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Preparation of fiber-microsphere scaffolds for loading bioactive substances in gradient amounts

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Gradient scaffolds are needed for interface tissue regeneration. In this study, a technique combining electrospinning and electrospraying was developed for preparing poly(L-lactide-*co*-glycolide) (PLGA) fiber-microsphere scaffolds for loading bioactive substances in gradient amounts. The gradient fiber-microsphere scaffolds contain two sheets of electrospun membranes and a sheet of microspheres loaded with bioactive substances in gradient amounts between the electrospun membranes. The morphologies of the gradient scaffolds were characterized and bovine serum albumin (BSA) was loaded as a model bioactive substance. The amount of BSA-loaded microspheres decreased gradually along the length of the gradient scaffold. The addition of poly (ethylene glycol) significantly improved the hydrophilicity of the gradient scaffold and the release behavior of BSA with respect to the gradient became apparent, with differences in the release amounts along the length of the gradient scaffold being observed. The biocompatibility of the gradient scaffold was verified using MC3T3-E1 pre-osteoblastic cells. The study demonstrated that the combination of electrospinning and electrospraying was a feasible method for the preparation of gradient scaffolds for potential applications in interface tissue engineering.

electrospinning, electrospraying, gradient scaffolds, bioactive substances

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Electrospinning has attracted much attention in recent years because of its potential applications in tissue engineering. Electrospun membranes have nanofibrous structures with large surface-to-volume ratios and interconnecting pores, and can be tailored to mimic the structure and composition of a natural extracellular matrix [1,2]. Several modified electrospinning techniques such as blending electrospinning [3,4], emulsion electrospinning [5,6], coaxial electrospinning [7,8], and emulsion-coaxial electrospinning [9] have been developed for delivering bioactive molecules via electrospun fibers. Electrospun fibers can protect active agents from environmental influences and achieve sustained and smooth release of these agents for an optimal period of time. However, to our knowledge, there have been few studies on the preparation of electrospun fibers loaded with bioactive molecule gradients.

Interface tissues such as ligament-to-bone, tendon-to-bone, and cartilage-to-bone exhibit anisotropic structural properties, which vary gradually from one tissue to another. Interface tissue engineering is an emerging field that aims to regenerate functional tissues in order to repair or regenerate diseased or damaged zones at tissue interfaces [10]. Gradient-based signal delivery strategies are gaining increasing attention in the field of interface tissue engineering [11–14]. *In vitro* and *in vivo* studies have indicated that biosignal gradients can promote biochemical production and enhance regionalized tissue formation within a single construct. Some researches on the fabrication of nanofibrous scaffolds with structural gradients have been performed. For example, Du et al. [15] prepared vertically graded chitosan/poly(ε-caprolactone) nanofibrous vessel scaffolds by sequential quantity grading co-electrospinning; the scaffold promoted rapid endothelial cell proliferation, which prevented restenosis caused by thrombus formation and stromal cell over-

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growth. In addition, both mechanical and adhesive gradients have been fabricated by mixing two solutions prior to electrospinning and fiber collection [16]. However, these studies only examined the structural properties or composition gradients of the nanofibers. The challenge of preparing nanofibrous scaffolds with bioactive molecule gradients remains.

Electrospraying is used to overcome the surface tension of a fluid meniscus at an orifice via a high-voltage supply, similar to electrospinning, resulting in atomization of the liquid into fine droplets [17]. The electrospraying technique has been widely used to produce protein-loaded polymer microparticles and nanoparticles [18–21]. The method is very flexible and different surfactants can be added to adjust the encapsulation efficiency and to tailor sustained release profiles. Mixed solutions, emulsions, and even solid particle dispersions can be electrosprayed into microspheres or nanoparticles [22].

The main aim of this study was to provide a feasible method for the preparation of ideal gradient scaffolds for interface tissue regeneration. Fiber-microsphere scaffolds for loading bioactive substances in gradient amounts were prepared using a combination of electrospinning and electrospraying. Poly (ethylene glycol) (PEG) was added to improve the hydrophilicity of the obtained gradient scaffold. Bovine serum albumin (BSA) was chosen as a model bioactive substance. The effect of PEG on *in vitro* BSA release from the gradient scaffold was examined in phosphate buffer saline (PBS) at 37°C. MC3T3-E1 pre-osteoblastic cells were seeded to evaluate the biocompatibility of the gradient scaffold.

1 Materials and methods

1.1 Materials

Poly(L-lactide-*co*-glycolide) (PLGA) (LA/GA=3:1, $\overline{M}_n$ = 8.95×10^4) and PLGA (LA/GA= 1:1, $\overline{M}_n$=3.05×10^4) were synthesized in our laboratory. The number-average relative molecular mass of PLGA was determined using gel permeation chromatography. BSA, 3-(4,5-dimethythiazol-2-yl)-2, 5-dipheyl tetrazolium bromide (MTT) and PEG ($\overline{M}_n$=2000) were purchased from Sigma-Aldrich Co., US. Fetal bovine serum (FBS) and Alfa modified minimum essential medium (α-MEM) was purchased from Gibco, US. Penicillin & streptomycin solution and trypsin were provided by Hyclone, US. Chloroform, dichloromethane, paraformaldehyde (PFA), glutaraldehyde (GA), trifluoroethanol and other chemicals were supplied by Tianjin Kemiou Chemical Reagent Co., Ltd., China and used as received.

1.2 Preparation of fiber-microsphere scaffolds for loading BSA in gradient amounts

The fiber-microsphere scaffolds for loading BSA in gradient amounts were prepared using a combination of electrospinning and electrospraying. A schematic diagram of the process is shown in Figure 1. First, a certain amount of PLGA (LA/GA = 3:1, with or without 20 wt% PEG) was dissolved in chloroform/trifluoroethanol ($CHCl_3$/TFE, 6:4) to obtain a solution of concentration 12% (w/v). The solution was electrospun at 14 kV, with a capillary collector distance of 18 cm and a solution flow rate of 0.8 mL/h. The nanofibers were collected using a rectangular piece of aluminum foil of size 6 cm × 3 cm. The electrospinning process was performed for 2 h. Then, a certain amount of BSA was dissolved in PBS and emulsified into the oil phase of a PLGA (LA/GA = 1:1, 8% w/v, with or without 20 wt% PEG) solution in $CHCl_3$. Emulsification was conducted by sonication using a probe sonicator (Scientz, JY92-IIN, China) at about 50 W for 5 min. The obtained water-in-oil

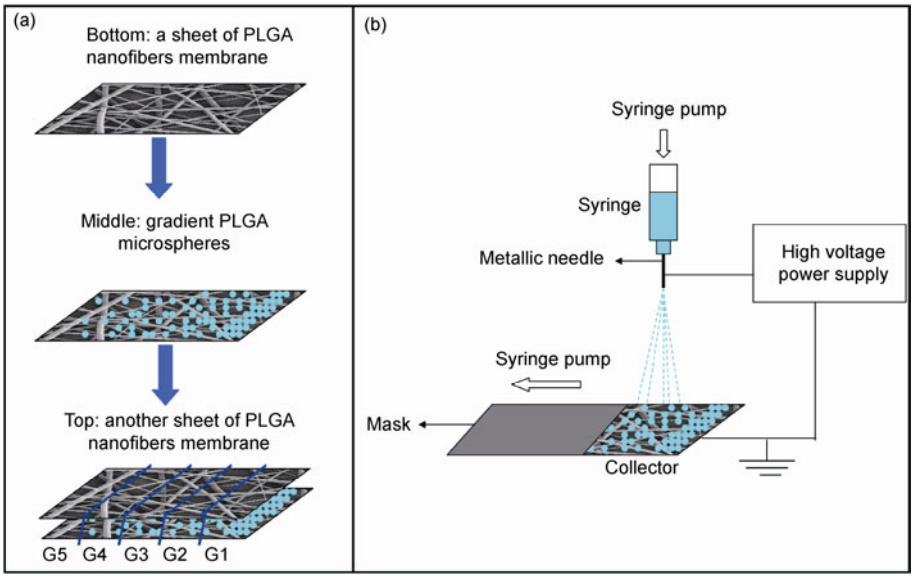

Figure 1 (Color online) Schematic illustration of the fiber-microsphere structure (a) and the preparation process of the gradient scaffold (b).

(W/O) emulsion was used to prepare BSA-loaded PLGA microspheres by electrospraying. A series of electrospraying experiments were performed at different combinations of voltages, flow rates, and W/O emulsion compositions. During the electrospraying process, a moveable paper mask, which was connected to a syringe pump and placed above the collector, moved continuously from one side of the rectangular collector to the other side, to achieve a gradient distribution of PLGA microspheres on the collector. Finally, on top of the collector, the PLGA (with or without 20 wt% PEG) solution in $CHCl_3$/TFE (6∶4) was again electrospun for 2 h to cover the BSA-loaded PLGA microspheres in gradient amounts.

As shown in Figure 1, the gradient scaffold was cut into five segments in the gradient direction; each segment was 1 cm in length. The gradient segments were designated as G1, G2, G3, G4, and G5, and these were characterized respectively to study the gradient properties of the scaffold.

1.3 Characterization of gradient scaffolds

The electrospun fibers, microspheres, and gradient scaffolds were coated with gold and examined using scanning electron microscopy (SEM, Philips XL30, Japan). The values of water contact angle of the gradient scaffolds were determined using an optical contact angle meter.

1.4 *In vitro* release of BSA from gradient scaffolds

In this study, the gradient scaffold carried BSA in gradient amounts along the length of the scaffold, so the scaffold was sliced into five gradient segments G1, G2, G3, G4, and G5; G1, G3, and G5 were selected at intervals to study the gradient release properties of the gradient scaffold. BSA was chosen as a model bioactive substance. The samples were suspended in 3 mL of PBS (containing 2.0 mmol/L NaN_3) and incubated at 37°C. At preset intervals, 1 mL of the supernatant liquid was collected and an equal amount of fresh PBS was added. The BSA content was analyzed using a Micro BCA™ Protein Assay Reagent Kit (Pierce Biotechnology, USA) for enzyme-linked immunosorbent assay (Synergy HT, US Biotech, USA). The amount of released BSA was calculated from a standard curve produced using standard BSA solutions of increasing concentrations.

The amounts of BSA remaining in the samples were determined using an extraction method. After *in vitro* release for 56 d, the samples were dried thoroughly and hydrolyzed in 1 mol/L NaOH, with vigorous shaking at room temperature for 2 h. After hydrolysis, the sample solution was neutralized with 1 mol/L HCl. The amount of BSA in the solution was determined using a Micro BCA™ Protein Assay Reagent Kit.

The total BSA loading was calculated using the following equation:

$$\text{total loading } (\mu g) = m_t + m_r,$$

where m_t and m_r are the cumulative released amount of BSA and the amount of BSA remaining in the sample at the end of the release time, respectively.

1.5 *In vitro* biological evaluation

(i) Cell culture. The biocompatibility of the gradient scaffold was determined by seeding MC3T3-E1 pre-osteoblastic cells on the selected gradient segments, i.e. G1, G3, and G5. A PLGA electrospun membrane was used as a control. The samples were cut into circular shapes (diameter, 1 cm), placed in a 24-well plate, and sterilized with ^{60}Co gamma irradiation in 15 kGy doses. MC3T3-E1 pre-osteoblastic cells (clone 5), isolated from nude mice embryos, were purchased from the National Platform of Experimental Cell Resources for Sci-Tech (China). The culture medium consisted of α-MEM supplemented with 10% (v/v) FBS and 100 U/mL penicillin-streptomycin. When the cells had grown to 90% confluence, they were detached using 0.05% trypsin and 0.05% EDTA. The cells were suspended in the culture medium at a concentration of approximately 4×10^4 cells/mL. Then 500 μL of the cell suspension were seeded directly on top of each sample. The medium was changed every other day, and the cultures were incubated at 37°C in a controlled atmosphere containing 5% CO_2.

(ii) Cell morphology. After seeding for 4 d, the samples with cells were removed to a new well, washed three times with PBS, and fixed in a 2% PFA-2% GA solution at 4°C for at least 4 h. Then the samples were dehydrated through a series of graded alcohols, freeze-dried, and sputter-coated with gold. Finally, the cell morphology of the sample was observed using SEM.

(iii) Cell proliferation. The cell viability was measured using the MTT assay, which is based on the mitochondrial conversion of tetrazolium salt. After culturing for 2, 4, and 7 d, the culture medium was removed and 50 μL of MTT (5 mg/mL) were added to each well, followed by incubation at 37°C in a controlled atmosphere containing 5% CO_2 for 4 h, for MTT formazan formation. After discarding the supernatant, the dark blue formazan crystals were dissolved in isopropyl alcohol. The absorbance at a wavelength of 570 nm was measured spectrophotometrically. The mean and standard deviation were obtained from six parallel samples.

2 Results and discussion

2.1 Preparation of PLGA microspheres and electrospun fibers

A series of experiments were carried out to examine the optimum conditions for the preparation of PLGA microspheres. The oil/water volume ratio of the W/O emulsion was a key factor. SEM micrographs of the prepared PLGA microspheres from W/O emulsions with different oil/water ratios are shown in Figure 2(a)–(c). It was found that when

the oil/water ratio was 20∶1, the microsphere surfaces were wrinkled. As the oil/water ratio increased to 30∶1 or 67∶1, the surfaces of the microspheres became much smoother. In the study, BSA was loaded into the microspheres to give a model of a bioactive substance to study the release of bioactive substances from a gradient scaffold. The concentration of BSA in the water phase remained constant, and when the oil/water ratio increased, the water phase was dispersed in the oil phase as much smaller drops, which made the electrospray process smooth. An oil/water ratio of 67:1 was therefore used to prepare the PLGA microspheres in the gradient scaffolds. Figure 2(d) shows the SEM micrograph of the PLGA electrospun fibers. As can be seen, the fibers were uniform and free of beads. The ultrafine fibers formed a fibrous membrane, which could mimic the extracellular matrix and serve as a good support for cell growth.

2.2 Gradient scaffolds

The goal of this work was to prepare fiber-microsphere scaffolds for loading bioactive substances in gradient amounts, for using in interface tissue engineering. The fibrous membranes on the top and bottom could support cell growth, while the bioactive-substance-loaded PLGA microspheres formed a gradient distribution, enabling a gradient distribution of bioactive substances to be achieved. In order to determine whether the microspheres were distributed gradually, after the microspheres were electrosprayed on the fibrous membrane, the morphologies of the microspheres were characterized using SEM, before preparation of the top fibrous membrane. The SEM micrographs of the PLGA microspheres on different segments of the gradient scaffold are shown in Figure 3. It can be clearly seen that the amount of microspheres decreased from G1 to G5. From the start of the electrospraying process, a moveable mask covered the whole collector. As the mask moved, one side of the collector was uncovered and could receive microspheres. The reception time decreased gradually as the mask moved from one side to the other. It was therefore supposed that the amount of microspheres deposited decreased gradually from one side of the membrane to the other. The total loading amount of BSA in the gradient scaffold (Figure 4) decreased from G1 to G5, which also indicated that a fiber-microsphere scaffold with a BSA gradient was successfully prepared.

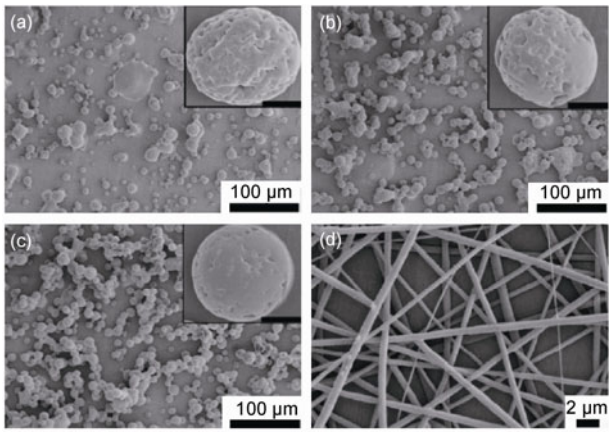

Figure 2 SEM micrographs of PLGA microspheres prepared by electrospraying at different oil/water ratios 20∶1 (a), 30∶1 (b), 67∶1 (c), and PLGA electrospun fibers (d). Inset micrographs (scale bars: 3 μm) were in larger magnification.

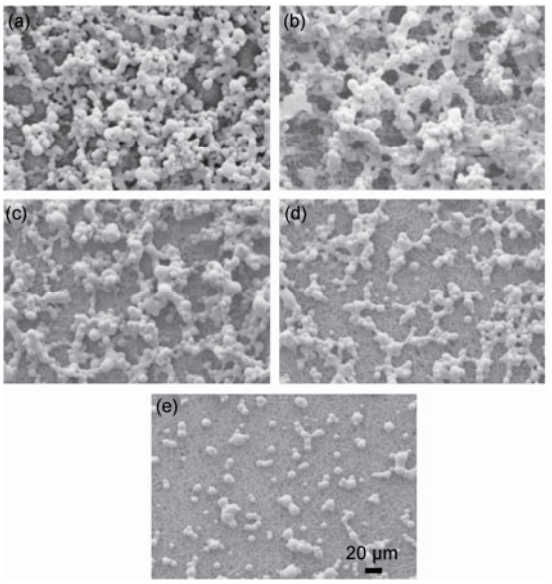

Figure 3 SEM micrographs of microspheres in the different segments of the gradient scaffold. (a) G1, (b) G2, (c) G3, (d) G4, (e) G5.

2.3 Effect of PEG on *in vitro* release of BSA from gradient scaffold

PLGA is hydrophobic, which is not favorable to cell adhesion and growth. In order to improve the hydrophilicity of the gradient scaffold, 20 wt% PEG was added to the solution used to prepare the fibers and microspheres. The water contact angles of the gradient scaffold before and after PEG

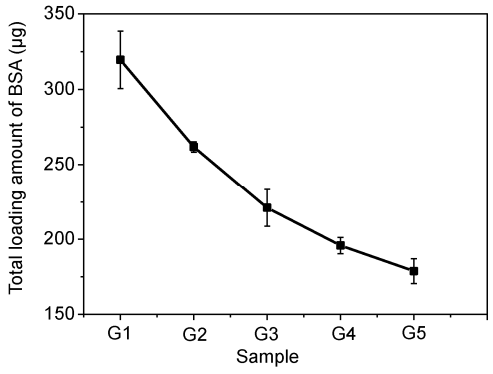

Figure 4 Total loading amount of BSA in the different segments of the gradient scaffold.

addition are shown in Figure 5. It is obvious that the water contact angle decreased significantly, from 130° to 20°, after PEG addition, suggesting that the gradient scaffold became hydrophilic.

From Figure 6, it can be seen that the microspheres and fibers with PEG still had good morphologies (Figure 6(a) and (d)), compared with those without PEG (Figures 2 and 3), showing that addition of PEG had no effect on the morphologies of the electrosprayed microspheres and electrospun fibers. In order to study the stabilities of the microspheres and fibers after PEG addition, microspheres and fibers with and without PEG were immersed in PBS at 37°C for 24 h. The SEM micrographs of the fibers are shown in Figure 6. It can be seen that the microspheres and fibers with PEG both kept their regular shapes quite well after immersion in PBS for 24 h. These results suggest that PEG addition significantly improved the hydrophilicity of the gradient scaffold, but did not affect the overall morphologies and stabilities of the microspheres and fibers in the gradient scaffold.

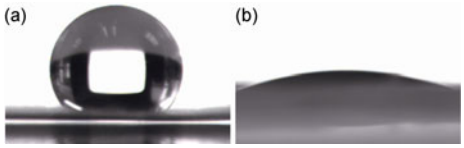

Figure 5 Water contact angle measurement of the gradient scaffold before (a) and after (b) PEG addition.

The effect of PEG on the *in vitro* release of bioactive substances from the gradient scaffolds was studied using BSA as a model bioactive substance. The *in vitro* cumulative release profiles of BSA from the gradient scaffolds before and after PEG addition are shown in Figure 7. For the gradient scaffold without PEG (Figure 7(a)), the amounts of BSA released from G1, G3, and G5 were similar, without significant differences in the first 10 d. After 10 d, there were differences among the amounts released; G1 had the highest value and G3 had the lowest release amount. The differences among the three segments increased with release time. The *in vitro* cumulative release profile of BSA from the gradient scaffold with PEG (Figure 7(b)) showed a similar tendency to that without PEG, but the differences among the three segments were relatively stable and the release amounts decreased gradually from G1 to G5 from the beginning to the end. In other words, gradient release behavior of BSA from the gradient scaffolds became apparent after PEG addition.

For the gradient scaffold without PEG, the PLGA fibers

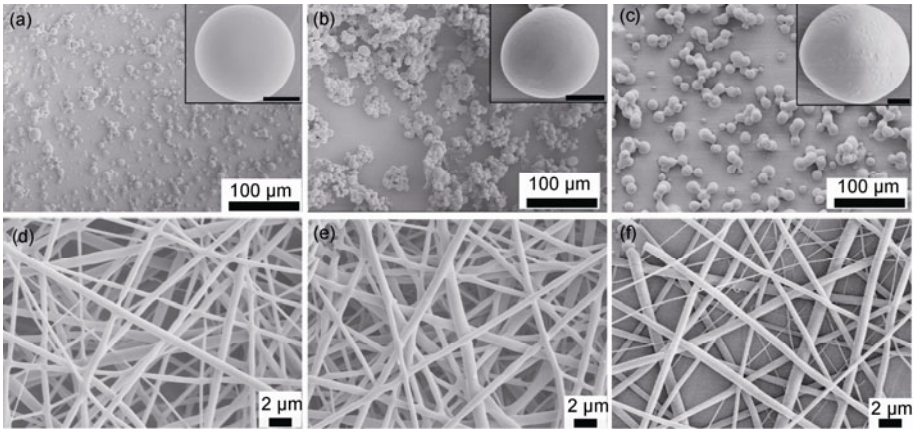

Figure 6 SEM micrographs of PLGA microspheres (a), (b) and electrospun fibers (d), (e) with PEG before (a), (d) and after (b), (e) being immersed in PBS for 24 h. SEM micrographs of microspheres (c) and electrospun fibers (f) without PEG after being immersed in PBS for 24 h were also shown for comparison.

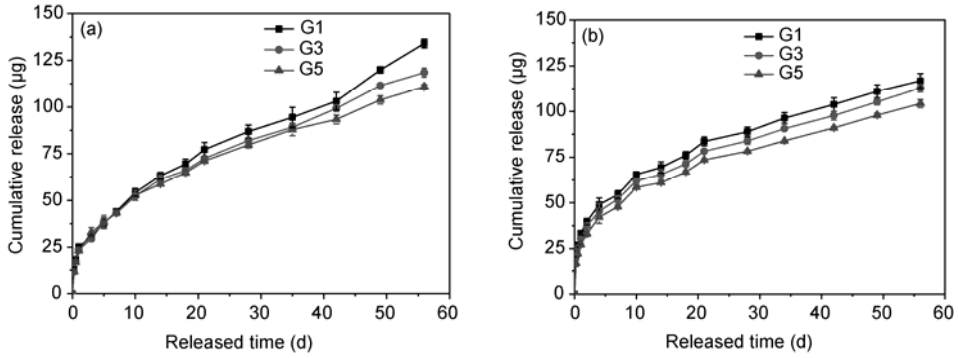

Figure 7 *In vitro* cumulative release profiles of BSA from the gradient scaffolds before (a) and after (b) PEG addition.

and microspheres were both hydrophobic. When the samples were incubated in the release solution, the fibers could not be wetted immediately, so an original burst release was not seen. It was also difficult to wet the inner microspheres. At the beginning, only microspheres on the surface could be soaked by the water. Since the amounts of microspheres on the surfaces of the three samples were the same, the release amounts were also the same. Although G1 had the highest amount of microspheres, the inner microspheres could not soak in the water. In the first 10 d, therefore, G1 released the same amount of BSA as the other two samples. As the release time increased, the inner microspheres began to be soaked by water. G1, which had the highest amount of microspheres, released the highest amount of BSA. As more and more microspheres began to soak in the water, the amount of microspheres began to control the amount released from the gradient scaffold. The differences among the amounts released by G1, G3, and G5 therefore increased with increasing release time.

In the case of the gradient scaffold with PEG, PEG has a hydrophilic group, so the hydrophilicity of the gradient scaffold was improved by PEG addition. When samples were incubated in the release solution, water could wet the fibers immediately, so the original burst release was more obvious than that for the gradient scaffold without PEG. The inner microspheres were also wetted by water in a shorter time. The release amount mainly depended on the amount of microspheres, so the amounts released by G1, G3, and G5 differed from the very beginning.

2.4 *In vitro* biological evaluation

After PEG addition, the hydrophilicity of the gradient scaffold improved significantly and the gradient release behavior of BSA became apparent, with differences in the release amounts being observed along the length of the gradient scaffold. The gradient scaffold with PEG was therefore chosen for the regeneration of interface tissues. MC3T3-E1 pre-osteoblastic cells were seeded on selected segments, i.e. G1, G3, and G5, to study the *in vitro* biocompatibility of the gradient scaffold. Figure 8 shows the proliferation data for MC3T3-E1 cells seeded on the samples. The MTT absorption of the gradient scaffold and PLGA membrane increased with increasing culture time, indicating cell proliferation. On day 7, the gradient scaffold and PLGA membrane showed a significantly higher proliferation rate than the other cell culture plates, indicating that MC3T3-E1 pre-osteoblastic cells were metabolically active on the gradient scaffold and PLGA membrane. There were no significant differences between the proliferation rates of the gradient scaffold and the PLGA membrane. The gradient scaffold did not carry any bioactive substances that could enhance cell proliferation, and the surface of the gradient scaffold was a sheet of PLGA membrane. The cell proliferation rate of the gradient scaffold was therefore more or less the same

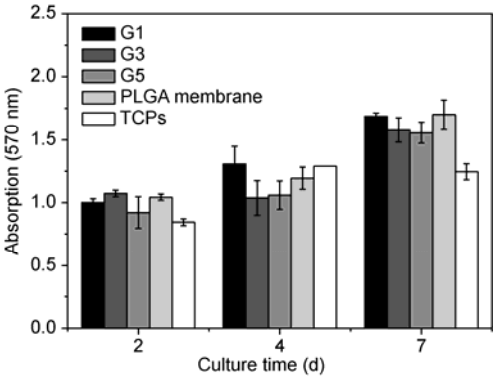

Figure 8 Proliferation of MC3T3-E1 cells seeded on the gradient scaffold and PLGA membrane with PEG addition.

as that of the PLGA membrane. We also observed the cell morphology using SEM and the SEM images are shown in Figure 9. After 4 d of cell seeding, the cells adhered well and spread extensively on all the samples. The gradient scaffold had good biocompatibility and proved to be an ideal support for cell adhesion and growth.

For tissue interfaces such as ligament-to-bone and tendon-to-bone, the transition zone is relatively large. Gradient scaffolds of large horizontal size are therefore needed. In this study, electrospinning combined with electrospraying was found to be a feasible way of obtaining functional gradient scaffolds for loading bioactive substances in gradient amounts. The gradient dimension of the gradient scaffold could be conveniently adjusted by changing the collector of the fibers and microspheres. Moreover, the microspheres could also load two or more bioactive substances to achieve different bioactive substances gradients in one scaffold.

3 Conclusions

PLGA fiber-microsphere scaffolds loaded with bioactive

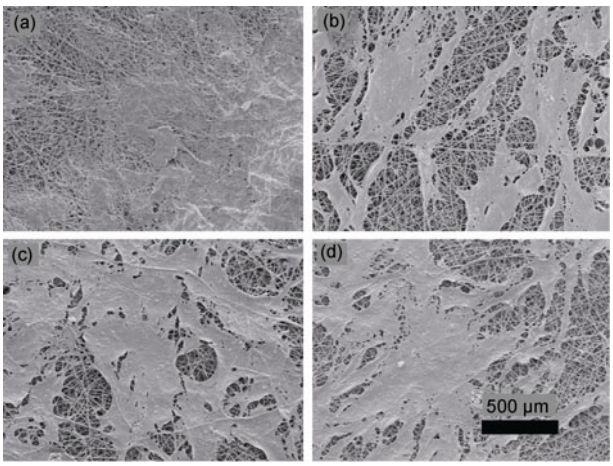

Figure 9 SEM micrographs of MC3T3-E1 cultured on the gradient scaffold and PLGA membrane with PEG addition for 4 d. (a) G1, (b) G3, (c) G5, (d) PLGA membrane.

substances in gradient amounts were successfully prepared using a combination of electrospinning and electrospraying. BSA was loaded as a model bioactive substance. The amount of BSA-loaded microspheres decreased gradually along the length of the gradient scaffold. The *in vitro* release profiles of BSA revealed that BSA showed gradient release along the gradient scaffold. After PEG addition, the hydrophilicity of the gradient scaffold improved significantly, and the gradient release behavior of BSA became apparent. *In vitro* biological evaluation verified good biocompatibility of the gradient scaffold. In future studies, two or more bioactive substances could also be introduced into the microspheres for gradient distribution, so the gradient scaffold could release two or more bioactive substances gradually. The combination of electrospinning and electrospraying is a feasible method for preparing gradient scaffolds for potential applications in the interface tissue engineering.

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