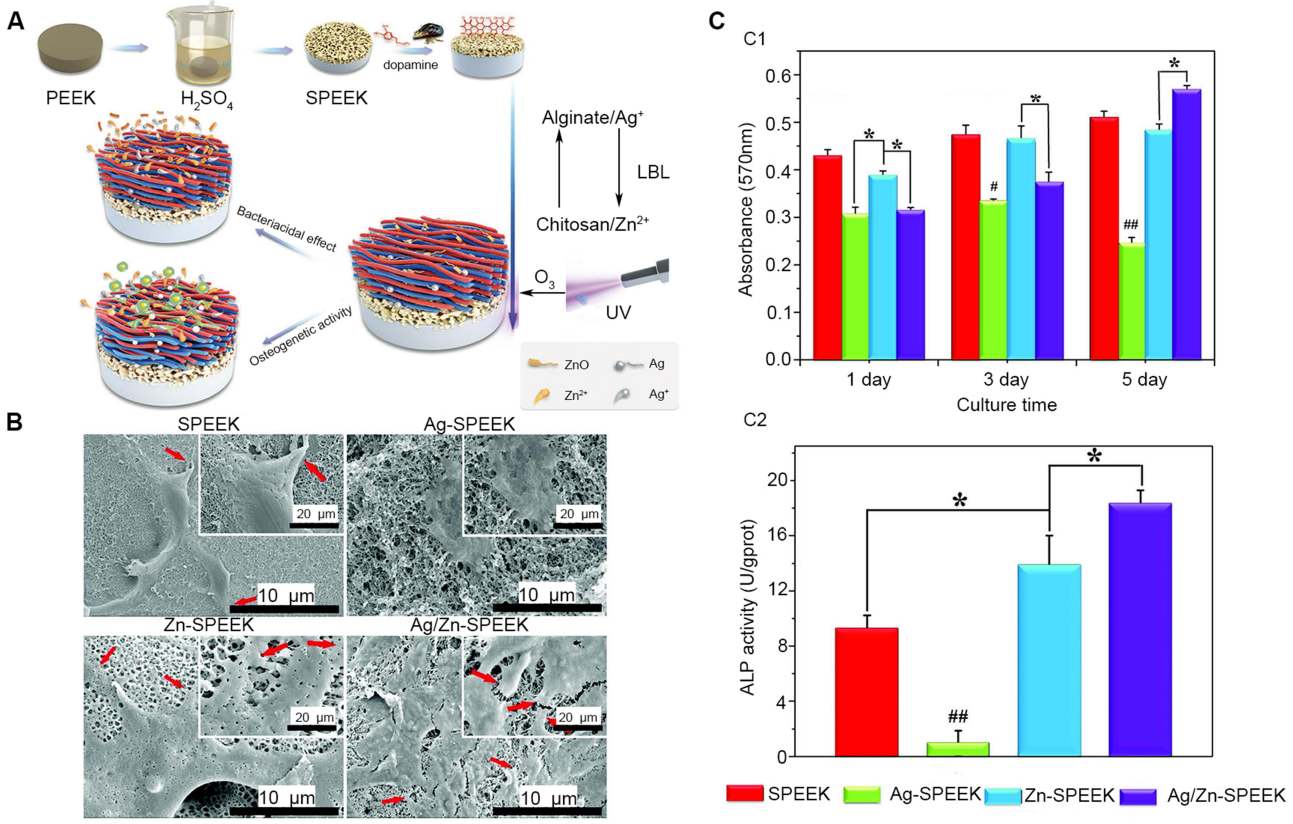

Figure 6 Combined application of ZnO-NPs and silver exerts a synergistic osteogenesis effect.

Notes: (**A**) Schematic of Ag/ZnO dual-decorated micro-/nanoporous SPEEK preparation process, and its bactericidal effect and osteogenic activity. (**B**) Scanning electron microscopy images of SPEEK, Ag-SPEEK, Zn-SPEEK, and Ag/Zn-SPEEK. (**C**) Biological activities of different samples: (c1) proliferation of MG-63 on various sample surfaces for 1, 3, and 5 days; (c2) ALP activity of MG-63 cells on various samples for 7 days. Data are presented as mean ± standard deviation: *P < 0.05, #P < 0.05 compared with other groups, and ###P < 0.01 compared with other groups. Reprinted from Deng Y, Yang L, Huang Xet al Dual Ag/ZnO-Decorated Micro-/Nanoporous Sulfonated Polyetheretherketone with Superior Antibacterial Capability and Biocompatibility via Layer-by-Layer Self-Assembly Strategy. Macromol Biosci. 2018;18(7): e1800028, with permission from Copyright (2018) John Wiley and Sons.

Abbreviations: ALP, alkaline phosphatase; SPEEK, sulfonated polyetheretherketone; ZnO-NPs, zinc oxide nanoparticles.

studies claim that Zn^{2+} can enhance chondrocyte proliferation, while also preventing cartilage loss by activating the P13/Akt pathway.[146,147] However, the mechanism by which ZnO-NPs promote cartilage growth is not clear, and further in vitro and in vivo experiments are needed.

Perspectives and Conclusion

Enhancing osseointegration and preventing post-implant prosthesis infections are major clinical challenges. ZnO-NP utilization may be one solution for these issues. This article summarized the preparation methods of ZnO-NPs, as well as the mechanisms, influencing factors, and the latest research progress into understanding their toxicity and osteogenic and antibacterial effects. However, several problems still remain. First, the specific mechanisms of antibacterial and toxicity are not yet well understood, so an effective non-toxic concentration range integrating multiple influencing factors cannot currently be determined. Second, ZnO-NPs exert synergistic antibacterial and osteogenic effects in combinations with other materials, and the specific mechanisms and optimal composition details need to be determined. Third, ZnO-NPs may promote cartilage formation, although this requires further exploration. Future research directions should focus on expanding non-toxic preparations (such as large-scale green preparations), reducing toxicity, clarifying specific mechanisms of antibacterial and osteogenic abilities, and determine how to enhance beneficial effects.

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Disclosure

The authors declare that they have no competing interests.

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