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A 3D graphene coated bioglass scaffold for bone defect therapy based on molecular targeting approach

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Development of a cell-free scaffold with excellent mechanical properties and osteoconductivity is a significant need for bone regeneration. Herein, a reduced graphene oxide (rGO) functionalized hierarchical macro-mesoporous bioactive glass scaffold integrated with osteoblast-specific aptamer is rationally designed to recruit and induce rapid differentiation of osteoblasts for bone regeneration. The scaffold exhibits a macroporous structure with fully interconnected open pores and shows excellent mechanical properties with Young’s modulus of ~80 KPa, which provides a strong scaffold to support the growth of osteoblasts and bone tissue regeneration. Furthermore, the scaffold displays good performance in accelerating osteoblast differentiation and promoting newly bone formation. The osteoblast recruitment is achieved since the osteoblast-specific aptamer can specifically target osteoblasts with strong binding affinity. Micro-computed tomography and histological tests confirmed that the large bone defects fully heal with new plate-like-pattern bone appearing both peripherally and centrally, suggesting the outstanding bone regeneration performance of this cell-free and graphene functionalized scaffold. Considering the promising bioapplications of the graphene functionalized bioactive glass scaffold with osteoblast recruitment capacity, our strategy paves a way for the design of new bioactive functional materials for tissue regeneration and shows attractive prospects in targeted therapy.

Introduction

Reconstruction of large bone defects resulting from trauma, infection and tumors remains a medical challenge. Concerning the risk of disease transfer, infection and chronic pain associated with autograft, synthetic bone substitute materials that are able to mimic the structural and biological properties of natural bone have been drawing a significant attention for bone regeneration in current clinical treatments. Compared with traditional bone substitutes, tissue engineering approaches aim to aid the regeneration of bone tissue by filling the bone defects with an osteoconductive scaffold loaded with growth factors and osteogenic cells (mesenchymal stem cells/osteoblasts). Osteoblasts are cells which originate in the bone marrow and contribute to the production of new bone. They are responsible for bone matrix synthesis and also play a role in subsequent mineralization. However, immune rejection, source limitation, pathogen transmission, and issues with packaging prevent the therapeutic use of cell-loaded scaffolds. Cell-free approaches utilizing a surface functionalized bioactive glass scaffold to induce bone regeneration by endogenous osteoblasts may provide a suitable alternative to improve bone regeneration. For enhancing the efficacy of bone regeneration, synthetic scaffolds need to capture osteoblasts and exhibit sufficient mechanical strength to withstand physiological loading, thereby providing a microenvironment suitable for osteoblasts to proliferate, differentiate and mineralize.

Graphene, the new allotrope of carbon, is defined as a two-dimensional honeycomb lattice composed of monolayer carbon atoms. Graphene and its derivatives have received tremendous attention in the field of materials science. The unique physicochemical and structural properties (e.g., high elasticity, excellent mechanical strength, and large surface area) of graphene make it attractive for application in composite materials. Due to its high Young’s modulus (0.5–1 TPa), the incorporation of graphene into brittle materials can surely improve their mechanical properties. It is worth noting that graphene induces no obvious toxic effects in vivo and exhibits favorable biocompatibility. Thus, graphene has emerged as an attractive bioactive component of biomaterials for tissue engineering. Furthermore, the ability of graphene to accelerate the osteogenic differentiation of mesenchymal stem cells and osteoblasts, as shown in recent studies, render it attractive for researchers in bone tissue engineering. In vitro studies demonstrated that graphene supports the proliferation of mesenchymal stem cells/osteoblasts and accelerates their differentiation into mature osteoblasts. Taking
into consideration both its excellent mechanical properties and favorable biocompatibility, graphene is a promising material in combination with synthetic scaffolds to enhance their mechanical strength and improve their bioactivity. In addition, reduced graphene oxide (denoted as rGO), which is usually denominated as chemically modified graphene, is rich in carboxyl groups on its surface. These functional groups provide reactive handles for surface modification to add biochemical functionality, such as recognition of specific targets. Similar to antibodies, aptamers, which are single-stranded DNA/RNA oligonucleotides, function primarily in molecular recognition, and have the capacity to recognize and bind with target molecules with high affinity and specificity. Aptamers have the additional advantages of small size, low production cost, facile chemical modification, low immunogenicity, low batch-to-batch variation, high chemical stability, rapid tissue penetration, and no toxicity. In order to enrich endogenous osteoblasts in the bone defect, an osteoblast-specific aptamer can be immobilized on the bioactive glass scaffold to give it capacity to capture endogenous osteoblasts around the bone defect.

This paper reports the use of rGO for surface coating of hierarchically macro-mesoporous bioactive glass (denoted as MBG) to construct a new three-dimensional bioactive porous scaffold. The raw material, MBG, is a biocompatible bone regeneration scaffold, which is capable of stimulating bone growth by its dissolution products and rapid bonding with the host bone without causing fibrous encapsulation. Graphene coating successfully improved the mechanical properties of MBG to support loads experienced by the newly formed bone in large bone defects. This architecture could provide a microenvironment to satisfy the requirements for osteoblasts to proliferate, deposit bone extracellular matrix and generate new bone formation.

Results and discussion

As shown in Scheme 1a, a MBG scaffold with hierarchically macro-mesoporous structure was first produced by the sol-gel method. Then, it was coated with rGO sheets. Finally, to achieve osteoblast enrichment in scaffolds, the scaffold surface was modified with osteoblast-specific aptamer CH6. This rGO-coated and aptamer-immobilized MBG scaffold (denoted as rGO-MBG-AP) was implanted in the bone defect, and it successfully fulfilled the requirement to capture osteoblasts to the scaffold surface (Scheme 1b). Furthermore, the rGO-coated and aptamer-immobilized MBG (denoted as rGO-MBG-AP) improved the mineralization of osteoblasts, as shown in a study of the interaction between the scaffold and rat femur osteoblasts. Through surface modification by rGO and osteoblast-specific aptamer, the bone substitute scaffold with sufficient mechanical strength displayed specific recognition to recruit osteoblasts, and achieved the integration of osteoblast enrichment, differentiation and bone tissue regeneration. Considering the promising bioproperties of graphene and the specific recognition of an aptamer for its target, our strategy paves the way to design new bioactive functional materials for tissue regeneration and shows attractive application prospects for targeted therapy.

![Scheme 1](image)

Scheme 1 (a) Overview of rGO-MBG-AP in critical bone defect repair. (b) Schematic diagram of the scaffold construction.
Fig. 1 (a) The highly magnified SEM image of MGB, (b) rGO-MBG, (c) rGO-MBG-AP surfaces. (d) The corresponding EDS spectra of MGB, (e) rGO-MBG, and (f) rGO-MBG-AP. (g) The atomic percent of C and P in the MBG, rGO-MBG, and rGO-MBG-AP scaffolds. (h) Low magnification of SEM images of rGO-MBG-AP. Inset: diagram of rGO-MBG-AP scaffold. (i) TEM image of the rGO-MBG-AP, Inset: diagram of rGO-MBG-AP scaffold. (j) Young’s Modulus of the MBG and MBG with different compositions of rGO. Inset: photographs of MBG and rGO-MBG-AP withstood 50 grams and 100 grams, respectively.

area can withstand the weight of 100 g without collapsing, while the MBG with the same surface area collapsed under 50 grams. Thus, the rGO-MBG-AP exhibited superior mechanical properties compared with MBG, demonstrating that graphene plays an important role in enhancing the mechanical properties of rGO-MBG-AP. Furthermore, the compressive strength of rGO-MBG-AP containing different amounts of rGO was measured (Fig. 1j and Fig. S8). The compressive Young’s modulus of the rGO-MBG-AP was ~80 KPa, while the MBG without rGO coating was ~2 KPa. In addition, the compressive Young’s modulus of the rGO-MBG-AP was significantly enhanced as the mass fraction of rGO increased, thus providing a stronger scaffold to support the growth of recruited osteoblasts and the newly formed bone tissue.

The cytotoxicity of the scaffold in vitro was visualized using a fluorescent live/dead assay. Live cells were stained green and appeared to adhere well on the scaffold, while dead cells were stained red and rounded. Little red cells were found in rGO-MBG-AP 3 days after seeding (Fig. 2a). Quantitative analysis based on the dead/live staining further verified the high survival rate of osteoblasts in the scaffolds (Fig. S10). Since cell morphology alteration is the first and most readily noticeable effect following exposure of cells to toxic materials, the morphology of cells in the rGO-MBG-AP was then observed. Phalloidine staining showed that cells had a osteoblast-like morphology (polygonal or spindle cells) with good attachment to the rGO-MBG-AP surface (Fig. 2b), verifying that the rGO-MBG-AP has little influence on the morphology alteration of osteoblasts. The proliferative activity of cells seeded in the rGO-MBG-AP scaffold was investigated at 1, 3 and 5 days. The number of cells increased as time progressed (Fig. 2c), suggesting that the cells maintained good proliferative activity after seeding in the rGO-MBG-AP.

It has been demonstrated that graphene can accelerate the osteogenic differentiation of mesenchymal stem cells and osteoblasts. Therefore, real-time PCR was performed on rat osteoblasts (rOBs) in the scaffolds to investigate the osteoblast differentiation in the rGO-MBG-AP scaffold. The total RNA of rOBs in the scaffolds after 7 and 14 days osteogenic induction was extracted to evaluate the mRNA expression level of four osteoblast markers, collagen type I alpha 1 (Col1a1, an important component of bone extracellular matrix), alkaline phosphatase (ALP, the enzyme playing an essential role in bone mineralization), runt related transcription factor 2 (Runx2, an essential protein for osteoblastic differentiation and skeletal morphogenesis) and bone gamma-carboxyglutamate protein (Bglap, a protein function in bone remodeling). As shown in Fig. 2d-2g, Col1a1, ALP, Runx2 and Bglap were significantly upregulated in the rGO-MBG-AP group at different levels when compared with those in MBG group, demonstrating that rGO-MBG-AP scaffolds can stimulate bone-related gene expression of rOBs. It is commonly agreed that the in

Fig. 2  (a) Confocal microscope images of dead/live cell staining in the rGO-MBG-AP scaffold. (b) Confocal microscope images of the cell morphology in rGO-MBG-AP scaffold. (c) The proliferation of rOBs in rGO-MBG-AP scaffolds. The expression of (d) Col1a1, (e) ALP, (f) Runx2, and (g) Bglap in rOBs on the MBG, rGO-MBG and rGO-MBG-AP scaffolds at days 7 and 14. (h) ALP activity of rOBs cultured in the MBG, rGO-MBG and rGO-MBG-AP scaffolds at days 7 and 14. (i) Illustration of osteoblast differentiation accelerated by rGO. All data represent the mean values ± standard deviation (*p < 0.05).
In this case, it is speculated that dexamethasone and β-glycerolphosphate was reported to improve the expression levels of proteins and enzymes needed in osteoblast osteogenic inducers functioned on immature osteoblasts to hydroxyapatite (HA) nanocrystals increased as the soaking time increased from 1 to 7 days. The X-ray diffraction (XRD) pattern of rGO-MBG-AP showed the characteristic peaks of the crystalline HA phase after immersing in SBF for 7 days (Fig. S14). It was observed that more apatite microparticles were present on the rGO-MBG-AP scaffold compared to the amount on the MBG scaffold surface after immersion in SBF for 7 days (Fig. S15), demonstrating that the rGO-MBG-AP scaffold has better in vivo bone-forming bioactivity than MBG. The ALP activity of rOBs in the scaffolds after culture in osteogenic medium was illustrated in Fig. 2h, and the rGO-MBG-AP scaffold showed higher ALP/Total protein ratio than that of MBG scaffold at days 7 and 14. Since ALP plays an important role in calcification during bone formation, our results indicate that the rGO-MBG-AP scaffold has better performance in inducing the calcification of rOBs. Recent studies have shown that rGO can act as a preconcentration platform for dexamethasone and β-glycerolphosphate, and the increase in concentration of dexamethasone and β-glycerolphosphate was reported to improve the expression levels of proteins and enzymes needed in osteoblast differentiation. In this case, it is speculated that dexamethasone and β-glycerolphosphate were first preconcentrated in the rGO-MBG-AP scaffold (Fig. 2i, left), and then the increased local osteogenic inducers functioned on immature osteoblasts to increase the osteoblast markers (Fig. 2i, middle). Furthermore, more hydroxyapatite was deposited on the rGO-MBG-AP scaffold as illustrated in the right part of Fig. 2i, thus providing a better osteogenic microenvironment for new bone formation. These results demonstrated that the rGO-MBG-AP holds great promise in accelerating osteoblast differentiation and mineral deposition in vitro and such bioactivity studies verified the great osteoconductivity of this scaffold.

Because an osteoblast-specific aptamer named as CH6 was conjugated to the rGO-MBG scaffold for faster recruiting of the immature osteoblasts, fluorescent microscopy and flow cytometry were used to verify the specific binding of CH6 aptamer and osteoblasts. As shown in Fig. 3a, the cells treated with this osteoblast-specific aptamer CH6 showed strong fluorescence. In comparison, the cells treated with FITC-labeled random ssDNA showed much less binding to osteoblasts (Fig. 3b), confirming the targeting role and high affinity of CH6 aptamer. The targeting specificity of CH6 aptamer toward rOBs was also confirmed by flow cytometry (Fig. S16). When CH6 aptamer was incubated with rOBs at 37 °C, strong fluorescence intensity was observed, demonstrating that CH6 aptamer can bind to rOBs with high binding affinity. In contrast, when incubated with fibroblasts, no fluorescence was observed. Therefore, CH6 aptamer exhibited stronger specific binding affinity for rOBs compared to random cells. What’s more, when CH6 aptamer was cultured with whole bone marrow, a slight fluorescence enhancement was observed compared to the fibroblasts group, indicating that CH6 aptamer can specifically bind with rOBs from whole bone marrow. It is widely accepted that recruitment of osteoblasts to the defect site is essential in the regeneration of bone defect, and that lack of osteoblast recruitment leads to poor healing. Thus, realization of osteoblast recruitment is a promising strategy to improve bone formation in bone defects. In this case, rOBs cultured from femoral cancellous bone were utilized to test the specific aptamer binding, and the rOBs recruitment ability of rGO-MBG-AP was performed by the transwell assay. As shown in Fig. 3c, the number of cells which migrated to the rGO-MBG-AP scaffold was much higher than the number in the rGO-MBG scaffold (Fig. 3d) and MBG (Fig. 3e). The statistical data showed that there were 108 ± 6 rOBs in the rGO-MBG-AP scaffold, while only 74 ± 6 rOBs were in the rGO-MBG scaffold (Fig. 3f). Therefore, the addition of osteoblast-specific aptamer CH6 has significantly improved the osteoblast migration toward the scaffold (p < 0.05), verifying that the rGO-MBG-AP scaffold possesses an outstanding capability for osteoblasts recruitment.

In vivo studies were carried out to evaluate the bone regeneration ability of this scaffold. Sprague Dawley rats were used to investigate the bone regeneration abilities of rGO-MBG-AP scaffolds (Fig. S17). A skin incision was made in the distal femoral epiphysis and blunt dissection was performed on the muscles to expose the femoral condyle. Then, an anteroposterior bicortical channel with a diameter of 2.8 mm was created in the rat femur perpendicular to the shaft axis, and the cancellous trabecular bone was destroyed using a trephine burr (left side of Fig. S17). The scaffolds were prepared to fit the defect size, and the macroanatomy of the bulk material is shown in the middle of Fig. S17. After that, the drilled defects were rinsed by injection with normal saline to remove bone fragments from the cavity. The
micro-architectural parameters were collected to show the parameters, including the ratio of bone volume/tissue volume mineralization of new bone formation within defects. The 3D morphology of newly regenerated bone and to quantify the tomography (μ-CT) to assess new bone formation. The bone defects were removed from the rats and examined by micro-computed tomography (μ-CT) to assess new bone formation. The bone defects treated with MBG and nothing were applied as control groups. After three dimensional (3D) reconstruction, μ-CT images and micro-architectural parameters were collected to show the morphology of newly regenerated bone and to quantify the mineralization of new bone formation within defects. The 3D reconstruction images show obvious differences in the defects treated with rGO-MBG-AP, rGO-MBG, MBG, and nothing. After 8 weeks healing, there was minimal new bone formation within the defect treated with nothing and the bone defect remained almost empty, indicating that the bone defect could not be regenerated by self-healing (Fig. 4a). As shown in Fig. 4b, when the bone defect was treated with MBG, there was newly regenerated bone around the defect border. However, the center of the defect still remained empty. It is obvious that the defects treated with MBG showed slightly increased new bone volume compared to the untreated defect. As shown in Fig. 4c, when the defect was treated with rGO-MBG, there was newly regenerated bone around the bone defect. Compared with the defect treated with MBG, the defect showed slight increased new bone formation in the bone defect, indicating that rGO possesses a potential for improving bone regeneration. Significantly, the bone defect treated with rGO-MBG-AP was found to fully heal with new plate-like-pattern bone appearing both peripherally and centrally, as shown in Fig. 4d. The amount of newly formed bone in the rGO-MBG-AP group was significantly higher than that in either the MBG group or rGO-MBG group. These results demonstrated that rGO-MBG-AP exhibited best performance in bone regeneration, largely due to the osteogenic induction of rGO and the high affinity of osteoblast-specific aptamer with rOBs. In accordance with the image observation, 3D micro-architectural parameters, including the ratio of bone volume/tissue volume (BV/TV), trabecular number (Tb. N), and trabecular separation (Tb. Sp), were assessed as quantitative indicators of new bone formation. BV/TV represents the amount of trabecular bone within the bone defect area and is evidence of new bone formation.40 The BV/TV of the rGO-MBG-AP group was the highest among four groups, indicating that the amount of trabecular bone in this group was maximum (Fig. 4e). Tb. N is an indicator of bone density.41 As shown in Fig. 4f, the rGO-MBG-AP group exhibited the highest Tb. N, together with the highest trabecular bone amount shown in Fig. 4e, demonstrating that the rGO-MBG-AP group has the highest bone density. Tb. Sp represents the spacing between bone trabecula and is another indicator of bone density.42 The trabecular microstructure of rGO-MBG-AP was found to be the best, because the Tb. Sp of this scaffold is the lowest among all groups in Fig. 4g, indicating a much more thickened regrown cancellous bone compared to other groups.

Histomorphological analysis by hematoxylin & eosi (H&E) staining was carried out to evaluate bone regeneration within the defect site. The new bone formation induced by the defect treated with rGO-MBG-AP was confirmed by the histological evaluation after 4 and 8 weeks implantation. In the histological image acquired in the fourth week, no appreciable bone formation was observed in the blank group treated with nothing (Fig. 5a). Moreover, the defect center of the blank group was filled with bone marrow-like tissue instead of new bone in the eighth week, demonstrating that the self-healing ability of bone defect is not sufficient (Fig. 5b). When the bone defect was treated with MBG for 4 weeks, loose connective fibrous tissues were largely populated in the defect site, and no obvious new bone formation was obtained (Fig. 5c). Compared with the results in the fourth week, new bone tissues were found to be dispersed and not connected with each other after treatment with MBG for 8 weeks (Fig. 5d). Meanwhile, the MBG scaffold failed to maintain its structure and degraded into small pieces due to its brittleness and fragility. Thus, the poor mechanical strength of MBG cannot provide a stable support for new bone regeneration. As for the rGO-MBG group, newly formed bone tissue can be observed in the defect area in the fourth week (Fig. 5e). An increasing volume of new bone tissues was observed after eight weeks healing, and a number of well mineralized bone tissues were formed (Fig. 5f). Newly formed bone tissues were much more arranged than those in the MBG group and lined beside the rGO-MBG surface. Therefore, the addition of rGO significantly improved the mechanical property of the scaffold to support the growth of newly regenerated bone and had a good performance in improving the bone regeneration. Notably, when the bone defect was treated with rGO-MBG-AP, a large scale of bone tissue and mineralized bone matrix deposition were displayed in the fourth week as illustrated in Fig. 5g. After 8 weeks implantation, the H&E staining of the group treated with rGO-MBG-AP showed that the defect was mainly filled with well-arranged new bone tissue and mineralized bone matrix deposition (Fig. 5h). These newly formed bone tissues directly integrated with the rGO-MBG-AP scaffolds (Fig. 5h, blue stars). Compared with the defect treated with MBG and rGO-MBG groups, much more new bone tissue along the rGO-MBG-AP interface was observed. In addition, an increased amount of osteoblastic cells and matrix were obtained in the defect treated with rGO-MBG-AP. The maximum new bone observed beside the
In this work, a graphene functionalized 3D bioactive glass scaffold with ability to recruit osteoblasts was constructed for regeneration of bone defects. The osteogenic differentiation of the osteoblasts was efficiently upregulated through the osteogenic inducer preconcentration of the rGO coating in the rGO-MBG-AP scaffolds. The mechanical strength of the scaffold has been significantly improved through rGO coating, thus providing a strong scaffold to support the growth of osteoblasts. Since the scaffold exhibited no negative effect on cell attachment, morphology and proliferation, it appears to be attractive for bioapplication. The osteoblast-specific aptamers were effectively immobilized on the surface of the 3D hierarchical structure, and osteoblast recruitment was achieved since the aptamers can specifically target osteoblast cells with high affinity. The rGO-MBG-AP scaffold exhibited outstanding results for bone regeneration in critical femur bone defects through recruiting and osteogenic induction of osteoblasts. To conclude, by applying rGO and aptamer in the 3D structure of MBG, increased mechanical stability and better osteogenic capacity were successfully obtained. This strategy can serve as an effective approach for cell recruitment in tissue engineering and is anticipated to find broad application in regenerating new tissues and organs.

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Notes and references


