Enhanced proliferation and osteogenic differentiation of human mesenchymal stem cells on biomineralized three-dimensional graphene foams

Ting Zhang, Ning Li, Kunyang Li, Ruifang Gao, Wei Gu, Chengcheng Wu, Ruigong Su, Liwei Liu, Qi Zhang, Jian Liu

A R T I C L E   I N F O
Article history:
Received 2 January 2016
Received in revised form 4 April 2016
Accepted 12 April 2016
Available online 14 April 2016

A B S T R A C T
Human mesenchymal stem cells (hMSCs) hold great promise for bone regeneration, yet the direction of MSC proliferation and induction of MSC differentiation remain challenging. Very recently, graphene provides a novel substrate for cell culture. Here, we report the utilization of mineralized three-dimensional graphene (3DG) scaffolds for hMSC growth. The nano-structured hydroxyapatite (HA) particles decorated 3D graphene (HA-3DG) scaffolds were developed by mineralization in 10 times concentrated simulated body fluid (10SBF) containing 10 mM of HCO$_3^-$/CO$_3^{2-}$. The HA-3DG scaffolds showed higher roughness and cell proliferation compared with the 2D graphene films. More importantly, the mineralized 3DG scaffolds exhibited faster osteogenic commitment and stronger osteogenic differentiation (13.7, 10.9 and 1.89 fold at 7d, 10d and 14d respectively from the western blot analysis). These findings demonstrated the potential of mineralized 3DG scaffold as a promising platform for hMSC culture and bone regeneration.

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1. Introduction

Human mesenchymal stem cell (hMSC) is an interesting cell source for a variety of groundbreaking therapies in regenerative medicine [1]. In particular, MSCs have been proposed as attractive candidates for bone regeneration since they have the potential to differentiate into osteoblasts, chondrocytes, and adipocytes [2,3]. However, one of the key objectives is to direct MSC’s proliferation and enhance their differentiation towards osteoblasts. Stem cell scaffolds have been developed to mimic the intrinsic characteristics of bone by creating physical and chemical microenvironment for the guidance of stem cell fate [4–6].

Graphene, an atomic-thick sheet of carbon atoms arranged in a honeycomb lattice, may offer a new opportunity to some of the challenges [7]. Owing to unique physical, chemical and mechanical properties as well as the good biocompatibility, graphene has attracted numerous interests as cell culture substrates [8–10]. To date, some efforts have been made to explore the interaction of graphene and MSCs [11]. It was found that graphene provided a new kind of coating materials that may confer the pro-osteodifferentiation capability of MSCs [12–14]. Nayak et al. studied the effect of graphene on MSC growth by investigating graphene coated-four different substrates, and they found that the graphene coating did not hamper the proliferation of hMSCs but accelerated their differentiation into bone cells at a rate comparable to differentiation under the influence of bone morphogenetic protein-2 (BMP-2) [15]. However, these studies are limited to 2 dimensional (2D) flat substrates. In the previous study, we reported the first utilization of three-dimensional graphene (3DG) scaffolds for neural stem cell culture, and enhanced cell proliferation and differentiation was found compared with that on 2D graphene films.
The 3DG scaffolds with interconnected pores offer a powerful platform for stem cell culture, which could provide 3D micro-environments to mimic the in vivo situation [17, 18]. Since hydroxyapatite (HA) is the main inorganic component of bone, HA and related calcium phosphates have been widely used in bone tissue engineering [19–21]. The incorporation of HA and calcium phosphates in 3D scaffolds could mimic bone materials and facilitate cell attachment and function [22]. A strategy of biomimetic mineralization in simulated body fluid (SBF) provides a straightforward route to introduce HA particles in the 3D scaffolds [23]. The mineralized scaffolds by SBF deposition method was found to be capable of inducing osteogenic differentiation of MSCs [24].

In this study, a modified SBF biomimetic mineralization system was used for producing porous mineralized 3DG with controlled mineral amounts. And further, we demonstrated the first example of mineralized 3DG scaffold for hMSC growth in vitro. The adhesion, proliferation, and differentiation of hMSCs on 3DG scaffolds were investigated, while 2D graphene (2DG) films were utilized as the control substrates.

2. Experimental

2.1. Fabrication of 3D graphene foams

The 3D graphene foam was fabricated as previous described with slight modification [16]. Nickel foam (Alantum Advanced Technology Materials, PPI 100 ± 10, density 320 ± 25 g/m², Shenyang, China), a porous structure with an interconnected 3D scaffold of nickel, was chosen as a template for the growth of graphene foams. In brief, carbon was introduced into the nickel foam by pyrolysis of CH₄ at 1000 °C under ambient pressure, and then graphene film precipitated on the surface of porous nickel foam. After etching away the nickel skeleton with 1M FeCl₃ and HCl solution, a monolith of continuous and interconnected graphene 3D network, was synthesized. 2D graphene film was fabricated according to previously reported chemical vapor deposition (CVD) method [25].

2.2. Mineralization of 3DG foams

For mineralization, the porous 3DG foams were initially treated with plasma for 3 min, followed by mineralizing in 10 times concentrated simulated body fluid (10SBF) [26]. Briefly, a supersaturated solution of 10SBF was prepared from NaCl, CaCl₂, MgCl₂, NaHCO₃, and Na₂HPO₄. The ion concentrations in 10SBF were 1000 mM for Na⁺, 25 mM for Ca²⁺, and 10 mM for HPO₄²⁻. To investigate the role of HCO₃⁻ in mineralization with 10SBF, the solutions were changed by varying the concentrations of HCO₃⁻ (5, 10, 15 and 20 mM). To remove the impurities, the solutions were filtered through a 0.22-μm-pore-size filter system. The 3DG foams were then immersed in 10SBF in a capped plastic tube and kept at 37 °C for 4 h. The 10SBF solution was changed every 4 h when necessary. After removal from 10SBF, the sample was gently washed with water and ethyl alcohol, dried in drying oven and sterilized by electromagnetic radiation.

2.3. Microscopic characterization

The morphologies and microstructures of the 2DG films and mineralized 3DG scaffolds were characterized by scanning electron microscopy (SEM, Quanta 200F, FEI) and transmission electron microscopy (TEM, CM 200, Philips). The surface topologies of the carbon foams were investigated by atomic force microscopy (AFM, Multi-Mode V, Veeco) using tapping mode at room temperature. The hydrophilicity of 2DG film and fragmented 3DG foam was measured by monitoring the water contact angles (CA, DataPhysics OCA) based on the sessile drop method. The surface chemistry of 3DG-GFs was examined by the X-ray photoelectron spectroscopy (XPS, ULTRA DLD, Shimadzu) and energy dispersive X-ray spectroscopy (EDX, Quanta 200F, FEI). The crystallinity and number of the layer presented within graphene scaffolds were measured by Raman spectrometer (lamboram HR8800, HORIBA) and XRD (XRD-6000, Shimadzu) equipped with Cu Kα radiation (λ = 0.15406 nm). The drift of Raman spectra was adjusted by simple baseline correction.

2.4. Culture of hMSCs

hMSCs were cultured following a method outlined in previous report [15]. Plastic adherent nucleated cells were separated from the aspirate and expanded using DMEM-F12 (Gibco) complete media at 37 °C and 5% CO₂. The 5th passage cells were used in the subsequent cultures. For 2DG and 3DG cultures, cell suspension with around ~2 × 10⁶ cells was seeded onto the substrates. The media were changed every other day and the cultures were maintained for 14d at 37 °C and 5% CO₂.

2.5. Cell viability assay

To evaluate the viability of the cells grafted into the 3DG scaffolds, hMSCs were seeded into 3DG slices with tissue culture polystyrene (TCP) petri dish as control. After 7 days of culture, the slices were rinsed with 0.1 M phosphate buffer (PBS, pH 7.4) for 30 min, and then incubated in PBS containing 2 μM of calcine-AM and 4 μM of ethidium homodimer 1 (EthD-I) (LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells, Invitrogen) for 1 h at 37 °C. The slices were rinsed three times (20 min for each), and then fixed with 4% formaldehyde in PBS for 30 min. The live-cells stained with calcine-AM show green color and the dead cells stained with EthD-I show red color under fluorescent microscope. Cell death rate was evaluated by counting the percentage of EthD-I-positive cells over total cell number. For each experimental group, at least five fields of each section (including 4 corners and one centre) were recorded.

2.6. Immunofluorescence staining and imaging of cultured hMSCs

After a certain time of culture, cells were fixed in 4% paraformaldehyde at room temperature for 30 min, blocked and permeabilized for 60 min. Primary antibodies were incubated overnight at 4 °C. Antibodies used in this study included primary antibody against OCA4-30 (Osteocalcin marker, abcam, 1:300), CD44 (osteogenesis marker, abcam, 1:300 dilution), Collagen II (chondrogenesis marker, abcam, 1:300), and fatty acid binding protein-4 (FABP4) (adipocytes marker, abcam, 1:500). Afterwards, cells were rinsed with PBS and incubated with Fluor®488-conjugated secondary antibody (1:500, Invitrogen) or Fluor®568-conjugated secondary antibody (1:500, Invitrogen) and DAPI (Invitrogen) in PBS for 60 min at room temperature. Immunofluorescence microscopy was performed using an UltraView VoX Confocal system (Perkin Elmer). At least 20 images of each staining condition were captured at 200× magnification.

2.7. Western blot

The cell-3DG construct was cultured for 7, 10 and 14 days and washed once with cold PBS. The cells were collected using a solution of 0.25% trypsin-0.03% EDTA and lysed in RIPA buffer for 30 min on ice. The cell lysates were harvested from the supernatant
by centrifugation at 14,000 rpm for 30 min at 4 °C. The protein concentration was determined by BCA protein assay kit (Ding Guo, Co.). Equal amounts of protein was separated by polyacrylamide gel, and transferred onto a PVDF membrane (Millipore). The membranes were blocked with 5% non-fat milk in TBST (25 mM Tris–HCl, 0.15 M NaCl, and 0.1% Tween 20) for 1 h at room temperature, and incubated with indicated primary antibodies, followed by HRP-conjugated second antibodies. The membranes were then reacted with ECL western blot substrate kit (Pierce) before exposure. All experiments were repeated three times. The bands of Western blot were quantified by using SenSciiCapture software (JS608B, P&Q, Shanghai) and further expressed as intensity changes compared with the control.

2.8. Statistics

All data were presented as mean ± standard deviation of at least 3 independent experiments. Statistical analysis was performed using one way analysis of variance (ANOVA). The significance levels were set at *p < 0.05 and **p < 0.01.

3. Results and discussion

In bone tissue engineering, how to effectively promote the proliferation and osteogenic differentiation of hMSCs has become a hot research issue. Previous studies have reported 2D graphene or graphene coating helped hMSCs differentiate to osteogenic phenotype [15,27]. Compared with the traditional two-dimensional culture, the function and properties of MSCs in 3D phenotype [15,27]. Compared with the traditional two-dimensional culture, the function and properties of MSCs in 3D phenotype [15,27]. Compared with the traditional two-dimensional culture, the function and properties of MSCs in 3D phenotype [15,27]. Compared with the traditional two-dimensional culture, the function and properties of MSCs in 3D phenotype [15,27]. Compared with the traditional two-dimensional culture, the function and properties of MSCs in 3D phenotype. Compared with the traditional two-dimensional culture, the function and properties of MSCs in 3D phenotype.

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2.3. Biomimetic hydroxyapatite coating on 3D graphene scaffolds

To enhance hMSC growth, many efforts have been made on the functionalization of 3D scaffolds [31]. It has been reported that the surface functionalization with specific chemical groups or extra-cellular matrix proteins could improve cell attachment and also influence lineage specification [32]. The CaP-based mineral coatings have been demonstrated to be efficient to enhance cellular adhesion, proliferation and differentiation [33,34].

In this study, 10 times concentrated SBF was used for the mineralization of graphene scaffolds. It has been reported that the HCO$_3^-$ concentration had a significant effect on mineral coating on poly(lactic-co-glycolic acid) fibers [35]. Therefore, we systematically controlled the mineral coating morphology by modulating the HCO$_3^-$ concentration (5, 10, 15, and 20 mM) in 10SBF. Fig. 1b-e shows morphological changes of 3D graphene scaffolds after incubation in 10SBF containing different concentration of HCO$_3^-$.

When the concentration of HCO$_3^-$ increased to 10 mM, tiny particles appeared on the surface (Fig. 1c). The TEM image revealed that ultrafine particles with a size of a few tens nanometers were homogeneously distributed throughout the skeleton surface (Fig. 2b). After incubation in 10SBF with 15 mM of HCO$_3^-$, the morphology changed significantly and the scaffolds were partially covered with agglomerates (Fig. 1d). After mineralization with an even higher concentration of HCO$_3^-$ in 10SBF, the scaffolds were fully encased in thick mineral sheaths (Fig. 1e). It has been reported that anionic groups such as —COO$^-$ and OH$^-$ could attract Ca$^{2+}$, resulting in local super saturation and nucleation [36]. The successful mineralization of 3DG scaffolds could be attributed to the oxygen-containing functional groups on the graphene surface by plasma treatment. These observations indicated that graphene foams could induce the nucleation of calcium phosphate on their surface, and facilitate the mineral crystallization and growth.

Previous study has reported that the mineral coatings composed of CaP could modulate the morphology of hMSCs. Many studies demonstrated that the nano- or micro-topographies could promote bone-to-implant contact and enhance osteoblast functions [38,39]. Thus, the 3DG scaffold decorated with mineral nanoparticles (the specimen with 10 mM HCO$_3^-$) was selected for further examination. AFM images showed that nanoparticles were observed on the mineralized 3DG debris, and the particle size was in the range of tens nanometers to hundreds nanometers, which were similar with those on 2DG films (Fig. S1). As determined by AFM, the root mean square deviations (Rq) was 7.0 ± 2.1 nm and 15.5 ± 4.5 nm for 2DG films and mineralized 3DG scaffolds respectively, suggesting that the surface roughness of mineralized graphene scaffolds was increased (Fig. 2c–f). Next, the hydrophilicity of the substrates was evaluated by using the sessile drop method. Fig. 2e and 2f insets showed that the contact angle of 2DG films and mineralized 3DG scaffolds were 78.9 ± 0.44° and 27.5 ± 1.95° respectively, indicating that the mineralized 3DG scaffolds became more hydrophilic due to the plasma treatment and introduction of mineral nanoparticles on the surface. In addition, the surface zeta potential of mineralized 3DG dropped from 0.7 to −4.7 mV, which confirmed the hydrophilic nature after mineralization (Fig. S2).

The EDX (Fig. 3a) and XPS (Fig. 3b) measurement were further employed to investigate the presence of calcium (Ca) and phosphorus (P) elements in the mineralized component on the graphene foams. Also, it was found that Ni and etching chemistry residual (FeCl$_3$) content, which would result in cytotoxicity, was undetectable. Moreover, Raman spectra of pristine graphene foams, pure HA crystal and mineralized 3DG were examined (Fig. 3c).

The weak intensity of Raman D band indicated that the grown graphene was of high quality, while the integrated intensity ratio between 2D and G band revealed the coexistence of few-layer or multi-layer domains [40]. In addition, for mineralized 3DG, it can be seen that besides the peaks assigned to graphene (1340 cm$^{-1}$ and 1580 cm$^{-1}$), the peak at around 960 cm$^{-1}$, which is characteristic of crystalline HA [35], appeared for the mineralized 3DG foams. Further, the crystal structure of the mineral phase was determined by XRD measurement (Fig. 3d). Compared with the reference data of pristine graphene and HA (ICPD No. 09-0432), the patterns of mineralized graphene foams almost matched those of pure graphene and HA preparations. The indexed crystal planes of HA in (211), (222), (222), (004), (322), (422) and (215) were shown in the
XRD profile of the mineralized graphene foams. It can be concluded that the deposition on the graphene foam was consist of HA.

3.3. Cell adhesion, viability and proliferation on HA deposited 3DG scaffolds

The hMSCs, which have great potential in bone tissue engineering, were cultured on the HA-3DG scaffolds. The cell viability on 3DG scaffolds with varied HA morphologies was investigated to evaluate whether the HA-3DG scaffolds composites satisfied the hMSC culture, and what level of mineralization was suitable for their growth. In Fig. 4a, MTT assay showed the mineralization with different concentration of $\text{HCO}_3^-$ in 10SBF, that is, 3DG scaffolds with different HA morphology, had a significant influence on cell growth. Specifically, the 3DG scaffolds after mineralization in 10SBF with 5 mM of $\text{HCO}_3^-$, had similar cell viability compared with the pristine graphene foams. While the 3DG scaffolds after mineralization in 10SBF containing 10 mM of $\text{HCO}_3^-$, which had HA nanoparticles on their surface (Fig. 1c), showed more than double the ability of proliferation compared with the pristine 3DG scaffolds.

Fig. 1. Influence of $\text{HCO}_3^-$ concentration on mineralization of 3DG scaffold. The SEM images illustrate the surface morphology of pristine 3DG and mineralized 3DG at low and high magnification. (a) Pristine 3DG, (b–e) 5, 10, 15 and 20 mM of $\text{HCO}_3^-$.
alone. Whereas, when the $HCO_3^-$ concentration increased to 20 mM, the graphene surface was fully covered by the HA precipitation (Fig. 1e), and the cell viability was decreased significantly. This observed change in cell viability on the HA-3DG scaffolds with varied morphology demonstrated that the 3DG scaffolds decorated with HA nanoparticles favored cell growth, which was consistent with the report by Choi et al. [37]. It is speculated that the 3DG scaffolds deposited with nano-sized HA particles could combine the advantages of graphene substrate and HA nanoparticles. Therefore, the 3DG scaffolds with mineralization in 10SBF containing 10 mM of $HCO_3^-$ were selected for the further study of stem cell culture.

In addition, the cell proliferation on HA-3DG scaffolds and 2DG films after 1, 3, 5, and 9 days of culture were studied. As shown in Fig. 4b, hMSCs propagated well in HA-3DG scaffolds, and the HA-3DG scaffolds exhibited faster cell proliferation than 2DG films, even than HA-2DG films (Fig. S3). Thus, the HA-3DG scaffolds enhanced the proliferation statistically compared with the 2DG films, which could attribute to the synergistic effect of large interspace for cell growth in 3D scaffolds [41] and incorporation of HA nanoparticles [42].

The morphology of hMSCs on HA-3DG scaffolds after 7d culture was further examined via confocal imaging and SEM analysis (Fig. 5). As shown in Fig. 5a, the hMSCs adhered and spread well on...
the wall of the scaffolds with a high population and good distribution. It should be noted that the cells could penetrate into the scaffolds due to the 3D structure with interconnected macro porosity. The skeleton surface was fully covered by confluent cells, which was in good agreement with the proliferation results (Fig. 4b). Fig. 5b, an enlarged image, showed that hMSCs maintained their spindle shape, and stretched long even up to 400 μm in length. Interestingly, some protrusions extended across the porous

Fig. 3. (a) EDX of HA-3DG. (b) XPS, (c) Raman spectra and (d) XRD patterns of pristine 3DG, HA-3DG and pure HA crystal. (A colour version of this figure can be viewed online.)

Fig. 4. hMSC proliferation on pristine 3DG and HA-3DG mineralized with different HCO_3/C0_3 concentration and proliferation at different culture time. (a) The cell density on pristine and mineralized 3DG scaffolds. (b) The cell proliferation at day 1, 3, 5 and 9. (n = 3/group, **p < 0.01). (A colour version of this figure can be viewed online.)
region in the HA-3DG scaffolds. It has been reported that the elongated MSC morphology may permit a higher proliferation rate [43]. The nanotopology and its secondary effects such as wettability and roughness (Fig. 2) enhanced cell–scaffold interactions and cell–cell contacts, and offered a suitable environment for hMSC's adhesion and proliferation.

Cell viability was further analyzed using Calcein-AM/EthD-I Live/Dead kit. Calcein-AM stained live cells (green) and EthD-I stained dead cells (red). In Fig. 5c, it can be seen that almost 90% of the cells were viable on the surface of the HA-3DG, indicating that the HA-3DG scaffolds did not exhibit cytotoxic activity against hMSCs. In an effort to obtain a clear observation about the interaction of the hMSCs and scaffolds, the hMSCs cultured on HA-3DG scaffolds were imaged by SEM. In Fig. 5d, it can be seen that hMSCs spread extensively on the skeleton and showed a spindle shape with noticeable filopodia extensions and cellular protrusions. These data collectively demonstrated that the porous 3D scaffolds composed of graphene and HA nanoparticles were biocompatible with hMSCs and strong filopodia/scaffold interaction was formed which may favor proliferation and osteogenic differentiation of hMSCs.

3.4. hMSC differentiation on HA-3DG scaffolds

hMSCs have been shown to differentiate into osteoblasts, chondroblasts, and adipocytes in standard in vitro cell culture-differentiating conditions. In this study, differentiation of hMSCs on HA-3DG scaffolds was investigated in the presence of an osteogenic medium [44]. Note that the conventional osteogenic medium usually contains dexamethasone [45], or BMP2 [46], which can lead osteogenic differentiation. In this study, none of any chemical inducers was administered, and the effect of substrate alone on hMSC differentiation was studied. Immunofluorescent staining was performed by using three typical protein markers, namely, OC4-30 (osteocalcin) for osteoblasts, Collagen II for chondrocytes and FABP4 for adipocytes. Both of 2DG films and HA-3DG scaffolds showed OC4-30- and Collagen II–positive staining, suggesting the directed osteogenic and chondrogenic differentiation of hMSCs (Figs. 6 and 7), but suppressed adipogenesis, which was consistent with the previous study [27]. In addition, it was found that the expression of OC4-30 on HA-3DG scaffolds was stronger than that on 2DG and HA-2DG (Fig. S4) films, indicating that the HA-3DG scaffolds were favorable for osteogenic differentiation. Since substantial change was observed for osteogenic differentiation, OC4-30 staining was studied for 7, 10 and 14 days of culture (Fig. 8). It can be observed that the OC4-30 expression was enhanced with an increased incubation time. At day 7 and day 10, the OC4-30 expression remained strikingly high on HA-3DG scaffolds but was barely detectable on 2DG films. These results showed that the osteogenic marker in the hMSCs was up-regulated on the HA-3DG scaffolds compared to those on 2DG films, indicating the dramatically enhanced osteogenic differentiation.

For quantitative analysis, hMSCs were harvested and subjected to western blot analysis. Fig. 9 shows the CD44 (the marker of hMSCs), OC4-30 and Collagen II expression at the intermediate time points (7, 10 and 14 days). The CD44 expression decayed with the increased time. And its value remained lower on the HA-3DG scaffolds, which is 67%, 35% and 17% of those on 2DG films after 7, 10 and 14 days of culture respectively, suggesting that the hMSCs on HA-3DG scaffolds spontaneously lost the pluripotency. Conversely, the expression of OC4-30 and Collagen II was remarkably high, and increased in a time-dependent manner on both of

Fig. 5. hMSC adhesion on HA-3DG scaffold. Low- (a) and high- (b) magnified fluorescence images of hMSCs proliferated on HA-3DG for 3 days, immunostaining markers were tubulin (green) for hMSCs and DAPI (blue) for nuclei. (c) Cell viability assay of hMSCs on HA-3DG scaffold, (d) SEM images of hMSCs cultured on HA-3DG scaffold. (A colour version of this figure can be viewed online.)
HA-3DG scaffolds and 2DG films. While, the FABP4 expression was too low to appear on Figure (data not shown). These observations suggested that the HA-3DG scaffolds and 2DG films could lead to osteogenic and chondrogenic differentiation, but not adipogenic differentiation over the whole duration of the experiment (14 days). Particularly, the HA-3DG scaffolds had a dominant influence on osteogenic differentiation. The OC4-30 expression was already very strong by day 7 on the HA-3DG scaffolds, and it became strikingly high at day 10 and 14. However, it was barely detectable on 2DG films at day 7 and day 10, and it was visible until the 14th day. Quantitatively, the OC4-30 expression on the HA-3DG scaffolds had 13.7, 10.9 and 1.89 fold increase at 7th, 10th and 14th day respectively as compared with those of 2DG films, exhibiting a faster osteogenic commitment and stronger osteogenic expression.

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**Fig. 6.** The differentiation of hMSCs on (a) HA-3DG scaffold and (b) 2DG film (control). Representative fluorescence images illustrated differentiated hMSCs under differentiation conditions, the cells were immunostained with DAPI for nuclei (blue), Collagen II for chondrocyte (green) and OC4-30 for osteocyte (red). (A colour version of this figure can be viewed online.)

**Fig. 7.** The fluorescence images of the differentiated hMSCs on (a) HA-3DG scaffold and (b) 2DG film (control) cultured under differentiation conditions, the cells were immunostained with DAPI for nuclei (blue), Collagen II for chondrocyte (green) and anti-FABP4 for adipocyte (red). (A colour version of this figure can be viewed online.)
which was further confirmed by the alkaline phosphatase (ALP) activity assay (Fig. S4).

Together with the immunostaining results, it can be concluded that mineralized graphene scaffolds accelerated and enhanced the hMSC differentiation towards osteoblasts significantly (Figs. 6–9). The chemistry and topography of biomaterials are critical parameters for directing cell fates [47,48]. Recently several studies have shown the osteoconductive and osteoinductive properties of calcium phosphate mineral and composites [33]. A study by Sargeant reported that the mineralized peptide amphiphile nanofibers, which had HA nanoparticles, promoted osteogenic differentiation of MSCs [49]. In agreement with these findings, the formation of HA particles on the graphene scaffolds via mineralization in this study showed a similar osteogenic activity. Besides chemical composition, the nanoscale topography and roughness of the substrates could tune the stem cell adhesion and subsequent differentiation. It has been well demonstrated that the subnano-, nano-, and submicron-surface features can trigger integrin receptors, and induce osteogenic differentiation of MSCs [50] or adipogenic differentiation of MSCs [51]. Khanget et al. systematically studied the MSC responses on controlled titanium surface features from the sub-nano to sub-micron scales, and they demonstrated that the most influential surface dimension in enhancing osteogenic differentiation was the nano-submicron hybrid surface structures [50]. Our work was in agreement with these findings that the mineralized graphene scaffolds with nano-to submicro-sized HA particles would promote MSC attachment and adhesion through contact guidance, and subsequent long-term function of stem cell differentiation. It’s worth to note that surface roughness [16,52] and dimensional difference [18] are also important cues for governing osteogenic differentiation of MSCs. In the present work, the mineralized graphene foam scaffolds, which had higher surface roughness (Figs. 1 and 2) and interconnected 3D architecture [16], could act a stronger supporter for osteogenic commitment of MSCs. Taken together, the enhanced osteogenic differentiation associated with the mineralized 3DG scaffolds could be due to the synergistic effect of compositional cue (i.e., HA), dimensional cue (Fig. S4) and topographical cue (i.e., nano-scale feature).

4. Conclusion

A class of hybrid scaffolds based on graphene and nano-sized HA particles has been developed through biomimetic mineralization of 3DG scaffolds in 10SBF. The mineral coating morphology changed from plat-like, to particles, to net-like with the increase of HCO₃⁻ concentration. It was found that after mineralization in 10SBF with 10 mM of HCO₃⁻, 3DG scaffolds was decorated with HA particles of tens nanometers. The mineralized 3DG scaffolds showed higher cell proliferation of hMSCs compared with those of 2DG films. More importantly, the HA-3DG scaffolds supported the cell adhesion and
el elongation, and subsequently enhanced their differentiation towards osteoblasts. The mineralized 3DG scaffold holds great potential for differentiating osteogenic stem cells, creating the ultimate building blocks for bone tissue-engineering scaffolds.

Acknowledgements

This work was supported by Collaborative Innovation Center of Radiation Medicine of Jiangsu Higher Education Institutions, National Natural Science Foundation of China (NSFC No. 31400806, 21505097, 11204349); NSF of Jiangsu Province (BK20130306, BK20150283); University Scientific Research Project of Jiangsu Province (15KJB150027). We thank Mr. Chuntao Chen and Dr. Zheng Ying for confocal imaging and helpful discussion.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.carbon.2016.04.027.

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