Enhancement mechanisms of graphene in nano-58S bioactive glass scaffold: mechanical and biological performance

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Graphene is a novel material and currently popular as an enabler for the next-generation nanocomposites. Here, we report the use of graphene to improve the mechanical properties of nano-58S bioactive glass for bone repair and regeneration. And the composite scaffolds were fabricated by a homemade selective laser sintering system. Qualitative and quantitative analysis demonstrated the successful incorporation of graphene into the scaffold without obvious structural damage and weight loss. The optimum compressive strength and fracture toughness reached 48.65 ± 3.19 MPa and 1.94 ± 0.10 MPa m⁰.⁵ with graphene content of 0.5 wt%, indicating significant improvements by 105% and 38% respectively. The mechanisms of pull-out, crack bridging, crack deflection and crack tip shielding were found to be responsible for the mechanical enhancement. Simulated body fluid and cell culture tests indicated favorable bioactivity and biocompatibility of the composite scaffold. The results suggest a great potential of graphene/nano-58S composite scaffold for bone tissue engineering applications.

58S bioactive glass (58S) (58% SiO₂, 33% CaO and 9% P₂O₅, based on mol%) has received special attention as scaffold material owing to its good biodegradability, excellent bioactivity and bone-bonding ability¹-². It reacts with physiological fluids to form direct bonds to bone tissue in the early time after implantation without toxicity, inflammation and foreign-body response³. The fast surface reactions in vivo lead to rapid ionic dissolution and formation of hydroxyl-carbonated apatite (HCA) layer⁴. The release of soluble Si, Ca, and P ions can activate gene expression and stimulate osteoblast proliferation for rapid bone formation⁵. Moreover, 58S in nano scale (nano-58S) exhibits better bioactivity in terms of cell growth, osteogenic differentiation and HCA formation⁶. The major hurdles of 58S are intrinsic brittleness, low fracture toughness and crack resistance to sustain the loads transmitted from surrounding bone tissue⁷, which is considered to be one of the main requirements of scaffolds during the period of new bone formation. Thus, there has been a strong impetus to improve the mechanical properties of 58S scaffold in the past years. Current attempts focused on improving the mechanical properties of 58S by incorporating second phase reinforcements including polymers and metallic oxides. O’Shea et al. developed a poly(lactic-co-glycolic acid) (PLGA)-coated 58S scaffold. The addition of PLGA coating improved the compressive strength of 58S scaffold to 0.25 MPa, which was twice that of uncoated 58S scaffold (0.12 MPa) but still lies toward the lower limit of cancellous bone⁸. Increasing efforts were also devoted to improve the mechanical properties by surface modification using organic molecular, which promoted the dispersion of 58S particles in the composites⁹,¹⁰. Nevertheless, this method was also accompanied by a weakened capability for calcium precipitation¹¹. So far, few of these scaffolds fulfill both the mechanical and biological requirements for load bearing applications.

Graphene possesses a unique two-dimensional structure with single-atom thickness and extensive conjugation, which endowed it with excellent mechanical, electrical and thermal properties¹². These exceptional properties make graphene a key enabler for the next-generation nanocomposites¹³. Moreover, it is far superior to other known reinforcements in transferring their mechanical properties to host material. Recent findings demonstrated no adverse effect of graphene incorporation on the microenvironment in vivo¹⁴. The combination of promising biocompatibility and outstanding mechanical properties indicate the potential application of graphene as reinforcing phase in 58S bone scaffold. However, till now researches have mainly focused on graphene/polymer composites and studies on graphene/ceramic composites are relatively limited. It is attributed to the greater...
requirements of individual patients. Up to now, there is a dearth of tailored shape and porous structure in order to match the specific needs. Furthermore, SLS is qualified for fabricating scaffolds with customized graphene and inhibit devitrification of 58S during the sintering process. The duration of laser beam at individual particle is typically between 0.5 and 25 ms, enabling a much shorter processing time of SLS compared with conventional sintering techniques. This is expected to avoid oxidative damage to graphene and inhibit devitrification of 58S during the sintering process. Furthermore, SLS is qualified for fabricating scaffolds with customized shape and porous structure in order to match the specific requirements of individual patients. Up to now, there is a dearth of literature in the use of graphene to improve the mechanical properties of nano-58S. The SLS fabrication of graphene/nano-58S composite scaffold and corresponding enhancement mechanism remain to be systematically explored.

In this study, nano-58S was combined with graphene in order to enhance its poor mechanical properties for bone tissue engineering applications. 3D porous composite scaffolds of graphene/nano-58S were fabricated using SLS technique. The microstructure and chemical composition were studied by scanning electron microscopy/energy dispersive spectroscopy (SEM/EDS), Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD) and field emission transmission electron microscopy (FE-TEM). The graphene in the scaffold was analyzed using Raman spectroscopy and thermogravimetric analysis (TGA). The compressive strength and fracture toughness were investigated by compression test and indentation method. The enhancement mechanism was discussed based on the test results. The biocompatibility and bioactivity were assessed after immersion in simulated body fluid (SBF) and by in-vitro cell culture using human osteoblast-like cells (MG-63).

Results
Good dispersibility of graphene in solvent can possess uniform distribution in matrix, which helps to maintain uniform properties throughout the composite. The dispersion states of the starting powders in NMP were shown in Fig. 1a. It can be seen that graphene suspension exhibited visually homogeneous and stable solution with black color. The forces keeping graphene dispersed in NMP arose from the hydrogen bonds formed between graphene and the solvent. TEM image showed many ripples and wrinkles on the as-received graphene surface (Fig. 1b), which was benefit for protein adsorption and cell adhesion. Besides, nano-58S powder was also well dispersed in the solvent and yielded a white suspension. The powder consisted of irregular particles with a size of 30–60 nm (Fig. 1c). After mixing with graphene suspension and ultrasonication, a composite solution was obtained with dark gray color. And nano-58S particles were found to uniformly and intimately anchor on the graphene surface, as can be seen in Fig. 1d.

Raman analysis was carried out to investigate the structure changes of graphene after the sintering process (Fig. 2). The spectrum of as-received graphene was dominated by a D peak at 1359 cm$^{-1}$ and a G peak at 1586 cm$^{-1}$ (Fig. 2a). The G peak referred to C–C stretching vibration while the D peak originated from the structural disorder and defects in graphene. A broad band was also observed around 2915 cm$^{-1}$ corresponding to 2D, D + G and 2D’ peaks. No peaks were found in the spectral region from 1200 to 3200 cm$^{-1}$ for 58S-0 (Fig. 2b). The aforementioned peaks were presented in the spectra of sintered samples with graphene, confirming the survival of graphene after SLS process. In comparison with the as-received graphene, there was only a slight increase of the intensity ratio of D peak to G peak (I_D/I_G) for graphene-containing samples (Figs. 2c–f), indicating a higher degree of disorder in the graphene lattice. The Raman results demonstrated the successful incorporation of graphene into nano-58S scaffold by SLS without obvious structural damage.

The TGA curves of as-received graphene and sintered samples were obtained (Fig. 3) in order to estimate the weight retention of graphene after the SLS process. The curves showed a weight loss below 200 °C, owing to the evaporation of adsorbed water. A weight loss (14.1 wt%) occurred between 200 and 500 °C in the curve of as-received graphene (Fig. 3a), which can be assigned to the oxidation of amorphous carbon. A significant weight loss (70 wt%) was noticed in the range of 500–675 °C, corresponding to the complete combustion of the carbon skeleton of graphene. In contrast, no obvious weight loss was found in this temperature range for 58S-0 (Fig. 3b). This indicated that the weight loss in the temperature range of 500–675 °C corresponded to the graphene content in the sintered samples. As can be seen in Figs. 3c–f, the weight losses in this temperature range were about 0.11, 0.48, 0.99 and 1.53 wt%, which

Figure 1 | Material preparation. (a) dispersion of the starting powders in NMP. TEM images of (b) as-received graphene, (c) as-received nano-58S powder and (d) graphene/nano-58S composite powder.
represented the amount of graphene in 58S-0.1, 58S-0.5, 58S-1, and 58S-1.5 respectively. As the temperature increased above 675 °C, the weight loss slowed down and plateau formed in the TGA curves. The TGA results demonstrated little weight loss of graphene after the SLS process in nitrogen atmosphere.

The FT-IR spectra of the starting powders and sintered samples were shown in Fig. 4. The spectrum of as-received nano-58S powder (Fig. 4a) was dominated by a strong absorption band in the region 900–1200 cm⁻¹, which was a combination of Si–O–Si stretching and P–O stretching vibrations in the glass network. The absorption peak at 466 cm⁻¹ was ascribed to the bending vibration of Si–O–Si. The small band appeared in the vicinity of 1498 cm⁻¹ was the typical absorption band of carbonates as a result of the admission of water and CO₂ on nano-58S powder. The spectrum of as-received graphene (Fig. 4b) exhibited an absorption band at 1558 cm⁻¹, associating with the C=C skeletal vibration. The peak for C–C stretching vibration was observed at 1630 cm⁻¹. The dispersive band located around 1069 cm⁻¹ corresponded to the C–O stretching vibration in the reduced graphene. The spectrum of composite powder was similar to that of nano-58S powder due to the low content of graphene (Fig. 4c). Absorption peak at 940 cm⁻¹ was observed in the spectrum of 58S-0 (Fig. 4d), which can be attributed to the formation of SiO groups with non-bridging oxygens. For the sintered samples with graphene (Figs. 4e–h), a double peak that arose from the bending vibration of P–O appeared at 567 and 605 cm⁻¹, indicating the formation of crystalline phosphate in the glasses.

The XRD patterns of the starting powders and sintered samples were shown in Fig. 5. It can be seen that the as-received nano-58S powder was amorphous, and no diffraction peaks were observed except for a broad band of 2θ between 26° and 32° (Fig. 5a). There was only a broad band at about 24° in the pattern of as-received graphene (Fig. 5b), which was attributed to the irregular arrays of atoms in three dimensions. After mixing with nano-58S powder, no trace of graphene was detected by XRD analysis because of its low amount in the composite powders (Fig. 5c). There was a similar XRD pattern among the sintered samples with and without graphene addition (Figs. 5d–h). Diffraction peaks of wollastonite (CaSiO₃) were identified at about 27.5°, 31.7° and 45.7° in all the sintered samples, indicating partial crystallization occurred in the nano-58S. The addition of graphene had no influence on the phase composition of sintered samples, since no obvious difference were found among the patterns with different graphene contents. Besides, the crystalline phosphate found in the FT-IR results were absent in the XRD patterns, which might be attributed to the trace amount of crystalline phosphate and insufficient detection limit of XRD method.

The mechanical properties of sintered samples were obtained by compressive tests and indentation technique, as shown in Fig. 6. It can be seen that the compressive strength firstly increased from 23.66 ± 3.90 MPa for 58S-0 to 48.65 ± 3.19 MPa for 58S-0.5, followed by a decrease to 44.19 ± 4.27 and 37.92 ± 3.84 MPa with increasing graphene content up to 1.0 or 1.5 wt%. The fracture toughness of sintered samples increased with the addition of graphene from 0 to 0.5 wt% and then diminished as graphene content reached 1.0 or 1.5 wt%. Incorporation of 0.5 wt% graphene improved the fracture toughness of nano-58S by 38% from 1.41 ± 0.07 to 1.94 ± 0.10 MPa m⁰. It was clear from the data that the 58S-0.5 displayed the highest compressive strength and fracture toughness among the samples tested. While the diminished enhancements of 58S-1 and 58S-1.5 might be attributed to the degradation in dispersion of graphene at high content.

Apart from the mechanical properties, bioactivity and biocompatibility of 58S-0.5 were studied by SBF and cell culture tests. SEM image revealed that the surface of 58S-0.5 scaffold was covered by a thick and well formed HCA layer after immersion in SBF for 7 days (Fig. 7a). Precipitated granules were found to distribute homogeneously on the surface in the magnified SEM image (Fig. 7b). EDS analysis of the precipitated granules revealed a mean molar Ca/P ratio of about 1.69 (Fig. 7c). XRD analysis (Fig. 7d) indicated that the diffraction peaks of (002) and (211) surfaces of HCA were superposed on that of CaSiO₃ at 2θ of 25.8° and 31.7°. The other peaks of HCA were observed at 2θ of 39° and 46°, corresponding to the (310) and (222) surfaces respectively. The apatite layer on 58S-0.5 scaffold was further...
analyzed using FTIR and shown in Fig. 7e. The double peak corresponding to P–O bending vibrations was observed at 558 and 605 cm$^{-1}$ in the spectrum, while the P–O stretching band was superposed on the Si–O stretching band of 58S at about 1082 cm$^{-1}$. Well-defined carbonate bands were presented at 875 (C–O bending vibration) and 1420–1480 cm$^{-1}$ (C–O stretching vibration)$^{42,43}$, indicating the incorporation of carbonate into the apatite layer. These findings further confirmed the formation of HCA layer on the surface of 58S-0.5 scaffold after immersing in SBF. In vitro cell culture tests revealed that MG-63 cells colonized and grew favorably on 58S-0.5 scaffold after 7 days (Fig. 7f). MG-63 cells showed a round shape with mineralised nodules and were anchored to the surface by elongated filopodia, suggesting a good cell biocompatibility of the scaffold.

A graphene/nano-58S composite scaffold was fabricated with the optimum graphene content (0.5 wt%) at laser power of 7.5 W, scan speed of 100 mm/min and spot diameter of 1 mm. The digital images of the scaffold (Figs. 8a–d) showed a 3D network of interconnected pores which were approximately 0.8 μm in size and isotropic in their distribution. SEM image indicated good connections between pore walls (Fig. 8e). The high degree of porosity and pore interconnectivity are beneficial for vascularisation in vivo and rapid osteogenesis$^{44}$.

Discussion

The typical toughening mechanisms for graphene-based materials can be summarized as follows: (a) Crack bridging. Graphene bridges the two surfaces of crack and provides a closure stress to counteract the applied stress, delaying further propagation of the crack. (b) Pull-out. Graphene is pulled out the matrix and slows down crack propagation by the interfacial friction between graphene and matrix. (c) Crack deflection. Crack deflects into a different plane when it encounters graphene, resulting in a tortuous path and more energy dissipation for crack propagation. (d) Crack tip shielding. The crack tip is restricted in the vicinity of graphene due to the insufficient energy required for interface debonding. The indentations and radial cracks were characterized by SEM to identify the mechanism responsible for the enhanced mechanical properties (Fig. 9). A representative microhardness indentation and induced radial cracks were prepared on the polished surface of 58S-0.5 (Fig. 9a). Magnified image in Fig. 9b showed the presence of graphene pull-out and crack bridging (labeled by white arrows) on the fracture line. Individual graphene bridged the gap between the crack surfaces, delaying the rupture of 58S matrix. EDS analysis confirmed the toughening effect by graphene since a strong peak referred to carbon element was observed in the spectrum. The direct evidence of graphene pull-out and crack bridging were also observed in Fig. 9c. The
Crack propagation was slowed down due to the loss of fracture energy by graphene pull-out. As a result, the reduced fracture energy was insufficient to pull out another graphene sheet and crack bridging formed in the propagation path. Characteristic crack deflections were visible in Fig. 9d and 9e. The crack firstly propagated in its own plane and then along graphene–58S interface when met with graphene, followed by the deflection to 58S matrix. Such crack deflection occurred several times in the propagating path, which would force the crack to propagate along a very tortuous path to release stress. Crack tip shielding by graphene was observed in Fig. 9f, in which graphene effectively prevented the further propagation of crack tip. These results indicated that graphene was highly effective in suppressing crack propagation in 58S matrix. The mechanisms of graphene pull-out, crack bridging, crack deflection and crack tip shielding were responsible for the enhancement of compressive strength and fracture toughness.

The enhancement mechanisms associated with the morphological evidence were schematically depicted in Fig. 10. Once a matrix crack is initiated and propagates, load is transferred from the matrix to graphene due to the difference in elastic modulus. The wrinkled surface texture of graphene enabled efficient mechanical interlocking and load transfer with the matrix. The toughening mechanism of crack bridging is shown in Fig. 10a where graphene bridges two crack surfaces. Graphene hampers the relative displacement between the opposite crack surfaces by alleviating the stress required for further crack propagation. The pull-out of graphene occurs when the shear stress at the interface exceeds the interfacial strength of graphene–58S (Fig. 10b). This mechanism requires high energy dissipation due to the high toughness and large surface area of graphene. The crack propagation can be effectively slowed down by additional resistance from the interfacial friction between graphene and 58S matrix. Fig. 10c demonstrates the toughening mechanism of crack...
deflection. A crack propagates firstly in 58S matrix and then deflects into a different plane when it encounters graphene. More energy is required for the further propagation of crack, since the crack plane is no longer perpendicular to the axis of stress. Moreover, the deflection process generates a tortuous path for crack propagation, which allows much more energy dissipation. Crack deflection may be highly effective for graphene-based materials in virtue of the large specific surface area. As shown in Fig. 10d, the crack tip is restricted

Figure 6 | Mechanical properties of sintered samples with different graphene contents. (# p < 0.05, ## p < 0.001 compared with 58S-0).

Figure 7 | Biological tests. (a) Low magnification and (b) high magnification SEM images, (c) EDS spectrum, (d) XRD pattern and (e) FT-IR spectrum of 58S-0.5 after immersion in SBF for 7 days. (f) surface morphology of 58S-0.5 after cultured with MG-63 cells for 7 days.
Figure 8 | Graphene/nano-58S composite scaffold by SLS. (a), (b) side views. (c) isometric view. (d) top view. (e) SEM image.

Figure 9 | Inhibition of crack propagation by graphene in the sintered samples. SEM images of (a) microhardness indentation and radial cracks, (b–e) Crack deflection, crack bridging and graphene pull-out, (f) Termination of crack growth at the crack tip.
in the vicinity of graphene due to the insufficient energy required for interface debonding. These mechanical enhancements get saturated with graphene content of 0.5 wt%, as more graphene are difficult to disperse homogeneously in the nano-58S powder.

In summary, this study evaluates graphene as a potential reinforcement to nano-58S bone scaffold for enhancing the mechanical properties. Uniform dispersion of graphene, nano-58S and composite powders were obtained using NMP solvent. Graphene was successfully incorporated into the scaffold by SLS without obvious structural damage in spite of a slight increase of IG/ID ratio. And there was little weight loss of graphene in the scaffold after the sintering process. The addition of graphene showed no influence on the phase composition of nano-58S scaffolds. The mechanical properties of nano-58S increased with the increase of graphene content (0–0.5 wt%) and then diminished with graphene content of 1.0 or 1.5 wt%. Incorporation of 0.5 wt% graphene improved the compressive strength of nano-58S by 105% and the fracture toughness by 38%. Microstructural results demonstrated that pull-out, crack bridging, crack deflection and crack tip shielding were responsible for the mechanical enhancement. A well-formed HCA layer with Ca/P ratio of 1.69 formed on the scaffold surface after immersing in SBF for 7 days. In vitro cell culture tests revealed that MG-63 cells colonized and grew favorably on the surface, suggesting a good cell biocompatibility of the composite scaffold. This study may pave the way for the mechanical enhancement of nano-58S scaffold in the application of bone tissue engineering.

**Methods**

**Materials and experiments.** Graphene (purity: 99.8%, 0.7–1.2 nm in thickness and 0.8–3 µm in diameter) was purchased from Nanjing JCNano Tech Co., Ltd. It was prepared by modified Hummers method with a metal impurity < 10 PPM. 58S bioactive glass was firstly prepared by sol-gel method. Then freeze-drying and grinding process were adopted to achieve nano-scale 58S powder (average particle size of 48.3 ± 6.6 nm). N-methyl-2-pyrrolidone (NMP) (Tianjin Kemiou Chemical Reagent Co., Ltd., China) was used as the solvent for dispersing graphene due to the well matched surface energy to graphene.

As-received graphene was firstly weighed using an electronic balance (Model FA1104, Changzhou Hengsheng Electronic Instrument Co., Ltd., China) and dispersed in NMP with concentration of 0.5 mg/ml using ultrasonic cleaning device (SK3300H, Shanghai Kudos Ultrasonic Instrument Co., Ltd., China) for 30 min. Then, a certain amount of nano-58S powder was added to the solution and dispersed ultrasonically for another 30 min. Subsequently, the resultant mixed solution was filtrated and dried by a rotary evaporator (RV 10 digital, IKA, Germany) at 60 °C for 24 h, followed by further drying in a vacuum oven (DZ-3, Tianjin Tasite Instrument Co., Ltd., China) for 24 h at 100 °C to remove any trapped solvent. Finally, the composite powder was carefully peeled off the filter paper and broken up using pestle. By accurately weighing the graphene and nano-58S powder, the composite powders with different graphene contents (0 wt%, 0.1 wt%, 0.5 wt%, 1.0 wt% and 1.5 wt%) were obtained.

Experiments were carried out on a home-made SLS system in nitrogen atmosphere with laser power of 7.5 W, scan speed of 100 mm/min, spot diameter of 1 mm. The sintered samples with different graphene contents were labeled as 58S-0, 58S-0.1, 58S-0.5, 58S-1 and 58S-1.5, respectively. The morphology of graphene, nano-58S and composite powder were observed with a field emission transmission electron microscope (JEM-2100F, JEOL Ltd., Japan) after the powders were ultrasonicated in NMP for 30 min to form homogeneous suspensions. The microstructure of sintered samples was characterized using a scanning electron microscope (JSM-6490LV, JEOL Ltd., Japan) at 20 kV accelerating voltage after the samples were vacuum coated with platinum (JFC-1600 auto fine coater, JEOL Ltd., Japan). The element composition on the sample surface was studied using EDS (Neptune XM4, EDAX Inc., USA).

The phase analysis was conducted on a X-ray diffractometer (D/Max 2500V, Rigaku, Japan) at 2θ range of 10–60° using CuKα radiation. FTIR spectra were recorded to identify the typical functional groups present in the samples using a Fourier transform infrared spectrometer (Nicolette TM 6700, Thermo Scientific Co., USA). The characterization was conducted with KBr pellets technique in a spectral region of 400–2000 cm⁻¹. In order to determine the structure changes of graphene, Raman spectroscopic studies were performed on a LabRAM HR800 spectrometer (HORIBA Jobin Yvon, France) with laser wavelength of 488 nm and spot size of 0.1 mm. TGA analysis was performed on a thermal analyzer (Netzsch STA 449C, Germany) to determine the weight retention with increasing temperature and further the amount of graphene in the samples. Prior to tests, all the samples were grinded to powders to ensure sufficient heat transfer. The measurements were conducted from ambient temperature to 750 °C at a heating rate of 10 °C/min under air flow of 30 mL/min.

**Microstructural characterization.** The morphology of graphene, nano-58S and composite powder were observed with a field emission transmission electron microscope (JEM-2100F, JEOL Ltd., Japan) after the powders were ultrasonicated in NMP for 30 min to form homogeneous suspensions. The microstructure of sintered samples was characterized using a scanning electron microscope (JSM-6490LV, JEOL Ltd., Japan) at 20 kV accelerating voltage after the samples were vacuum coated with platinum (JFC-1600 auto fine coater, JEOL Ltd., Japan). The element composition on the sample surface was studied using EDS (Neptune XM4, EDAX Inc., USA).

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**Mechanical properties assessment.** Fracture toughness was determined by the indentation technique using a digital microhardness tester (HXD-1000TM/LCD, Shanghai Taiming Optical Instrument Co., Ltd., China). The samples were firstly inlayed by a mounting press (XQ-2B), followed by surface grinding with abrasive paper and polishing using diamond pastes. The indentations and cracks were made on the samples by loading to the maximum load (500 gf) in 10 s, subsequently holding for 15 s and then unloading to zero load in 10 s. The fracture toughness was calculated based on the crack length according to equation (1). A total of ten indentation data points were collected for each sample to check the reproducibility.

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K_{IC} = 0.0824 \frac{P}{\sqrt{c}} \tag{1}
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Biological properties assessment. The bioactivity of a scaffold is directly related to its ability to induce HCA formation. The HCA formation behavior of samples was evaluated in vitro by immersing them in SBF. The ion concentration (mmol/L) of SBF is similar to that of human plasma and presented as follows: Na⁺ 142.0, Ca²⁺ 2.5, K⁺ 5.0, Mg²⁺ 1.5, CI⁻ 147.8, HCO₃⁻ 4.2, HPO₄²⁻ 1.0, and SO₄²⁻ 0.5. The samples were immersed in SBF (pH 7.4) with a surface area (cm²) to solution volume (mL) ratio of 0.1 cm²/mL at 37 °C for 7 days. The solution was renewed every 3 days to keep the ion concentration and pH constant for better simulation of the in vivo conditions. After immersing, the samples were extracted from the solution, gently rinsed with acetone, and dried at ambient temperature before further characterization.

Cell culture test was conducted to study the biocompatibility of scaffolds using MG-63 cells (American Type Culture Collection, Rockville, MD) isolated from human osteosarcoma. The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, containing 10% fetal bovine serum (FBS), 50 IU/mL penicillin and 50 mg/mL streptomycin) under a humidified atmosphere of 5% CO₂ at 37 °C. The samples were further sterilized with ultraviolet radiation and preconditioned for 24 h in DMEM to prevent the sudden release of high ion concentrations from SBF. Then MG-63 cells were seeded on the samples and incubated in 12-well cell culture plates for 7 days. The medium was changed every 3 days to guarantee a constant nutrition supply. After the cell culture, the samples were rinsed twice in phosphate buffered saline (PBS) and fixed with 2.5% glutaraldehyde. Dehydration was performed by sequential immersion in a graded series of ethanol solutions (70%, 80%, 90%, 95% and 100%). Afterwards, the samples were dried and sputtered with platinum for SEM observations.

Statistical analysis. Data analysis was performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) and the data were expressed as mean ± standard deviation (SD). Statistical analysis was conducted by two-tailed Student’s t-test between groups with different graph contents (n = 10). Differences were considered statistically significant when p < 0.05.
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Author contributions
C.G. and C.S. conceived and designed the research. C.G., T.L. and S.P. performed experiments. C.G. and C.S. analyzed the data and wrote the manuscript. All authors reviewed the manuscript.

Additional information
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