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

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Experimental Details

Chemicals. Calcium nitrate tetrahydrate ($Ca(NO_3)_2 \cdot 4H_2O$), tetraethyl orthosilicate (TEOS), triethyl phosphate (TEP), nonionic block copolymer $EO_{20}PO_{70}EO_{20}$ (P123), 1-ethyl-3-(3-dimethyllamino-propyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), Phalloidin-FITC, Dexamethasone (≥98%), Ascorbic acid (≥99.0%), β-glycerophosphate were purchased from Sigma-Aldrich. Polyurethane foam (PUF) was obtained from Alibaba Group. Natural graphite flakes were purchased from Qingdao Jinrilai Company. Sodium azide (NaN_3), magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$, analytical pure), paraformaldehyde [$(CH2O)_n$), analytical pure], ethylene diamine tetraacetic acid (EDTA, analytical pure) were provided by Sinopharm Chemical Reagent Company. Calcein-AM, and CCK-8 assays were purchased from Dojindo Molecular Technologies. 4', 6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) were provided by Beyotime Institute of biotechnology. The dulbecco's phosphate buffered saline (D-PBS), α-modified eagle's medium (α-MEM) and fetal bovine serum (FBS) were obtained from Thermo Fisher Scientific Inc.

Aptamers. Aptamers were all synthesized on an ABI3400 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA, USA). The as synthesized aptamers were purified by HPLC (Agilent, 1260 GC, Japan) with a C-18 column.[1]

FITC-labeled Osteoblast specific aptamer:

5'-FITC-AGTCTGTTGGACCGAATCCCGTGGACGCACCCTTTGGACG-3'

Amino-modified osteoblast specific aptamer:

5'-$(A)_9$-NH_2-AGTCTGTTGGACCGAATCCCGTGGACGCACCCTTTGGACG-3'

FITC-labeled Random ssDNA:

5'-FITC-GGGAGCTCAGCCTAAACGCTCAAGGATCGTTCGCAACGGTTCG-3'

Fabrication of hierarchically porous bioactive glass scaffolds

Calcium nitrate tetrahydrate ($Ca(NO_3)_2 \cdot 4H_2O$), tetraethyl orthosilicate (TEOS), triethyl phosphate (TEP), nonionic block copolymer $EO_{20}PO_{70}EO_{20}$ (P123), and Polyurethane foam (PUF) were used in this process. The hierarchically porous bioactive glass scaffolds were synthesized by using the nonionic block copolymer $EO_{20}PO_{70}EO_{20}$ (P123) and polyurethane foam (PUF) with an open porous structure as the templets for mesoporous and macroporous. Firstly, mesoporous bioactive glass (MBG) sol was prepared through an evaporation-induced self-assembly process following a previously reported method by Zhao et al.[2] Afterwards, PUF was completely immersed into the sol and stirred for 10 minutes. The MBG sol loaded PFU was then removed into a Petri dish and the excess sol was squeezed out, followed by a drying process at 30 °C for one day. After the sample was completely dry, it was calcined in air at 700 °C for 5 h to remove the templets of PUF and block copolymer.

Preparation of rGO coated MBG scaffolds (rGO-MBG)

GO was prepared and purified from natural graphite flakes according to a previously reported modified Hummers' method.[3] Then, a series of concentrations of GO suspension (1 mL, 0.8, 1.6, 2.4, 3.2, 4, 4.8 mg mL^{-1}) were mixed with ascorbic acid (1.6, 3.2, 4.8, 6.4, 8, 9.6 mg) and dispersed under ultrasound for 10 min. The MBG scaffold (7 × 7 mm surface area and 4 mm

height) was soaked in the GO/ascorbic acid suspension for 10 min. Afterwards, the soaked scaffolds were heat-treated at 90 °C for 1.5 h to reduce the GO into reduced GO (denoted as rGO)[4], and the rGO was coated on the MBG scaffold through the heat-treated reduction process. The resulted rGO-MBG scaffolds were weighted. Finally, we used the mass fraction to determine the percentage of rGO, and calculated that the mass fraction of the successfully coated rGO was 1%, 2%, 3%, 4%, 5%, and 6%, respectively.

Conjugation of rGO-MBG with osteoblast specific aptamer

Typically, a single rGO-MBG scaffold was immersed in 2 mL of activation buffer (0.1 M MES, pH=6) containing 8 mg of EDC and 12 mg of NHS. After reacting for 15 min at room temperature, the carboxyl groups on the surface of rGO were activated. The activation buffer was removed and the material was washed with the coupling buffer (0.1 M PBS, pH=7.2) for 2 times and then added 2 mL of PBS. For the conjugation of aptamer, 4 nmol of amino-modified osteoblast specific aptamer was added, and reacted for 12 h in a reciprocating oscillator. The aptamer modified rGO-MBG scaffold was purified by washing with coupling buffer twice.[5]

Sample characterization.

Transmission electron microscopy (TEM) images were recorded on a by a JEM–2100 transmission electron microscope (JEOL) with an accelerating voltage of 200 kV. The morphology and element mapping of the materials was characterized by a field emission scanning electron microscope (Sigma, Zeiss, Germany). Mechanical measurements on MBG and rGO-MBG-AP were carried out by a single-column static instrument (Instron 5843) equipped with two flat compression stages and a 10 N load cell. The rGO-MBG-AP scaffolds with 7 × 7 mm surface area and 4 mm height were used to perform the mechanic test. Three tested specimens for each type of scaffold were used for parallel group. The compression speed was 0.08 mm s^{-1}. The wide angle X-ray diffraction (XRD) of the materials was carried out using a D8 Advance X-ray diffractometer (Bruker) with a Cu-Kα radiation (λ = 1.5406 Å).

The observation of cell viability and morphology was carried on confocal laser scanning microscope (FV1200, Olympus, Japan). The optical density was measured on a plate reader (PowerWave XS2, BioTek, USA). The femurs were evaluated by the μ-CT imaging system (μ-CT50, Scanco Medical, Switzerland) at 70 kVp, 114 μA, 24 μm. The binding of FITC-labeled aptamers and rat osteoblasts (rOBs) were verified by an Olympus D71 fluorescence microscope (Olympus Co, Japan) and by FACSCalibur (Becton, Dickinson and Company, USA). The histological analysis was recorded by an Olympus DP72 microscope (Olympus, Japan).

The isolation of primary rat osteoblasts

The rat osteoblasts used in this experiment were isolated from rat femur as Heidi Declercq described[6] with some modification. Briefly, skin, soft connective tissue were removed. Then the epiphysis was cut free of epiphyseal cartilage. Then the cancellous bone in the epiphysis was cut into pieces of around 2 × 2 mm^2 and washed for several times. Then the bone pieces were incubated for 30 min at 37 °C on the bottom of T25 tissue culture dish and left to adhere before culture medium (α-MEM containing 10% FBS) was added. Approximately 20 bone pieces were put in every T25 tissue culture dish and were cultured until confluence in the incubator at 37 °C, 95% air and 5% CO_2.

***In vitro* cellular biocompatibility of rGO-MBG-AP scaffolds**

Each scaffold placed in 48-well culture plates was added 10^5 of rOBs. The cells were then incubated in culture medium at 37 °C in a 5% CO_2 incubator. To investigate the attachment and morphology of rOBs in rGO-MBG-AP (Length of side: 8 mm, height: 5 mm), the scaffold was fixed with 4% paraformaldehyde after cultured with rOBs for 3 days. Then the cytoskeleton and nuclei were stained with phalloidin-FITC and DAPI, respectively. Confocal images were obtained by a confocal laser scanning microscope. The rOBs were cultured in scaffolds for 3 days, then fixed with 4% paraformaldehyde.

For cell cytotoxicity assays, the rOBs cultured on scaffolds for 3 days were stained with 3',6'-Di(O-acetyl)-4',5'-bis[N,N-bis(carboxymethyl) aminomethyl]fluorescein, tetraacetoxymethyl ester (Calcein-AM) and propidium iodide (PI) at the same time, and then observed by confocal laser scanning microscope.

The cell proliferation of rOBs on scaffolds was evaluated by CCK-8 assays. The scaffolds are shifted to a new 48-well plates, then 500 μL of CCK-8 mixture (reagent: culture medium = 1:10) was added into each well for 4 hours. Then the mixture was extracted for reading at 450 nm on a plate reader. The assays are carried out on 1, 3 and 5 days, normal culture medium was added into the wells after each test.

***In vitro* osteogenic differentiation of rOBs in rGO-MBG-AP scaffolds**

For osteogenic differentiation assays, the rOBs were cultured to the 2–4 passage and then each scaffold placed in 48-well culture plates was added 10^5 of rOBs. The cells were then incubated in osteogenic inducing medium (α-MEM containing 10% FBS, 10 nm dexamethasone, 50 μg mL^{-1} ascorbic acid, and 10 mm β-glycerophosphate) and changed every 3 days at 37 °C in a 5% CO_2 incubator. The osteogenic related gene expression of rOBs in the scaffolds was evaluated at days 7 and 14. The total RNA was extracted using the AxyPrep™ Multisource Total RNA Miniprep Kit (Axygen, USA) under the instruction. Then 1 μg of total RNA was utilized to synthesize the complementary DNA following the manufacturer's protocol using a First strand cDNA synthesis kit (GeneCopoeia, China). The alkaline phosphatase (ALP), collagen I (Col1a1), runt-related transcription factor 2 (Runx2) and osteocalcin (OCN) was chosen to evaluate the osteogenic property of rGO-MBG-AP. The glyceraldehyde-3-phosphate -dehydrogenase (GAPDH) was used as a control.

The alkaline phosphatase activity of rOBs in the scaffolds was detected at day 7 and 14 to investigate the osteogenic differentiation. ALP activity was evaluated using the PNPP method.[7] Cell lysates were fixed for 30 min with 2-amino-2-methyl-1-propanol. p-Nitrophenylphosphate and $MgCl_2$ in darkness. Then the reaction was terminated by NaOH.

The absorbance was measured by a spectrophotometer at 405 nm. The ALP levels were normalized according to the total protein content.

***In vitro* assays in SBF**

In vitro assays were performed soaking the rGO-MBG-AP scaffold in SBF at 37 °C for 3, 5 and 7 days (containing 142 mM Na^+, 5.0 mM K^+, 1.5 mM Mg^{2+}, 2.5 mM Ca^{2+}, 147.8 mM Cl^-, 4.2 mM HCO_3^-, 1.0 mM HPO_4^{2-}, 0.5 mM SO_4^{2-}). The chemical composition was similar to that of human plasma and the solution had a PH of around 7.3.

The binding of aptamer and rat osteoblasts

10^5 osteoblasts were seeded onto glass slides one day before imaging. Cells were washed with phosphate buffered saline (PBS) and incubated with 10 nM FITC-labeled aptamer in 200 μL buffer A (adding 1 mg mL^{-1} BSA and 10% FBS into D-PBS containing 4.5 mg mL^{-1} glucose and 5 mM $MgCl_2$) for 1 h at 37 °C. After three washes with buffer B (Adding 0.1% NaN_3 into D-PBS containing 4.5 mg mL^{-1} glucose and 5 mM $MgCl_2$), cells were imaged using a fluorescence microscope.

10^6 osteoblasts were trypsinized and the cells were centrifuged at 1000 rpm for 5 minutes, then the cells were washed with PBS for three times. Then the cells were incubated with 10 nM FITC-labeled aptamer in 200 μL buffer A for 1 hour at 37 °C. After three washes with buffer B, cells were evaluated using a flow cytometry. 5 mL of fresh rat bone marrow was lysed with ammonium chloride then incubated with 10 nM FITC-labeled aptamer in 200 L buffer A for 1 hour at 37 °C as control.

***In vitro* cell migration assay**

The ability to recruit osteoblasts of rGO-MBG-AP scaffold was assessed using a Transwell system (Costar, 24-well plate, 8 μm) as reference.[8] Briefly, 10^4 cells resuspended in 100 μL of serum-free DMEM was placed in the upper chamber, and the scaffold with 200 μL of DMEM containing 10% FBS was placed in the lower chamber for 36 h. Then the cells was

fixed with 4% paraformaldehyde and stained with crystal violet. The results were exhibited as mean number of cells per field ± standard deviation.

***In vivo* bone regeneration of aptamer modified scaffolds by using rat model**

The critical femoral defect model in Sprague Dawley rat was used to investigate the bone regeneration ability of MBG, rGO-MBG and rGO-MBG-AP scaffolds. The femoral defect was made as described before.[9] The scaffolds were gently filled into the bilateral femoral defects of 2.8 mm in diameter and about 4 mm in height. After 4 and 8 weeks healing, the rats were sacrificed. The rats were divided into five groups (blank defects with no scaffold were used as control group) with each 4 samples reduplicated for a total of x animals per time point. All surgical procedures used in this experiment were approved by the Ethics Committee for Animal Research, Wuhan University, China.

μ-CT analysis of bone regeneration in the femoral defect

After the rats were sacrificed at each time point, the femoral area was removed and fixed in 4% paraformaldehyde for 24 h at 4 °C. The new bone regeneration was evaluated by the μ-CT imaging system as previously described.[10] In brief, the femoral samples were put in the position that the long axis of the drilled channel was perpendicular to the axis of the X-ray beam. The scanning parameters, reconstructed parameters and analysis parameters were entered into the μ-CT scanner according to our previous studies.[11] The region of interest (ROI) of 2.8 mm diameter region was defined to evaluate the level of bone regeneration. Besides, the mineralized bone tissue was differentially segmented with a fixed low threshold (value = 184) to exclude the interference of non-mineralized tissue and the scaffolds themselves. After 3D reconstruction, the bone volume fractions (BV/TV), trabecular number (Tb. N) and trabecular separation (Tb. Sp) were automatically collected and analyzed using the built-in software of the μ-CT.

Histological and immunohistochemical analysis

Following μ-CT scanning, femoral samples were immersed in 10% EDTA which was changed twice weekly for 4 weeks until decalcification, then gradient dehydrated for embedding in paraffin. Serial sections of 5 mm were cut and mounted on polylysine-coated slides and then H&E staining was performed.

For immunohistochemical assessment, the expression of Col1a1 which is expressed in the osteoid matrix was detected according to the following procedure. Following the process of deparaffinating, rehydrating and washing with PBS, the sections were incubated with 0.3% hydrogen peroxide for 20 min followed by incubation with BSA. Then the sections were incubated with the primary antibody for Col1a1 (1:100; Boster SA2005, Boster Co., China) overnight at 4 °C. In accordance with the manufacturer's protocol, the sections were incubated using the SP 9000 immunohistochemical kit (Zhongshan Biotechnology Co., Ltd, China) and visualized by 3,3-diaminobenzidine tetrahydrochloride (DAB) (Zhongshan Biotechnology Co., Ltd, China). Lastly, the sections were counterstained with hematoxylin. Finally, the images of all stained sections were captured with a light microscope.

References

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Supplementary Figures

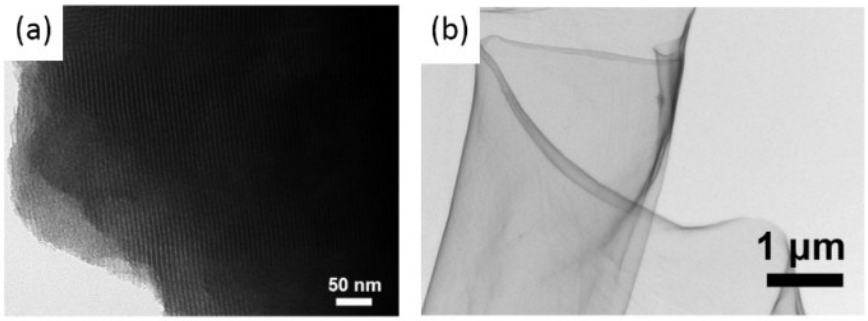

Fig. S1 TEM image of MBG(a) and rGO(b).

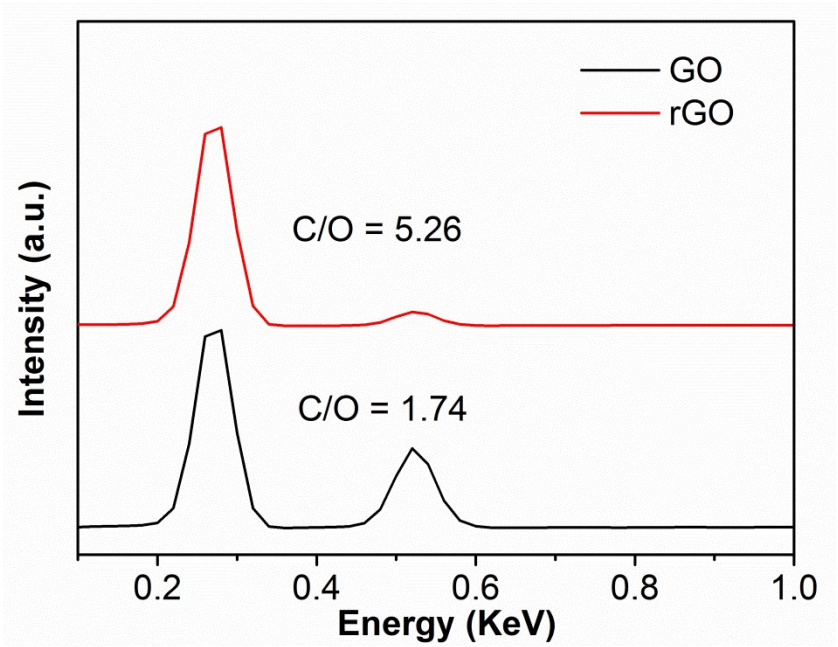

Fig. S2 The typical EDS analysis of the GO and rGO.

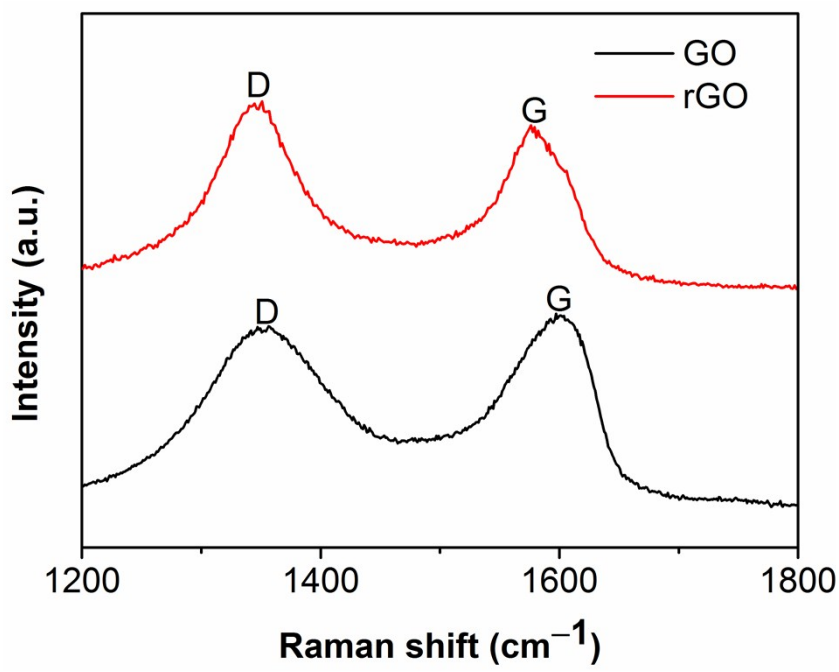

Fig. S3 Raman spectra of GO and rGO.

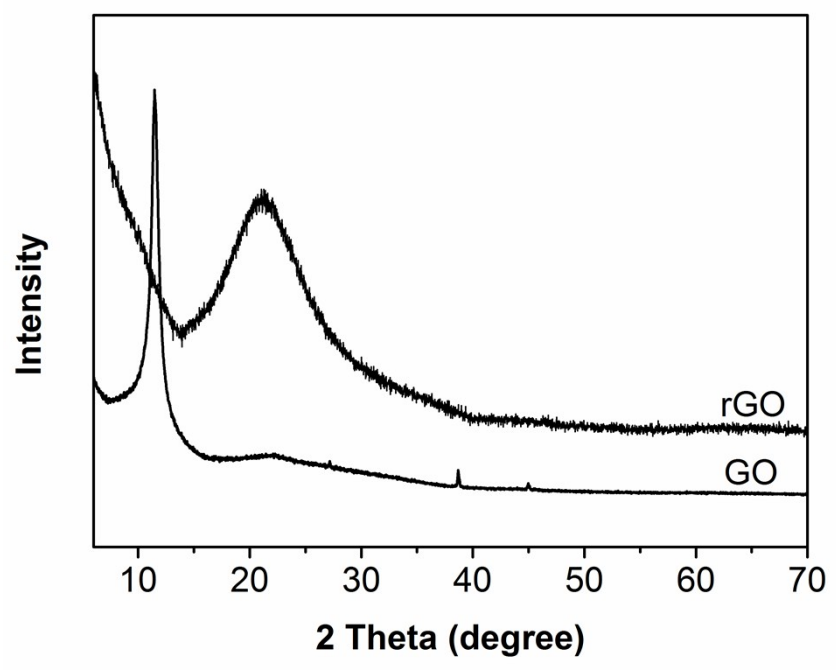

Fig. S4 XRD patterns of GO and rGO.

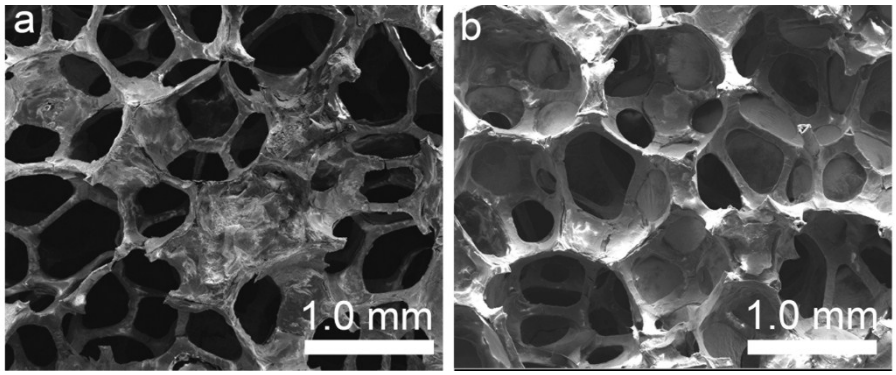

Fig. S5 The low magnification SEM images of the MBG and rGO-MBG scaffolds.

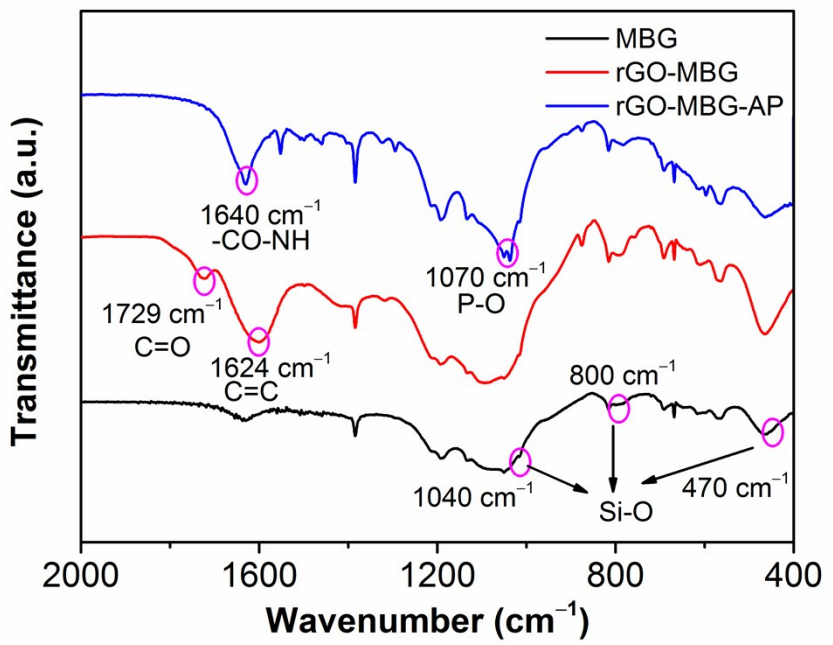

Fig. S6 FT-IR spectra of MBG, GO-MBG, and rGO-MBG-AP.

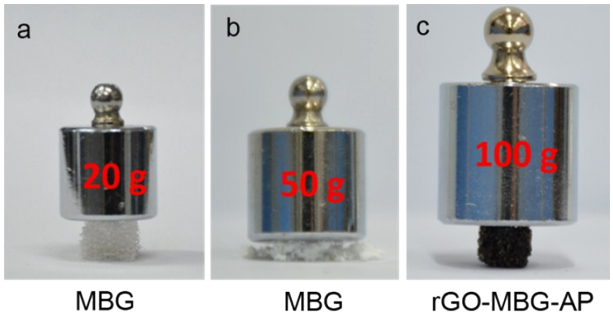

Fig. S7 The mechanical properties of MBG (a, b) and rGO-MBG-AP (c).

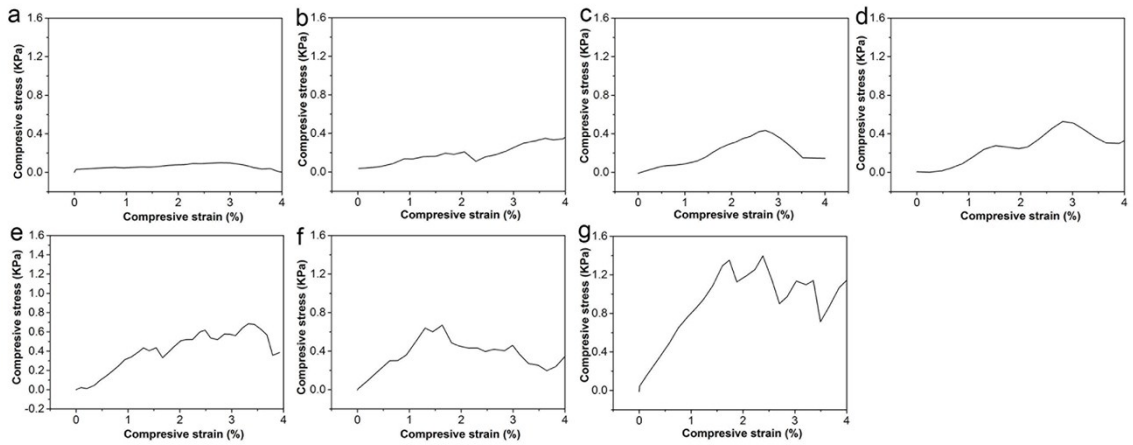

Fig. S8 Stress-strain curves of each type of scaffold with (a) 0, (b) 1%, (c) 2%, (d) 3%, (e) 4%, (f) 5%, and (g) 6% of rGO coating.

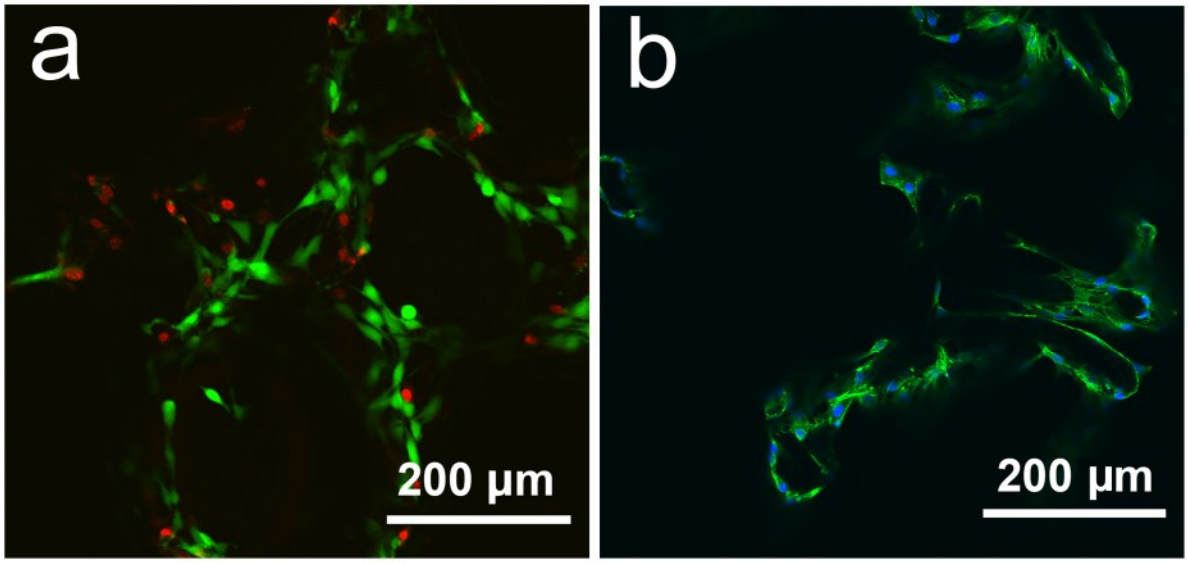

Fig. S9 (a) Confocal microscope image of dead/live cell staining in the MBG scaffold. (b) Confocal microscope image of the cell morphology in MBG scaffold.

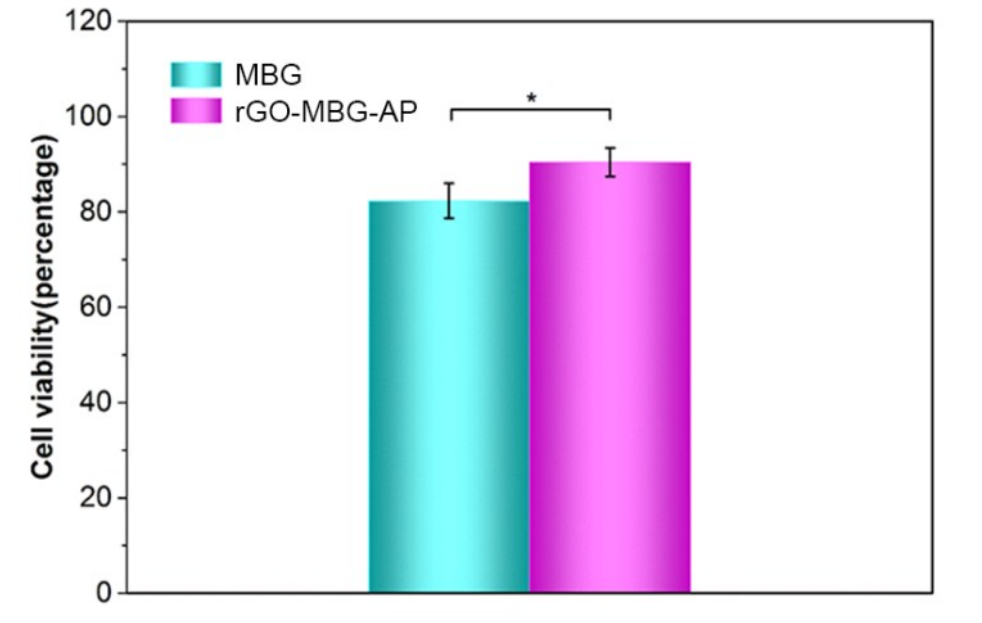

Fig. S10 The survival rate of rOBs in MBG and rGO-MBG-AP scaffolds.

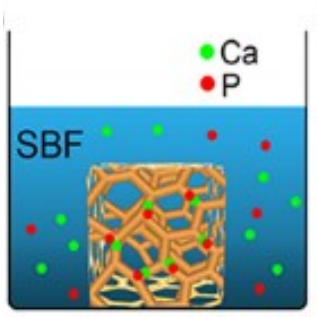

Fig. S11 Schematic illustrate of in vitro apatite deposition.

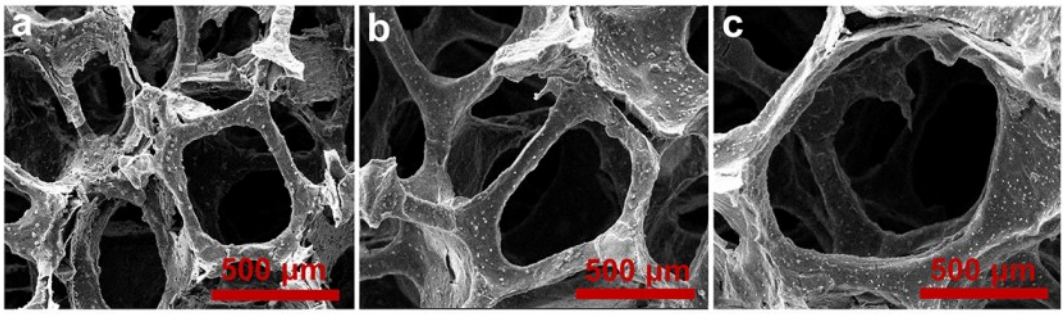

Fig. S12 SEM images of rGO-MBG-AP immersed in SBF for (a) 3, (b) 5 and (c) 7 days.

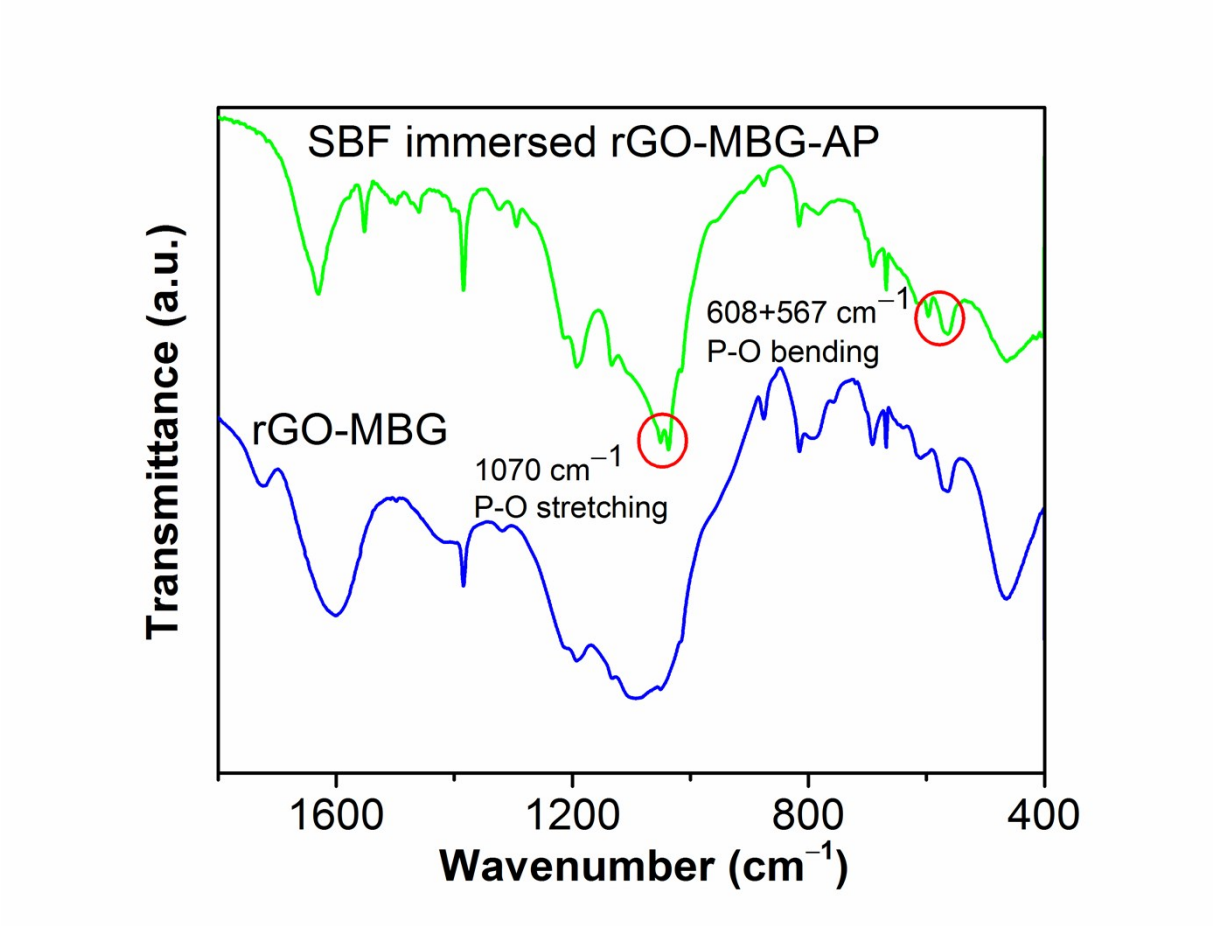

Fig. S13 FR-IR spectra of rGO-MBG and SBF immersed rGO-MBG-AP.

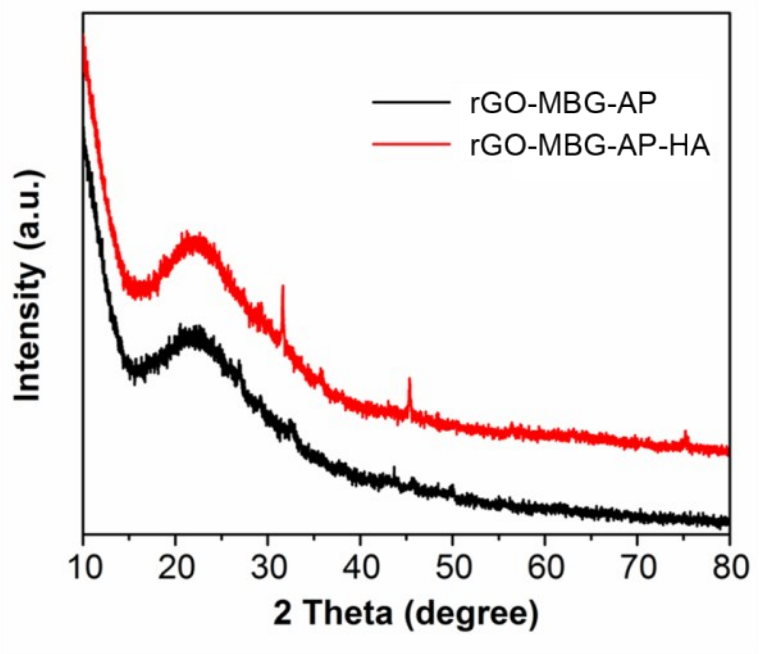

Fig. S14 XRD patterns of rGO-MBG-AP and rGO-MBG-AP-HA (rGO-MBG-AP immersed in SBF for 7 days).

Fig. 15 SEM image of MBG (left) and rGO-MBG-AP (right) immersed in SBF for 7 days.

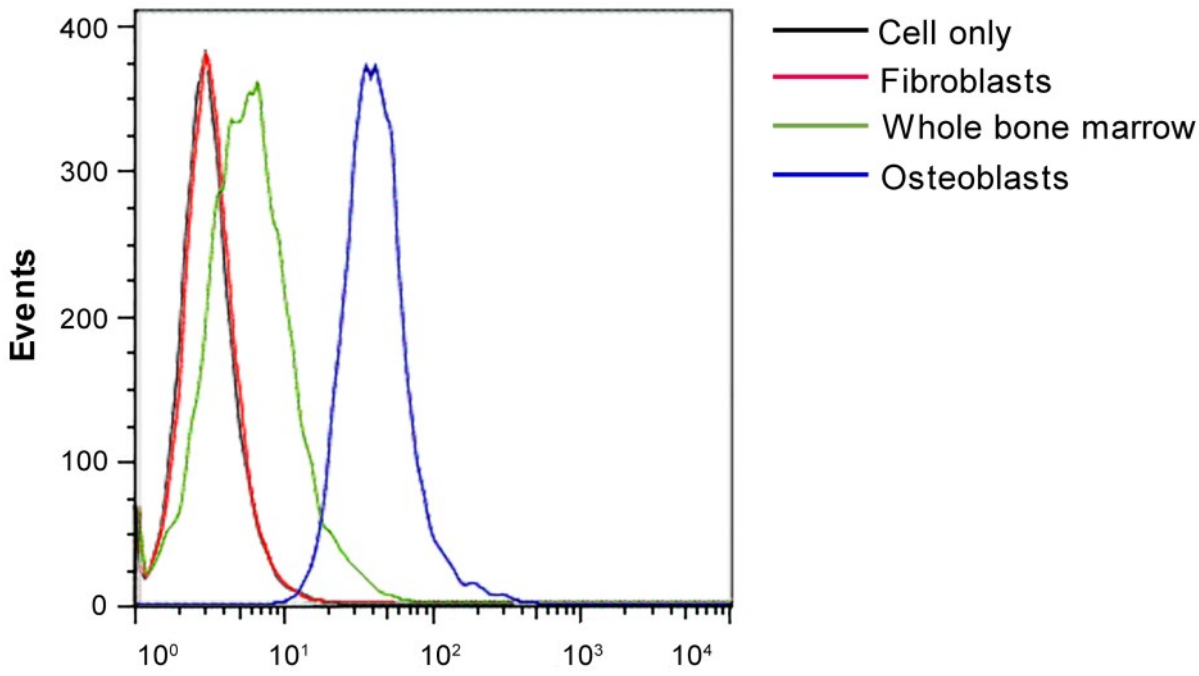

Fig. S16 Flow cytometry analysis of osteoblasts, fibroblasts and whole bone marrow after incubation with FITC-labeled CH6 aptamer, respectively.

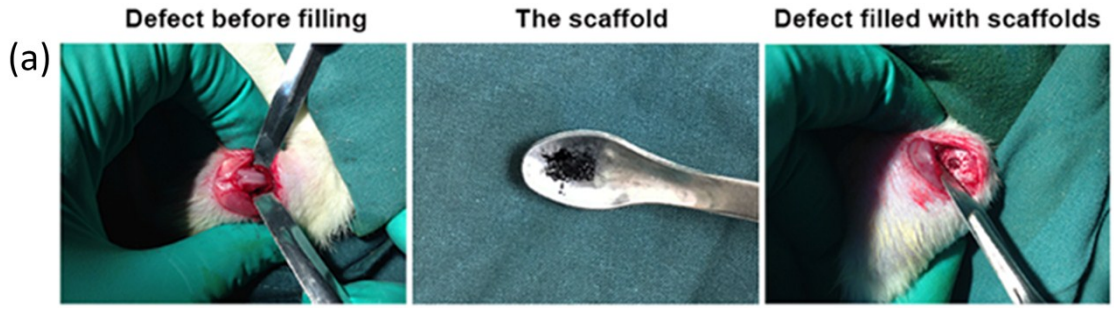

Fig. S17 The implantation process of the scaffolds.

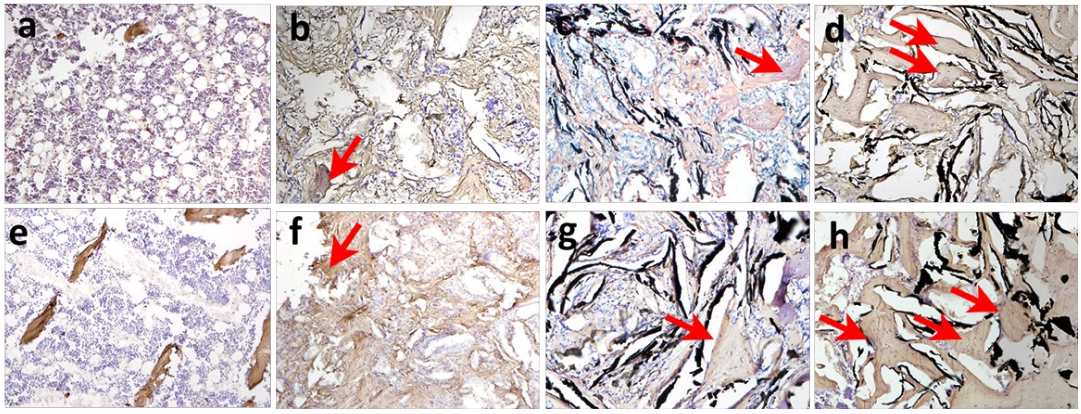

Fig. S18 The defect treated with (a) nothing, (b) MBG, (c) rGO-MBG, and (d) rGO-MBG-AP in the fourth week. The defect treated with (e) nothing, (f) MBG, (g) rGO-MBG, and (h) rGO-MBG-AP in the eighth week. The red arrow refers to the newly formed bone stained positive with type 1 collagen.