Electrospun Three-Dimensional Silk Fibroin Nanofibrous Scaffold

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ABSTRACT: Practical application to three-dimensional (3-D) tissue culture has been limited by the structural restriction of two-dimensional (2-D) nature of electrospun nanofiber mat. In this study, for constructing 3-D nanofibrous structure as real 3-D tissue engineering scaffold, we developed new fabrication process with silk fibroin (SF) by electrospinning and evaluated the features of this SF nanofiber scaffold (SFNS) through morphological and cell-culture analyses. Foam type of the SFNS exhibited high porosity as well as large pores and its cell proliferation well occurred inside (inner spaces of pores), which makes this suitable for 3-D cell-culture scaffold. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 106: 3922–3928, 2007

Key words: structure; fibers; biomaterials; biological applications of polymers

INTRODUCTION

For the regeneration of a new tissue *in vitro* or *in vivo*, a scaffold is essential because it makes up room formed by absence of tissue and supports cells.[1–3] The ideal structure of a scaffold is to mimic a natural extracellular matrix (ECM), which consists of collagen nanofibers, glycoproteins, glycosaminoglycans (GAGs), etc. As a point of structural view, ECM is very similar to skeleton of building. Nanoscale collagen fibers are randomly entangled and there are many small pores in which new cells can be embedded.[3–5]

Many researches on an artificial scaffold have been focused on mimicking ECM structure and various techniques to fabricate such a scaffold have been so far tried. Generally, the freeze-drying and salt-leaching methods are widely used because they are very simple to make the porous structure[6–8] although the structure is not similar to that of real natural ECMs. Their pores are formed by surrounded polymeric walls based on 2-D surface. However, the natural ECM is constructed on the fiber-based structure to induce the cell attachment and sufficient flow of nutrients and wastes. In the recent years, a nanofibrous 3-D structure by electrospinning has received much interest as a promising material in tissue engineering.[9–16] The electrospun nanofibrous scaffold offers a highly porous structure as well as large surface area, a similar structural environment to the natural ECM to seeded cells.

In spite of a similar structure to the natural ECMs, the use of electrospun fibers as tissue engineering scaffold is limited in the sheetlike shapes collected by a metal plate or mandrel. The size of pores in the electrospun sheet is very small due to the random deposition of nanofibers on the metal surface during the electrospinning process. Such small pores are not proper for seeded cells to migrate into the pores existing at the inside of a scaffold.

In this study, we suggest a novel electrospinning method to make a 3-D nanofiber scaffold with a large pore size as well as high porosity using silk fibroin (SF), known as an excellent biopolymer.[12–14,17–22] SF dope solution was electrospun using typical electrospinning apparatus except collecting part of the process. The SF nanofibers were dropped directly into coagulation bath containing methanol below the spinneret. Fairly well dispersed nanofiber assemblies were lyophilized and foam type of SF nanofiber scaffold (SFNS) could be prepared. Morphological structure of the 3-D SFNS was examined and compared with conventional 2-D SF nanofiber mat for evaluation of fibroblast culture. And this structure may be much

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more similar to the natural ECM than any other artificial scaffolds ever made.

EXPERIMENTAL

Materials

Bombyx mori cocoons were boiled in a mixed aqueous solution of marseillus soap of 0.3% and sodium carbonate of 0.2% to remove sericin. Then, the degummed cocoons were rinsed thoroughly in warm distilled water and dried at 50°C in an oven for obtaining SF. The SF was dissolved in a ternary solvent of calcium chloride/water/ethyl alcohol (mole ratio: 1/8/2) solution at 85°C for 3 min and dialyzed to remove salt and alcohol in cellulose membrane tube (MWCO: 12,000–14,000) against distilled water for 3 days at room temperature. Finally, aqueous SF solution was lyophilized to obtain a spongelike form of regenerated SF, which was used for electrospinning afterward. About 98% formic acid (Kanto, Japan), calf skin type I collagen (Sigma-Aldrich, USA), and 99% methanol (Daejung, Korea) were used without further purification.

Electrospinning of SF

To prepare the SF dope solution, the regenerated SF sponge was dissolved in 98% formic acid at room temperature for an hour and the solutions were filtered to remove impurities.[12,13,22] The concentration of the dope solutions was controlled in the range of 12–18% (w/v). The completely dissolved SF solution was moved in 10-mL syringe with 22G syringe needle (spinneret), which was loaded with a syringe pump (KDS-10, KD Scientific, USA). The flow rate of the dope was carefully controlled to fit the electrospinning speed. The applying voltage was 15 kV, and the methanol bath was set below 20 cm from the spinneret.

As DC voltage applied, the SF solution on the spinneret started to be spun, and the spun fibers were projected to methanol bath surface. Then, the SF nanofibers were deposited and dispersed uniformly in the methanol bath. Because a lot of very fine fibers were dispersed, the dispersion seemed to be like colloidal liquid. For lyophilizing the SF nanofiber dispersion, the methanol was substituted by distilled water using cellulose membrane. And then, the dispersion was moved into tissue culture Petri dish and lyophilized after freezing at −70°C. Finally, the foam type of 3-D SFNS could be prepared. And a sheet type of 2-D SF nanofiber mat was also made using rotating mandrel as a collector. The electrospun SF nanofiber mat was immersed in methanol for an hour to recrystallize and insolubilize the SF.

Characterizations

SF nanofiber structure was observed by scanning electron microscope (SEM) (JSM-5410LV, JEOL, Japan) after gold coating. The fiber size was determined by using image analysis software (ImagePro, USA). The porosity and density of the SFNS were determined by a liquid displacement method with ethanol.[23] At first, sample of weight W was immersed in a graduated cylinder containing a known volume (V_1) of ethanol. The sample was kept in the ethanol for 5 min, and then a brief evacuation purging was performed so that the ethanol could perfectly penetrate into the scaffold. And the total volume (V_2) of the ethanol and scaffold was measured. The volume difference ($V_2 - V_1$) was the volume of the skeleton of the scaffold. Then, the scaffold was removed from the cylinder, and the residual ethanol volume (V_3) was recorded. Therefore, the porosity (ε) and density (ρ) could be calculated by the following equations:

$$\varepsilon = (V_1 - V_3)/(V_{21} - V_3)$$

$$\rho = W/(V_2 - V_3)$$

where $V_1 - V_3$ is the pore volume and $V_2 - V_3$ is the total volume of the scaffold.

Cell culture and analysis of cell spreading

3T3 (mouse fibroblast cell line) purchased from ATCC was cultured to evaluate the SFNS. Cells were cultured in RPMI1640 (Gibco Laboratories, USA) supplemented with 10% fetal bovine serum (FBS) 4 m*M* L-glutamine, 7.5 mL HEPES, adjusted to contain 100 μg/mL streptomycin at 37°C in a humidified 5% CO_2 atmosphere. When the cells reached 80% confluence, they were subcultured. Then, the fibroblasts were used in the cell seeding.

Each SF scaffold was cut into 2 cm × 2 cm and soaked in 70% ethanol to sterilize for 20 min. Then, the scaffolds were rinsed three times with PBS. The sterilized scaffolds were coated by immersing in 50 μg/mL of type-I collagen solution during overnight. The collagen coated SF scaffolds were rinsed three times with PBS again and placed in 40 mm diameter Petri dish for the cell culture. The cells on the flask were detached by 0.25% trypsin-EDTA solution, and cell dispersion was diluted to 1×10^5 cells/mL in medium. Subsequently, 100 μL of the diluted cell solution was added to the 3D SFNS, 2D SFNS, and a solution cast SF film as control groups. The cell seeded scaffolds were incubated for 7 days in a condition mentioned before, and the medium was exchanged into a fresh one every 2 days.

For evaluating cell adhesion and spreading, the scaffolds were gently rinsed with PBS and fixed with 3% paraformaldehyde for 30 min, followed by postfixing with 1% osmium tetraoxide for 15 min. The cells were then dehydrated using a graded series of ethanol (10, 20, 30, 50, 70, 80, 90, and 100%). After drying and gold coating, the samples were observed by SEM (JSM-5410LV, JEOL, Japan).

RESULTS AND DISCUSSION

Electrospun nanofiber scaffolds may exhibit an excellent performance of cell adhesion and migration due to its large surface area and structural similarity to ECM. The nanofiber mat, which is prepared using conventional electrospinning technique, can be presumed as a 3-D structure. However, it is not a real three dimension and it is rather a 2-D sheet form with very small thickness. The pores and interstices formed between nanofibers in the mat are very small and stacked nanofiber layers are so dense that the seeded cells are not able to migrate into inner space of nanofiber mat. This makes an obstacle to the practical use of electrospun nanofiber scaffold in tissue engineering, and many researchers are trying to develop the scaffold of highly porous structure. Here, it may be asked whether a real 3-D scaffold having nanofibrous structure with high porosity can be made. Furthermore, it is more useful if the scaffold can be manufactured in various shapes.

Figure 1 shows an apparatus for manufacturing 3-D nanofiber scaffold, which has been used in this study. The electrospun SF nanofibers were deposited in methanol bath instead of collecting on flat metal plate. Because the bath is stainless steal and grounded, the charged SF nanofiber jet was easily projected down to the coagulation bath. Using methanol, the SF nanofibers were dispersed uniformly in the coagulation bath. To solidify the nanofiber dispersion by keeping its dispersed state intact, lyophilizing process was necessary. However, the methanol suspension could not be allowed in general lyophilizing system because of an extremely low freezing point of methanol (about $-98^{\circ}C$). Therefore, exchanging the methanol for water was carried out using cellulose membrane. In lyophilizing process, the water between SF nanofibers was removed, and the initial volume of the SF nanofiber dispersion was almost kept constant during the process.

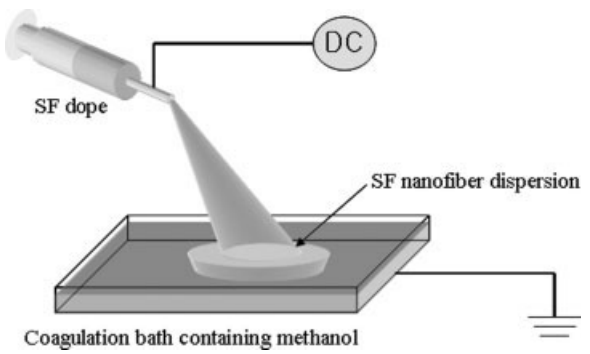

Figure 1 Scheme of electrospinning apparatus for manufacturing 3-D SF nanofibrous scaffold.

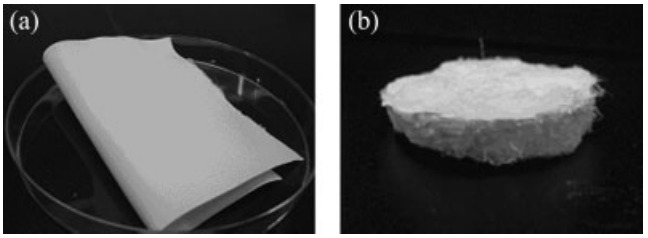

Figure 2 Photographs of (a) 2-D SF nanofiber mat and (b) 3-D SF nanofiber foam.

Foam type of SF nanofiber is shown in Figure 2. When compared with conventional nanofiber mat, it exhibits 3-D geometry in spongelike structure with certain thickness, which can be easily controlled by varying a mold vessel and the amount of SF nanofiber dispersion. Various shapes of real 3-D SFNS could be prepared and shown in Figure 3. It suggests that this SFNS is more suitable as tissue engineering scaffold than conventional SF nanofiber mat because scaffold must fit into the shape and size of deficient tissue.

Moreover, the 3-D SFNS has large pores, which can allow to easy penetration of nutrients and wastes and cell migration. Figure 4(a,c) shows the SEM micrographs of SF nanofiber mat and 3-D SF nanofiber foam, respectively. And cross-section of SF nanofiber mat is shown in Figure 4(b). The pore size of SF nanofiber foam is much lager than that of SF nanofiber mat. The pore size of SF nanofiber mat is about 1–5 μm. The pore is so small that seeded cell is unable to spread into inner space of the scaffold. To maximize the surface area of nanofiber mat for

Figure 3 Photographs of various shapes of 3-D SF nanofibrous scaffolds.

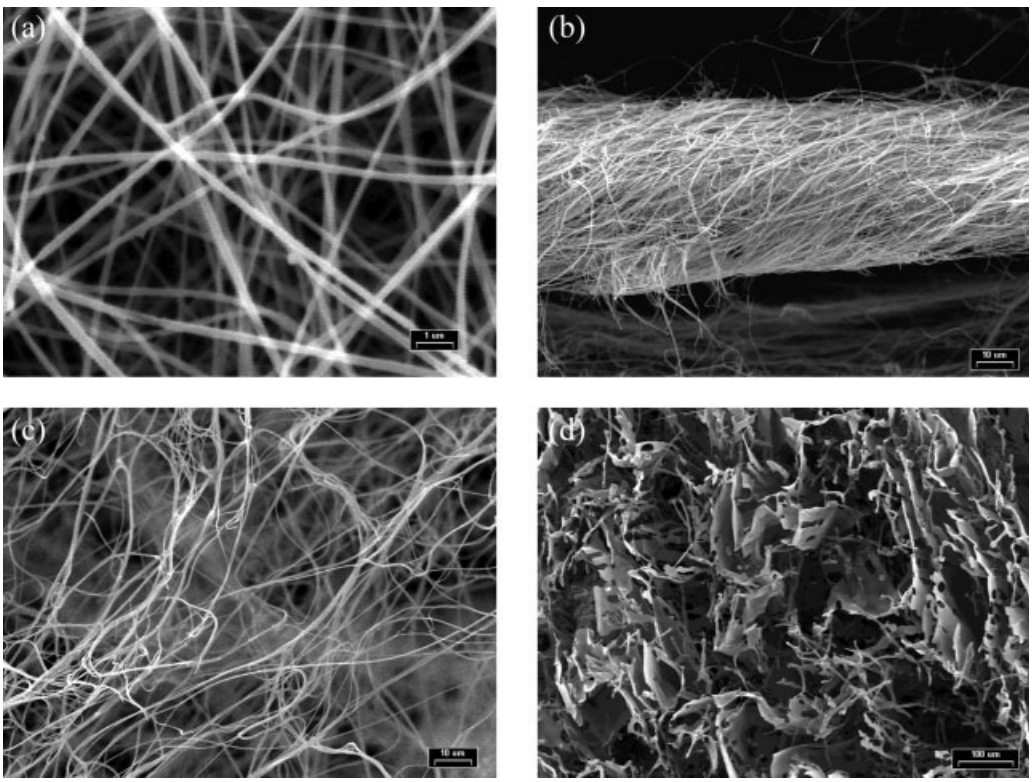

Figure 4 SEM micrographs of various SF scaffolds: (a) surface structure of 2-D nanofiber mat (magnification: ×10,000), (b) cross-section of 2-D nanofiber mat (magnification: ×1000), (c) morphological structure of 3-D nanofiber foam (magnification: ×1000), and (d) lyophilized SF sponge (magnification: ×150).

enhancing cell adhesion, the fiber size has to be minimized as possible. However, the decrease of fiber size induces the decrease of pore size and, consequently, the regeneration of 3-D tissue on scaffold comes to be impossible. On the other hand, the inter space of the nanofibers is as large as 10–100 μm for 3-D SF nanofiber foam, and this can be controlled by varying nanofiber density in the dispersion during preparation. Such a large pore structure maybe allow to easy migration of cells when the cells are seeded on the surface of the scaffold.

To compare the 3-D SFNS with typical 3-D SF scaffold, SF sponge was prepared by lyophilizing SF aqueous solution. This lyophilizing method has been generally known for making the sponge type of SF scaffold, whose pore size and porosity are easily controllable.[8] Figure 4(d) shows highly porous structure of lyophilized SF sponge. However, its pores were shaped by surrounding polymeric walls while the pores of SF nanofiber foam were constructed by many entangled SF nanofibers similar to natural ECM. Therefore, it can be inferred that the 3-D SF nanofiber foam is structurally more suitable for tissue engineering scaffold than either SF sponge or 2-D SF nanofiber mat.

The concentration effect on the morphological structures of SF nanofibers is depicted in Figure 5. The fibers in 3-D SF nanofiber foam are more

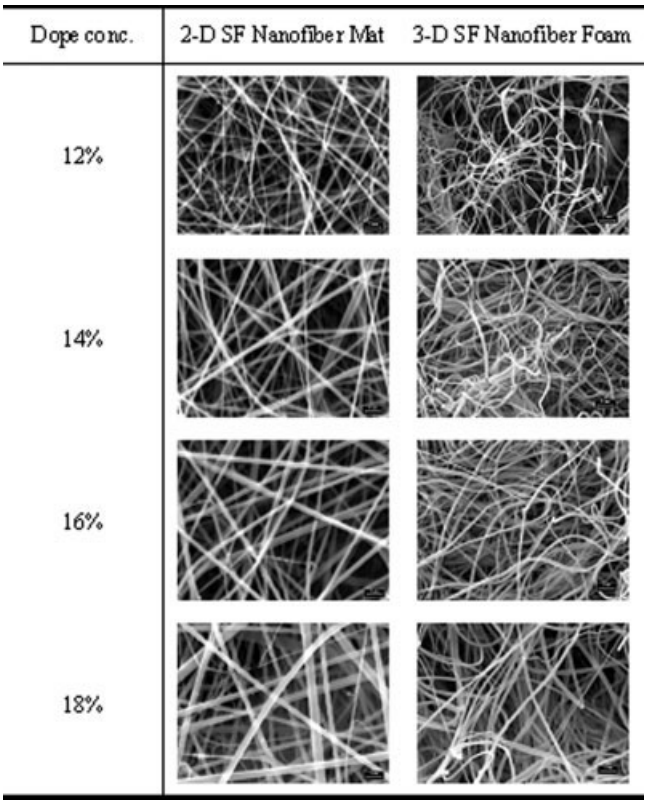

Figure 5 SEM micrographs of electrospun 2-D SF nanofiber mat and 3-D nanofiber foam prepared from various dopes concentrations (magnification: ×10,000).

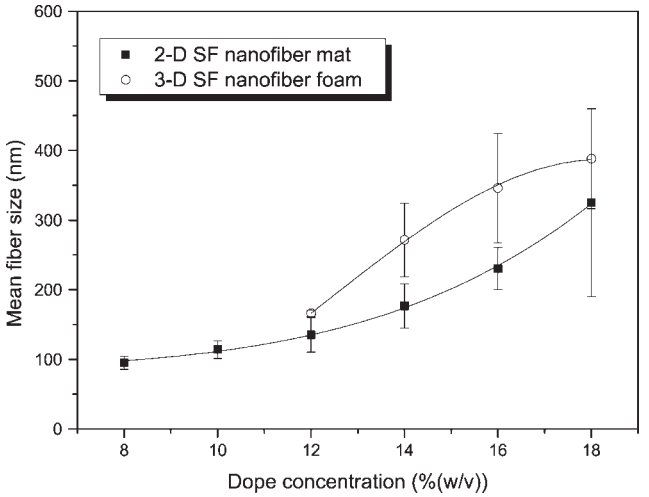

Figure 6 Mean diameters of electrospun nanofibers of 2-D SF nanofiber mat and 3-D nanofiber foam prepared from various dope concentration.

entangled and coiled than those in 2-D SF nanofiber mat because of different collecting method in electrospinning. Moreover, interspaces between the nanofibers are much larger in 3-D SF nanofiber foam due to the freely dispersed nanofibers in methanol. The diameter of the SF nanofiber was in the range of 100–400 nm. At a low concentration (less than 12%), bead formation occurred and proper SF concentration for electrospinning was observed as 12–18%. The morphological structure of nanofiber, especially nanofiber size, is related to solution viscosity and surface tension depending on the dope concentration.[24] Figure 6 shows the change of mean diameter of SF nanofiber with the dope concentration. As expected, the mean diameter increased with increasing the SF dope concentration. The difference between 2-D SF nanofiber mat and 3-D foam existed in nanofiber size and its distribution. In the case of 3-D SF nanofiber foam, they were larger at same dope concentration due to swelling effect for collecting process in methanol bath. Considering that the nanofiber size is controlled by the dope concentration, it seems to be capable to tailor fiber size accurately suiting a specific cell type.

A desirable scaffold has high porosity and sufficient pores of appropriate sizes due to spaces for living cells and cell migration. Furthermore, highly porous structure is not only profitable for exchanging nutrients and wastes, but also for vascularization. Nanofiber scaffold prepared from electrospinning process can be recognized as a high porous scaffold because numerous pores are formed in nanofiber assemblies. However, as mentioned earlier, it is difficult to expect an adequate pore structure for cell culture, and the porosity does not reach a level of conventional scaffold. As shown in Figure 7, the porosity of 2-D SF nanofiber mat was measured about 84%. However, nanofiber layer was stacked very densely and the large pores enough to permit cell migration were not observed in cross-sectional view [Fig. 4(b)].

On the other hand, 3-D SF nanofiber foam had much higher porosity, about 94%, than 2-D mat. This porosity value competed with or was even better than that of lyophilized SF sponge. Moreover, large inter spaces as well as uniform pore structures were formed in 3-D nanofiber network of SF.

Figure 8 shows SEM micrographs of various SF scaffolds whose cell adhesion and proliferation abilities were assessed by cultivating mouse fibroblasts. The 2-D SF nanofiber mat [Fig. 8(b)] exhibited an excellent cell adhesion and proliferation abilities, and this result was coincident with other researchers previously reported. After 7 days, the fibroblast entirely covered the surface of 2-D SF scaffold while the number of cells seeded on SF film did not proliferate and even the adhered cells were scarcely observed. This phenomenon is due to the difference of specific surface area between SF nanofiber mat and film. The larger contact area of scaffold to surface of cells gives easy cell adhesion, and, consequently, the capability of focal adhesion of the cells can be enhanced in nanofiber mat. The seeded cells, however, did not migrate into the inner space of the 2-D SF scaffold. As mentioned earlier, cell migration was not possible due to the small pore and dense layered structure but the fibroblasts well proliferated only on surface of 2-D SF nanofiber mat. Therefore, it can be said that 2-D electrospun nanofiber mat is less suitable for 3-D cell culture and tissue regeneration.

On the other hand, the seeded cells on 3-D SFNS located at the surface as well as inside inner spaces

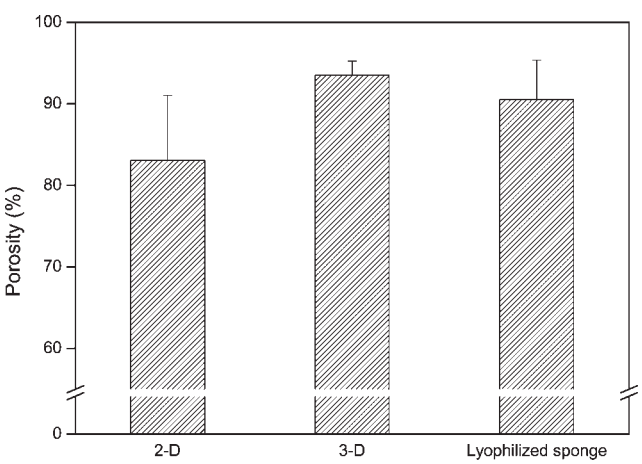

Figure 7 Porosity of SF nanofibers assemblies and lyophilized SF sponge.

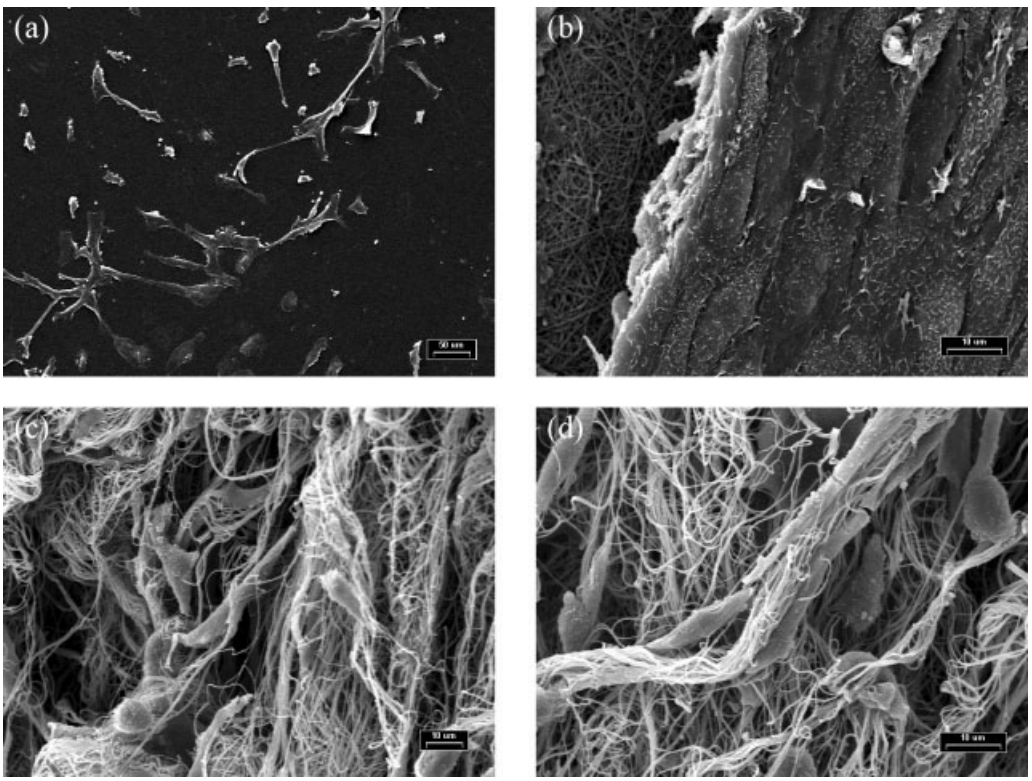

Figure 8 SEM micrographs of cultured mouse fibroblasts after 7 days incubated at 37°C in 5% CO_2 atmosphere on: (a) SF film, (b) 2-D SF nanofiber mat, and (c) and (d) 3-D SF nanofiber foam.

due to its relatively larger pores in 3-D structure. Figure 8(c,d) shows the mouse fibroblast cells adhered and proliferated in 3-D SF nanofiber foam. The circumstance for cell adhesion, migration, and proliferation is entirely different from the case of 2-D SF scaffold. It seems that the 3-D SF nanofiber foam can offer the most similar structural circumstance to natural ECM in living organisms by making suitable nanostructure scaffold for 3-D tissue culture.

CONCLUSIONS

The 3-D nanofibrous structure of scaffold was built by modifying conventional electrospinning process. It could be achieved by which electrospun SF nanofibers were collected in coagulation bath containing methanol. The 3-D SFNS exhibited very highly porous structure and large pores compared with 2-D SF nanofiber mat. Moreover, the size and shape of SFNS could be easily constructed for fitting target tissue shape. As a result of cell adhesion and proliferation of mouse fibroblast, it was confirmed that the seeded cells were well proliferated in inner spaces (pores) of 3-D SF nanofiber foam. Because of the structural features of 3-D SF nanofiber foam and its structural similarity to natural ECM, the 3-D SF nanofiber foam may be considered as an excellent scaffold for 3-D cell culture and tissue regeneration.

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