Magnesium and Gallium co-loaded microspheres accelerate bone repair through osteogenesis and antibiosis

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Abstract

Bone defects have serious economic and clinical impacts; however, despite improvements in their management, their outcome s are limited. A variety of biomaterials have been used to treat complex bone defects. However, the final bone repair outcome might be adversely affected by poor osteogenic capacity and risk of infection. Consequently, therapeutic methods are required that reduce bacterial contamination and increase the osteogenic properties of biomaterials. Herein, poly (lactic acid-co-glycol ic acid) (PLGA) microspheres co-loaded with magnesium ions (Mg^{2+}) and gallium ions (Ga^{3+}) (Mg-Ga@PLGA) were prepare d, which could fill irregular bone defects and display good biosafety. *In vitro*, Mg-Ga@PLGA not only showed a synergistic e ffect on promoting osteogenic differentiation but also inhibited osteoclastic differentiation. We found that Mg-Ga@PLGA de monstrated a superior antibacterial effect. *In vivo*, Mg-Ga@PLGA exhibited a good osteogenic ability *in situ*. In conclusion, Mg-Ga@PLGA has good potential to treat bone defects at risk of infection.

Keywords: microsphere, osteogenesis, antibacterial, magnesium, gallium

Abbreviations:

PLGA: Poly (lactic acid-co-glycolic acid); Mg: Magnesium; Ga: Gallium; GN: Gallium nitrate (GN); RAS: Rat sarcoma viral oncogene; RAF: V-raf murine sarcoma viral oncogene; MEK: Mitogen-activated protein kinase; ERK: Extracellular signal-regulated kinase;Bmp2: Bone morphogenetic protein 2; Runx2: Runt-related transcription factor 2; PVA: Poly (vinyl alcohol); MC3T3-E1s: Mouse embryo osteoblast precursor cells; BMMs: Bone marrow-derived macrophages; DCM: Dichloromethane; ICP-OES: Inductively coupled plasma optical emission spectrometer; SEM: Scanning electron microscope; XPS: X-ray photoelectron spectroscopy; CCK-8: Cell counting kit-8; PBS: Phosphate-buffered saline; AM/PI stain: Calcein-AM/propidium iodide double staining; CLSM: Confocal laser scanning microscopy; BHI: Brain heart infusion; TRAP: Tartrate-resistant acid phosphatase; CFU: Colony forming units; ELISA: Enzyme linked immunosorbent assay; IF: Immunofluorescence; ALP: Alkaline phosphatase; OCN: Osteocalcin; OPN: Osteopontin; COL I: Collagen type I; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; ARS: Alizarin red s; H&E Stain: Hematoxylin-eosin staining; IHC: Immunohistochemistry. TNF-α: Tumor necrosis factor-α, IL-6: Interleukin-6.

Introduction

Bone defects caused by infection, tumor resection, and mechanical trauma [1] are major contributors to reduced quality of life through functional failure. Although bone grafting [2-3] is now considered one of the main clinical approaches to treat bone defects, this solution has technical problems [4], including restricted sources, potential risk of infection, and immune rejection [5]. Bone grafting also has socioeconomic challenges, such as high cost, multiple invasive surgeries, and a general lack of donors [6].

For proper bone regeneration, a designed bionic bone material based on cells should recruit stem or progenitor cells to the site of tissue injury through a biological scaffold and provide an osteo-friendly microenvironment for the recruited cells [3,7] to colonize *in situ*, promoting their differentiation into a specific cell lineage [8]. It is also important for such a scaffold to be biocompatible and show no significant host tissue inflammation [9]. Recently, delivery systems comprising bioresorbable microspheres have received increased attention for multiple drug delivery [10-12]. Microspheres containing bioactive components create a microenvironment that is suitable for cell differentiation [13]. In addition, microspheres can be used not only as an injectable material for tissue therapy, but also as a freeze-dried powder for filling irregularly shaped bone defects [14-15].

In this study, we designed an efficient protocol to generate poly (lactic acid-co-glycolic acid) (PLGA) microspheres encapsulating magnesium ion and gallium ion (Mg-Ga@PLGA).. The incorporation of Mg^{2+} into bone mineral (i.e., hydroxyapatite, HA) lattices markedly affected the natural bone mineral density and biomechanics [16-17]. Molecular level studies found that Mg^{2+} might promote osteogenic gene expression through the RAS/RAF/MEK/ERK signaling pathway (RAS: Rat sarcoma viral oncogene; RAF: V-raf murine sarcoma viral oncogene; MEK: Mitogen-activated protein kinase; ERK: Extracellular signal-regulated kinase) and Bmp2/Runx2 (Bmp2: Bone morphogenetic protein 2; Runx2: Runt-related transcription factor 2) signaling pathway [18-20]. However, the use of Mg^{2+} alone as a bioactive ingredient only leads to osteogenic differentiation-promoting effects, which are relatively homogeneous in function and limited in solving clinical problems, making it important to find effective bioactive combinations [21-22]. Gallium (Ga) is located in the thirteenth main group of the fourth period of the periodic table and has two valences, 2 and 3. It has two stable isotopes and 20 radioisotopes in nature. To date, gallium has been widely used in communications, energy, computers, and space exploration [23]. Ga-containing biomaterials offer more options for the repair of osteoporotic bone defects due to its inhibition of osteoclastic effect, and might improve the repair outcome while avoiding liver and kidney damage [24-25]. In addition to the negative effect on the osteoclastic cells, Gallium nitrate (GN) also exerts antibacterial activities against methicillin-resistant *Staphylococcus aureus* [26], *Escherichia coli*, and *Pseudomonas aeruginosa* [27-29]. Ga, as a bioactive metal with antimicrobial properties, could be used as a direct wound-filling antimicrobial agent to prevent surgical failure caused by bacterial infection during the placement of biological scaffolds in the body [30-31]. Based on Mg^{2+} induced osteogenesis and the antibacterial effects of Ga^{3+}, we hypothesized that Ga^{3+} and Mg^{2+} co-doped into PLGA microspheres could enhance the repair of bone defects.

Materials and Methods

Materials

Aladdin (Shanghai, China) provided dichloromethane (DCM, HPLC grade, ≥ 99.9%, MW: 84.93 Da), Poly (vinyl alcohol) (PVA, viscosity: 3.5-4.5 mPaS, Alcoholysis degree: 97.5–99%, MW: 44.05 Da), $Ga(NO_4)_3$, $MgSO_4$, and PLGA (50:50, Mw: 38000-54000).

Synthesis of microspheres

A modified water-in-oil-in-water (W1/O/W2) emulsion method was used to prepare the Mg@PLGA microspheres. Briefly, PLGA (100 mg) was added cautiously and dropwise into 4 mL of DCM, as 'O'. 20 (*wt%*) $MgSO_4$ and 2% $Ga(NO_4)_3$ powders were dispersed into 1% (*wt%*) PVA to form the 'W2'. Then, 1mL of ultrapure water, which is the internal aqueous

phase (W1), was added drop by drop to the oil phase in a blender, and stirred to obtain the W1/O mixture. The W1/O was quickly poured into 100 mL of the W2, and stirred to obtain the compound emulsion. Stirring was continued for 24 h until the DCM had completely evaporated. The suspension was centrifuged at 3000 rpm, the supernatant was discarded, the pellet was cleaned, and centrifuged again (3000 rpm) to collect the spheres. The microspheres were freeze dried.

Morphological characterization.

Scanning electron microscopy (HITACHI SU8100, Tokyo, Japan) was used to characterize the morphology of the microspheres, while the mean radius was obtained by counting spheres with multiple morphologies using the Image J software analysis (NIH, Bethesda, MD, USA).

Elemental analysis

Elemental mapping was carried out scanning electron microscopy (HITACHI SU8100). Elemental mapping only yields qualitative results; therefore, the metallic elements encased within the spheres were further quantified using X-ray photoelectron spectroscopy (XPS) (ESCALAB 250XI, Thermo Fisher Scientific, Waltham, MA, USA).

Release pattern of Mg^{2+} and Ga^{3+}

To assess the release kinetics of Mg^{2+} and Ga^{3+}, we immersed 30 mg of Mg-Ga@PLGA in 10 mL of phosphate-buffered saline (PBS), incubated at 37 °C on a magnetic mixer and mixed thoroughly (60 rpm). Then, 2 mL of the supernatant was removed at 7, 10, 20, 30, and 60 d, respectively, and the Mg and Ga concentrations were measured using Inductively Coupled Plasma Optical Emission spectroscopy (ICP-OES) (Agilent 7800, Santa Clara, CA, USA) while supplementing with equal amounts of PBS.

Assay of cytotoxicity and cell adhesion

To detect the state of cell death during co-culture, live and dead cells were labeled with Calcein-AM/propidium iodide (PI) double staining (YEASEN, Shanghai, China) after 3 days of co-culture. Calcein-AM/PI double staining uses two colored molecular probes, Calcein-AM, which labels live cells in green, and PI, which labels dead cells in red. The results are observed using fluorescence microscopy (Leica, Wetzlar, Germany) and quantified using ImageJ.

To observe the cytoskeleton, Mouse embryo osteoblast precursor cells (MC3T3-E1s) and Bone marrow-derived macrophages (BMMs) were fixed in 4% paraformaldehyde at the 3rd day and BMMs were fixed at the 3rd day after inoculation with 5mg microspheres in 1mL medium, washed with PBS, stained with 50 μg/mL Cy3-ghost cyclopeptide for 40 min, and observed using confocal laser scanning microscopy (CLSM; Carl Zeiss, Oberkochen, Germany) to determine the number of cells at the early stage of adhesion, their cell morphology, pseudopod extension, and cytoskeletal aggregation.

The vertical migration of cells was assessed using transwell assay. To assess the effect of the slow release of Mg^{2+} and Ga^{3+} from the microspheres on the migration of MC3T3-E1s, we placed the 5 mg microspheresin 1 mL medium in the lower transwell chamber and placed the cells in the upper chamber. After 48 h, the cells above the porous membrane were mechanically removed and the cells on the lower surface of the membrane were fixed using 4% (*v/v*) paraformaldehyde and stained using hematoxylin (Solarbio) solution for 10 min. Images were obtained under a microscope and the cells were quantified using ImageJ.

Evaluation of cell apoptosis and proliferation

MC3T3-E1s or BMMs were inoculated and co-cultured with 5 mg microspheres in 1 mL medium. On day 3, the cells were centrifuged, and the supernatant was removed. The cells were then stained using BD Annexin V (BD Pharmingen, San Jose, CA, USA) by gently resuspending the cells in the Annexin V solution and incubating them for 20 min at 37 °C in the dark. The cells were then analyzed using flow cytometry with a BD LSRFortessa instrument (BD Biosciences, San Jose, CA, USA). FlowJo software (TreeStar, Ashland OR, USA) was used to analyze the data.

A Cell Counting Kit-8 (CCK-8; Biosharp) was used to determine cell proliferation. 5×10^3 MC3T3-E1s or 1×10^5 BMMs were inoculated into 96-well plates and 5 mg microspheres in 1 mL medium were added and cocultured with the cells after 24 hours of cell apposition. CCK-8 assay solution was added at various times according to the supplier's instructions and the optical density (OD) values were recorded at 450 nm using an enzyme marker (Spectra Max, Molecular Devices, Berlin, Germany).

Assessment of osteogenesis effect and osteoclast differentiation *in vitro*

Alkaline phosphatase (ALP) activity was detected using a 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) Chromogen Kit (Solarbio). According to the kit's instructions, the provided solutions were mixed and added to each plate to stain the cells for 8 h in the dark. Thereafter, the cells were observed using a microscope and quantified using ImageJ.

At the 21st day, the cells were washed using PBS three times, and fixed using 95% (*v/v*) ethanol for Alizarin Red S (ARS) Stain Solution (Beyotime, Jiangsu, China). The cells were photographed under an inverted microscope and quantified using ImageJ.

Osteogenic markers were detected using immunofluorescence after the cells were cultured for 5 days. ß-tubulin was chosen as a morphological marker, and Bmp2 and Runx2, were detected as osteogenic markers. Primary antibodies recognizing these proteins (1: 200, Abcam, Cambridge, UK) were incubated with the cells overnight at 4 °C and secondary antibodies comprising goat anti-rabbit IgG conjugated with corallite 594 or corallite 488 (1: 200, ProteinTech, Wuhan, China). Nuclei was labeled using DAPI. CLSM was used to obtain fluorescence images.

The levels of osteogenic proteins were determined using western blot. The total proteins were extracted and quantified. Following the standard protocol, we incubated with primary antibodies recognizing Bmp2, ALP, and Runx2 (1:1000, Affinity Biosciences, Jiangsu, China) and the appropriate secondary antibodies (1:1000, ProteinTech) for 1 h at room temperature. Protein levels were reported relative to that of the internal control GAPDH (1:10000, Abcam). Visualization of the immunoreactive proteins was achieved using BeyoECL Plus (Beyotime) and quantified using ImageJ.

BMMs was seeded at 1×10^5 per well in 24-well plates. The specific differentiation protocol was described earlier. On the 5th day of differentiation, cells were fixed in paraformaldehyde for 10 min, rinsed three times using PBS and then stained using a tartrate-resistant acid phosphatase (TRAP) staining kit (Sigma, St. Louis, MO, USA) according to the manufacturers' protocol.

Analysis of anti-bacterial effect *in vitro*

Bacteria are linked with common infections; therefore, *S. aureus* (gram-positive) and *E. coli* (gram-negative) were used to observe antibacterial activity. In broth medium, the concentrations of the two types of bacteria were adjusted to a density of 1×10^6 colony forming units (CFU)/mL. 50 mg of sterilized microspheres were immersed in 5 mL sterile deionized water to prepare the extracts. After 6 hours of shaking at 37 °C, we removed 100 µL of the bacterial solution, spread it onto brain heart infusion (BHI) agar, incubated for 24 h, and then counted the CFU.

For the inhibitory zone experiment, 100 µL of a solution containing bacteria at 1×10^9 CFU/mL was spread BHI agar plates and incubated for 30 minutes at 37 °C with 10 mg of sterilized microspheres were immersed in 1 mL sterile deionized water. After colonization, holes were made in the agar plates to which were added 30 µL of sample extracts; a hole containing PLGA served as the experimental control. After 24 h of culture at 37 °C, the inhibitory zone was measured.

The Live/Dead staining revealed the microspheres' antibacterial action. 100mg four types of microspheres were placed in 10 mL of a solution containing 1×10^6 CFU/mL of bacteria and cultivated at 37 °C. The bacterial solution was diluted 100 times using medium. In addition, after 24 h of growth, the bacteria were stained using Live/dead staining kit, and fluorescence images were acquired using CLSM.

Assessment of *in vivo* bone regeneration

The animal experiments followed the protocol for the care and use of the experimental animals approved by the Institutional Animal Care and Use Committee of Yi Shengyuan Gene Technology (Tianjin) Company (YSY-DWLL-2022108). Male Wistar rats (6-8 weeks old) were selected to establish a critical cranial defect model (8 mm) and were divided into five groups: A blank (untreated) control group, and groups whose defect was treated using 20 mg microspheres respectively. The animal models were established according to the standard protocol. Under aseptic conditions, a defect of 8 mm in diameter was made in the median line of the skull using a ring drill and microspheres were implanted into the defects, covered with a barrier membrane, and the skin was sutured and sterilized. The skin sutured and disinfected again. We ensured that the rats' airway was open to prevent asphyxia. All rats were free to move around, eat, and drink.

At 12 weeks after surgery, the rats were sacrificed and their skulls were obtained and fixed using 4% paraformaldehyde. Micro-CT (Perkin-Elmer, Waltham, MA, USA) was used to scan and reconstruct images of the fixed skulls. CTvox software (Bruker, Billerica, MA, USA) was used to view the images and CTAn software (Bruker) was used to analyze. The fixed cranial samples were subjected to decalcification and dehydration, before being embedded and cut into section with 5 μm thickness. The sections were then subjected to hematoxylin and eosin (H&E) stain, Masson stain and Immunohistochemistry (IHC). Samples were stained with anti-Bmp2, COL I, OCN, and OPN antibodies (1:200, Abcam, UK), and then with the appropriate secondary antibodies. And imaged under an inverted microscope (Olympus, Tokyo, Japan).

To assess the biosafety *in vivo*, IL-6 and TNF-α levels in blood were measured using commercially available ELISA kits (Kexing, Jinan, China) to analyze systemic inflammation.

Evaluation of the antibacterial effect *in vivo*

Based on the model establishment of the bone defect, we placed *S. aureus* bacterial solution (10^7 CFU in 100 μL of sterile saline) covering the defects to achieve infection [32], mixed with or without 20 mg microspheres and closed the wound with sutures. After 3 days, the cranial bone was re-exposed to collect the granulation tissue (1 mm^3).The granulation tissue was transferred to 10 mL of sterile saline, and then 100 μL of the liquid was transferred and spread to an agar plate. The agar plates were incubated at 37℃ for 24 h, and then counted the CFU. In addition, we extracted the granulation tissue (1 mm^3) and assessed the infection by performing qRT-PCR for TNF-α and IL-6. The primers for genes were shown in Table S1.

Statistical analysis

The mean ± the standard deviation (SD) was used to express the quantitative data. One-way analysis of variance (ANOVA) and t-tests were used to carry out the analyses. Differences between groups of $p < 0.05$ was considered to have statistical difference and $p < 0.01$ indicated a very significant difference.

Results and Discussion

The characteristics of the Mg-Ga@PLGA microspheres

Figure 1a shows the SEM characterization of the morphology of the microspheres. While the Mg-Ga@PLGA microspheres showed rough surfaces because of the deposition of inorganic salts. Figure 1b shows the qualitative results of elemental mapping of Mg-Ga@PLGA microspheres. A good scaffold not only needs to be loaded with key factors, but also needs to provide space for cells to recruit and grow stem cells *in vivo* [33]. In addition to cell growth, the microsphere scaffold needs a gap to provide space for the initial capillaries to grow. After the initial local recruitment of cells to form bone tissue, the growth of tiny blood vessels and capillaries is required to transport oxygen and nutrients to the deeper tissues [34], thereby ensuring that the new bone produced has quality. The diameter of prepared microspheres was around 50μm (Figure 1c). The microspheres were allowed to fill in any irregular defects, and to facilitate cell adhesion and provide support for new bone tissue [35].

To further analyze the distribution of Mg^{2+} and Ga^{3+} in the PLGA microspheres, XPS was used. Figure 1d shows the loading content of Mg^{2+} and Ga^{3+}. From the enlarged spectra, we found that Ga^{3+} and Mg^{2+} were both loaded into the PLGA microspheres. The atom percent of Mg representing the loading efficiency in the PLGA microspheres was 6.99%, which was 16 times higher than that of Ga.

Subsequently, the release patterns of Mg^{2+} and Ga^{3+} on the microspheres were shown in Figure S1. After 10 days, the release rate was slower, reaching a plateau; however, the release rate did not reach 100%, which was possibly related to the non-specific adsorption of ions on the microspheres and attrition resulting from the microsphere preparation conditions (organic solvents and sonication). In general, the system guarantees a slow release of ions and maintains the local concentration of bioactive components, thus facilitating the biological functions of Mg^{2+} and Ga^{3+}.

Biocompatibility

To test the biocompatibility of the Mg^{2+} and Ga^{3+} loaded PLGA microspheres, we cultured MC3T3-E1s and BMMs with the microspheres directly or indirectly. None of the four groups of microspheres inhibited the proliferation of MC3T3-E1s and BMMs compared with the blank control. Using Calcein-AM/PI probes, we detected few dead cells (Figure 2a), suggesting that the microspheres were not cytotoxic.

Cytoskeleton stain showed that the cells were intact, with a fully expanded polygonal shape, consistent with the fibroblast-like morphology of MC3T3-E1s [36]. Confocal images showed that fibroblasts adhered to the microspheres showing a star-like phenotype [37]. MC3T3-E1s co-cultured with microspheres had well-stretched bundles of actin (red) with clear lines comprising actin filaments, which also extended pseudopods, indicating that the PLGA microspheres acted as a safe scaffold for cell extension and cytoskeleton development [38]. BMMs also showed intact morphology, with a rounded cytoskeleton near the nucleus and no obvious expansion, exhibiting the primitive state of inactive macrophages (all Figure 2b).

Transwell assays were used to examine the migration and recruitment abilities of MC3T3-E1s. After 48 h of incubation, the co-loaded of Mg^{2+} and Ga^{3+} group showed higher numbers of migrating cells compared with that in the PLGA and blank groups (Figure 2c). Quantitative analysis (Figure S2) showed that the number of migrating cells was significantly different between the Mg-Ga@PLGA group and the blank group ($p < 0.0001$). Endogenous stem cells exert crucial functions in the overall bone healing process; the cytokines produced by trauma and the initial inflammatory process have a poor stem cell recruitment ability [39-40]. The Mg^{2+} and Ga^{3+} groups showed marked promotion of MC3T3-E1s migration *in vitro* and a more prominent pro-migration effect after their co-loading, which might facilitate the accumulation of endogenous stem cells at the site of injury *in viv*o [41].

As shown in Figure 3, the results of CCK-8 assay and flow cytometry incubated with the four types of microspheres and the blank control were similar, indicating that while the microspheres did not enhance cell proliferation, neither did they show obvious cytotoxicity. Moreover, the MC3T3-E1s and BMMs in each group showed logarithmic proliferation. Cell populations undergo apoptosis to preserve the stability of the internal environment. No statistically significant difference in the proportion of apoptotic cells was noted between the control and the experimental groups, suggesting that the microspheres had no toxic effects on MC3T3-E1s and BMMs. Among all groups, there were few early apoptotic cells (Q2) and late apoptotic cells (Q3). This demonstrated the high biosafety of PLGA microspheres and ensured cellular status, which formed the basis for the later study of the effect of the microspheres on cell differentiation.

Mg-Ga@PLGA increased osteoblast differentiation and inhibited osteoclast differentiation

At day 7 of differentiation induction, western blot was used to determine the levels of osteogenesis-related proteins (Figure 4a). Mg-Ga@PLGA showed the most significant increase in protein levels related to osteogenic differentiation. (Figure 4b), demonstrating that co-loading of the two elements resulted in a superior osteogenic differentiation effect

compared with either Mg^{2+} or Ga^{3+} alone. At day 14 of induction of differentiation, we still observed an increase in the expression of osteogenesis-related proteins, except for ALP, similar to the results at day 7. One explanation might be that, as an early osteogenic differentiation protein, ALP expression peaks at day 10 [42-43]and then gradually decreases, leading to no significant difference in its levels among the groups. To directly observe the location of typical osteogenesis-related proteins, we used immunofluorescence. At day 5 of osteogenic differentiation, Runx2 (red) was distributed in the cytoplasm and nucleus, with the Mg-Ga@PLGA group showing an increased level of Runx2 in the nucleus, indicating that Mg^{2+} and Ga^{3+} might synergistically promote Runx2 function in the nucleus [44] (Figure 4c), while Bmp2 showed aggregation in the cytoplasm. The Mg-Ga@PLGA group showed the strongest fluorescence intensity of osteogenic differentiation-related proteins (Figure 4d), which was consistent with the western blotting results. Compared with the other groups, the co-loaded group showed a larger contribution to osteogenic differentiation (Figure 4e), suggesting a possible synergistic effect of Mg^{2+} and Ga^{3+} in the osteogenic differentiation process.

To further validate, ALP staining and ARS staining were used to infer the osteogenic differentiation in the presence of Mg-Ga@PLGA. The Mg-Ga@PLGA group showed the most obvious ALP and ARS staining, indicating higher levels of ALP expression and more calcium deposition (Figure 5a-c, e-g).

The Mg-Ga@PLGA microspheres not only showed a superior osteogenic effect, but also showed an inhibitory effect on osteoclastic differentiation. TRAP staining (Figure 5d, h) showed that Ga^{3+} had a significant inhibitory effect on the BMMs differentiation, which was consistent with the results of a previous study. Moreover, the presence of Mg^{2+} was found to enhance the inhibitory effect of Ga^{3+} on osteoclastic differentiation [31]. At the 5th day of induction of osteoclastic differentiation, BMMs cultured with Mg-Ga@PLGA microspheres were still at the undifferentiated stage compared with the other groups, indicating that Mg-Ga@PLGA inhibits in the formation of osteoclasts. This suggested that the microspheres can promote bone formation and inhibit bone resorption, suggesting their utility in repairing bone defects.

The *in vitro* antibacterial activity of Mg-Ga@PLGA microspheres

We used live/dead staining, the total antibacterial activity test, and the inhibition zone test to evaluate the microspheres' antibacterial properties (Figure 6). There were few inhibition zones in the blank and Mg@PLGA groups; however, the inhibition zones in the Ga@PLGA and Mg-Ga@PLGA groups were significant for *S. aureus* and *E. coli* (Figure 6a). In the inhibition zone test, the Mg-Ga@PLGA microspheres exhibited the most significant antibacterial effect. Treatment with Ga@PLGA and Mg-Ga@PLGA resulted in significantly reduced numbers of bacterial colonies compared with those in the blank and Mg@PLGA groups, with only sporadic bacterial colonies observed (Figure 6b). The live/dead staining (Figure 6c) showed that almost all bacteria survived in the PLGA and Mg@PLGA groups (green), whereas the Ga@PLGA and Mg-Ga@PLGA groups had far more dead bacteria. Relevant statistical results shown in Figure S3. These experiments demonstrated that Ga^{3+} showed a good anti-bacterial effect and Mg^{2+} combined with Ga^{3+} retained this antibacterial activity. This inherent antibacterial property of Ga^{3+} might prevent bacteria from damaging bone healing and reduce risk of infection occurring after bone damage.

It has been reported that the antimicrobial effect of Ga^{3+} may be related the similar ionic radii with iron ions (Fe^{3+}) [45]. Fe^{3+} are involved in the survival of most micro-organisms and in metabolic processes such as oxidative stress defense, respiration, electron transfer, and protein and DNA. However, bacteria employ various strategies for host environmental iron acquisition, including iron carrier-based systems and transferrin/lactoferrin receptors. Importantly, many microbial uptake systems are unable to distinguish between Ga^{3+} and Fe^{3+} because of the similarity of their ion radii [29]. Therefore, bacterial infections could be inhibited by exploiting their nutrient vulnerability [46], for example, Ga^{3+} could compete with Fe^{3+} to disrupt Fe^{3+} metabolism. [47].The antibacterial effect can reduce ameliorate inflammatory responses and inducing osteogenic microenvironment for enhanced osteogenic properties [48].

In vitro assays, the inhibition effect of Mg-Ga@PLGA microspheres on bacteria showed significant differences unsteadily, which may be related to the way of bacteria to absorb magnesium ions without limitation. And bacteria exhibited variability in their ability to adapt to stress [49].

The repair ability of Mg-Ga@PLGA in rat skull defect models

The ability of Mg-Ga@PLGA to promoting the repair of bone defects was evaluated using an 8 mm rat skull defect model. Micro-CT images of samples collected 12 weeks after surgery in each group showed the formation of new bone that progressed from the edge toward the center of the defect (Figure 7a). Among the five groups, Mg-Ga@PLGA exhibited the best repair ability according to the micro-CT scans and bone parameters (Figure 7b). For BV/TV, PLGA had the lowest bone volume fraction of 23.45%; Mg@PLGA and Ga@PLGA induced better bone repair, of 28.57% and 27.32% respectively; while Mg-Ga@PLGA had the best bone repair ability, showing a bone volume fraction of 33.41% ($p < 0.01$). BMD and BS/TV assessments showed similar results. Overall, the results for the bone parameters showed good consistency with the micro-CT findings. Contrastingly, the Tb. sp values showed no differences among the groups, indicating that the average width of the trabecular medullary cavity in the newly formed bone was consistent, reflected by the uniform particle size of the filling microspheres.

H&E and Masson stainings were used for histological analysis and to observe the growth of collagen, new bone tissue, and lymphocyte infiltration. Collagen is a major constituent of bone. Osteoblasts secrete the extra cellular matrix (ECM), which includes type I collagen, which transforms from an initial amorphous state to a more crystallized form, which slowly promotes osteogenesis. In addition, mineralization in osteoblasts is the main process of bone formation promotion. Collagen acts as a template to initiate and reproduce mineralization. Therefore, bone formation is closely associated with the collagen content. The defects in the blank and PLGA groups mainly comprised fibrous tissue, with only a few (blue) collagen fibers, and we observed no prominent indications of new bone formation in these groups (Figure 7c). By contrast, the Mg-Ga@PLGA groups had abundant collagen in the bone defects and the stump was surrounded by new bone formation, with little fibrous connective tissue infiltration. In addition, H&E staining showed that all groups had low levels of lymphocyte infiltration, suggesting an absence of an inflammatory reaction [50] (Figure 7c). Masson and H&E staining also demonstrated that mature bone tissue grew in a lamellar pattern with a parallel bone plate structure, whereas the new bone was mainly composed of collagen fibers that were undergoing mineralization and showed mainly round-like mineral deposits without a clear parallel hemipelagic bone structure. Peripheral blood serology also proved that there was no significant increase in inflammation in the rats (Figure 7d).

Immunohistochemistry detected the expression of Bmp2, collagen type I (COL I), osteocalcin (OCN), and osteopontin (OPN) (Figure 8). At 12 weeks post-surgery, no significant OCN and COL I staining was seen in the control group, whereas high levels were observed in the Mg@PLGA, Ga@PLGA, and Mg-Ga@PLGA groups, with the highest levels in the Mg-Ga@PLGA group, which was consistent with the Micro-CT results. The above results indicated that the co-loading of Mg^{2+} and Ga^{3+} produced microspheres with excellent bone regenerative ability, which could help to solve the various problems associated with bone defects. Moreover, the anti-bacterial ability of Mg-Ga@PLGA can prevent infection and its associated tissue inflammation, which will promote the repair infected bone defects.

Antibacterial activity *in vivo*

To verify the antibacterial effect of Mg-Ga@PLGA microspheres in animal models, we established infected bone defects with *S. aureus*, and collected the granulation tissue around the defects to analyze. As shown in Figure S4 a, the tissue derived from the defects filled with PLGA microspheres exhibited significant bacterial clones. Clearly, a large number of bacterial colones formed in the Mg@PLGA group. The filling of Ga@PLGA and Mg-Ga@PLGA microspheres significantly

reduced the survival proportion of *S. aureus* at the site compared to the PLGA microspheres and Mg@PLGA group (Fihure S4 b). It is noticed that the inhibition of *S. aureus* was more pronounced in the Mg-Ga@PLGA group.

The results of qRT-PCR analysis (Fig. S4 c) showed that the Mg-Ga@PLGA microspheres exhibited the lowest expression of TNF-α and IL-6 *($p < 0.0001$).* The significant decrease in the gene expression of TNF-α and IL-6, which were regarded as common indicators of infection [46], suggested that the Mg-Ga@PLGA microspheres could inhibit inflammatory responses caused by bacterial infection. These results verified the antibacterial efficiency of Mg-Ga@PLGA microspheres *in vivo*. Interestingly, the microspheres co-loaded of Mg^{2+} and Ga^{3+} showed a more significant antibacterial effect compared to the group of adding Ga^{3+} only, suggesting that the presence of Mg^{2+} enhanced the antibacterial effect of Ga^{3+}. Moreover, the expression of IL-6 was inhibited more significantly than that of TNF-α. Its potential antimicrobial mechanism needs further exploration.

Conclusion

In this study, PLGA microspheres co-loaded with Mg^{2+} and Ga^{3+} were prepared successfully with good biocompatibility and biosafety. We demonstrated that the Mg-Ga@PLGA microspheres have antibacterial effects, reducing the risk of infection at the bone defect and providing a microenvironment for the repair of bone defects. Meanwhile, Mg^{2+} and Ga^{3+} were observed to play a synergistic role in promoting osteogenic differentiation and inhibiting osteoclastic differentiation. Thus, Mg-Ga@PLGA microspheres have potential for use as a biomaterial to repair bone defects.

Author contributions

Conceptualization, Jin Bai; Methodology, Jin Bai, Si Shen, Yan Liu, Tianqi Li; Investigation, Jin Bai, Si Shen, Yan Liu, Tianqi Li; Writing- Original Draft, Jin Bai; Writing- Review & Editing, All Authors;
Funding Acquisition, Xinxing Wang; Resources, Tianqi Li, Zirou Wang, Weili Liu, Lingling Pu; Supervision, Gang Chen, Xinxing Wang.

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Declarations

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

The animal experiments in thiswork have obtained the permission from Yi Shengyuan Gene Technology (Tianjin) Company.

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Figures

Figure 1 Characteristics of the Mg-Ga@PLGA microspheres. (a) Under SEM observation, the microspheres showed a smooth surface and microstructure. (b) Elemental mapping of the microspheres. (c) Particle analysis size of the microspheres. (d) XPS spectra of the Mg-Ga@PLGA microspheres with enlarged spectra.

Figure 2 Mg-Ga@PLGA microsphere biocompatibility. (a) Staining of live (Calcein-AM, green) and dead (PI, red) cells when cultured with various microspheres. (b) Staining of the cytoskeleton at 3 days post-seeding in each sample. (c) Effect of microsphere treatment on the migration of MC3T3-E1cells.

Figure 3 Apoptosis caused by Mg-Ga@PLGA *in vitro*. (a, c) Flow cytometry analysis of Annexin-V staining of MC3T3-E1s and BMMs on day 3 of microsphere treatment. (b, d) According to CCK-8 assays, treatment with Mg-Ga@PLGA microsphere extracts caused no cytotoxicity toward MC3T3-E1s or BMMs.

Figure 4 Effects of Mg-Ga@PLGA on the expression levels of proteins according to the osteogenic differentiation of MC3T3-E1s. (a) Osteogenesis-related protein levels after 7 and 14 days of microsphere treatment. (b) Quantitative data derived from the western blotting results. (c) Images of immunofluorescence for Runx2 and (d) Bmp2, and (e) fluorescence intensity quantification after MC3T3-E1s were cultured with microspheres for 5 days. ***$p < 0.001$, ****$p < 0.0001$

Figure 5 Mg-Ga@PLGA's effects on MC3T3-E1 cell osteogenic differentiation. (a,b) Representative images of MC3T3-E1s treated with microspheres for 5 days and 7 days and then stained for ALP. (e, f) the quantification of relative ALP activities. (c) Images of ARS staining of MC3T3-E1s treated with microspheres for 21 days. (g) The quantification of ARS staining. (d) TRAP staining images of BMMs cultured with microspheres for 5 days and (h) quantification of TRAP-positive polynucleated (≥ five nuclei) osteoclasts derived from BMMs. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

Figure 6 Evaluation of the antibacterial properties of the microspheres. (a) *E. coli* and *S. aureus* inhibition zones when co-cultured for 24 h with microsphere extracts. [(a) blank, (b) Mg@PLGA (c) Ga@PLGA, and (d) Mg-Ga@PLGA]. (b) Total antibacterial activity (c) Live/dead staining of *E. coli* and *S. aureus*.

Figure 7 Evaluation of bone formation at 12 weeks after implantation of PLGA, Mg@PLGA, Ga@PLGA, and Mg-Ga@PLGA microspheres. The control comprised the blank group. (a) Rat skulls reconstructed in three dimensions. (b)

Quantification of the bone volume fraction in the defect site. (c) H&E and Masson trichrome staining at 12 weeks post-surgery. (d) Peripheral blood serology for the detection of inflammatory factors. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure 8 Assessment of the osteogenesis effect of protein levels *in vivo*. (a)Immunohistochemical (IHC) analysis of Bmp2, COL I, OPN, and OCN to assess *in vivo* bone formation at 12 weeks post-surgery. (b) Quantification of the IHC results. (NB: new bone; and MB: mature bone) *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.