



Electrospinning of PLGA/gelatin randomly-oriented and aligned nanofibers as potential scaffold in tissue engineering

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ABSTRACT

Electrospinning technique can be used to produce the three-dimensional nanofibrous scaffold similar to natural extracellular matrix, which satisfies particular requirements of tissue engineering scaffold. Randomly-oriented and aligned poly(lactic-co-glycolic acid) (PLGA) and PLGA/gelatin biocomposite scaffolds were successfully produced by electrospinning in the present study. The resulting nanofibrous scaffolds exhibited smooth surface and high porous structure. Blending PLGA with gelatin enhanced the hydrophilicity but decreased the average fiber diameter and the mechanical properties of the scaffolds under the same electrospinning condition. The cell culture results showed that the elongation of the osteoblast on the aligned nanofibrous scaffold was parallel to the fiber arrangement and the cell number was similar to that of randomly-oriented scaffold, indicating that the aligned nanofibrous scaffold provide a beneficial approach for the bone regeneration.

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1. Introduction

In recent years, there have been a large number of patients who suffered from the bone defects caused by tumor, trauma or other bone diseases. Generally, autogenetic and allogenic bones are used as substitutes in treatment of bone defects. However, secondary surgery for procuring autogenetic bone from patient would bring donor site morbidity and allogenic bone would cause infections or immune response [1,2]. Thus, it is necessary to find new approach for the bone regeneration. As a promising approach, the tissue engineering develops the viable substitutes capable of repairing or regenerating the functions of the damaged tissue. For the bone tissue engineering, it requires a scaffold system to temporarily support the cells and direct their growth into the corresponding tissue in vivo [3]. Among existing methods for fabricating tissue engineering scaffold, electrospinning technique has many advantages that can satisfy the particular requirements. Electrospinning is a simple and convenient method to produce an ultrafine fiber with a diameter ranging from nanometer to micron scales. The electrospun scaffold possesses reticular structure with high specific surface area, high porosity and interconnect pores,

which is similar to the natural extracellular matrix (ECM) and can enhance the adhesion, proliferation and growth of cells [4]. The corresponding mimetic electrospun nanofibers with high orientation can be fabricated through a special collector such as rotating drum [5], which is helpful for guiding the growth of nerve [6] or muscle cells [7]. Although there are many reports on bone tissue engineering scaffolds, most of them were randomly-oriented electrospun nanofibers [8–10]. As we know, the natural bone behaves significant anisotropic mechanical properties, with highly oriented ECM and bone cells. Therefore, it is desirable to introduce aligned electrospun nanofibers as bone tissue engineering scaffold to investigate the guidance for growth of bone cell.

So far, hundreds of polymers have been directly electrospun into nanofibers [4,11–13]. However, synthetic and natural polymer alone cannot meet all the requirements for tissue engineering. Cell affinity towards synthetic polymers is generally poor because of their low hydrophilicity and lacking of surface cell recognition sites. There is also the limitation of weak mechanical property for the natural polymers [14]. Therefore, it is desirable to prepare electrospun composite fibrous membranes including both the synthetic polymer for the backbone and the natural polymer for the cellular attachment, which might possess not only suitable mechanical properties but also the bioactive surface [15,16].

Poly(lactic-co-glycolic acid) is a FDA-approved biodegradable polymer which attracts much interests since it has controllable microstructure, degradation rate and mechanical properties for different applications [17,18]. However, the cell surface adherence on PLGA is poor because the PLGA material lacks of surface cell

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discrimination points and has poor hydrophilicity and cellular affinity. Gelatin is a natural biopolymer derived from the collagen which is the main component of the natural extracellular matrix in human body [19,20]. Therefore, in this study, gelatin was blended into the PLGA as a component of PLGA/gelatin composite system with different weight ratios, and the randomly-oriented and aligned PLGA/gelatin nanofibrous scaffolds were fabricated through electrospinning. Various properties were investigated by scanning electron microscopy (SEM), FT-IR, swelling ratio measurements and tensile tests. The adhesion and proliferation of MC3T3-E1 mouse osteoblasts on aligned and randomly-oriented PLGA and PLGA/gelatin nanofibrous scaffolds were also studied.

2. Materials and method

2.1. Materials

Poly (D,L-lactide-co-glycolide) (LA/GA 85/15, $M_w = 200,000$) was purchased from Jinan Daigang Biomaterial Co. Ltd. (China). Gelatin was purchased from Tianjin Bodi Chemical Co. Ltd. (China). 2,2,2-trifluoroethanol (TFE) was selected as the solvent and was purchased from Shanghai Aladdin Co. Ltd. (China). Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 nutrient mixture (DMEM/F-12) were purchased from Sigma-Aldrich. All chemicals were used directly without further purification. Aqueous solutions were prepared with doubly distilled water.

2.2. Electrospinning

Initially the PLGA and gelatin were dissolved in TFE and the solutions with concentration of 10% w/v were prepared, respectively. For the preparation of the composite solutions used in electrospinning process, the PLGA and the gelatin solutions were mixed, and the mixtures with different weight ratios of 10/0, 9/1 and 7/3 were obtained under gentle stirring for 12 h at room temperature, respectively.

In this study, the electrospinning apparatus consisted of an infusion pump (TS2-60, Baoding Longer Precision Pump Co. Ltd., China), high voltage power supply (HB-F303-1-AC, China) and a grounded target. The composite solution was fed into a 5 mL plastic syringe fitted with a stainless-steel blunt needle of 0.5 mm in diameter and an injection rate of 0.5 mL/h using an infusion pump. A high voltage of 7 kV was applied to the composite solution. Randomly-oriented PLGA/gelatin nanofibers were collected on a flat collector wrapped with aluminum foil which was kept at a distance of 10 cm from the needle tip. Aligned nanofibers were formed using a rotating drum with 50 mm diameter at the rate of 3000 rpm. The scaffolds were dried overnight under vacuum at room temperature.

2.3. Characterization

The morphologies of the electrospun randomly-oriented and aligned PLGA/gelatin nanofibers were characterized by scanning electron microscopy (SEM, CamScan MX2600FE, UK) at an accelerating voltage of 20 kV. All samples were coated with a thin layer of platinum in two 30 s consecutive cycles at 45 mA to reduce charging and produce a conductive surface. The diameters of resulting nanofibers were analyzed using software Image J.

The apparent density of the electrospun scaffold was accurately measured using density bottle method. An average of three measurements was taken for each sample. The porosity of electrospinning nanofibrous scaffold was calculated by using the following equation [21]

$$\text{Porosity (\%)} = \left(1 - \frac{\rho}{\rho_0}\right) \times 100\% \quad (1.1)$$

where ρ is the density of the electrospun scaffold, ρ_0 is the density of the bulk polymer.

Chemical characteristics of the electrospun PLGA/gelatin nanofibrous scaffolds were evaluated by an attenuated total reflection Fourier transform infrared (ATR-FT-IR) spectrophotometer (Perkin-Elmer Spectrum One, Perkin-Elmer Co., USA). The spectra were obtained in the range of 650–4000 cm^{-1} with a resolution of 4.0 cm^{-1} and 16 scans.

Mechanical properties of the electrospun nanofibrous scaffolds were characterized using a uni-axial testing machine (Instron 3365) with a 100 N load cell under a cross-head speed of 10 mm/min. All the samples were cut into rectangular shape with two-dimensions of 40 × 5 mm^2 . At least five samples were tested for each type of electrospun fibrous scaffold.

The water adsorption capacity was determined by swelling ratio [20]. Firstly, the rectangular shape sample was cut and dipped in deionized water for 12 h. Then the water on the specimen surface was removed with filter paper and the specimen was weighed in wet condition. The swelling ratio was calculated according to the following equation:

$$\text{Swelling ratio (\%)} = \left(\frac{W - W_0}{W_0}\right) \times 100\% \quad (1.2)$$

where W_0 is the initial weight and W is the wet weight of sample. Each swelling experiment was repeated three times.

2.4. Cell culture

Murine calvarial preosteoblasts (MC3T3-E1) were cultured in DMEM/F-12 medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin at 37 °C with 5% CO_2 in humidified incubator. After reaching 80–90% confluence, the cells were trypsinized and counted with a hemocytometer. The electrospun nanofibrous scaffolds were cut into small slices and placed in the cell culture plate. After sterilization under UV radiation for 2 h, they were washed several times with phosphate buffered saline and soaked in DMEM/F-12 for 24 h before cells seeding. Subsequently, the cells were seeded on electrospun PLGA, PLGA/gelatin nanofibrous scaffolds and DMEM/F-12 medium containing supplement as a negative control with seeding density of 5000 cells/well, respectively, and were cultured in a humidified atmosphere with 5% CO_2 at 37 °C up to desired time points.

Adhesion and proliferation of the cells on the nanofibrous scaffolds were measured by cell counting kit-8 (CKK-8, Dojindo: Japan) after 1, 2, 4 and 7 days of cell culture. At desired time points, the CKK-8 solution were added to each well and incubated for 2 h in incubator with 5% CO_2 at 37 °C. The absorbance was measured at 450 nm by microplate reader (Bio-RAD680, Bio-rad Co., USA).

Morphological study of *in vitro* cultured MC3T3-E1 on aligned and randomly-oriented PLGA and PLGA/gelatin nanofibrous scaffolds were performed. Firstly, the scaffolds with cells after 1 and 4 days of cell culture were taken out and washed twice with PBS to remove unattached cells, then the cells fixed for 12 h using 3% glutaraldehyde solution. The scaffolds were then dehydrated in ethanol solution with a series of concentration (30, 50, 70, 90 and 100% v/v) for 15 min each concentration and subsequently were immersed in isoamyl acetate for 15 min in order to replace ethanol solution, followed by a period of standing in the air until dry. Finally, the specimens were sputter coated with gold and then characterized by environmental scanning electron microscopy (ESEM, Quanta 200 F).

2.5. Statistical analysis

All the data in this paper were presented as mean ± standard deviation and were analyzed using Student's *t*-test for calculation of

significance level of the data. Differences were considered significant when $P \leq 0.05$.

3. Results and discussion

3.1. Morphology

There are many important factors influencing the cell adhesion and proliferation such as surface properties, porosity and chemical composition which should be considered in the study of tissue engineering scaffold. Fig. 1 shows the morphology of the randomly-oriented and aligned electrospun PLGA/gelatin with three different ratios (10/0, 9/1 and 7/3 w/w), and the insets display the corre-

sponding diameter distribution. As can be seen, highly uniform and smooth nanofibers were formed without the occurrence of bead defects for all the randomly-oriented and aligned nanofibrous scaffolds. Among them, the randomly-oriented nanofibrous scaffold depicted the interconnected structure and the mean fiber diameters were 1174 ± 350 nm, 774 ± 181 nm and 479 ± 307 nm corresponding to the PLGA/gelatin ratio of 10/0, 9/1 and 7/3, respectively. The nanofiber diameter decreased sharply and the diameter distribution became broader with increasing of gelatin concentration. Similar result was reported by Lee et al. [14] that when the gelatin ratio increased from 0 to 70 wt.% in PLCL/gelatin blend system, the electrospun fiber diameter decreased from $1.3 \mu\text{m}$ to 200 nm. A possible explanation is that the increasing gelatin content decreased

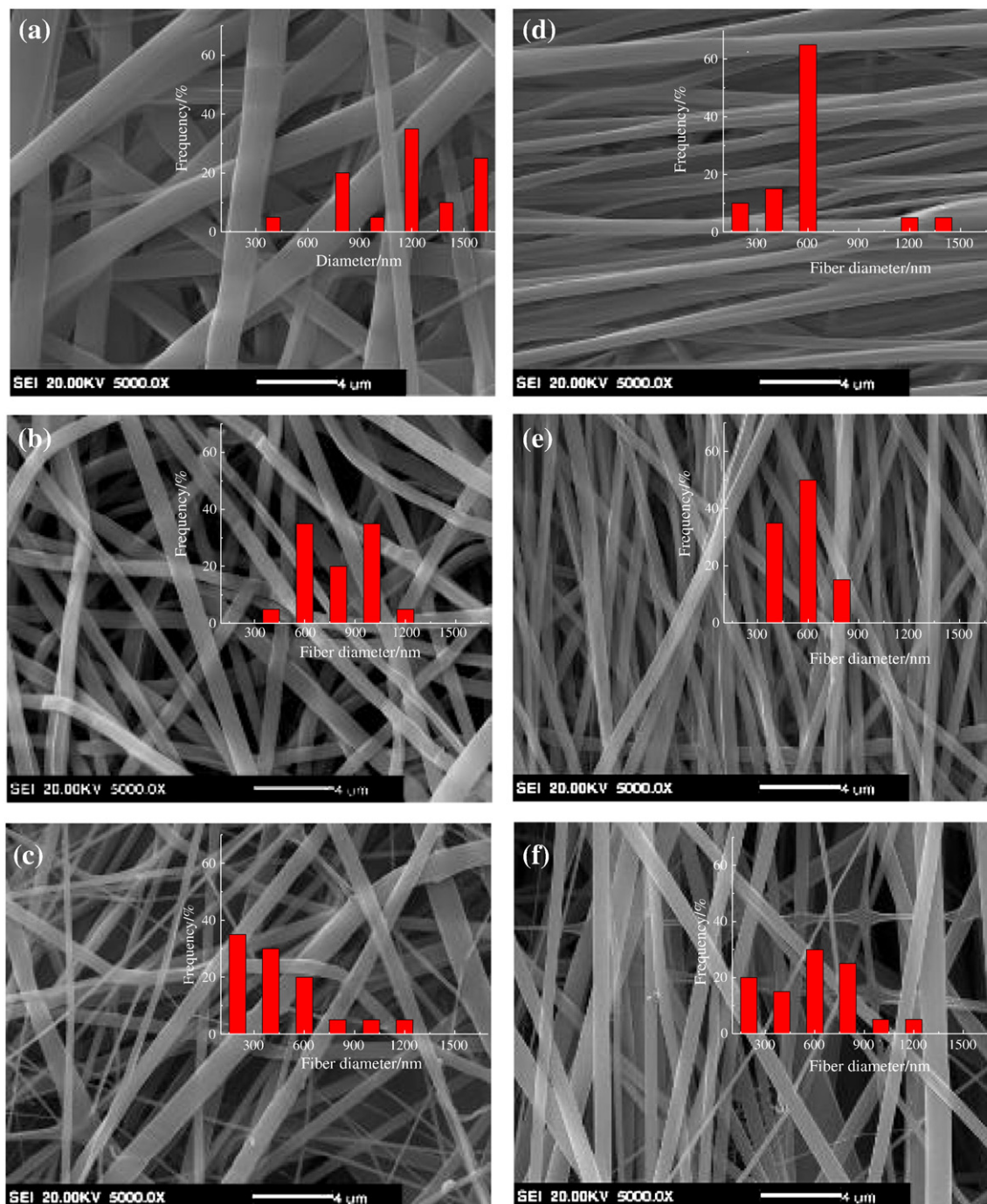


Fig. 1. Morphology of randomly-oriented (a, b, c) and aligned (d, e, f) electrospun nanofibers at weight ratios of PLGA/gelatin of (a, d) 10/0, (b, e) 9/1, (c, f) 7/3.

the viscosity of the composite solution and the amino acids of the gelatin improved the stretching force and the self-repulsion [22] due to the increasing charge density of the jet during the electrospinning process, which resulted in the smaller fiber diameter and the broader distribution.

In order to prepare the aligned scaffold, a high speed rotating drum is used as the collector with a rotating speed of 3000 rpm. The aligned PLGA and PLGA/gelatin nanofibers possessed smooth surface with diameter of 594 ± 226 nm, 560 ± 124 nm and 568 ± 280 nm corresponding to PLGA/gelatin ratio of 10/0, 9/1 and 7/3, respectively. Compared with the randomly-oriented nanofibers, the aligned nanofibers possessed smaller diameter due to the drawing effect given by the rotating drum while the jet contacted the surface of the drum [23]. Degree of alignment decreased with increasing the gelatin content because the increased charge improved the instability of whipping, resulting in the nonuniform splitting of the jet [24]. The fiber diameter distribution of the aligned nanofibers increased with increasing the gelatin content which was similar to that of the randomly-oriented nanofibers.

In addition, it can be seen from Fig. 1 that both the randomly-oriented and the aligned nanofibers electrospun from a solution with PLGA/gelatin ratio of 9/1 exhibited most favorable morphology, thus these nanofibrous scaffolds are further studied for cellular compatibility in the following.

Porosity is an important parameter when selecting the scaffold for the cell culture experiment. As seen from Table 1, the porosity of both randomly-oriented and aligned scaffolds was over 70%, indicating that they were highly porous and were beneficial for the adherence and proliferation of the cells. However, the randomly-oriented nanofibrous scaffolds showed higher porosity than the aligned ones, probably due to the intertwined structure of the randomly-oriented nanofibrous scaffold. Moreover, the increased gelatin content decreased the fiber diameter, which caused the decreased porosity of scaffolds.

3.2. FT-IR spectrum

The FT-IR spectrum of the electrospun PLGA/gelatin nanofibers with different weight ratios are shown in Fig. 2. For pure PLGA nanofibers, the strong characteristic absorption bands at about 1752 cm^{-1} attributes to the stretching vibration of C=O bond, and the bands at 1182 cm^{-1} can be assigned to the C–O–C ether group stretching, and the bands at 1130 cm^{-1} and 1452 cm^{-1} arise from C–O bond and methyl group C–H bond of PLGA respectively [25]. These characteristic absorption bands were also observed in the FT-IR spectra of PLGA/gelatin nanofibers. In addition, two absorption peaks appeared at 1650 cm^{-1} and 1540 cm^{-1} corresponded to amide I band and II band of the gelatin, respectively [26,27]. Among them, the amide I band is caused by C=O stretching vibrations of peptide linkages in the backbone of protein and the amide II band is caused by the combination of N–H in plane bending and C–N stretching vibrations. Moreover, the broad absorption at about 3300 cm^{-1} was observed in the FT-IR spectra of PLGA/gelatin 7/3 nanofibers, which attributed to the N–H and OH–O stretching vibration and the intermolecular hydrogen bonding.

Table 1
Fiber diameter and porosity of PLGA/gelatin scaffolds.

Sample of PLGA/gelatin	Randomly-oriented fiber		Aligned fiber	
	Average diameter (nm)	Porosity (%)	Average diameter (nm)	Porosity (%)
10/0	1174 ± 350	83.55	594 ± 226	78.57
9/1	774 ± 181	82.98	560 ± 124	75.14
7/3	479 ± 307	78.41	568 ± 280	74.85

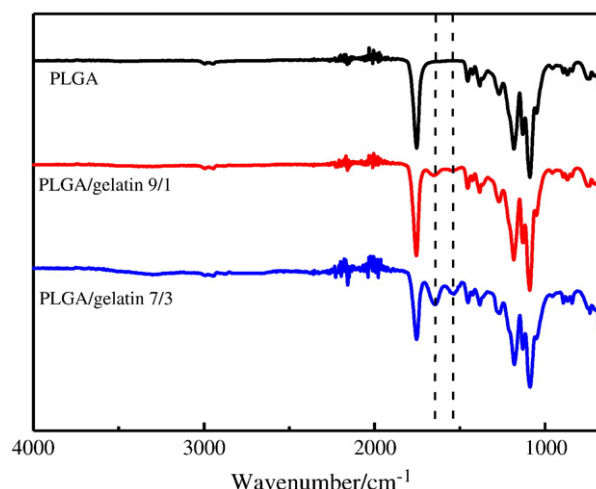


Fig. 2. FT-IR of electrospun nanofibers from PLGA, PLGA/gelatin 9/1, PLGA/gelatin 7/3.

3.3. Hydrophilicity

The characteristic of hydrophilicity is important to tissue engineering scaffold, which would enhance the cell viability and proliferation [28]. Fig. 3 shows the swelling ratio of the randomly-oriented and the aligned PLGA/gelatin scaffolds by the weight percentage of gelatin increase. Compared to 140.88% and 102.28% of the pure randomly-oriented and the aligned PLGA scaffold, the swelling ratio of the randomly-oriented and the aligned PLGA/gelatin scaffolds increased from 378.03% to 380.52% and 323.19% to 363.52% when the PLGA/gelatin ratio increased from 9/1 to 7/3, indicating that the addition of gelatin improved the hydrophilicity of the scaffold. This attributed to the amine and carboxylic functional groups in the gelatin structure. Additionally, according to the report of Li et al. [29], the capillary effect of higher porosity can lead to the better water adsorption. Therefore, it can be seen from Fig. 3 that the randomly-oriented scaffolds had a higher swelling ratio than the aligned scaffolds.

3.4. Mechanical properties

Fig. 4 shows the typical stress–strain curves of the randomly-oriented and the aligned electrospun PLGA and PLGA/gelatin

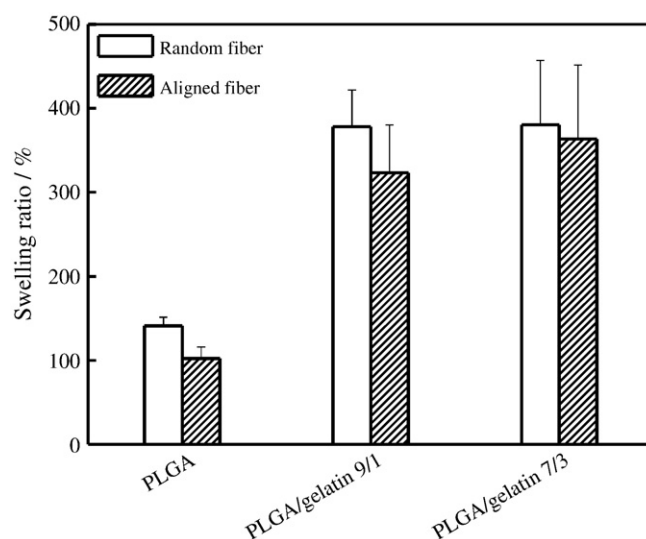


Fig. 3. Swelling ratio of electrospun nanofibers from PLGA, PLGA/gelatin 9/1, PLGA/gelatin 7/3.

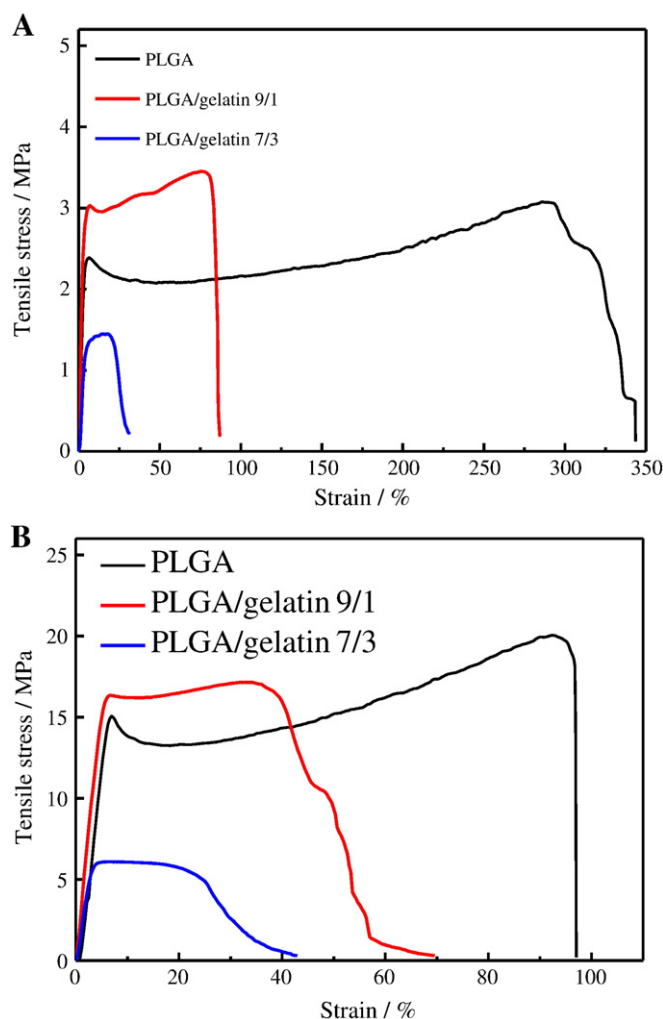


Fig. 4. Stress–strain curve of randomly-oriented (A) and aligned (B) electrospun PLGA, PLGA/gelatin 9/1 and 7/3 nanofibrous scaffolds.

nanofibrous scaffolds. The averages of the mechanical properties, such as the data of Young's modulus, tensile strength and elongation at break are summarized in Table 2. Compared with the pure randomly-oriented PLGA scaffold, the tensile strength and modulus of the randomly-oriented PLGA/gelatin scaffold increased slightly when 10 wt.% gelatin was added, but sharply decreased when the gelatin content continually increased to 30 wt.%. However, for the aligned electrospun scaffold, the tensile strength and elongation at break of PLGA/gelatin nanofibers decreased with increasing of the gelatin content, which was similar to the previous reported effect [14]. Furthermore, the elongation of both the randomly-oriented and the aligned electrospun PLGA/gelatin nanofibrous scaffolds declined with the increasing gelatin content, which was similar to the relevant report [26]. Thus it could conclude that the addition of the gelatin decreased the mechanical properties of PLGA/gelatin composite nanofibrous scaffold. On the other hand, compared with the

randomly-oriented nanofibrous scaffold, the aligned nanofibrous scaffold exhibited higher tensile strength and lower elongation at break, which may be due to the high degree of orientation.

3.5. Cell morphology and viability

The electrospun scaffold may provide a favorable matrix for the cell adhesion and proliferation because it physically mimics the structure of ECM of native tissues. However, most of the previous electrospun scaffolds were with two-dimensional thin-film structure, only a few report involved the three-dimensional electrospun bulk structure, for example, multilayered hybrid structure formed by depositing electrospun membrane layers [30]. In this study, we studied the adhesion and proliferation of MC3T3-E1 onto the randomly-oriented and the aligned electrospun PLGA and PLGA/gelatin nanofibrous scaffolds based on two-dimensional structure, which was visualized using SEM observation and CCK-8 assay. The PLGA/gelatin nanofibrous scaffolds with different weight ratio of 10/0, 9/1 and 7/3 were prepared by electrospinning. However, PLGA/gelatin 7/3 scaffold was not suitable to meet the requirements of tissue engineering scaffold because of its lower fiber alignment and weaker mechanical property, although it had higher hydrophilicity. Therefore, the electrospun PLGA and PLGA/gelatin 9/1 nanofibrous scaffolds were chosen for the further cell culture study.

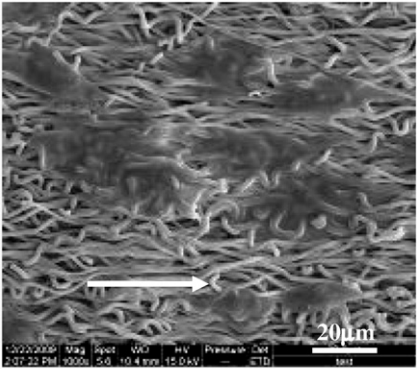
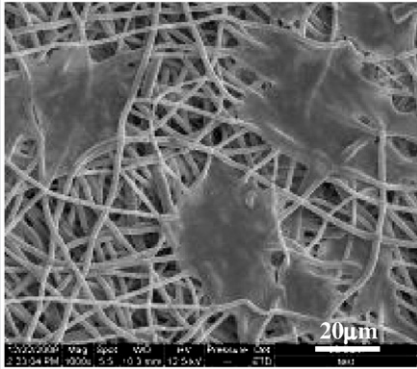
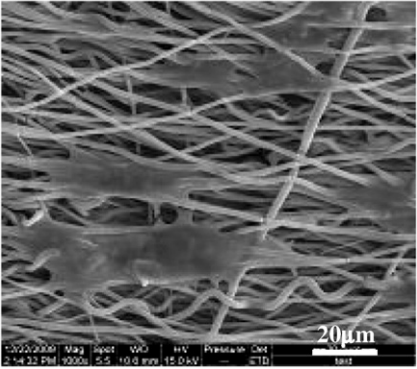
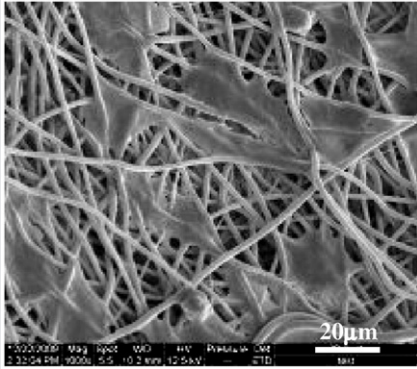
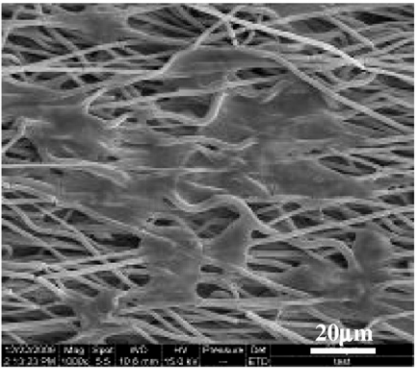
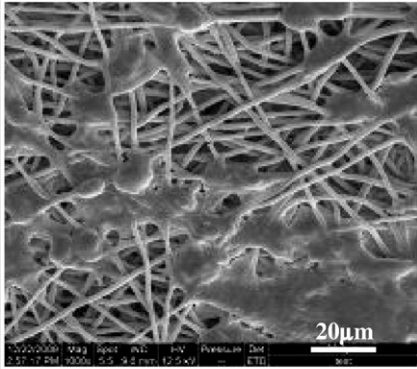
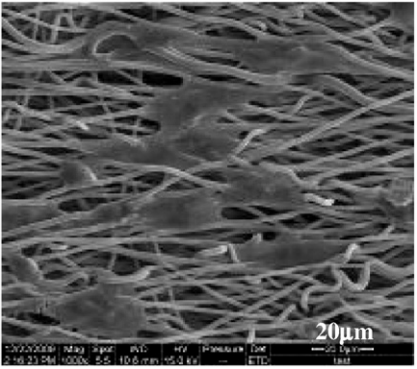
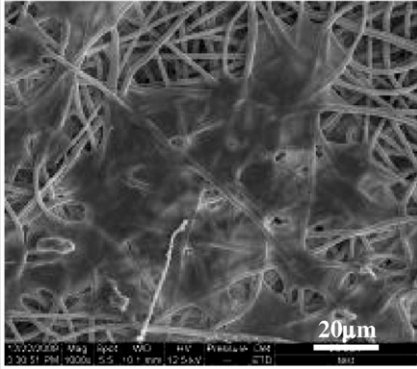
The cell proliferation onto both randomly-oriented and aligned electrospun PLGA and PLGA/gelatin nanofibrous scaffolds after 1 and 4 day of cell culture are shown in Table 3. After 1 day, the cells adhered well on the surface of all electrospun scaffolds, and direction of the cell growth on the aligned scaffolds was parallel to the direction of fiber alignment, whereas it was random on the randomly-oriented scaffolds. After 4 day, more cells were clustered around the nanofibers. Many researchers reported that the gelatin composite nanofibrous scaffolds were crosslinked after electrospinning because crosslinked gelatin could improve the mechanical properties and decrease the dissolution in the aqueous solution [14,31]. However, the crosslinking not only decreased the hydrophilicity and the bioactive of scaffold but also syncrized the fibers [32], which inhibited the cell adhesion and growth into the matrix. In this study, PLGA/gelatin scaffolds were not crosslinked, which showed the loosely interlaced fibrous structure and induced cells to attach onto the scaffolds easily.

Fig. 5 shows the cell viability cultured on the randomly-oriented and aligned PLGA and PLGA/gelatin nanofibrous scaffolds for 7 days using CCK-8 assay. It is well known that initial cell adhesion could be affected by the surface hydrophilicity of scaffold, and the hydrophilic surface would lead to higher cell adhesion than the hydrophobic surface [33]. From Fig. 5 it can be seen that on the first day after cell seeding, there was a significant increase ($P \leq 0.05$) in cell proliferation on PLGA/gelatin scaffolds when compared with the case of PLGA scaffolds, which was due to the higher hydrophilicity of PLGA/gelatin scaffold. Moreover, on the first day, the number of cell attachment on randomly-oriented scaffolds was higher than that on the aligned scaffolds, which may because the higher porosity of randomly-oriented scaffolds (see Table 1) and the rough surface caused by randomly-oriented nanofibers. However, after 7 days of culture, there was no significant difference ($P \leq 0.05$) for the number of the cell on both electrospun scaffolds, which indicated that the proliferation of

Table 2
Mechanical properties of randomly-oriented and aligned PLGA/gelatin scaffold.

Sample of PLGA/gelatin	Randomly-oriented fiber			Aligned fiber		
	Elastic modulus (MPa)	Tensile stress (MPa)	Elongation at break (%)	Elastic modulus (MPa)	Tensile stress (MPa)	Elongation at break (%)
10/0	0.67 ± 0.01	3.26 ± 0.12	324.65 ± 78.49	2.11 ± 0.35	20.36 ± 0.58	110.83 ± 30.22
9/1	0.96 ± 0.02	3.59 ± 0.42	93.83 ± 27.54	3.10 ± 0.18	17.15 ± 0.40	69.54 ± 19.23
7/3	0.29 ± 0.02	1.44 ± 0.18	29.18 ± 2.85	1.84 ± 0.06	6.43 ± 0.37	40.75 ± 5.74

Table 3
Morphology of osteoplasts on aligned and randomly-oriented electrospun PLGA and PLGA/gelatin 9/1nanofibers after 1 and 4 day.

Time	Materials	Aligned	Random
1 day	PLGA		
	PLGA/gelatin 9/1		
4 days	PLGA		
	PLGA/gelatin 9/1		

cells increased in aligned nanofibrous scaffold was much higher than that in randomly-oriented nanofibrous scaffold. The results indicated that aligned PLGA/gelatin nanofibrous scaffold is a more suitable substrate than randomly-oriented PLGA/gelatin nanofibrous scaffold, in terms of cell proliferation and guidance.

As a tissue engineering scaffold, aligned PLGA/gelatin nanofibrous scaffold could also be used in other tissue engineering with highly oriented ECM and cells. However, for further application, more research would be done, such as cytotoxicity and hemocompatibility tests, which are the next work we planning to do.

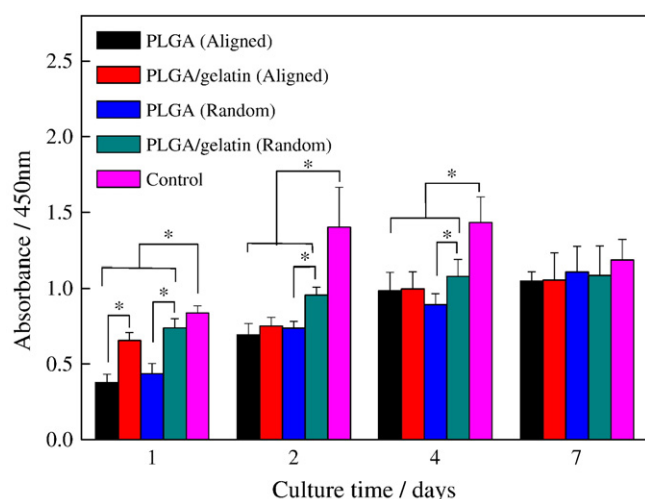


Fig. 5. CCK-8 result of MC3T3-E1 on PLGA and PLGA/gelatin 9/1 nanofibrous scaffolds after 1, 2, 4 and 7 days of cell culture. (* $P \leq 0.05$).

4. Conclusions

In this study, both the randomly-oriented and the aligned PLGA and PLGA/gelatin scaffolds were fabricated through electrospinning. The morphology showed smooth and bead free surface feature of nanofibers, and the nanofiber diameter decreased with increasing of gelatin content. Moreover, the addition of gelatin increased the hydrophilicity of the composite scaffolds but decreased the mechanical properties. However, compared with the randomly-oriented nanofibrous scaffolds, the aligned ones exhibited a higher tensile strength and a lower elongation at break. Finally, the addition of gelatin enhanced the adhesion and proliferation of the cell, and the aligned orientation of nanofibers guided cells growth along the longitudinal axis of nanofibers, which would provide a beneficial approach for bone regeneration.

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