Strong and biocompatible three-dimensional porous silk fibroin/graphene oxide scaffold prepared by phase separation

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A B S T R A C T
Silk fibroin (SF) is blended with graphene oxide (GO) to prepare the strong and biocompatible three dimensional porous SF/GO blended scaffold via phase separation. GO could be well dispersed in SF solution and GO could also be well distributed in the SF scaffold. Furthermore, the introduction of GO can lead to structural change in the blended scaffold. Higher concentration of GO resulted in more compact structure and smaller pore size of the composite scaffolds without decreasing their porosity. Scanning electron microscopy and energy dispersive spectrometry results also reveal that SF and GO are homogeneous blended together. Analysis of chemical structures of the scaffold shows that addition of GO do not affect the crystalline structure of SF and it is evenly blended with SF. The blended scaffold has significantly higher breaking strength than the pure SF scaffold. In vitro study indicates that both pure SF scaffold and SF/GO composite scaffold support proliferation of MC3T3-E1 osteoprogenitor cells. However, the addition of GO contribute to the proliferation of MC3T3-E1 osteoprogenitor. The testing results show that the blended scaffold is an appropriate candidate for tissue engineering.

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1. Introduction

Bombyx mori silk extracted from silkworm is a biopolymer fiber which has been extensively used in textiles for thousands of years due to its exceptional mechanical properties and luster [1, 2]. Native Bombyx mori silk is composed of silk fibroin (SF) coated with silk sericin proteins. Due to favorable biocompatibility, biodegradable and minimal inflammatory reaction [3, 4], SF has been fabricated into a variety of silk-based materials, such as gel, fiber, powder, sponge, film and tube, and so on [5–10], and these silk-based materials have been widely used for tissue engineering scaffold [11–16].

During the past three years, there are many reports about SF scaffolds prepared by phase separation, which are used for the tissue engineering [17–19]. As we known, tissue engineering scaffolds have high requirements for mechanical performance and biocompatibility of materials. However, in practical applications, there are problems that the strength of SF scaffolds is not high enough and the biocompatibility needs to be further improved. Therefore, SF scaffold is often modified to improve the strength and biocompatibility [20–21]. In our previous research, we have also explored the related research of improving the mechanical properties and biocompatibility of SF scaffold. One research is that the SF scaffold is crosslinked with organic alcohols to enhance the strength [22, 23], but the scaffold is hard and brittle after crosslinking with organic alcohols. Furthermore, organic alcohol treatment may decrease the biocompatibility of scaffolds. Another research, gelatin is blended with SF to improve the biocompatible of SF [24, 25], the results show that blending of gelatin does enhance the biocompatibility of SF scaffold. However, there is a problem that the mechanical strength of SF scaffold is not high enough.

Graphene oxide (GO) contains a large number of functional groups and an extremely large surface-to-volume, which gives some unique properties of the GO in reinforcement, biomaterials and other fields widely [26–28]. Recently, there are a few reports about the GO based biomaterials. However, till now, there has been no report on the SF/GO composite scaffold. In particular, there is no report on GO resulted in the improvement of both strength and biocompatibility of the scaffold. In this paper, the GO is prepared, and then GO is dispersed in SF solution. The three dimensional porous SF/GO blended scaffold is fabricated by phase separation. The fluid property of the SF/GO blended solution is investigated by rheometer. Macroscopic and microscopic morphology of the blended scaffold are observed by scanning electron microscopy (SEM), energy dispersive spectrometry (EDS), X-ray diffraction (XRD). In particular, the mechanical property and biocompatibility...
are characterized by electronic strength tester and cell culture. The testing results show that the blended scaffold is an appropriate candidate for tissue engineering.

2. Results and discussion

2.1. Morphology and microstructure of GO

The as-prepared GO sheets have lateral dimensions of several micrometers and a thickness of about 0.8 nm (Fig. 1a–b), which are characteristics of single layer GO sheets [29]. The chemical structure of GO was studied by Raman and XPS. The Raman spectrum has two prominent peaks at 1340 and 1580 cm\(^{-1}\) (Fig. 1c) and they are assigned to the D-band and G-band of carbon, respectively. The G-band is related to graphitic carbon and the D-band is associated with the structural defects or partially disordered structures of graphitic domains [30]. The C/O atomic ratio of GO was measured to be about 2.2 by XPS examinations and the GO sheets have four types of carbon bonds: C−C/C\(_2\)C (284.6 eV), C−O (286.6 eV), C=C (287.7 eV), and O−C=O (289.0 eV) (Fig. 1d). These hydrophilic oxygenated groups of GO sheets make them dispersible in water to form a stable colloidal suspension [31]. It was also believed that the electrostatic repulsion between GO sheets caused by the ionization of carboxyl groups prevented their aggregation in aqueous medium [32]. The above results indicate that the GO sheets are prepared successfully.

2.2. Morphology and microstructure of the three-dimensional porous SF/GO scaffold

Fig. 2a shows photo of a series of SF/GO blended aqueous solution with different GO concentrations. The concentration of the prepared aqueous SF solution was determined as 5 wt%. It can be seen that the pure aqueous SF solution appears to be transparent while the SF/GO blended aqueous solution is in a brown colour. By increasing the concentration of GO in the blended solution, the brown colour of the blended solution is gradually getting darker. Apparently, as the concentration of GO is lower than 5 wt%, GO is well dispersed in the SF solution without occurrence of precipitation or suspended solids, which shows that GO in a proper concentration can be added in the SF aqueous solution in an uniform dispersion. However, as the concentration of GO increases to 5 wt%, slight agglomeration of GO occurs, which indicates that the content of GO in the blended solution can’t be increased further. As shown in Fig. 2b, an increase in the viscosity of the solution can be observed with the addition of GO, and this increase is more clear upon increasing the content of GO. This may be the consequence of active groups such as hydroxyl (−OH), carboxyl (−COOH) and epoxy (−O−) in GO and nano-size effect of GO. The large surface area, nano-sheets structure and active groups of GO can increase the interaction between components of the solution system, which is favor to increase the viscosity of the solution [33]. Thus it is easier to prepare scaffolds with improved mechanical properties.

The SF/GO blended aqueous solution was poured into the Petri dish with a level about 2/3 of the height of the dish and freeze-dried to obtain scaffolds. Macrographs of those prepared scaffolds are shown in Fig. 3. It is observed that pure SF scaffold displays a white three dimensional structure while SF/GO composite three dimensional scaffolds show a homogeneous brown colour, indicating uniform distribution of GO in the composite scaffolds. The colour of the composite scaffolds turns gradually darker on increasing the amount of GO in the composite scaffolds.

Fig. 4 shows SEM micrographs of the SF and SF/GO composite scaffolds. It is noted that the pure SF scaffold exhibits an irregular loose and porous honeycomb structure, together with large pore sizes (average pore diameter is about 56 ± 9 μm), which can facilitate more cell growth, differentiation and proliferation. Beyond that, it can be seen that the surface of the pure SF scaffold is covered by small holes due to volatilization of n butyl alcohol. N butyl alcohol was used as porogen to achieve pore interconnectivity in the preparation process of composite scaffolds. Introduction of GO resulted in compact and porous honeycomb structure and small pore size in the composite scaffolds. At higher

![Fig. 1. Characterizations of the GO. (a)–(b) SEM and AFM images, (c)–(d) Raman and XPS spectra.](image-url)
GO concentration in the composite scaffolds, the structure of the composite scaffolds become more compact and the pore size of the composite scaffolds decreases, and the average pore diameter of the SF/GO composite scaffold decreases to about 35 ± 7 μm when the GO concentration is 5 wt%. Moreover, pore interconnectivity disappears, adherence between pores occurs, as the concentration of GO increases to 5 wt%. This is possibly due to increase in the viscosity of the composite SF/GO solution caused by concentration increase of GO. From Fig. 4f, the pure GO forms a loose lamellar structure after freeze-drying process. It is clear that a loose lamellar structure is not observed in composite SF/GO scaffolds, which implies homogeneous blending of SF and GO (Fig. 4b–e).

EDS Distribution maps of carbon, oxygen and nitrogen elements on the surface of SF/GO composite scaffolds are shown in Fig. 5. Elements on the surface of all scaffolds display homogenous distribution. Nitrogen on the surface of SF/GO composite scaffolds distributes uniformly without blank or discontinuous areas, which proves that SF and GO were evenly blended as there is almost no nitrogen in the surface of GO. This finding is consistent with the SEM result.

2.3. Chemical structure

FTIR spectra of SF/GO composite scaffolds are shown in Figs. 6 and 7. SF is mainly composed of three main conformational states: random coils, α-helix and β-sheets, and α-helix and β-sheets are called as silk I and silk II, respectively by Kaplan DL et al. [34]. Secondary structures of SF can be determined by the location of characteristic bands related to amide I, amide II. According to Fig. 6, SF/GO composite scaffolds have absorption bands at 1625 cm⁻¹ (Amide I), 1520 cm⁻¹ (Amide II), corresponding to the existence of Silk II conformation (β-sheet). This is the result of n-butyl alcohol treatment, since pure SF scaffold without treatment is mainly in random coil conformation [35].

From Fig. 7, amide I band and amide II band for pure SF scaffold without n-butyl alcohol treatment show peaks at 1654 cm⁻¹ and 1545 cm⁻¹ respectively, corresponding to random coil structure. After treated by the n-butyl alcohol, the amide I band and amide II band for SF scaffold shifted to lower values, and the conformation of SF transited from random coil to β-sheet. As shown in Fig. 6, after being blended with GO, no shifts of characteristic absorption peaks of amide I band and amide II band for SF is observed and other main characteristics remains same, indicating that the addition of GO does not damage the chemical structure of the SF. Furthermore, characteristic absorption bands at 1731 cm⁻¹ (C=O stretching vibration) and 1064 cm⁻¹ (C–OH stretching vibration) of GO can’t be traced in the FTIR spectra of SF/GO composite scaffolds. This, combine with the SEM and EDS results, demonstrates that SF and GO were evenly blended.

XRD spectra of SF/GO composite scaffolds are shown in Fig. 8. All composite scaffolds containing SF are characterized by diffraction peaks at 2θ = 20.6° (4.5 Å), attributing to the silk II structure (β-sheet). This is assumed to be the result of n-butyl alcohol treatment, since pure SF scaffold without treatment is mainly in random coil conformation.
conformation. Besides, the FTIR analysis reveals that the secondary structure of SF converted into β-sheet after n-buty alcohol treatment. As Fig. 9 shows, the pure SF scaffold without n-buty alcohol treatment shows a smooth XRD curve without diffraction peaks, while the pure SF scaffold with n-buty alcohol treatment showing obvious characteristic peaks, which further confirm the above analysis.

From Fig. 8, it is possible to observe that there are no significant differences between all scaffolds samples except GO in respect to the peak positions and peak intensity at 2θ = 20.6°, which is an indication that the addition of GO didn’t affect the crystalline structure of SF. From Fig. 8, it is possible to observe that the pure freeze-drying GO scaffold exhibits distinct peak at 2θ = 11°. According to Bragg equation 2sin θ = nλ, the interlayer spacing of our synthesized GO can be calculated as 0.8 nm. Besides, it can be seen that the diffraction angles of the composite scaffolds are almost similar to that of the pure SF, and the diffraction peaks corresponding to GO is not observed, which indicates that SF and GO were evenly blended and the addition of GO didn’t affect the crystalline structure of SF.

2.4. Mechanical properties

Graphene is known for its incomparable advantages such as special surface properties and excellent mechanical properties. Graphene is usually prepared by chemical reduction of its precursor GO. GO contains a wide range of hydrophilic groups including hydroxyl groups, carboxyl groups and epoxy groups. These functional groups allows good dispersion of GO in water. GO also shows the same high mechanical properties as graphene, which allow it to be potential reinforcing materials for polymers [36, 37] and inorganic non-metallic materials [38, 39]. Herein, the effect of GO on mechanical properties of SF scaffold was investigated, and results are shown in Fig. 10 and Table 1. As shown in Fig. 10, the breaking strength, Young’s modulus and elongation of the pure SF scaffold was calculated as (1.44 ± 0.28) MPa, (53.33 ± 12.2) % and (2.71 ± 0.46) MPa, respectively. GO incorporation significantly increases the breaking strength and Young’s modulus of the composite scaffolds, while decreases their elongation. The breaking strength and Young’s modulus gradually increases on increasing the amount of GO into the composite scaffold, the value reaches to (3.79 ± 0.33) MPa and (10.32 ± 2.31) MPa respectively at 5 wt% GO concentration. Clearly, the breaking strength is almost 3 times higher, and the Young’s modulus is about 4 times higher, which combines to the fact that the addition of GO effectively improves the mechanical strength of the scaffold. This is due to the introduction of GO resulted in compact the composite scaffolds, which is analyzed in Section 2.2. And another reason, it is probably that there are some hydrogen bonds between the SF and GO, which will be analyzed by molecular docking of SF and GO.

2.5. Biocompatibility

Due to their unique physicochemical structure and novel electrical, mechanical, and thermal properties, graphene and GO have shown great potential in a wide range of applications. Graphene and GO have been found can promote the growth of neuroblastoma cells and can induce the neural stem cells differentiate into neural functional cells [40–42]. Growth and proliferation of MC3T3-E1 osteoprogenitor cells on the pure SF scaffold and the SF/GO composite scaffold with different GO concentration (0.5, 1, 3 and 5 wt%) were studied. Results shown in Fig. 11 demonstrates that MC3T3-E1 osteoprogenitor cells can adhere and proliferate on both pure SF scaffold and SF/GO composite scaffold after 1 day, 3 days and 7 days of cultivation. The MTT assays demonstrates that the viability of MC3T3-E1 osteoprogenitor growing on SF/GO composite scaffold is higher than on pure SF scaffold after 1 day’s culture. But statistical results shows no significant difference since cells haven’t finished attachment onto the scaffolds after 1 day’s culture. After 3 days’ culture, more cell proliferation is observed on the SF/GO composite scaffold than on the pure SF scaffold. Furthermore, proliferation of the cell increases with the increase of the concentration of GO. The MTT assays result remains the same after 7 days’ culture. The result of MTT shows that the addition of GO contributes to the proliferation of MC3T3-E1 osteoprogenitor cells.

SEM is carried out to study the dynamics of cell adhesion, spreading, and proliferation on the pure SF and SF/GO blended scaffolds as shown in Fig. 12. MC3T3 cells are adhered to and spread on the SF and SF/GO scaffold after 1 day in culture. After 3 days of cell culture, there is an
increase in cell densities compared with the first day, and the cells are also observed to migrate and proliferate in certain patterns. After 7 days, the cells increase in number significantly and almost reached confluence on the scaffolds. Moreover, the cells on the SF/GO blended scaffold show greater cells adherence and a higher degree of spreading than that of the pure SF scaffold. Furthermore, proliferation of the cell
increases with the increase of the concentration of GO, which is consistent with result of MTT analysis. It further confirms the analysis of MTT. The above result shows that the blending of GO contributes to the proliferation of the cells. This is probably due to that blending of GO leads some new combinations between the SF and GO, such as hydrogen bonds. The theoretical binding mode of GO in the binding site of the SF is illustrated in Fig. 13. As shown in Fig. 13, GO adopts a compact conformation to bind on the surface of SF. Detailed analysis shows that the four key hydrogen bond interactions are observed between the GO and the residues Lys-79 (bond distance: 3.2 Å), Glu-99 (bond distance: 3.5 Å) and Glu-56 (bond distance: 2.7 and 3.0 Å), which is the main interactions between the SF and GO. And these new combinations increases the number of hydrophilic group and oxygen-containing groups, such as aldehyde, carbonyl and carboxyl, and this is verified by XPS, as shown in Fig. 14.

Fig. 14 shows the high resolution carbon XPS of the SF and SF/GO (5 wt%) blended scaffolds. It could be seen that the carbon element mainly exists in alkyl, aldehyde and carbonyl group the in pure SF scaffold. However, it exists in alkyl, aldehyde, carbonyl and carboxyl groups in the SF/GO blended scaffold, and the aldehyde group and carboxyl group occupy the main part of the carbon element.

3. Conclusions

Three dimensional SF/GO composite scaffolds with different GO content were successfully prepared by phase separation method in this work. Rheological characterization of SF/GO blended aqueous solutions indicates that GO is well dispersed in SF solution and the viscosity of the solution gradually increases on increasing the amount of GO. Macromorphology, micromorphology and distribution of GO for all samples were measured by SEM and EDS, which illustrate that introduction of GO can lead to structural change in composite scaffolds. Higher concentration of GO resulted in more compact structure and smaller pore size of the composite scaffolds without decreasing their porosity. SEM and EDS results also reveals that SF and GO were homogeneous blended together. Chemical structures of all samples were analyzed by FTIR and XRD, which show that the secondary structure of SF has transited into β-sheet after n butyl alcohol treatment. Addition of GO didn’t affect the crystalline structure of SF and it was evenly blended with SF. The composite scaffolds have significantly higher breaking strength and Young’s modulus but lower elongation than the pure SF scaffold. Higher breaking strength and Young’s modulus can be achieved with a higher GO content. In vitro study indicates that both pure SF scaffold and SF/GO composite scaffold support growth and proliferation of MC3T3-E1 osteoprogenitor cells. MTT and SEM result shows that the addition of GO contribute to the proliferation of MC3T3-E1 osteoprogenitor.

4. Materials and methods

4.1. Preparation of GO

GO was prepared from natural graphite powder by a modified Hummers method [43, 44]. The details are described as follows. Graphite powder (3 g) was added to concentrated H2SO4 (70 mL, 98 wt%) under stirring at room temperature, then NaN3 (1.5 g) was added, and the mixture was cooled to 0 °C in an ice bath. Under vigorous agitation, KMnO4 (9.0 g) was added slowly and the temperature of the suspension was kept lower than 20 °C. Successively, the reaction system was transferred to a 40 °C water bath and stirred for 30 min. Then, 150 mL of water was added, and the solution was stirred for 15 min at 90 °C. Additional 500 mL of water was added, followed by a slow addition of H2O2 (15 mL, 30 wt%), turning the colour of the solution from dark brown to yellow. The mixture was filtered and washed with 1:10 HCl aqueous solution (250 mL) to remove metal ions followed by washing with 200 mL of water to remove the residual acid. The resulting solid was dried in air and dissolved in distilled water with the help of ultrasonication to make a GO aqueous dispersion. And then it was purified by dialysis for one week to remove the remaining metal species. Finally, it was centrifuged at 4000 rpm to remove the unoxidized or incompletely oxidized graphite powder.

4.2. Preparation of SF/GO three-dimensional porous scaffolds

Silkworm cocoons were boiled for 40 min in an aqueous solution of 0.5% (w/v) Na2CO3 and then rinsed thoroughly with distilled water to remove silk sericin. The purified SF fibers were dissolved in 9.3 mol/L LiBr solution at 60 °C for 1 h. The solution was dialyzed in deionized water using a cellulose dialysis membrane (MWCO 6000–8000 Da, Spectra/Por, U.S.A.) at room temperature for 3 days to remove LiBr. The dialyzed SF aqueous solution was collected and stored at 4 °C until further use. Different contents of the prepared GO (0.5, 1, 3 and 5 wt%) were added into the prepared SF aqueous solution. The resulting mixed solutions were placed under sonication until the GO was well dispersed in the mixture solutions. Samples were pre-cooled at −20 °C for 4 h and frozen overnight at −80 °C, followed by lyophilization for 48 h. The prepared lyophilized scaffolds were cut into different sizes prior to use. The process of the preparation is shown in Fig. 15.
4.3. Characterizations

The macro and micro-morphology of the prepared GO were observed with Canon digital SLR camera and atomic force microscope (AFM, multimode8&bioscope, Bruker, America). Samples for AFM tests were prepared by depositing a diluted GO aqueous solution onto a freshly cleaved mica. The images were taken at a scan rate of 1 Hz using the phase mode and operating in air. Micro-morphology analysis of the prepared scaffolds was carried out on the SEM (S-4800, Hitachi, Japan) at a magnification of 60–1000. Utilizing SEM photographs, the surface area of the pores were calculated and then normalized to a circular area. The circular diameter was regarded as the pore diameter. Prior to the analysis, samples were fixed on the specimen stage by conductive adhesive and sputter coated with gold for 120 s. Rheological study of the aqueous SF solution and the composite SF/GO solution were run on a rheometer (AR2000, TA Instruments, America). All the rheological data were obtained by using a 8 mm diameter parallel plate with a 0.5 mm plate-plate gap. EDS mapping of C, O and N elements in the SF/GO composite scaffolds were studied by energy dispersive spectrometry (TM3030, Hitachi). The infrared spectra of the SF/GO composite scaffolds were recorded on a FTIR spectroscopy (Nicolet 5700, PE Co., America) in the range of 4000–400 cm$^{-1}$, the powdered scaffolds were pressed into potassium bromide (KBr) pellets before testing. An X-ray diffractometer (X’Pert-Pro MRD, Philips, Holland) was used to analyze the crystallinity of the prepared scaffolds on powder. Data were collected for 2θ values of 5–60°.

Molecular docking between SF and GO was performed to investigate the binding mode between GO and the *Bombyx mori* SF using Autodock vina 1.1.2 [45]. The three-dimensional structure of the *Bombyx mori* SF (PDB ID: 3UA0) was downloaded from Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). The three-dimensional structure of the GO was drawn by ChemBioDraw Ultra 14.0 and ChemBio3D Ultra 14.0 softwares. The AutoDockTools 1.5.6 package [46, 47] was employed to generate the docking input files. The search grid of the SF scaffolds was 1.5–2.5 nm.

### Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Breaking strength (MPa)</th>
<th>Elongation (%)</th>
<th>Young's modulus (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF</td>
<td>1.44 ± 0.28</td>
<td>53.33 ± 12.21</td>
<td>2.71 ± 0.46</td>
</tr>
<tr>
<td>SF/GO (0.5 wt%)</td>
<td>1.85 ± 0.41</td>
<td>50.96 ± 7.41</td>
<td>3.63 ± 0.62</td>
</tr>
<tr>
<td>SF/GO (1 wt%)</td>
<td>2.21 ± 0.19</td>
<td>43.55 ± 4.65</td>
<td>5.07 ± 0.27</td>
</tr>
<tr>
<td>SF/GO (3 wt%)</td>
<td>2.84 ± 0.51</td>
<td>41.49 ± 9.86</td>
<td>6.85 ± 0.98</td>
</tr>
<tr>
<td>SF/GO (5 wt%)</td>
<td>3.79 ± 0.33</td>
<td>36.74 ± 14.25</td>
<td>10.32 ± 2.31</td>
</tr>
</tbody>
</table>

Fig. 10. Mechanical properties of SF/GO composite scaffolds: (A) stress-strain curve, (B) breaking strength, (C) Young’s modulus, and (D) elongation.

Fig. 11. Proliferation of cells on pure SF and SF/GO blended scaffolds after 1, 3 and 7 days respectively.
was identified as center_x: 14.4, center_y: 37.595, and center_z: 20.661 with dimensions size_x: 30, size_y: 30, and size_z: 30. The value of exhaustiveness was set to 20. For Vina docking, the default parameters were used if it was not mentioned. The best-scoring pose as judged by the Vina docking score was chosen and visually analyzed using PyMol 1.7.6 software (http://www.pymol.org/).

4.4. Mechanical properties

Mechanical properties of the composite scaffolds (10 × 30 mm, thickness was measured by a vernier caliper at different places for 5 times, and the average value was calculated) were measured on a universal tensile tester (Instron3365, America) equipped with a 0.2 cN capacity load cell. All samples were equilibrated in a constant temperature and humidity chamber for 24 h at 23 °C, 70% relative humidity prior to testing. The gauge length and the speed were set as 20 mm and 20 mm/min, respectively. The average values of the breaking strength, elongation and the Young’s modulus were evaluated for three tested specimens. Breaking strength and elongation at break are calculated based on the generated tensile stress-strain curves.

4.5. Biocompatibility evaluation

MC3T3-E1 osteoprogenitor cells (Chinese Academic of Science, Shanghai, China) were cultured in minimum essential medium α (αMEM, A1049001, Thermo Fisher, America) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution in a humidified incubator (HF90, Heal Force, China) at 37 °C and 5% CO2. The medium was replaced every 2 days. The inverted microscope was employed to observe the growth of the cells. The cells were passaged after 80% of the bottom of the bottle was covered by them.

Adhesion and proliferation of the cells on the scaffolds were determined by mitochondrial metabolic (MTT) activity assay. The prepared scaffolds were steam sterilized at 121 °C for 20 min and transferred to
96-well plates after cooling down. Digested MC3T3-E1 cells in the logarithmic phase of growth were diluted with the culture medium into $5 \times 10^5$ cells/ml. Cell suspension containing $5 \times 10^4$ cells/well was seeded onto the scaffolds. After culture in an incubator at 37 °C, 5% CO$_2$ for 1 day, 3 days, and 7 days, respectively, CCK-8 solution were added in (10 μL/well). Then, all samples were incubated for 4 h followed by measuring absorbance of the samples on a microplate reader (Synergy4, Bio-Tek, America) at 570 nm. The results were analyzed by Statistics software SPSS10.0. Values were presented as means ± standard deviation (SD). Statistical significance was determined by Student’s t-test with $p < .05$.

To determine the morphology of cell growth, cell-seeded samples were examined under scanning electron microscopy (SEM, S4800, Japan) at desired time points. Cell-seeded samples were gently washed three times with cold 0.1 M phosphate buffer, fixed by 2.5% glutaraldehyde for 2 h at 48 °C, washed with 0.1 M phosphate buffer (4 °C) three times at 15 min intervals each, and then dehydrated through a series of graded alcohols. The samples were dried in a critical point drier (HCP-2 HITACHI, Japan) and sputtered with a thin gold film for SEM observation.

4.6. Statistical analysis

All data were expressed as means ± standard deviations (SD). The statistical significance of differences among each group was examined by the t-test. Significance was set at $p < .05$ level.

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