Cu/Gd co-doped hydroxyapatite/PLGA composites enhance MRI imaging and bone defect regeneration

Lu Wei Lu  
Second Affiliated Hospital of Dalian Medical University

Xin Xia  
Second Affiliated Hospital of Dalian Medical University

Yihang Ma  

Hongtao He  
Second Affiliated Hospital of Dalian Medical University

O Kikkawa Don

Lu Zhang  
Second Affiliated Hospital of Dalian Medical University

Bo Zhang

Liu Xiangji Liu Xiangji  (✉️ 13174462143@163.com)  
Second Affiliated Hospital of Dalian Medical University

Research Article

Keywords: Hydroxyapatite, Bone regeneration, MRI Imaging, PLGA, Co-doping

Posted Date: August 17th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3245811/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Background

The hydroxyapatite (HA)/poly(lactide-co-glycolide) acid (PLGA) composite material is one of the most widely used orthopedic implant materials with good biocompatibility and plasticity. In recent years, cation doping has increased the number of its possible biological applications. Conventional HA/PLGA composite cannot be observed using X-rays after implantation in vivo and does not lead to good osteogenic induction results. Cu can regulate the proliferation and differentiation of osteoblasts, while Gd can effectively enhance the magnetic resonance imaging ability of materials.

Methods

In this study, a Cu/Gd@HA/PLGA composite was prepared to explore whether the introduction of Cu and Gd into a HA/PLGA composite could enhance the osteogenic ability of osteoblasts, the in vivo bone defect repair ability, and the magnetic resonance imaging (MRI) characteristics.

Results

The characterization of materials confirmed that the Cu/Gd@HA has HA morphology and crystal structure. The Cu/Gd@HA/PLGA composite material has excellent nuclear magnetic imaging ability, porosity and hydrophilicity, which can promote cell adhesion and implant detection. The results of in vitro experiments confirmed that the Cu/Gd@HA/PLGA composite enhanced the proliferation, differentiation, and adhesion ability of MC3T3-E1 cells and upregulated the expression of COL-1 and BMP-2 at the gene and protein levels. In vivo, the Cu/Gd@HA/PLGA composite still showed good T1-weighted MRI abilities and effectively enhanced the bone defect healing rate in rats.

Conclusion

These findings indicate that the Cu/Gd@HA/PLGA composites can effectively improve the T1-weighted magnetic resonance imaging ability of the materials, promote the proliferation and differentiation of osteoblasts in vitro, and increase the rate of bone defect healing in vivo.

Background

The hydroxyapatite (HA)/poly(lactide-co-glycolide) acid (PLGA) composite material is one of the most widely used orthopedic implant materials with good biocompatibility and plasticity. In recent years, cation doping has increased the number of its possible biological applications. Conventional HA/PLGA composite cannot be observed using X-rays after implantation in vivo and does not lead to good osteogenic induction results. Cu can regulate the proliferation and differentiation of osteoblasts, while Gd can effectively enhance the magnetic resonance imaging ability of materials.

Results
Characterization of the nanoparticles

HA has a dense hexagonal structure with typical crystal characteristic peaks, while doped HA leads to crystal lattice transformation due to the introduction of foreign ions, which is reflected in the peak position shift, and in peak width and size changes, according to the XRD results. According to the XRD results, two main classes of diffraction peaks appeared, representing the two-phase composition (hydroxyapatite and amorphous calcium phosphate) of the material. From the diffraction peak distribution in Fig. 1A, we can see that the peak at 31.779 corresponding to 0.5Gd@HA shifted, and that all the characteristic peak fractions shifted due to the lattice size changes, indicating that the nanoparticles appeared as an amorphous phase, which may represent the transition of nanoparticles from the crystalline phase to the amorphous one, which was caused by excessive doping.

Regarding the 0.5Cu@HA nanoparticles, the three strong characteristic peaks of HA were sharp, and that of the substrate was narrow, indicating that no crystalline phase other than HA was present in the nanoparticles. The height of the peak corresponding to the 0.5Cu/Gd@HA nanoparticles at 30.5779 was significantly lower than that corresponding to the 0.5Cu@HA nanoparticles. This indicates that the introduction of the Gd element has an impact on the crystal structure of HA, making it looser. However, the diffraction peak of the 0.5Gd@HA nanoparticles, but not that of the 0.5Cu/Gd@HA nanoparticles, shifted to 31.779, indicating that the co-introduction of the Cu and Gd elements can change the main peak position. This is because the ionic radius of Gd is slightly lower than that of Ca, and the introduction of Gd alone leads to a smaller lattice of the synthesized 0.5Gd@HA nanoparticles and to a rightward shift of the diffraction peak. Among the co-doped components, the ionic radii of Gd and Cu were smaller than that of Ca. However, because the doping of elements with a small ionic radius is difficult, the doping amount of Gd and Cu decreased, and the doping amount of Ca increased; thus, the main peak position remained unchanged, and the crystal structure was stabilized.

The FTIR results are shown in Fig. 1B, and show the absorption peaks of different groups at different wavelengths. The products obtained from the hydrothermal reaction exhibit absorption peaks near 3,570 cm\(^{-1}\) and 1,640 cm\(^{-1}\), corresponding to the stretching and bending vibrations of the -OH group, indicating the presence of -OH in the sample. The absorption peaks at 565 and 607 cm\(^{-1}\) are consistent with the bending vibration mode of O-P-O, which demonstrates the presence of phosphate root groups in the material. However, the strong absorption peak at 1,640 cm\(^{-1}\) corresponds to the P-O antisymmetric stretching vibration mode. The positions of these functional groups were consistent with the infrared absorption peak wavelengths of HA reported in the literature, and no other miscellaneous peaks were present, indicating that the main groups in the product were hydroxyl and phosphate root groups, which is in line with the HA infrared absorption spectrum results.

To further analyze the morphology of the product, SEM analysis of the nanoparticles was performed. Figure 1C shows the SEM images of the products obtained via hydrothermal reaction at 180°C for 12 h at different doping ratios. In the fraction doped with only Cu, 0.5Cu@HA exhibited a short-rod-like structure inherent to hydroxyapatite. When the doped component was 0.5Gd, the morphology of nano-hydroxyapatite was slightly irregular and the hydroxyapatite crystal structure slightly changed, in line with
the XRD results.\textsuperscript{(2, 3)} The difference was that 0.5Cu/Gd@HA maintained a better short-rod morphological structure. Image J was used to analyze the size of the nanoparticles in the picture, and the results are shown in Fig. 1D. The 0.5Cu/Gd@HA, 0.5Cu@HA, and 0.5Gd@HA particle size was mostly 110–120, 100–110, and 80–100 nm, respectively. This indicates that the addition of nanoparticles within the lattice affects the lattice size structure of the nanoparticles, further affecting the structure of the nanoparticles.

Characterization of the composites

When the curve shows a $K$ (susceptibility) $> 0$, it means that the sample has paramagnetism, that is, the direction of magnetization is the same as that of the magnetic field.\textsuperscript{(6, 25)} In Fig. 2B, the magnetic force curve of the samples with different components in the magnetic field environment shows that when the external magnetic field acts on a material containing Gd, the electronic magnetic moment of Gd atoms that does not completely cancel it out is disturbed by the magnetic field environment, and the disordered state is transformed into an ordered state, thus having magnetic properties.\textsuperscript{(26)} In magnetic resonance imaging technology, the location of implantation of materials can be determined by observing the implants containing Gd elements. These applications play an important role in the \textit{in vivo} observation of implants.

The hydrophilicity of the implant material determines the strength of the cell adhesion on the surface of the material. Figure 2C and 2E characterize the contact angle between the material and water, with smaller contact angles indicating a higher hydrophilicity.\textsuperscript{(2)} The results show that the contact angle of 0.5Gd@HA/PLGA nanoparticles was the largest, and was similar to that of 0.5Cu/Gd@HA/PLGA nanoparticles, indicating that these two nanoparticles have the highest hydrophilicity. The main reason for the change in the contact angle is that the crystal structure of the 0.5Gd@HA/PLGA nanoparticles is partially destroyed and the crystallinity decreases, which is consistent with the SEM and XRD results.\textsuperscript{(3, 27)} Similarly, all the doped components exhibited higher hydrophilicity than that exhibited by the HA/PLGA material, and a higher hydrophilicity contributed to cell adhesion on the composite surface.

The surface morphology of the HA/PLGA composite was further analyzed using SEM. Figure 2F shows the SEM images of each component material doped with different elements. No significant difference was observed in the surface morphology of each component, indicating a porous structure. \textsuperscript{(7)} The results of the micro-CT scanning of the composite material showed that the material presented a porous structure with an average size of 1–5 µm, which is the characteristic microscopic morphology of the PLGA composite prepared using the typical phase conversion method.\textsuperscript{(28, 29)} The nanoparticles did not agglomerate and were uniformly distributed in the direct structure. The porosity of the composites was analyzed using CTAn software.\textsuperscript{(2)} The results (Fig. 2D) showed that the porosity of all samples was within the 50% range, and no statistically significant differences among groups were observed. A porosity of 50% is conducive to the entry of cells, nutrients, blood, and air, and can promote the growth, proliferation, and differentiation of cells in the material, which provides a theoretical basis for the material to be used as a bone tissue substitute and bone repair material \textit{in vivo}.\textsuperscript{(7)}
To evaluate the imaging capabilities of the nanocomposites, MRI was performed using scaffolds immersed in deionized water. In Fig. 2G, the T1-weighted images show that the 0.5Cu/Gd@HA/PLGA and 0.5Gd@HA/PLGA composites had a better short T1-weighted MRI ability. Owing to the high proportion of Gd doping, the 0.5Gd@HA/PLGA material exhibited the highest signal intensity. These results indicate that Gd$^{3+}$ doping can improve the MRI ability of the composite, and that the imaging intensity depends on the Gd content of the material. However, no T1-weighted imaging manifestations were observed for the HA/PLGA and Cu@HA/PLGA composites, which is consistent with the susceptibility curve results. Currently, X-rays are commonly used in clinical practice to detect the position, size, and shape of implants. However, most implants are metal alloys, which may produce artifacts in X-rays or disenable the observation of bone healing because of their strong X-ray shielding ability. Gd@HA/PLGA can be observed using magnetic resonance, which efficiently prevents X-ray shielding, effectively solving this problem.

Biological evaluation of the Cu/Gd@HA/PLGA Nanocomposites

Cell adhesion is one of the early behaviors of cells, when in contact with a material. Good adhesion properties contribute to further cell proliferation and differentiation. In our previous work, we analyzed the hydrophilicity of materials by measuring the contact angle. After MC3T3-E1 cells were co-cultured on the material for 24 h, phalloidin/DAPI fluorescence staining was performed to evaluate cell adhesion onto the material surface. When the cells were cultured for 24 h, the 0.5Cu/Gd@HA/PLGA sample showed the highest number of attached cells, which was significantly higher than that of the other groups, and the cell adhesion to both 0.5Cu@HA/PLGA and 0.5Gd@HA/PLGA materials was similar, which is in agreement with the cell proliferation results (Fig. 3A). These results indicate that Cu and Gd co-doping contributes to early cell adhesion. In addition, the cells of the co-doped fraction spread more obviously, and their nuclear cytoplasmic ratio was the highest, indicating that the cells were more spread on the material, which promoted diffusion and interactions between cells.

Calcein-AM, a membrane-permeable fluorescent dye that can be used to observe living cells, exhibits green fluorescence (Ex/Em = 490 nm/515 nm). Once inside the cell, calcein is hydrolyzed by intracellular esterase to calcein, which can bind to intracellular calcium ions and produce intense green fluorescence. Since dead cells lack esterase, calcein-AM can only be used to label live cells. The cells cultured in Cu/Gd@HA/PLGA nanocomposites were stained with calcein to observe the short-term toxicity of HA/PLGA. From day 1 to day 3, the number of cells in all composites gradually increased, indicating that the release of the material did not result in obvious toxicity, since no large area containing dead cells (absence of fluorescence) was observed (Fig. 3B-D). The growth density of the 0.5Cu/Gd@HA/PLGA fraction was the most obvious among all fractions, similar to the cell proliferation proliferation results, confirm the cell proliferation ability of the 0.5Cu/Gd@HA/PLGA fraction was the highest.

HA is a calcium phosphate implant material widely used in clinical practice. It can degrade calcium and phosphate ions in vivo and promote cell proliferation and differentiation. Metal Gd is a lanthanide element and heavy metal which may harm human health. In recent years, studies have shown that Gd$^{3+}$
can promote the proliferation and differentiation of osteoblasts, indicating that Gd$^{3+}$ has no cytotoxicity within a certain concentration range. (26) Nanoparticle extracts were obtained by immersing nanoparticles in DMEM for 24 h, and cells were cultured for 24 h in medium containing different concentrations of extracts to observe the cell proliferation efficiency of different fractions and to assess the cytotoxicity of the materials. None of the samples showed cytotoxicity without dilution; 80% of the cells were able to survive, and the proliferation efficiency increased slightly as the concentration of the extract decreased (Fig. 3E). Therefore, the introduction of Cu and Gd did not cause obvious toxicity to the cells, and the doping ratio of the two elements in HA was within the biosafety range.

To examine the effect of Cu/Gd@HA/PLGA nanocomposites on cell proliferation, MC3T3-E1 cells (pre-osteoblasts) were used. After the cells were seeded on 24-well plates, the effect of Cu/Gd@HA/PLGA nanocomposites on their proliferation was analyzed using the CCK-8 method. (11) When the PLGA group was used as the control group and the cells were cultured for 7 days, the cell proliferation rate for all the composites was greater than 100% (Fig. 3F), indicating that the new HA@PLGA material synthesized in this study promotes cell proliferation at 7 days. No significant difference in cell proliferation was observed between the 0.5Cu@HA/PLGA and 0.5Gd@HA/PLGA nanocomposites. On day 3 of cell culture, the Gd-doped fraction exhibited a slightly higher proliferation efficiency than the Cu-doped fraction, which may be related to the low crystallinity of 0.5Gd@HA nanocomposites. The SEM and XRD results showed that the crystallinity of 0.5Gd@HA nanocomposites was the lowest, and this lower crystallinity promoted the early degradation of the nanoparticles, increased the ion concentration in the solution, and promoted cell proliferation at 3 days. (2)

Osteogenic differentiation

To investigate the effect of Cu/Gd@HA/PLGA nanocomposites on gene and protein expression during osteogenic differentiation, the gene and protein expression of collagen type I (COL-1) and bone morphogenetic protein 2 (BMP-2) was analyzed after 14 days of culture on the surface of different materials. COL-1 is the most important collagen protein pre-bone formation and is involved in multiple gene pathways related to bone growth and development. BMP-2 participates in the entire process of osteogenesis and has been approved for clinical application. As shown in Fig. 4A-1 and 4A-2, the BMP-2 gene expression of the metal element-doped fraction increased after 14 days of culture on the surface of the composite, and was higher than that of the HA/PLGA group. Among them, 0.5Cu/Gd@HA/PLGA with single doping exhibited a high degree of BMP-2 gene expression. The COL-1 gene expression in the 0.5Gd@HA/PLGA and 0.5Cu/Gd@HA/PLGA groups was higher than that in the other groups. As shown in Fig. 4B–C-2, the composites doped with metals all showed high BMP-2 and COL-1 protein expression levels, indicating that the doping with Cu and Gd contributed to cell differentiation and bone formation. (31)

The degree of osteogenic differentiation of osteoblasts was observed via ALP staining and synthesis on the surface of the materials after 7 and 14 days of cell culture. Figure 5 shows that the ALP expression of cells cultured on the materials of each group on day 14 was much higher than that on day 7, and the ALP
expression of cells in the co-doped nanocomposites was higher than that in the single-doped composites for the same ratio, indicating that co-doping can better promote osteogenic differentiation in the early stages. No significant difference in ALP expression was observed between the 0.5Cu@HA/PLGA and 0.5Gd@HA/PLGA groups. (32)

Alizarin red (ARS) can chelate calcium ions to form an orange complex, which can be used to evaluate the level of calcium deposition by observing the color depth of calcium nodules and by quantitatively detecting calcium. The degree of alizarin red staining is proportional to calcium deposition, which indicates a high level of osteogenic differentiation. The calcium deposition levels of MC3T3-E1 cells cultured for 14 and 21 days were detected using ARS. As shown in Fig. 5, the surface of the cells cultured for 14 days was slightly stained with alizarin red, whereas the cells cultured for 21 days showed abundant calcium nodules, with obvious changes in the staining intensity and area of stained cells compared to those of the cells cultured for 14 days. The staining results of the single-doped fractions showed that the calcium deposition of HA doped with Cu and Gd was better than that of pure HA, indicating that both elements could promote calcium deposition in cells. However, at the same culture time, the staining intensity and size of the calcium nodules in the co-doped fraction were larger than those in the other groups. These results indicate that simultaneous Cu and Gd doping can better promote the maturation of pre-osteoblasts.

Animal studies

3.5.1. HE staining and Sirius Red staining of the composites (4w/8w)

HE and Masson staining can be used to observe the formation of new bone and cartilage, as well as the number and distribution of collagen fibers. In this study, HE and Masson staining were used to observe the differences in new bone ingrowth among the different groups. As shown in Fig. 6A, at 4 weeks, an obvious material degradation performance occurred inside the 0.5Cu/Gd@HA/PLGA and 0.5Gd@HA/PLGA composite materials, and collagen was formed at the edge of the 0.5Cu/Gd@HA/PLGA composite material, which corresponds to an early osteogenesis performance. At 8 weeks, new bone grew into the center and edge of the Cu- and Gd-doped fractions, especially in the 0.5Cu/Gd@HA/PLGA fraction, which was completely infiltrated by new bone tissue, indicating that the co-doped fraction highly induced new bone formation; however, no significant difference in bone formation was observed between the metal-doped and the pure metal-doped fractions.

As shown in Fig. 6B, collagen encapsulation occurred at 4 weeks around the site of implantation of the 0.5 Cu/Gd@HA/PLGA and 0.5Gd@HA/PLGA composites, and the collagen formation in the other composites was not significant. These results indicate that co-doping induces collagen expression, while the 0.5Gd@HA/PLGA composite degraded rapidly at the early stage, and the released Gd element promoted collagen enrichment. The area of the defect that was covered with collagen increased with time; thus, the area at eight weeks was higher than that at 4 weeks for the composites doped with a mixture of the two metal elements. The use of the 0.5Cu@HA composite resulted in a bone defect site with high amounts of collagen and bone tissue formed. Furthermore, the amount of collagen fibers
obtained from the composites doped with Gd alone was similar to that obtained from the composites doped with Cu alone. This indicates that the two ions induced osteogenic differentiation, and the 0.5Cu/Gd@HA/PLGA fraction resulted in the highest collagen formation, indicating that ion co-doping can better promote the formation of collagen(11).

Protein secretion in the defect was observed via immunofluorescence staining of the animal tissues. As shown in Fig. 7, the protein fluorescence intensity for all the composite bone defects at 8 weeks was higher than that at 4 weeks. On one hand, the protein gradually accumulated at the defect site and, on the other hand, the bone gradually matured at the defect site, leading to an increase in protein expression. Moreover, the protein immunofluorescence intensity of the 0.5Cu/Gd@HA/PLGA group was higher than that of the HA/PLGA and single metal element doping groups, indicating that the amount of BMP-2 and OCN secreted by the 0.5Cu/Gd@HA/PLGA implanted bone tissue was the highest. The introduction of ions promoted the in vivo expression of BMP-2 and OCN and promoted bone healing(30, 33).

In order to evaluate the formation of Bone Tissue within orbital bone, we scanned orbital bone with Micro-CT, and analyzed Bone Volume/Tissue Volume (BV/TV) and bone trabecular Number (Tb.N), Trabecular Thickness (Tb.Th) and Trabecular Separation (Tb.Sp) with imaging software.

As shown in Fig. 8(A), in the sagittal map at 8 weeks, circular bone defects in the 0.5Gd@HA/PLGA and 0.5Cu/Gd@HA/PLGA groups were transformed into irregular edges, and bone components grew into the circular interior, indicating degradation of the two materials, and the degraded space was filled by new bone. It was confirmed that the material had a good ability to promote osteogenic differentiation, among which 0.5Cu/Gd@HA/PLGA group showed a better osteogenic ability. At 8 weeks of MRI examination, 0.5Gd@HA/PLGA and 0.5Cu/Gd@HA/PLGA showed high brightness of implants in the orbital lining at T1, indicating that the composite was not completely degraded, which was consistent with the results of CT. However, there were no obvious imaging features in the orbital wall of 0.5Cu and HA groups at 8 weeks, which was consistent with the results of hysteresis curve, indicating that the introduction of Gd element can enhance the MRI imaging features of composite materials in animals and can be used for postoperative implant observation. It is known that BV/TV, Tb.Th and Tb.N are proportional to the number of bone formation, while Tb.Sp is inversely proportional to the degree of bone formation. Figure 8 (B) shows that the BV/TV values of HA/PLGA, 0.5Cu@HA/PLGA, 0.5Gd@HA/PLGA and 0.5Cu/GdHA/PLGA composites are 12%, 18%, 22% and 25%, respectively. The Tb.N values were 1.1/mm, 1.523/mm, 1.889/mm and 2.112/mm, respectively. The Tb.Th values were 0.167µm, 0.255µm, 0.299µm and 0.312µm, respectively. The values of Tb.Sp were 0.724µm, 0.656µm, 0.5µm and 0.278µm, respectively, indicating that the bone formation effect of HA/PLGA alone was poor, while the bone formation effect of metal ions was better, among which 0.5Cu/Gd co-doped component showed the best bone formation effect.

To verify whether the ion release of the material in vivo produces toxicity, organs such as the heart, liver, spleen, lungs, kidneys, and brain were collected and tissue sections were prepared and stained with HE to observe tissue toxicity. According to the HE staining results at 8 weeks (Fig. 8), no inflammatory reaction
was observed in the organs of the animals, indicating that the materials had no obvious toxic effect on the animals. (4)

**Discussion**

In this study, 0.5Cu/Gd@HA and 0.5Cu/Gd@HA/PLGA composites were prepared using the hydrothermal method and the phase conversion method, respectively. HA has a typical dense hexagonal structure, in which Ca ions can be replaced by other metal elements. When Gd was used for doping, the 0.5Gd@HA/PLGA and 0.5Cu/Gd@HA/PLGA composite materials became paramagnetic, which enhanced the MRI ability, enabling us to observe the implantation of materials using more imaging methods. Moreover, the in vitro experiments showed that 0.5Cu/Gd@HA/PLGA effectively promoted the proliferation and differentiation of osteoblasts after the introduction of Cu and Gd. The in vivo results showed that the 0.5Gd@HA/PLGA and 0.5 Cu/Gd@HA/PLGA composites could be observed after implantation using MRI, whereas HA/PLGA and 0.5Cu@HA/PLGA could not be observed using MRI. The histological staining and immunofluorescence results showed that the composites containing Cu and Gd ions could better promote bone formation, and the 0.5Cu/Gd@HA/PLGA composite had the best bone formation promoting effect. The 0.5Cu/Gd@HA/PLGA composite promotes bone growth and MRI simultaneously; thus, it can be used to confirm the morphological changes occurring in bone implants after implantation and be used as a bone implant material in the future, since it promotes bone defect repair.

**Materials and Methods**

**Materials**

PLGA (LA/GA = 80:20; Mn = 1 x 10^5 Mw) was synthesized in our laboratory via the ring-opening co-polymerization of L-lactide (LA) and glycolide (GA), which were purchased from Changchun (China). Chloroform, nitric acid, gadolinium, ammonium hydroxide, cupric nitrate, calcium chloride, and ethanol were purchased from Beijing Chemical Works (Beijing, China).

Preparation of Cu/Gd co-doped hydroxyapatite

Cu- and Gd-cation-substituted HA was synthesized using a hydrothermal method, which can be described by the following equation:

\[(20 - 3x - 2y)/2Ca^{2+} + xGd^{3+} + yCu^{2+} + 6PO_4^{3-} + 2OH \rightarrow Ca_{(20-3x-2y)/2}Gd_xCu_y(PO_4)_6(OH)_2\]

Four samples, denoted as HA, 0.5Cu@HA, 0.5Gd@HA, and 0.5 Cu/Gd@HA, were prepared via hydrothermal synthesis (1–3). Briefly, a hydrothermal solution (Ca + Cu + Gd/P molar ratio = 1.67) was prepared by mixing calcium chloride, nitric acid, gadolinium, copper nitrate, and diammonium phosphate solutions, as shown in Table 1. (4) With the aid of the dilute ammonia, the pH of the hydrothermal solution was maintained at 10.0–11.0 until a white suspension was formed. (5) Subsequently, fresh HA liquid was
placed in a reactor with 50 mL of the previously prepared hydrothermal solution. The reaction was performed in a sealed autoclave at 180°C for 12–24 h.

Table 1
Chemical composition analysis of Ca, Cu, and Gd in the Cu/Gd@HA and HA nanoparticles using the ICP method.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ca/P in theory</th>
<th>(Ca + Gd + Cu)/P in theory</th>
<th>Molecular formula in theory</th>
<th>Ca/P molar ratios in reality</th>
<th>Chemical formula in reality using the ICP method</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>1.67</td>
<td>1.67</td>
<td>Ca(_{10})(PO(_4))(_6)OH(_2)</td>
<td>1.67</td>
<td>Ca(_{10})(PO(_4))(_6)OH(_2)</td>
</tr>
<tr>
<td>0.5Cu@HA</td>
<td>1.583</td>
<td>1.67</td>
<td>Ca(<em>{9.5})Cu(</em>{0.5})(PO(_4))(_6)OH(_2)</td>
<td>1.41</td>
<td>Ca(<em>{9.72})Cu(</em>{0.28})(PO(_4))(_6)OH(_2)</td>
</tr>
<tr>
<td>0.5Gd@HA</td>
<td>1.542</td>
<td>1.792</td>
<td>Ca(<em>{9.25})Gd(</em>{0.5})(PO(_4))(_6)OH(_2)</td>
<td>1.62</td>
<td>Ca(<em>{8.44})Gd(</em>{1.56})(PO(_4))(_6)OH(_2)</td>
</tr>
<tr>
<td>0.5Cu/Gd@HA</td>
<td>1.458</td>
<td>1.625</td>
<td>Ca(<em>{8.75})Gd(</em>{0.5})Cu(_{0.5})(PO(_4))(_6)OH(_2)</td>
<td>1.08</td>
<td>Ca(<em>{6.5})Gd(</em>{1.87})Cu(_{0.7})(PO(_4))(_6)OH(_2)</td>
</tr>
</tbody>
</table>

Fabrication of the Cu/Gd@HA/PLGA composites

N-methylpyrrolidone (NMP) was used as the solvent, and HA and PLGA were used as the solutes. HA/PLGA scaffolds were prepared using the phase conversion method, and HA/PLGA composite films were prepared using the solvent evaporation method. Specifically, according to a mass ratio of 20 wt%, the HA powder was poured into a mixture of PLGA and NMP, and the mixture was homogenized via magnetic stirring in a water bath at 50°C. The HA/PLGA/NMP suspension was extracted using a 2.5 mL syringe and placed in a freezer at -80°C for 12 h. After removal, the HA/PLGA composite scaffolds were placed in deionized water, which was changed every 8 h. After 120 h, the HA/PLGA composite scaffolds were removed and dried using a vacuum dryer to obtain dry HA/PLGA scaffolds.

HA was evenly dispersed in a chloroform solution and dissolved in PLGA. After stirring using a magnetic stirrer for 12 h, the blended HA/PLGA/chloroform mixture was poured onto a clean circular glass slide and slowly dried using a dryer at 50°C to obtain HA/PLGA composite film glass slides.

Characterization of the Cu/Gd@HA nanoparticles

The microstructure of the Cu/Gd@HA/PLGA scaffold was observed and measured using SEM. The contact angles of the Cu/Gd@HA/PLGA composites were measured using a picture contact angle instrument to assess their hydrophilicity or hydrophobicity. The microstructure of the Cu/Gd@HA/PLGA composite scaffold was measured using micro-CT by using the companion software CTAn. T1-weighted MR images of the composites were obtained using a 3.0T MRI machine.

In vitro cell study
MC3T3-E1 mouse pre-osteoblasts were used as the target cell line, and DMED-HG containing 10% fetal bovine serum, 100 mg/L streptomycin, and 63 mg/L penicillin was used as their medium. MC3T3-E1 cells were seeded on the nanocomposites (films) by $20 \times 10^4$ and stored in an incubator containing 5% CO$_2$ at 37°C in a humidified environment.

*In vivo* rat study

Twenty-four female SD rats weighing 250–300 g were provided by the Dalian Medical University. Rats were divided into four groups: HA/PLGA, 0.5Cu@HA/PLGA, 0.5Gd@HA/PLGA, and 0.5Cu/Gd@HA/PLGA. Before performing the animal experiments, all nanomaterials and medical devices used in surgery were sterilized using ultraviolet light for 6 h, and then immersed in iodophor 6 h then immersed in 75% alcohol for preservation for 24 h.

The rats were anesthetized using a subperitoneal injection of pentobarbital sodium (0.3ml/100g) and placed on a surgical plate. A skin incision under the orbit was made, and subperiosteal dissection was performed to expose the medial orbital wall. The ethmoid bed was gently pressed using vascular forceps, and its width and height were measured in the 1.0–1.5 cm range. The bone wall defects were created using a grinding drill. Blunt dissociation disrupts the periosteum and orbital septum, depositing the orbital content in the ethmoid sinus. After the wound was closed, the rats were intramuscularly injected with penicillin (300000 units/day) for the next 3 days. The rats were euthanized at weeks 4 and 8, and samples were collected for examination and testing. Micro-CT and magnetic resonance imaging were performed to analyze the rat orbital bone defects at eight weeks. CTVol software was used to analyze the CT results of the rats, and the area of the new bone was measured. The bone volume/tissue volume (BV/TV) was analyzed using the CTAn software, and MRI analysis of the implants was performed using an MRI machine. Orbital bone defects from sacrificed rats were harvested at 8 weeks and immersed in a 10% buffered formalin solution for 7 days. The heart, liver, spleen, lung, and kidney tissues were dehydrated in 70%, 95%, and 100% ethanol solutions, and then transferred to an intermediate pure xylene solution for 4 days. After gradient dehydration, the orbital bone, heart, liver, spleen, lungs, kidneys, brain, and other sections were stained with H&E to evaluate the *in vivo* toxicity of the implanted material. At 4 and 8 weeks, the bone defects tissues were decalcification, the stained with HE, Masson trichrome, and Sirius red, and the amount and distribution of COL-1 and BMP-2 in the defects was analyzed using immunofluorescence. The slices were washed in xylene 10min-xylene 10min-anhydrous ethanol 5min-anhydrous ethanol 5min-95% alcohol 5min-90% alcohol 5min-80% alcohol 5min-70% alcohol 5min-distilled water in turn. Sections were dyed with Harris hematoxylin for 3-8min, washed with tap water, differentiated with 1% hydrochloric acid alcohol for several seconds, rinsed with tap water, returned blue with 0.6% ammonia water, and rinsed with running water.

Slice into eosin dye solution and stain for 1-3min. The sections were then dehydrated and transparent in 95% alcohol I 5min- 95% alcohol II 5min-anhydrous ethanol 5min - anhydrous ethanol 5min - xylene 5min-xylene 5min, and the sections were taken out of xylene to dry slightly and sealed with neutral gum.
Statistical analysis

Three duplicate measurements were performed, unless otherwise stated. Data are expressed as the mean ± standard deviation. T test analysis of variance was performed using the Origin software. A p-value < 0.05, marked with an asterisk (*), indicates a significant difference.

Declarations

Ethics approval and consent to participate

Animal experiments have been approved by Dalian Medical University for Animal Experiment Ethics

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that there is no conflict of interest.

Funding

This work was funded by Natural Science Foundation of Liaoning Province, The Second Affiliated Hospital of Dalian Medical University (2021-MS-291) and Hospital cultivation fund of The Second Affiliated Hospital of Dalian Medical University.

Author Contributions

Conceptualization, LW, ZL, LXJ and MYH; Formal analysis, LXJ and ZL; Funding acquisition, ZL and LXJ; Investigation, WH and GW; Methodology, WH, GW, YC and QL; Writing—original draft, WH, GW; Writing—review & editing, WH, GW, YC and YW All authors have read and agreed to the published version of the manuscript.

Acknowledgements

Not applicable.

References


Figures

(A) XRD step scan ranging from 20° to 55° and (B) Fourier transform infrared spectra. (C) SEM images of the nanoparticles and (D) diameter distribution histograms of HA, 0.5Cu@HA, 0.5Gd@HA, and

Figure 1
0.5Cu/Gd@HA nanoparticles. Scale bar, 200 nm.

Figure 2

(A) ICP of Nano-HA (B) Hysteresis curve results (C) Contact angle (D) Porosity (E) Composite contact angle (F) Composite SEM image. (G) T1-weighted images and parametric mapping of HA/PLGA, 0.5Cu@HA/PLGA, 0.5Gd@HA/PLGA, and 0.5Cu/Gd@HA/PLGA.
Figure 3

(A) Morphology of the MC3T3-E1 cells co-cultured with the composite material for 24 h was observed using phalloidin/DAPI staining, scale bar = 20 μm. Calcein((B)) staining and immunofluorescence intensity quantitative analysis of Cu/Gd@HA/PLGA composites co-cultured with MC3T3 cells at day 1 (C) and day 3 (D). (E) Proliferation of MC3T3-E1 cells cultured in suspensions of HA, 0.5Cu@HA, 0.5Gd@HA, and 0.5Cu/Gd@HA nanoparticles at different concentrations for 24 h. (F) The proliferation efficiency of MC3T3-E1 cells co-cultured with HA/PLGA was measured at 1, 3, and 7 days using the CCK-8 method. One-way analysis of variance was used to analyze the differences among multiple groups, and the results are expressed as the mean ± SD. *P < 0.05.
PCR results (A-1) (A-2) and protein fluorescence results (B) of BMP-2 and COL-1 in MC3T3-E1 cells cultured on the Cu/Gd@HA/PLGA composites for 14 days, and the differences in the fluorescence quantitative results (C-1) (C-2) among multiple groups were analyzed using one-way ANOVA. The results are expressed as the mean ± SD. *P < 0.05.
Figure 5

Macroscopic and microscopic observations of the MC3T3-E1 cells cultured on the Cu/Gd@HA/PLGA composite membrane for 7 and 14 days (A) and alizarin red staining after 14 and 21 days (B) (100×).
Figure 6

(A) HE/MASSON staining results of the composites implanted in animals and cultured \textit{in vivo} at 4 and 8 weeks. NB, new bone; OB, old bone; MT, the implant material. Sirius red staining results of the composites implanted after 4 and 8 weeks of \textit{in vivo} culture (scale bar = 100 μm). Sirius red staining was used to observe the distribution of collagen in the bone (scale bar = 20 μm). Red triangle = new collagen; orange triangle = composite material; blue triangle = old collagen.
Figure 7

Protein immunofluorescence results of the composites implanted in the animals and cultured for 4 and 8 weeks. The expression of BMP-2 and OCN in different experimental groups was analyzed via immunofluorescence staining at 4 and 8 weeks. Scale bar = 50 μm. One-way analysis of variance was used to analyze the differences among multiple groups, and the results are expressed as the mean ± SD. *P < 0.05.
Figure 8

Results of the staining of the heart, liver, spleen, lung, kidney, and brain tissues of the animals in which the composite materials were implanted after 8 weeks of culture. Scale bar = 20 μm.