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Enhanced bone tissue regeneration using 3D printed microstructure incorporated with hybrid nano hydrogel

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Abstract

Three-dimensional (3D) functional constructs with biomimetic mechanical and chemical properties are ideal for various regenerative medicine applications. Within a 3D fabricated construct these properties are intrinsic characteristics of the materials and fabrication method. In this respect, the current use of hydrogels for musculoskeletal tissue repair are not perfect due to the lack of suitable mechanical properties, as well as the high biomimetic requirement for success. To overcome this limitation, we developed a novel functionalized hydrogel with bioactive gold nanoparticles (GNPs) and reinforcing 3D printed microstructure via fused deposition modeling (FDM) for bone tissue regeneration. We used the biodegradable thermoplastic polylactic acid (PLA) as the 3D printed microstructure in combination with photo-curable gelatin hydrogels as the encapsulation matrix for the incorporation of cyclicRGD conjugated GNPs (RGNP), and investigated their mechanical properties. In addition, human adipose-derived stem cells (ADSCs) were encapsulated within the gelatin hydrogel and examined for viability, morphology, and osteogenic differentiation in vitro. The results showed that the stiffness of the composite hydrogel with reinforcing 3D printed microstructure can be readily modulated to simulate the stiffness of the human mandibular condyle. ADSCs encapsulated in the composite structures remained viable within the hydrogel and showed excellent spreading on the 3D printed PLA microstructure. More importantly, osteogenic differentiation with incorporated RGNP promoted significantly higher gene expression of osteogenic specific factors. Therefore, reinforced composite hydrogels are suitable for stem cell differentiation control and bone tissue regeneration.

Introduction

Within the biomedical and biotechnological field, biodegradable hydrogels have been proven as an important class of biomaterials due to their excellent biocompatibility,

biodegradability, water content, and soft mechanical properties which mimic natural tissue.¹⁻³ Recently, there has been a great deal of research focusing on cell encapsulated hydrogels and 3D printing to engineer various tissues ranging from liver to bone.^{4, 5} 3D bioprinting of cell-laden hydrogels for the fabrication of 3D functional living tissues analogs is an effective method capable of transferring a large amount of desired cells into a damaged tissue.^{6, 7} Many groups have developed these approaches to improve, replace or regenerate damaged/diseased tissue, but the low mechanical properties of hydrogels remain an inherent limitation. In most systems, insufficient mechanical properties of the hydrogel compromise the structural integrity of the scaffold leading to compromised results especially in musculoskeletal tissue repair.^{4, 8, 9} In addition, the loss of cell viability during processing of 3D bioprinting such as increased dispensing pressure, nozzle gauge and fabrication time are disadvantageous for the production of 3D functional constructs.^{10, 11} When attempting to extrude a viscous hydrogel solution within living cells, higher compressive stresses inflicted upon the 3D printed material results in decreased cell viability. Although highly viable 3D cell-laden constructs can be fabricated using low dispensing pressure and larger nozzles, other drawbacks such as decreased structure resolution and printing speed arise.¹²⁻¹⁴ To address these shortcomings, many studies have dedicated significant effort to develop the combination of cell-laden hydrogel with woven or non-woven thermoplastic polymers,^{15, 16} nano- or microfiber meshes,^{17, 18} and inorganic materials.^{19, 20} The mechanical properties of composite hydrogels are enhanced and can support cell viability and function from mechanical stress during the regeneration process. However, even though hybrid/composite hydrogels are mechanically strong, several disadvantages still remain for various reasons, such as the limited control over 3D architecture,²¹ toxic degradation products,²² and insufficient mechanical properties compared to native bone tissue.^{23, 24}

In recent years, focus has shifted to improve bone tissue regeneration using osteo-conductive or inductive agents.²⁵ With regards to the successful delivery system of osteogenic agents, it is necessary to retain the agent at the defected area for periods of time needed to effectively treat the defect.²⁶ Therefore, various types of nano- and micro carriers have been used as biomolecule delivery systems.²⁷ Amongst these, gold nanoparticles (GNPs) are very attractive materials for systemic delivery due to several inherent benefits including non-cytotoxicity, ease of preparation with defined sizes, high reproducibility, and easy surface modification with various thiol-containing biomolecules through gold-thiol bonding.²⁸ In particular, GNPs are the most efficient carrier in the field of bone tissue regeneration because of their effects on the bone remodeling process by enhancing osteogenic differentiation while inhibiting osteoclast activity.²⁹⁻³⁴

In light of these findings, we developed a functionalized and reinforced composite hydrogel with 3D printed microstructure and bioactive gold nanoparticles (GNPs). 3D printed microstructures with different porosities were fabricated using the biodegradable thermoplastic polylactic acid (PLA) by fused deposition modeling (FDM). Cyclic peptide containing the cell-adherent arginine-glycine-aspartate (RGD) sequence were conjugated upon the surface of GNPs (RGNPs) to enhance human adipose-derived stem cell (ADSC) behavior for bone remodeling. Then, functional cell-laden hydrogels incorporated with methacrylated gelatin (GelMA) and RGNPs were combined with the PLA microstructure by infusing and UV-induced chemical crosslinking. We demonstrated that ADSC encapsulated

within reinforced composite hydrogels can be exploited to produce biomimetic 3D structures with both high mechanical strength and osteogenic differentiation potentiality. Therefore, this strategy may be a useful platform for regulating stem cell functions and treating defected bone tissues.

Experimental

3D PLA microstructure design and fabrication

The 3D PLA microstructure was designed and printed based on our previously reported method.³⁵ For embedding the hydrogel inside the 3D PLA microstructure, the computer-aided design (CAD) based architectural models with the square open pore were designed with various fiber spacing from 0.6 mm to 1.4 mm, and printed using a FDM 3D printer. The 3D PLA microstructures were composed of parallel aligned fibers measuring 200 μm thickness at a 250 μm layer height. The CAD models of various microstructure were used to calculate their porosity.

Preparation and characterization of GNPs and RGNPs

GNPs were synthesized by the reduction of chloroauric acid with trisodium citrate as described previously.²⁹ The GNP solution was used without further purification in all experiments. To prepare RGD-conjugated GNPs, Tween 20 solution (1 mM, 500 μl) was first added into 5 mL of GNPs solution obtained from the previous step and then the mixture was stirred for 10 min. After stirring, 500 μl of RGD peptide (1 mM) was added and then reacted for 2h to allow the chemical exchange of the citrate with thiol. The peptide-conjugated GNPs (RGNPs) were purified by centrifugation (12,000 rpm for 10 min) twice. After decanting the supernatant, the pellet was re-dispersed in distilled water. To determine the surface plasmon resonance of GNPs and RGNPs, ultraviolet-visible (UV-vis) absorbance was measured using a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific, USA). To determine the size of GNPs and RGNPs, atomic force microscopy (AFM) was performed using a CypherTM AFM (Asylum Research, USA) in conventional tapping mode with an Olympus Micro Cantilever model AC160TS.

Preparation of reinforced composite hydrogel

GelMA was synthesized by methacrylation of gelatin derived from type A porcine skin, as described previously.³⁰ Synthesized GelMA was dissolved in dulbecco's phosphate-buffered saline (DPBS) containing 0.1 wt% photo-initiator (Irgacure 2959, Sigma-Aldrich) at 8 wt%. Subsequently, 1 mL of these mixed solutions were injected in the 3D PLA microstructure and exposed to UV light for 60 sec (gelatin hydrogel, Gel). To compare the functionality of the GNPs and RGNPs, 1 mL of aqueous GNPs and RGNPs solution were centrifuged and the pellets were then added into 4 mL of the above mixture and labelled as 'Gel-GNP' and 'Gel-RGNP'. All samples were incubated in DPBS at 37 °C for 24 h and used for further experiments.

Mechanical characterization

For the characterization of mechanical properties, a biopsy punch was used to take cylindrical samples measuring 5 mm diameter and 2 mm height (10 layer). The compressive

modulus of GelMA hydrogel, PLA scaffolds and the reinforced composite hydrogels was measured using a MTS criterion universal testing system (MTS Corporation, USA) in single uniaxial uniform compression mode ($n = 8$). The samples were placed on the platen and a static force was applied at a rate of 5 mm min^{-1} . The compressive modulus was calculated from the linear elastic region of the stress-strain curve.

ADSC culture

ADSCs (Thermo Fisher Scientific, USA) were culture in MesenPRO RS™ medium composed of MesenPRO RS™ growth supplement, 2 mM glutamine, 1% penicillin/streptomycin (PS). The cells were cultured under standard cell culture conditions in a humidified atmosphere with 5% CO_2 at 37°C . For cellular experiments and analyses, ADSCs were harvested, collected by centrifugation at 1200 rpm for 5 min and then used at six cell passages or less. For osteogenic differentiation studies, ADSCs were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 1% PS, $50 \mu\text{g mL}^{-1}$ L-ascorbic acid, 10 mM β -glycerophosphate and 10 nM dexamethasone.

Cell viability assay

Cell viability of the various scaffolds was evaluated by using a cell counting kit (CCK, Dokindo Molecular Technologies INC., Japan) assay. After seeding the cell in well plates, the cells were treated with various concentrations of RGD, GNPs and RGNPs and their optical density (OD) was measured at 450 nm by. The cell viability was conducted 24 and 48 h.

Cell adhesion and proliferation

To evaluate the effect of the Gel-RGNP composite hydrogels on cell adhesion, spreading and proliferation, cell-laden hydrogels were prepared by UV-induced chemical crosslinking using the various kinds of hydrogel solutions with ADSCs (2×10^6 cells per ml). After encapsulating the cells, the morphologies of attached and spread cells were examined after 24 h. The cytoskeletal organization was identified with actin staining using fluorescein (FITC)-phalloidin. The samples were rinsed three times with DPBS, fixed in 10% formalin for 60 min, permeabilized in 0.1% Triton X-100 for 30 min, and blocked with 2.5% BSA for 60 min at room temperature. Next, the samples were incubated with FITC-phalloidin for 60 min and washed three times with DPBS. The stained samples were observed and imaged using a Zeiss 710 confocal laser scanning microscope (CLSM). ImageJ (NIH Freeware) was used to quantify total area of cell spreading within the hydrogels.³⁶ To confirm the cell proliferation rate, cell-laden hydrogels were incubated for 1, 4, and 7 days, and quantified using the CCK assay as previously described.

Quantitative real-time polymerase chain reaction (real-time PCR)

To evaluate the osteogenic gene expression profiles of type I collagen (COL1), alkaline phosphatase (ALP), runt-related transcription factor 2 (RUNX2) and bone sialoprotein (BSP), real-time PCR was performed after 7 and 14 days of osteogenic differentiation. At each time point, the total RNA of ADSCs encapsulated with Gel, Gel-GNP and Gel-RGNP was isolated by using an RNeasy Plus Mini Kit (Qiagen, USA) per manufacturer's

instructions. The primers of the measured mRNA genes were as follows: COL1 (Forward: ATG ACT ATG AGT ATG GGG AAG CA, Reverse: TGG GTC CCT CTG TTA CAC TTT), ALP (Forward: AGC TGA ACA GGA ACA ACG TGA, Reverse: CTT CAT GGT GCC CGT GGT C), RUNX2 (Forward: AAC CCA CGA ATG CAC TAT CCA, Reverse: CGG ACA TAC CGA CGA GGG ACA TG), BSP (Forward: AAC GAA GAA AGC GAA GCA GAA, Reverse: TCT GCC TCT GTG CTG TTG GT), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Forward: ATG GGG AAG GTG AAG GTC G, Reverse: GGG GTC ATT GAT GGC AAC AAT A). Real-time PCR was analyzed by using SsoFast™ EvaGreen® Supermix (Bio-Rad, USA). Threshold cycle values were calculated by using a comparative cycle threshold method. The fold change of ADSCs cultured in standard cell culture conditions was set at 1-fold and the ratio of the normalized fold change was calculated, all results were normalized by GAPDH.

Statistical analysis

All values were presented as the mean $\pm$ standard deviation (SD). A one-way analysis of variance (ANOVA) with Student's *t*-test was used to verify statistically differences amongst the groups. The differences with *p*-values (**p* < 0.05, ***p* < 0.01) were considered statistically significant.

Results and discussion

Fabrication of 3D-printed PLA scaffolds infilled with hydrogel and GNP

A schematic diagram of the fabrication process of 3D PLA scaffolds infilled with hydrogel and GNP is shown in Figure 1. First, 3D PLA scaffolds were pre-designed with various fiber spacing from 0.6 mm to 1.4 mm and fabricated with CAD-based architectural designs by FDM (Figure 2A). The resultant scaffolds had porosities varying from 74.2 % to 86.8 % (Figure 2B). All 3D PLA scaffolds had multi layers of parallel aligned fibers with orthogonal orientation, as shown in the optical images (Figure 2C). Prior to infilling the hybrid hydrogel into the 3D PLA scaffolds, cRGD conjugated GNPs (RGNPs) and photo-curable gelatin (GelMA) were synthesized for use as an osteo-inductive agent and incorporating material with RGNPs, respectively. For the synthesis of RGNPs, cRGD was added into the aqueous solution of GNPs, which was synthesized by citrate reduction. After addition of cRGD, the color of the solution changed from light red to pale purple, due to the immobilization of cRGDs on the surface of GNPs. This was in good agreement with the results of UV-vis spectroscopy (Figure 3A). The UV spectral absorption of the GNP appeared at 522 nm, due to its surface plasmon resonance band. However, the absorption peak of RGNP shifted to the right at 549 nm. It is well known that surface modification of GNPs with thiol-containing biomolecules is easy and well-controllable due to their strong gold-thiol bonding strength.²⁸ Therefore, the results indicated that the cRGD is well conjugated on the GNP surface, thereby increasing the size of RGNP than GNP. The morphology of RGNPs was examined by atomic-force microscopy (AFM) and compared to GNPs (Figure 3B). AFM images showed that the synthesized RGNPs have a near-spherical shape with well-dispersion in the range of 20–40 nm. After synthesis of RGNPs, the mixed solutions of GelMA and RGNPs were poured into the 3D PLA scaffolds and photo-cured by UV irradiation.

Effects of cRGD, GNPs and RGNPs on the viability of ADSCs

The cell viability at different concentrations of RGD, GNPs and RGNPs for ADSCs was evaluated by CCK assay at 24 and 48 h (Figure 4). It was found that all components showed no significant cytotoxic effects up to 48 h. On the contrary, GNPs and RGNPs exhibited a slight positive effect on ADSCs proliferation where the cell viability increased at a dose-dependent manner after 48 h. It was reported that the surface ligand of the GNPs have an important influence on the cytotoxicity.^{29–31, 37} In this study, we used two kinds of GNPs covered with citrate and RGD, and they showed no cytotoxicity and even more enhanced cell proliferation. Therefore, this result indicates that the prepared RGNPs could be useful as a biocompatible osteo-inductive agent.

Stiffness of 3D-printed PLA scaffolds infilled with hydrogel and GNP

Compressive mechanical testing was carried out using a universal testing machine at a crosshead speed of 5 mm min⁻¹. The stiffness of PLA scaffolds alone decreased from 729.5 ± 78.3 MPa for 0.6 mm fiber spacing to 303.6 ± 36.7 MPa for 1.4 mm fiber spacing (Figure 5). After infilling with Gel and GNP, the stiffness of the scaffolds showed no significant difference: from 791.3 ± 76.9 to 296.6 ± 17.2 MPa for PLA infilled with Gel and from 704.3 ± 151.1 to 291.5 ± 46.2 MPa for PLA infilled with Gel/GNP. For comparative purposes, the compressive modulus data of human mandibular bone was included in Figure 5.³⁸ This demonstrated that all PLA scaffolds with 0.6 mm fiber spacing (401.0 ± 29.2 MPa for PLA scaffold, 378.9 ± 27.7 MPa for PLA infilled with Gel, 402.0 ± 26.9 MPa for PLA infilled with Gel/GNP) have a similar compressive modulus to human mandibular bone (431.0 ± 217.0). In contrast to PLA scaffolds, Gel has relatively ultra-low compressive modulus with 7.1 ± 1.0 kPa, which is much softer and weaker than human mandibular bone. The mechanical property of Gel is not suitable to fabricate 3D functional human constructs with mechanical properties for clinical restoration of hard bone tissue.^{9, 39} Therefore, reinforcement is required to maintain their 3D shape after implantation. As confirmed by mechanical testing, 3D printed PLA scaffolds after infilling with Gel might help to enhance the mechanical properties and provide initial mechanical strength over a long period of implantation time.

ADSC spreading and proliferation after encapsulation in the various hydrogel component with the 3D printing PLA structure

To verify cytoskeletal organization, spreading, and proliferation of cells, ADSCs were cultured in the various hydrogel component with the 3D printing PLA structure, and determined by F-actin staining and CCK assay at different time intervals (Figure 6). Cell spreading on the outside and inside of ADSC encapsulated hydrogel was confirmed using optical microscopy and confocal laser scanning microscope (CLSM). The cells seeded on the hydrogel surfaces had well-spread and branched morphologies, whereas the cells encapsulated in the hydrogel showed granular shapes (Figure 6A). In addition, cell spreading morphology was noticeably difference and depended on the composition of the hydrogel component. The CLSM images shown in Figure 6B indicate that the adhered cells on the outside of hydrogel become more highly stretched in all specimens. Among these specimens, Gel-RGNP showed the highest cytoskeletal spreading, large lamellar

morphology and numerous stress fibres. It is well known and recognized that the cRGD peptide is an integrin-binding site, which has beneficial effects on cell adhesion, spreading, and actin-filament formation.⁴⁰ Moreover, structurally cRGDs have many advantages such as high affinity interaction with cell surface integrin and low biodegradability than linear RGDs.⁴¹ Therefore, Gel-RGNP provided a greater positive effect on surface cell adhesion when compared to Gel and Gel-GNP groups. Also, we focused on quantitative analyses of cell spreading area to each hydrogel layer and analyses of the cell proliferation rate to investigate the effect of the RGNPs. To confirm actin filament formation and spreading of ADSCs encapsulated in various hydrogel structures, cells were stained with fluorescence labeled phalloidin (Figure 6C). In both types of Gel and Gel-GNP groups, encapsulated ADSCs appeared morphologically granular even after 24 h but Gel-RGNP seemed to induce better spreading throughout the hydrogel network. In addition, the average cell spreading area in Gel-RGNP was twofold higher than in Gel and Gel-GNP under the same culture condition (Figure 6D). With cRGD-induced increases in cell spreading area from Gel-RGNP, cell proliferation rate was significantly increased the inside of Gel-RGNP than that of Gel and Gel-GNP (Figure 6E). Thus, Gel-RGNP appeared to provide a more positive effect on their structure inside to enhance actin filament formation, cell spreading, and proliferation.

Effects of ADSCs encapsulated Gel-RGNP hydrogels on the osteogenic differentiation

To investigate the gene expression profiles of ADSCs encapsulated within various hydrogels during osteogenic differentiation, the mRNA expressions of COL1, RUNX2, BSP and ALP were determined by real-time PCR. COL1, which is an early stage osteogenesis marker, is present in the bone extracellular matrix and expressed at high levels during bone development.^{42, 43} Its maximum level of mRNA expression was reached on day 7, and decreased at day 14 (Figure 7A). Other early osteogenic transcription factors, ALP and RUNX2, displayed an increased expression level over time (Figure 7B, C). These markers are crucial osteogenic transcription factors for the generation of a mineralized bone tissue matrix.^{44, 45} The mRNA expressions of the early osteogenic specific markers, COL1, ALP and RUNX2, significantly increased in the order of Gel, Gel-GNP and Gel-RGNP during the whole period of osteogenic differentiation. Amongst these specimens, Gel-RGNP showed much higher expression than all other sample groups. The expression level of BSP which is a late osteogenic specific marker, increased in all specimens over the course of time. It significantly increased in the following order: Gel < Gel-GNP < Gel-RGNP similar to the ALP and RUNX2 results (Figure 7D). The highest level of BSP was observed with Gel-RGNP at whole time period. Furthermore, the highest amount of stained calcium deposition at 21 days of culture was observed in Gel-RGNP group (Figure 8). Overall in osteogenic differentiation studies, it was found that Gel-RGNP showed the highest mRNA expression of all osteogenic differentiation markers.

In summary, we found that, in order to provide suitable mechanical strength over time for long-term implantation, reinforced composite hydrogels with 3D printed microstructure provide positive effects to achieve 3D functional constructs with suitable mechanical properties when compared to hydrogel constructs alone. 3D printed microstructures provide increased stiffness with a wide range of mechanical strength including the human

mandibular condyle (Figure 5). Also, embedding RGNP into reinforced hydrogels leads for further enhanced biological effects. Before cell encapsulation into the hydrogel, cell viability tests confirmed that all components had no significant toxicity *in vitro* (Figure 4). Moreover, although here we did not examine the *in vivo* toxicity of GNPs, previous studies showed that GNPs did not cause any biological toxicity after *in vivo* injection.^{46, 47} Therefore, GNPs are suitable materials for use as a biocompatible osteo-inductive agent. At the cellular level, RGD can be useful as a critical regulator of cell adhesive response. Previous reports have shown that RGD enhances the attachment of numerous cell types on the different types of biomaterials.^{48–50} As shown in cell study, the Gel-GNP group did not show any effect on cell spreading within a hydrogel layer as compared to the Gel group. In contrast, when cells were encapsulated in Gel-RGNP, a very high rate of cell spreading area and proliferation rate was observed (Figure 6). As a result, increasing cell adhesion, spreading and proliferation in Gel-RGNP will promote the osteogenic differentiation of ADSCs under osteogenic culture conditions (Figure 7, 8). Interestingly, Gel-GNP showed an enhanced osteogenic differentiation without significant difference on cell spreading and proliferation rate. These results were attributed to the incorporated GNPs, which can be used as osteogenic promoters *in vitro* and *in vivo*.³⁰ Taken together, our composite constructs with biomechanical and biomimetic properties served two main advantages of this approach to provide strength reinforced microstructure and enhanced cell behavior, including cell attachment, spreading, proliferation and osteogenic differentiation. As mentioned earlier, the only hydrogel is hard to maintain their 3D shape by the process of surgical operation and other handling factors.^{51–54} In these cases, our approach to overcome the weak mechanical strength of hydrogel can eliminate its limitation for hard tissue regeneration as tissue scaffolds by reinforcement via 3D printed microstructure.

Conclusions

In conclusion, we have successfully designed and developed a novel functional hydrogel incorporating RGNPs, which reinforced the mechanical strength through the combination of 3D printed microstructure and hydrogel. The stiffness of the composite microstructure was significantly increased to a similar level of native bone tissue. Stem cell viability tests suggest that all components had no significant cytotoxic effects while RGNPs showed a dose-dependent cell proliferation manner. Moreover, the addition of RGD to the composite constructs enhanced the cellular behavior. The *in vitro* experiments showed that encapsulated ADSCs in the Gel-RGNPs promoted significantly higher cell adhesion, spreading and proliferation, which led to an increased gene expression of osteogenic specific markers. Our findings suggest that reinforced composite hydrogels can be a useful strategy for improving the bone tissue regeneration.

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Fig. 1. Patient-specific bone tissue regeneration using 3D Printing PLA incorporated with hybrid hydrogel composed of gold nanoparticles and human adipose derived stem cells.

Fig. 2. (A) Pre-designed 3D scaffolds and (B) their porosity. (C) Optical images of different 3D PLA scaffolds with various fiber spacing, Scale bars = 500 μm .

Fig. 3. Synthesis and characterization of cRGD conjugated gold nanoparticles (RGNP) by (A) UV spectrometer and (B) AFM.

Fig. 4. Viability of ADSCs in the presence of RGD, GNP and RGNP at different concentrations, investigated by CCK. “*” indicates significant difference of $p < 0.01$ as compared with the control at respective hours.

Fig. 5. Compressive modulus of 3D printing PLA scaffolds, Mandible, and Gelatin hydrogel. (Data of mandibular bone reproduced from Reference [38]) There are no significant differences between PLA scaffold and PLA infilled with hydrogel and GNP. “*” indicates significant difference of $p < 0.01$.

Fig. 6.

The effect of altering hydrogel component with the 3D printing PLA structure on ADSC spreading and proliferation. (A) optical images of ADSC spreading outside and inside of the hydrogel structures. (B) Actin cytoskeletal organization of representative ADSCs to exist their outside after 24h of seeding. Scale bars = 100 μm. (C) Cell outlines of twelve representative ADSCs to exist their inside after 24h of seeding. Scale bars = 100 μm. (D) Quantification of ADSCs spreading area of the hydrogel inside. (E) Proliferation of ADSCs cultured on the Gel, Gel-GNP, and Gel-RGNP, investigated by CCK. “*” indicates significant difference of $p < 0.01$.

Fig. 7.

Gene expression levels of ADSCs culture in the Gel, Gel-GNP, and Gel-RGNP at 7 and 14 days for osteogenic differentiation markers: (A) COL1, (B) ALP, (C) RUNX2, and (D) BSP. Relative gene expression of each gene (mean $\pm$ SD), normalized to the expression of the housekeeping gene GAPDH, are compared with ADSCs cultured in standard cell culture conditions. “*” indicates significant difference of $p < 0.05$. “**” indicates significant difference of $p < 0.01$.

Fig. 8. Deposition degree of calcium from ADSCs culture in the Gel, Gel-GNP, and Gel-RGNP at 21 days. (a) Optical images of ARS stained complex structures and (b) quantification of mineralization by UV spectrometer. “*” indicates significant difference of $p < 0.01$.