Cell adhesion and proliferation enhancement by gelatin nanofiber scaffolds

Shih-Ching Wu[1,2], Wei-Hong Chang[1], Guo-Chung Dong[3], Kuo-Yu Chen[4,†], Yueh-Sheng Chen[5,6,†] and Chun-Hsu Yao[5,6,*,†]

[1]Institute of Biomedical Engineering and Materials Science, Central Taiwan University of Science and Technology, Taichung 406, Taiwan, ROC

[2]Department of Dental Technology and Materials Science, Central Taiwan University of Science and Technology, Taichung 406, Taiwan, ROC

[3]Division of Medical Engineering Research, National Health Research Institutes, Miaoli 350, Taiwan, ROC

[4]Department of Chemical and Materials Engineering, National Yunlin University of Science and Technology, Yunlin 640, Taiwan, ROC

[5]Department of Biomedical Imaging and Radiological Science, China Medical University, Taichung 404, Taiwan, ROC

[6]School of Chinese Medicine, China Medical University, Taichung 404, Taiwan, ROC

*** Corresponding author:**

Chun-Hsu Yao, Department of Biomedical Imaging and Radiological Science, China Medical University, Taichung 404, Taiwan, ROC; Tel: +886-4-22053366 ext. 7806; Fax: +886-4-2207-6976; Email: chyao@mail.cmu.edu.tw

[†]**These authors contributed equally to this work**

Abstract

Gelatin nanofibers prepared by electrospinning were cross-linked with glutaraldehyde vapor to improve their water-resistant ability. After cross-linking treatment, the form of the fibers expressed no substantial change, but the average diameter of the fibers increased with increasing cross-linking time. The swelling induced by the moisture during cross-linking process could be moderated when the cross-linking time reached 45 min. The result of contact angle measurements displayed that the electrospun gelatin fibers was more hydrophilic than the gelatin film. Moreover, increasing the cross-linking time did not alter the hydrophilic properties of the gelatin fibers. Furthermore, cell compatibility was evaluated based on [3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide] assay, scanning electron microscope and confocal microscope observations and Western blot analysis by culturing MG-63 cells on the gelatin films and gelatin nanofibers. It was found that nanofibrous structure fabricated by an electrospinning technique could enhance cell adhesion and proliferation. The aforementioned study displays a cost-effective simulation of gelatin nanofiber structures and forecasts promising applications on scaffold preparation for tissue engineering.

Keywords

gelatin, nanofibers, electrospinning, cross-link, cell compatibility

Introduction

Tissue engineering provides an emerging approach for replacing or repairing the function of damaged or diseased tissues. Scaffold preparation is an important part of medical tissue engineering, and plays an important role in the guidance of tissue regeneration. An ideal scaffold should mimic the physical and chemical structure of the native extracellular matrix (ECM).[1,2] Mammalian cells evolve *in vivo* in close contact with ECM comprised of fibers, pores, and ridges with nanoscale features.[3] Nanostructures such as nanoholes, nanoislands, nanofibers, and nanoparticles can be introduced to mimic the environment of ECM, regulating the physiology reaction, and promoting the cell adhesion, proliferation, and differentiation.[4,5]

Among the various methods of preparing nanostructured scaffolds, electrospinning provides an effective and simple way to prepared nano- or submicron fibrous scaffolds similar to the structure of natural ECM.[6-9] Moreover, nanofibrous structure can provide huge surface areas and appropriate porosity for cell growth. Up to present, a variety of synthetic polymers (such as polyvinyl alcohol (PVA), polylactic acid, polylactic-co-glycolic acid, and polycaprolactone) and natural polymer (such as collagen, gelatin, chitosan, and silk fibroin) have been electrospun as scaffolds for tissue engineering. These researches have demonstrated that nanofibrous structure formed by electrospinning could promote cell attachment, proliferation, and differentiation.[10-14]

A number of physiological ECM proteins have been used as scaffolds for bone tissue engineering, such as collagen, hyaluronan and fibrin.[15] Collagen, the major structural protein found in ECM of many tissues, is rich in arginine-glycine-aspartic acid (RGD) sequences and able to promote cell adhesion and proliferation.[16] It is widely known that the RGD sequence refers to particular integrin joint locations of focal adhesion, outside the cell membrane. In recent years, various researchers have employed gelatin, a partially

hydrolyzed collagen, as a scaffold for tissue engineering application.[17,18] Gelatin possesses the RGD sequences of collagen, making it highly effective in cell adhesion.[19] Moreover, gelatin has a lower antigenicity than collagen. However, gelatin degrades rapidly at body temperature.[20] It will reduce its utility as a scaffold in tissue regeneration. Various cross-linking treatments, including chemical and physical methods, have been proposed to increase the structural stability of gelatin scaffolds. Physical cross-linking methods such as dehydrothermal treatment, plasma treatment, ultraviolet irradiation, and γ-ray irradiation do not cause potential harm to cell and tissue, but the desired amount of cross-linking is difficult to achieve due to the cross-linking occurring only at the surface of the material.[21-23] Various cross-inking reagents, including glutaraldehyde, carbodiimides, caffeic acid, tannic acid, and genipin, have been used to cross-link gelatin to prolong the absorption of the gelatin in the living tissue.[21,24-26] Among these cross-inking reagents, most investigators used glutaraldehyde vapor to cross-linked electrospun gelatin fibers due to its high efficiency in stabilizing gelatin molecules.[21,27-29] Moreover, using glutaraldehyde vapor can reduce solubility of electrospun gelatin fibers during cross-linking. However, glutaraldehyde is highly cytotoxic at high concentration. Most studies investigated the factors influencing the production of electrospun gelatin fibers including spinning temperature, potential, and concentration of gelatin solution.[21,28] Zhang et al. evaluated the effect of cross-linking time on the water-resistant behaviors of gelatin nanofibers cross-linked with glutaraldehyde vapor.[29] However, the effects of cross-linking time on the cytotoxicity of electrospun gelatin fibers cross-linked with glutaraldehyde vapor have not yet been reported upon.

The aim in this study was to (1) prepare gelatin nanofiber scaffolds via electrospinning process to mimic the structure of the natural ECM, (2) optimize the cross-linking time for the electrospun gelatin fibers cross-linked with saturated glutaraldehyde vapor to improve

their water-resistant ability and to reduce their cytotoxicity, and (3) evaluate the effect of nanofibrous structures on cellular response. The effects of cross-linking time on the degree of cross-linking, morphology, hydrophilicity, swelling, and *in vitro* degradation of electrospun gelatin fibers were examined. MTT assay was used to investigate the influence of cross-linking time on the cytotoxicity of electrospun gelatin nanofibers and gelatin films. The behavior of cell adhesion to gelatin films and gelatin nanofibers was investigated by scanning electron microscope and confocal microscope observations and Western blot analysis to assess how nanofibrous structures influenced cell adhesion.

Materials and methods

Materials

Gelatin (Bloom number 300) and glutaraldehyde were purchased from Sigma-Aldrich (Saint Louis, MO, USA). PVA (1,400 Mw) was obtained from Showa (Japan). All chemicals were used without further purification.

3-(4,5-Dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT), mouse monoclonal anti-β-actin, and anti-mouse IgG conjugated Cy3 were all purchased from Sigma. Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, New York, USA). 4',6-diamidino-2-phenylindole (DAPI) was purchased from Invitrogen (USA). Osteoblast-like MG-63 human osteosarcoma cells (BCRC no. 60279) were purchased from the Food Industry Research and Development Institute, Taiwan.

Preparation of electrospun gelatin nanofibers

Gelatin powders were dissolved in formic acid (88 wt%) to form a 17 wt% gelatin solution. PVA powders were dissolved in heating de-ionized water to form a 10 wt% PVA aqueous solution. The PVA aqueous solution was then added into the gelatin solution with gelatin/PVA ratio of 9/1 v/v and the mixture was magnetically stirred for 1 h at room temperature.[30] The solutions were transferred to a plastic syringe fitted with a needle (G22, diameter = 0.41 mm) and set up in the electrospinning apparatus (SC-80H, Xian Xing Electric Co., Taiwan). The syringe was controlled by a syringe infusion pump (KDS220, KD Scientific, USA). A high-voltage electric field was created between a needle capillary end and a collector by applying a high voltage to the needle, using a power supply apparatus. A piece of grounded aluminum foil used as a collector was located 10 cm apart from the capillary tip. The applied voltage was controlled at 20 kV and electrospinning was performed at room temperature.

After electrospinning, the gelatin fibers were cross-linked in glutaraldehyde (50%) vapor for a predetermined time at 25°C.[30] The cross-linking times were 0, 15, 45, 90 and 360 min, and the specimens were denoted by GN0, GN15, GN45, GN90 and GN360, respectively. Solvent casting gelatin/PVA film served as the control substrate in cell adhesion studies.

The degree of cross-linking of electrospun gelatin nanofibers

A ninhydrin assay was used to evaluate the degree of cross-linking of the electrospun gelatin nanofibers. Ninhydrin (2,2-dihydroxy-1,3-indanedione) was used to determine the amount of amino groups of each test sample. The samples were heated with a ninhydrin solution for 20 min. After heating with ninhydrin, the optical absorbance of the solution was recorded using a spectrophotometer (ELISA reader, Model Genesys™ 10, Spectronic Unicam, New York, USA) at 570 nm (wavelength of the blue-purple color) using glycine at various known concentrations as the standard.[31] The amount of free amino groups in the residual gelatin, after heating with ninhydrin, is proportional to the optical absorbance of the solution. The degree of cross-linking of electrospun gelatin nanofibers was then determined.

Morphological evaluations

Morphology of the electrospun gelatin nanofibers was observed by scanning electron microscope (SEM, Hitachi S-3000N, Tamura, Japan) with an accelerating voltage of 20 kV. Before SEM observation, all of the samples were sputter-coated with gold.

Determination of swelling and in vitro degradation

After cross-linking, the electrospun gelatin nanofibers were punched into 37°C de-ionized water for soaking test. Samples were taken from the de-ionized water after 6 h, 12 h, 4 days and 7 days. Subsequently, the samples were dehydrated in a graded series of ethanol

solutions, critical-point dried with carbon dioxide, spattered with gold film, and examined with a SEM. The diameters of fibers were observed from SEM for understanding the wetting and degradation of the electrospun gelatin nanofibers.

Contact angle test

The surface hydrophilicity of each test specimen was examined by measuring its water contact angle. The static contact angle was obtained on a Data Physics OCA 30 plus goniometer and imaging system (Filderstadt, Germany) at room temperature using the sessile drop method. The measurement of each specimen was performed on a 20 μL water spot. When the liquid is dropped on a solid specimen surface, the liquid droplet will spread or make a spherical globule. Five different points of each sample were performed to calculate the mean contact angle and its standard deviation.

MG-63 cells cultured with electrospun gelatin nanofibers and gelatin films

In this study, the cellular compatibility of each test sample was evaluated by an *in vitro* cell-culture assay. The samples were first sterilized using UV light irradiation, and 15 mm diameter specimens were then cut from each sterilized test sample and glued to the bottom of each well in a 24-well plate (the diameter of each well is about 16 mm) using a sterilized collagen solution. Subsequently, osteoblast-like MG-63 human osteosarcoma cells at 10^4 cells/well were seeded evenly on the surface of each test specimen in 1 mL DMEM with 10% FBS in an incubator containing 5% CO_2 at 37ºC. Using the MTT assay, the viable cells cultured on each test specimen were determined at 2 days after cell seeding.

In the MTT assay, the cells cultured on each test specimen were washed with phosphate buffered saline (PBS) twice and viable cell numbers were then determined indirectly by MTT dye reduction. The MTT assay is based on the reduction of MTT, a yellow soluble

dye, by the mitochondrial succinate dehydrogenase to form an insoluble dark blue formazan product. Only viable cells with active mitochondria reduce significant amounts of MTT to formazan. During the cell-culture period, the growth medium was changed daily.

In the MTT test, 200 μL MTT solution (0.5 g/L in medium, filter-sterilized) was added to the culture wells. After incubation for 4 h at 37°C in a 5% CO_2 atmosphere, the MTT reaction medium was removed and blue formazan was solubilized by acidic isopropyl alcohol (0.04 M of HCl in isopropyl alcohol). Optical density readings were then performed using a multi-well scanning spectrophotometer (MRX Microplate Reader, Dynatech Laboratories Inc., Chantilly, Virginia, USA) at a wavelength of 570 and 650 nm.

The morphology of the cells cultured on each test specimen was also examined by a SEM. After 2, 4, and 8 h of culture, the specimens used for the SEM examination first were briefly rinsed with PBS and fixed with 2% glutaraldehyde. Subsequently, the samples were dehydrated in a graded series of ethanol solutions, critical-point dried with carbon dioxide, and spattered with gold film. The examination of the cells cultured on each test specimen was performed with a SEM.

Confocal microscope

Immunofluorescent labeling was performed to observe and compare the difference of MG-63 cells seeded onto the gelatin film (GF) and the electrospun gelatin nanofibers (GN). After sterilizing with UV light irradiation and 2 h of culture with MG-63 cells, the specimens were rinsed with PBS twice and fixed in acetone and methyl alcohol (1:1 v/v) at –20ºC. Non-specific labeling was blocked by incubating with 3% skim milk. The samples were incubated in primary antibody: mouse monoclonal anti-β-actin and then kept in anti-mouse IgG conjugated Cy3. Finally, they were stained with DAPI and the stained cells were examined using a confocal microscope.

Western blot analysis

After culturing with samples for 2 h, the cells were lyzed in lysis buffer. These lysates were then centrifuged at 4ºC, 12000 rpm for 2 min. The supernatant was recovered and protein concentrations were determined ($R^2 = 0.9926$). Typically, 30 µg/µL of protein was resolved by electrophoresis in a sodium dodecylsulfate (SDS) – 10% polyacrylamide gel and blotted onto a polyvinylidene difluoride membrane. The membranes were blocked by incubating with 3% skim milk for 30 min and then they were incubated in primary antibody mouse anti-β-actin and γ-tubulin, respectively for 1 h. Then, they were kept in goat-anti mouse conjugate HRP for 1 h. Finally, they were detected using ECL kit (Millipore, Immobilon TM Western, chemiluminescent HRP substrate) and showed by X-ray film (Konica Minolta, Japan).

Statistical analysis

All quantitative data were presented as mean ± standard derivation. Statistical analysis were conducted using one-way analysis of variance followed by *post hoc* Fisher's LSD multiple comparison test. A difference was deemed significant at $p < 0.05$.

Results

The degree of cross-linking and morphological observation of electrospun gelatin nanofibers

Figure 1 shows the SEM morphology of electrospun gelatin nanofibers before and after cross-linking with glutaraldehyde vapor. It demonstrates that the form of the fibers did not change substantially. However, the diameter of the fibers increased after cross-linking, indicating that the fibers were swollen with water vapor present in the cross-linking solution. The diameter of electrospun gelatin nanofibers before cross-linking is 144.9 ± 7 nm. The diameters of electrospun gelatin nanofibers cross-linked for 15, 45, 90, and 360 min (denoted as GN15, GN45, GN90, and GN360) were 279.2 ± 19.2, 384.8 ± 9.8, 405.2 ± 20.3, and 403.2 ± 20.9 nm, respectively. After ninhydrin tests were conducted, the degree of cross-linking was 31.6% for GN15, 53.4% for GN45, 56.9% for GN90, and 91.4% for GN360. These measurements support the fact that the degree of cross-linking increased with cross-linking duration.

Determination of swelling and in vitro degradation

Cross-linked gelatin nanofibers were then sent for soaking tests. The SEM pictures of the nanofibers soaked in de-ionized water for 7 days are shown in Figure 2. The non-cross-linked nanofibers show no observable fiber structure after soaking (Figure 2(a)), which means that the fibers had completely dissolved in the water. For the cross-linked specimens, the fiber diameter of GN15 swelled to a remarkable degree (Figure 2(b)) due to insufficient cross-linking. However, the fiber diameters of GN45, GN90, and GN360 (Figure 2(c), 2(d), and 2(e), respectively) did not exhibit noticeable swelling. Figure 3 illustrates statistical analysis of the above results. The diameter of GN15, which had undergone 15 min of cross-linking duration, increased with soaking time. With cross-linking times exceeding 45

min, no noticeable change was observed in the diameter of specimens after 12 h of soaking.

Contact angle test

Figure 4 displays the contact angles of the gelatin film (GF) and electrospun gelatin nanofibers (GN). In the current study, both samples had undergone glutaraldehyde vapor cross-linking for 45 min. The results show that the electrospun material had improved hydrophilic properties over that of gelatin film. Furthermore, the contact angles after 15, 45, 90, and 360 min of cross-linking duration are 39.8° ± 2.4°, 41.2° ± 3.2°, 45.6° ± 3.8°, and 43.9° ± 3.7°, respectively, indicating that the cross-linking time did not significantly affect the hydrophilicity of the gelatin fibers.

Proliferation of MG-63 on gelatin films and electrospun gelatin nanofibers

After analysis of the properties of the materials, samples with different cross-linking durations were evaluated for their cyotoxicity. Solvent casting gelatin film served as a control group. Figure 5 shows the results of MTT assay after culturing for 2 days. The following conclusions can be drawn. First, a comparison between electrospun nanofibers and the control group reveals that gelatin nanofibers had superior cell viability over that of solvent casting gelatin film. Second, the overall cell activity in the 45 min cross-linked case (GN45) was better than that of the other three sets. The inhibition of cell expansion on GN90 and GN360 suggested some cytotoxic effect of the residual glutaraldehyde on the cells.

Morphological observation of MG-63 adhesion on gelatin films and electrospun gelatin nanofibers

Cell adhesion is an important cellular process because it directly influences cell growth, differentiation and migration. Based on the above results, sample GN45 was selected for the

investigation of cell adhesion performance. The gelatin film sample processed with 45 min of glutaraldehyde vapor cross-linking (GF45) was used as a control group. As shown in Figure 6, the cell adhesion characteristics of GN45 was obviously superior to that of GF45 after culturing for 2 h. When the culture time reached 8 h, it became more obvious that the cells had spread and adhered to the surface of electrospun gelatin nanofibers (Figure 6(f)). The cells were integrated with the surrounding fibers, indicating that GN45 has a high biocompatibility. For GF45 thin film materials, the cells had not adhered well or stretched, and were still considered as a ball-shaped cell. Clearly, the GN nanofibrous structure had superior cell adhesion to that of GF gelatin film. Additionally, Figure 6 demonstrates that the fibrous morphology of gelatin nanofiber scaffolds did not change significantly after cell culture, indicating that a 45-minute cross-linking time is sufficient.

Confocal microscope observation

The highest quality electrospun nanostructures (glutaraldehyde vapor cross-linking for 45 min, GN45) were chosen for immuno-fluorescence assay testing. Figure 7 shows the differences in cells using gelatin film and gelatin nanofibers through a confocal microscope. Figure 7(a) illustrates how cells adhere to the gelatin film (GF45); the red luminescence indicates the cytoskeletal protein; and blue luminescence indicates the location of the cell nucleus. Figure 7(b) shows cells adhering to nanostructured gelatin nanofibers (GN45). Green, blue, and red luminescence indicates the locations of the gelatin nanofiber, cell nucleus, and cytoskeletal protein, respectively. A high concentration of cytoskeletal protein appears within the center of the nanofibers, indicating an intrusion of filopodia into the system. Serial scanning results by the confocal microscopy indicated the location at which the cell adhered to the material (Figure 7(c)). Figure 7(d) is also a scanned picture from the location beneath that of Figure 7(b), showing the internal structure of the nanofibers. Figure

7(c) and (d) proved that the cell filopodia had extended to the interior of the material.

Western blot analysis

The above results indicate that for the electrospun nanofibers, cytoskeleton had been generated after cell flattening and stretching. The western blotting analysis was then used to explore the quantity of cytoskeleton created after cell adhesion. Figure 8 shows that cytoskeletal protein with nanofibrous structure had more γ-tubulin and β-actin than those of the gelatin film without nanostructural features.

Discussion

Electrospinning has recently been introduced in the preparation of scaffolds for tissue-engineering application. It has received a great deal of attention due to the ability of the produced nanofibers to mimic natural ECM. In this study, gelatin nanofibers had been prepared through the process of electrospinning followed by cross-linking with glutaraldehyde vapor with different durations to retain fibrous structure. The cell adhesive properties on gelatin nanofibers were compared with those on solvent casting gelatin film.

Glutaraldehyde can react with amine groups of collagen and gelatin molecules via formation of Schiff bases.[32] Zhao et al. used glutaraldehyde vapor and glutaraldehyde absolute ethanol solution to cross-link electrospun gelatin fibers with average diameter of 327 ± 59 nm.[27] An average diameter of gelatin fibers increased to 528 ± 76 nm (vapor cross-linked) and 690 ± 89 nm (solution cross-linked) after cross-linking. Therefore, in this study, the cross-linking was carried out by vapor-solid reactions to avoid gelatin fibers dissolving and to reduce the degree of swelling of gelatin fibers during cross-linking. The results showed that the degree of cross-linking was only 53.4% for the electrospun gelatin nanofibers cross-linked with glutaraldehyde vapor for 45 min. However, SEM pictures proved that the moisture induced swelling phenomena of nanofibers could be moderated after applying cross-linking for 45 min (Figure 2). There was no noticeable change in diameter after more than 45 min of cross-linking. This degree of cross-linking had been sufficient to maintain the fibrous structure during 7 days of soaking (Figure 3). Moreover, SEM images also showed the fibrous morphology of the electrospun gelatin nanofibers did not change significantly after cell culture (Figure 6). Zhang et al. prepared gelatin nanofibers with averaged fiber diameters around 200-300 nm.[29] It exhibited that the cross-linking time of gelatin nanofibers cross-linked with glutaraldehyde (25%) vapor at room temperature should be at least 3 days to generate a cross-linking extent sufficient to preserve the fibrous

morphology tested by soaking in 37 ºC water. In this study, the average diameter gelatin nanofibers is 145 nm and the concentration of glutaraldehyde is 50%. The greater surface area of nanofibers and higher concentration of glutaraldehyde could improve the cross-linking efficiency of glutaraldehyde vapor.

In this study, a cell culture process was performed for the gelatin nanofibers and gelatin films with various cross-linking times to evaluate their biological adaptability. The results showed that gelatin nanofibers and gelatin films cross-linked with glutaraldehyde vapor for 45 min had a higher cell proliferation activity than those cross-linked for 90 and 360 min, indicating that the longer cross-linking time would exert a cytotoxic effect on cells. The study of Zhang et al. demonstrated that the initial inhibition of cell expansion on the cross-linked gelatin fibrous scaffold, indicating some cytotoxic effect of the residual glutaraldehyde on the cells due to long cross-linking time (3 days).[29]

The results of MTT assay and cell adhesion studies showed that the nanostructured material promoted cell proliferation and adhesion over that of the gelatin film. The architecture of the fibrous gelatin scaffold was similar to the natural ECM. Moreover, nano-sized fiber can provide huge surface areas for cell growth. Many studies have showed that nanofibrous scaffold has a higher cell proliferation than solvent casting film with a smooth surface. For example, Guarino et al. exhibited that the nanostructured surfaces of polycaprolactone/gelatin electrospun membranes enhanced human mesenchymal stem cells adhesion, spreading, and proliferation.[10] Shih et al. reported a higher cell viability of mesenchymal stem cells on electrospun type I collagen nanofibers compared to smooth surfaces.[13] Chu et al. found that the nanofibrous structure of the chitosan scaffold could improve hepatocyte adhesion and function.[33]

Confocal microscope pictures demonstrated that a high concentration of cytoskeletal protein existed in the nanofibrous scaffold, indicating an intrusion of filopodia into the

scaffold (Figure 7). According to the experimental result of Maurin et al., it was demonstrated that the appearance of the so-called focal adhesion, referred to as the adhesion process was accomplished by the cell filopodia with RGD motif of ECM.[34] The cell adhesion had been produced with the following process. Centosome was used to promote the formation of microtubes, and then it was turned into the integrins of the transmembrane. An interactive reaction then occurred between the integrins and actin-filament, in which the filopodia were encouraged to contact with ECM. As the cell adhesion process completed, the shape of the cells was flattened due to the rearrangement of cell structure. Experimental results of Pugacheva and Golemisl demonstrated that the behavior of cytoskeletal protein was related to cell mitosis.[35] When cell mitosis is at the stage of G2 to M, hydrolysis of cytoskeletal protein occurred, causing it to react with the AurA and Nek2. This reaction was favorable to the formation of microtube from γ-tubulin as well as the completion of the mitosis process. In this study, the quantity of cytoskeletal protein attached to the gelatin nanofibers was greater than the gelatin film (Figure 8). In other words, the nanofibers had encouraged such cell adhesion. The quantity of γ-tubulin and β-actin in this process was greater than in the process with thin films. Since gelatin films had poor adhesive properties, the quantity of cytoskeleton was low and the shape of the cells remained circular. In addition, there were too few γ-tubulin synthesized microtubes to assist in the process of mitosis. Therefore, gelatin nanofiber scaffold had a better cell proliferation rate than the gelatin film due to highly efficient cell adhesion process for nanofiber.

Conclusions

Gelatin scaffold with fiber diameter of about 145 nm was successfully fabricated by electrospinning. The nanofiber material degradation could be postponed after cross-linking with glutaraldehyde vapor. Increasing the cross-linking time increases the degree of cross-linking of gelatin fibers. However, increasing the cross-linking time did not alter the hydrophilic properties of the material. When the cross-linking time was 45 min, the gelatin fibers had a higher cell activity. Moreover, the structure of nanofibers prepared by electrospinning could enhance cell adhesion and proliferation. Electrospining is an efficient and cost-effective method for preparing gelatin nanofibers as a scaffold for bone tissue engineering.

Acknowledgments

The authors would like to thank the National Science Council of the Republic of China, Taiwan (contract No. NSC98-2622-E-039-002-CC3) and the China Medical University (contract No. CMU98-S-48) for financially supporting this research.

References

1. Puppi D, Chiellini F, Piras AM and Chiellini E. Polymeric materials for bone and cartilage repair. *Prog Polym Sci* 2010; 35: 403-440.
2. Lu H, Chen WJ, Xing Y, Ying DJ and Jiang B. Design and preparation of an electrospun biomaterial surgical patch. *J Bioact Compat Polym* 2009; 24: 158-168.
3. Engel E, Michiardi A, Navarro M, Lacroix D and Planell JA. Nanotechnology in regenerative medicine: the material side. *Trends Biotechnol* 2008; 26: 39-47.
4. Zhang L and Webster TJ. Nanotechnology and nanomaterials: promises for improved tissue regeneration. *Nano Today* 2009; 4: 66-80.
5. Smith LA and Ma PX. Nano-fibrous scaffolds for tissue engineering. *Colloids Surf B Biointerfaces* 2004; 39: 125-131.
6. Volpato FZ, Ramos SLF, Motta A and Migliaresi C. Physical and *in vitro* biological evaluation of a PA 6/MWCNT electrospun composite for biomedical applications. *J Bioact Compat Polym* 2011; 26: 35-47.
7. Puppi D, Piras AM, Detta N, Ylikauppila H, Nikkola L, Ashammakhi N, Chiellini F and Chiellini E. Poly(vinyl alcohol)-based electrospun meshes as potential candidate scaffolds in regenerative medicine. *J Bioact Compat Polym* 2011; 26: 20-34.
8. Zhang H and Chen Z. Fabrication and characterization of electrospun PLGA/MWNTs/hydroxyapatite biocomposite scaffolds for bone tissue engineering. *J Bioact Compat Polym* 2010; 25: 241-259.
9. Lannutti J, Reneker D, Ma T, Tomasko D and Farson D. Electrospinning for tissue engineering scaffolds. *Mater Sci Eng C* 2007; 27: 504-509.
10. Guarino V, Alvarez-Perez M, Cirillo V and Ambrosio L. hMSC interaction with PCL and PCL/gelatin platforms: A comparative study on films and electrospun membranes. *J Bioact Compat Polym* 2011; 26: 144-160.

11. Schofer MD, Boudriot U, Wack C, Leifeld I, Gräbedünkel C, Dersch R, Rudisile M, Wendorff JH, Greiner A, Paletta JRJ and Fuchs-Winkelmann S. Influence of nanofibers on the growth and osteogenic differentiation of stem cells: a comparison of biological collagen nanofibers and synthetic PLLA fibers. *J Mater Sci-Mater Med* 2009; 20: 767-774.

12. Woo KM, Jun JH, Chen VJ, Seo J, Baek JH, Ryoo HM, Kim GS, Somerman MJ and Ma PX. Nano-fibrous scaffolding promotes osteoblast differentiation and biomineralization. *Biomaterials* 2007; 28: 335-343.

13. Shih YRV, Chen CN, Tsai SW, Wang YJ and Lee OK. Growth of mesenchymal stem cells on electrospun type I collagen nanofibers. *Stem Cells* 2006; 24: 2391-2397.

14. Fujihara K, Kotaki M and Ramakrishna S. Guided bone regeneration membrane made of polycaprolactone calcium carbonate composite nano-fiber. *Biomaterials* 2005; 26: 4139-4147.

15. Cancedda R, Dozin B, Giannoni P and Quarto R. Tissue engineering and cell therapy of cartilage and bone. *Matrix Biol* 2003; 22: 81-91

16. Godbey WT and Atala A. *In vitro* systems for tissue engineering. *Ann NY Acad Sci* 2002; 961: 10-26.

17. Rungsiyanont S, Dhanesuan N, Swasdison S and Kasugai S. Evaluation of biomimetic scaffold of gelatin-hydroxyapatite crosslink as a novel scaffold for tissue engineering: biocompatibility evaluation with human pdl fibroblasts, human mesenchymal stromal cells, and primary bone cells. *J Biomater Appl* 2011; 22: 1-15.

18. Hu X, Ma L, Wang C and Gao C. Gelatin hydrogel prepared by photo-initiated polymerization and loaded with TGF-beta 1 for cartilage tissue engineering. *Macro Biosci* 2009; 9: 1194-1201.

19. Rosellini E, Cristallini C, Barbani N, Vozzi G and Giusti P. Preparation and

characterization of alginate gelatin blend films for cardiac tissue engineering. *J Biomed Mater Res A* 2008; 91: 447-453.

20. Michon C, Cuvelier G, Relkin P and Launay B. Influence of thermal history on the stability of gelatin gels. *Int J Biol Macromol* 1997; 20: 259-264.
21. Ratanavaraporn J, Rangkupan R, Jeeratawatchai H, Kanokpanont S, Damrongsakkul S. Influences of physical and chemical crosslinking techniques on electrospun type A and B gelatin fiber mats. *Int J Biol Macromol* 2010; 47: 431-438.
22. Bhat R and Karim AA. Ultraviolet irradiation improves gel strength of fish gelatin. *Food Chem* 2009; 113: 1160-1164.
23. Cataldo F, Ursini O, Lilla E and Angelini G. Radiation-induced crosslinking of collagen gelatin into a stable hydrogel. *J Radioanal Nucl Chem* 2008; 275: 125-131.
24. Zhang X, Do MD, Casey P, Sulistio A, Qiao GG, Lundin L, Lillford P and Kosaraju S. Chemical cross-linking gelatin with natural phenolic compounds as studied by high-resolution NMR spectroscopy. *Biomacromolecules* 2010; 11: 1125-1132.
25. Solorio L, Zwolinski C, Lund AW, Farrell MJ and Stegemann JP Gelatin microspheres crosslinked with genipin for local delivery of growth factors. *J Tissue Eng Regen Med* 2010; 4: 514-523.
26. Zhang S, Huang Y, Yang X, Mei F, Ma Q, Chen G, Ryu S and Deng X. Gelatin nanofibrous membrane fabricated by electrospinning of aqueous gelatin solution for guided tissue regeneration. *J Biomed Mater Res A* 2009; 90: 671-679.
27. Zhao J, Zhao Y, Guan Q, Tang G, Zhao Y, Yuan X and Yao K. Crosslinking of electrospun fibrous gelatin scaffolds for apatite mineralization. *J Appl Polym Sci* 2011; 119: 786-793.
28. Sisson K, Zhang C, Farach-Carson MC, Chase DB, Rabolt JF. Fiber diameters control osteoblastic cell migration and differentiation in electrospun gelatin. *J Biomed Mater*

Res A 2010; 94: 1312-1320.

29. Zhang YZ, Venugopal J, Huang ZM, Lim CT and Ramakrishna S. Crosslinking of the electrospun gelatin nanofibers. *Polymer* 2006; 47: 2911-2917.
30. Yang D, Li Y and Nie J. Preparation of gelatin PVA nanofibers and their potential application in controlled release of drugs. *Carbohydrate Polymers* 2007; 69: 538-543.
31. Chen YS, Chang JY, Cheng CY, Tsai FJ, Yao CH and Liu BS. An *in vivo* evaluation of a biodegradable genipin-cross-linked gelatin peripheral nerve guide conduit material. *Biomaterials* 2005; 26: 3911-3918.
32. Olde Damink LHH, Dijkstra PJ, Van Luyn MJA, Van Wachem PB, Nieuwenhuis P and Feijen J. Glutaraldehyde as a cross-linking agent for collagen-based biomaterials. *J Mater Sci Mater Med* 1995; 6: 460-472.
33. Chu XH, Shi XL, Feng ZQ, Gu ZZ and Ding YT. Chitosan nanofiber scaffold enhances hepatocyte adhesion and function. *Biotechnol Lett* 2009; 31: 347-352.
34. Maurin B, Cañadas P, Baudriller H, Montcourrier P and Bettache N. Mechanical model of cytoskeleton structuration during cell adhesion and spreading. *J Biomech* 2008; 41: 2036-2041.
35. Pugacheva EN and Golemisl EA. The focal adhesion scaffolding protein HEF1 regulates activation of the Aurora-A and Nek2 kinases at the centrosome. *Nat Cell Biol* 2005; 7: 937-946.

Figure captions

Figure 1. Morphologies of the electrospun gelatin nanofibers (a) before and (b) after cross-linking treatment were observed by SEM

Figure 2. The SEM morphology results of the electrospun gelatin nanofibers after a 7-day soaking test are displayed above at (a) 0; (b) 15; (c) 45; (d) 90; and (e) 360 min cross-linking times

Figure 3. Diameter variation of the electrospun gelatin nanofibers immersed in 37°C de-ionized water for 7 days

Figure 4. Optical images showing the contact angles of water droplet in contact with (a) gelatin film and (b) electrospun gelatin nanofibers

Figure 5. Results from the MTT assay, display cell culture with gelatin film and electrospun gelatin nanofibers

Figure 6. SEM photographs of MG-63 cells attached and adhered to the surface of (a)-(c) gelatin film and (d)-(f) electrospun gelatin nanofibers cross-linked with glutaraldehyde vapor for 45 min after culturing for (a), (d) 2; (b), (e) 4; and (c), (f) 8 h.

Figure 7. The morphologies of MG63 cells were observed by confocal microscope. The cells were cultured with (a) gelatin film and (b) electrospun gelatin nanofibers for 2 h. Color: gelatin nanofiber (green), β-actin (red), and DAPI nuclear (blue)

Figure 8. Western blot analysis results of gelatin film (GF) and electrospun gelatin nanofibers (GN)

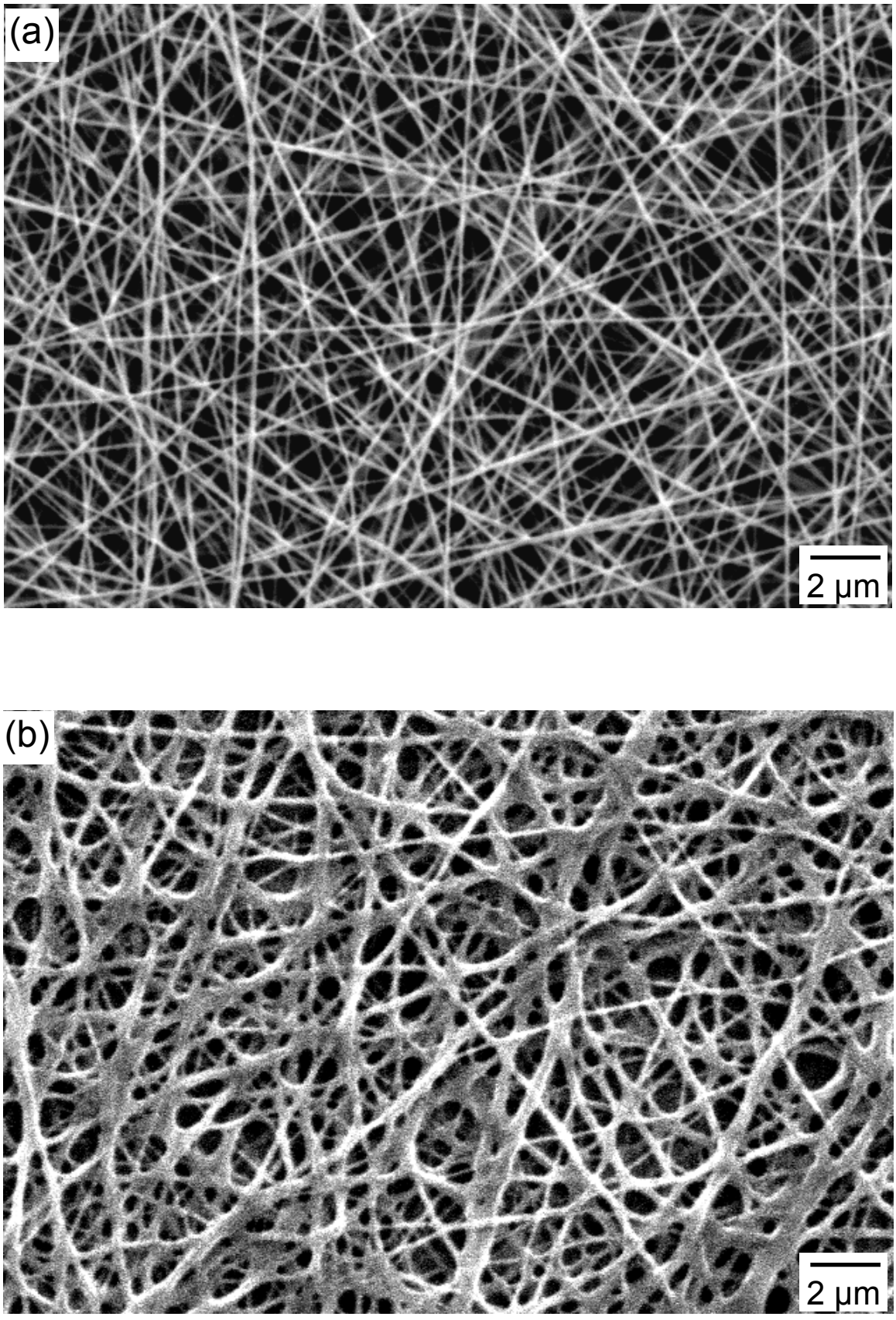
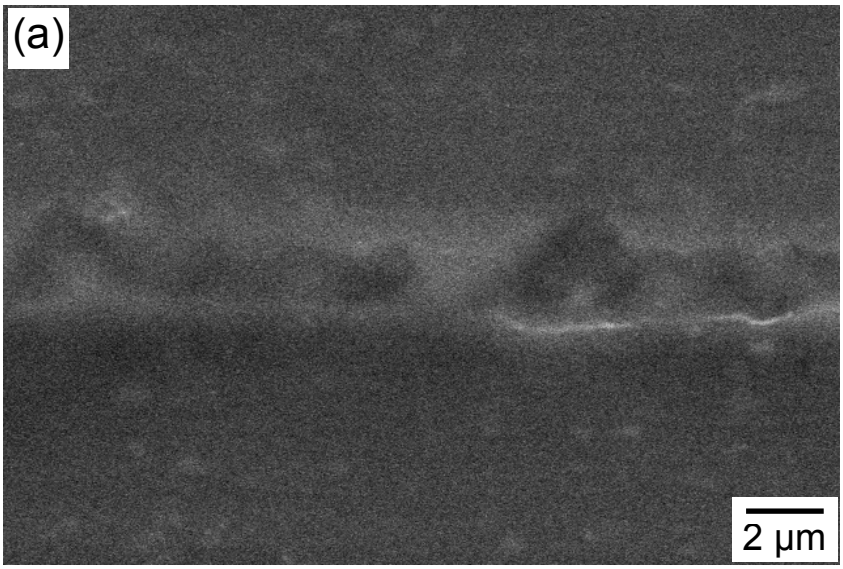
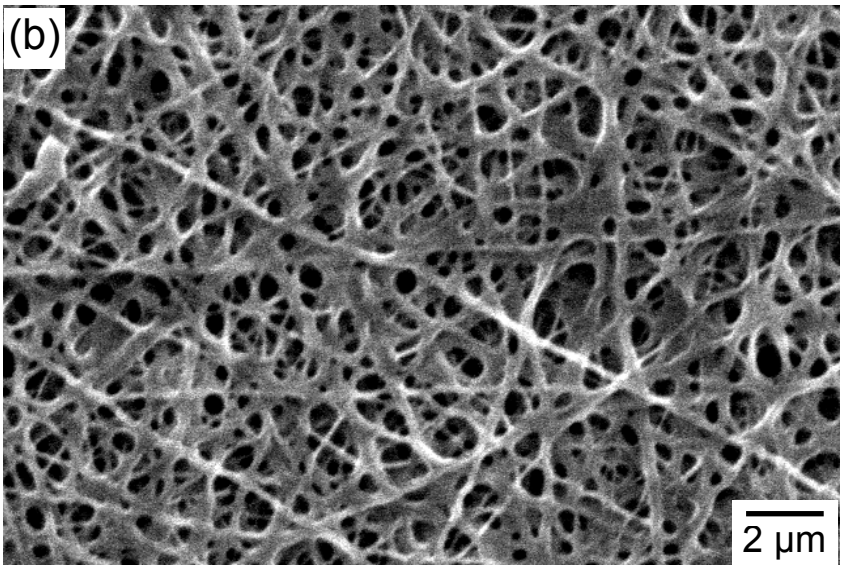
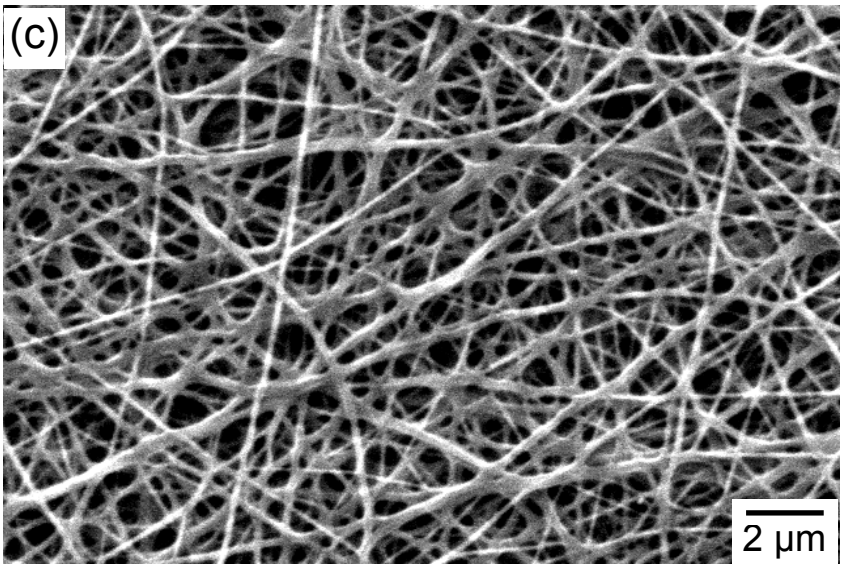


Figure 1.


(a)
2 μm


(b)
2 μm


(c)
2 μm

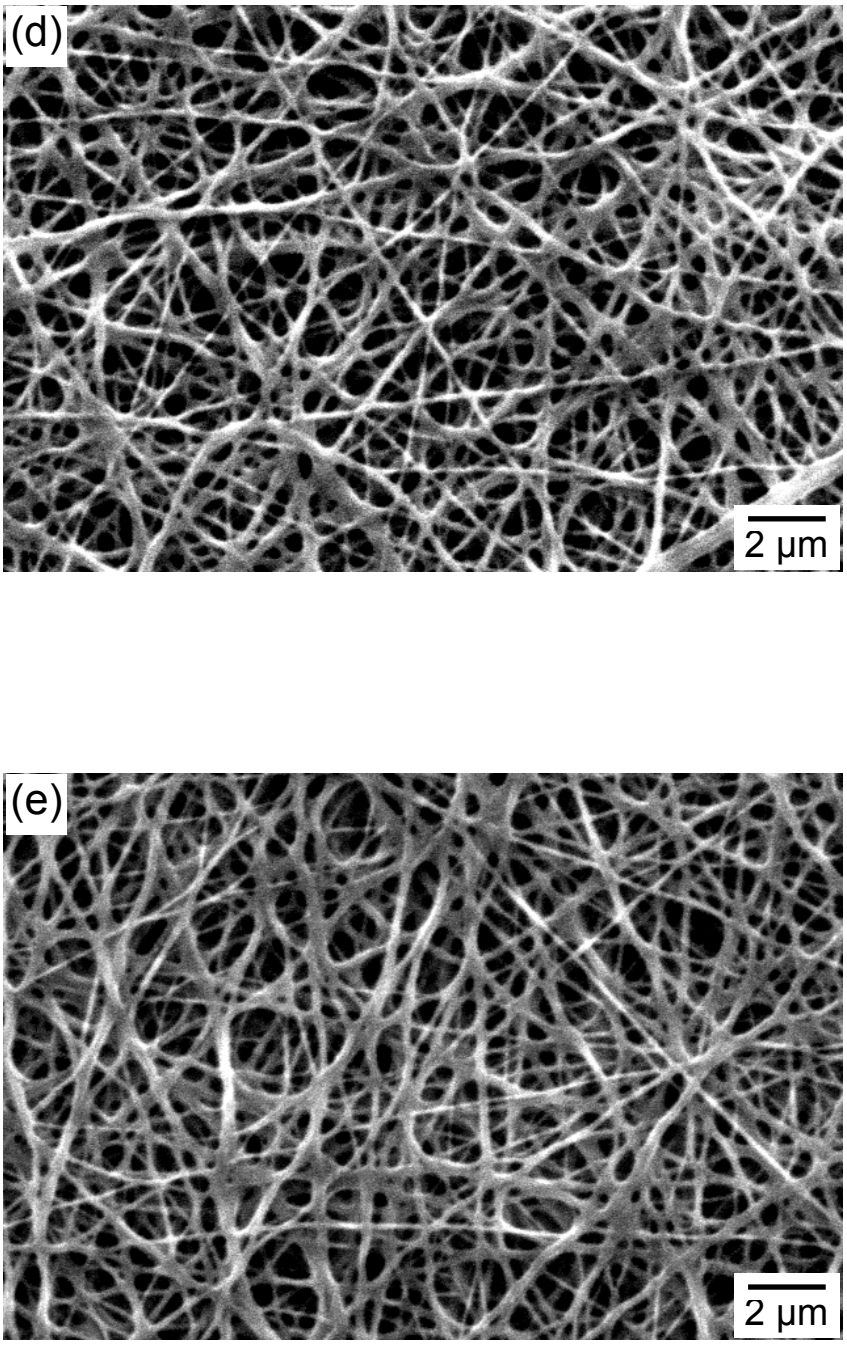


Figure 2.

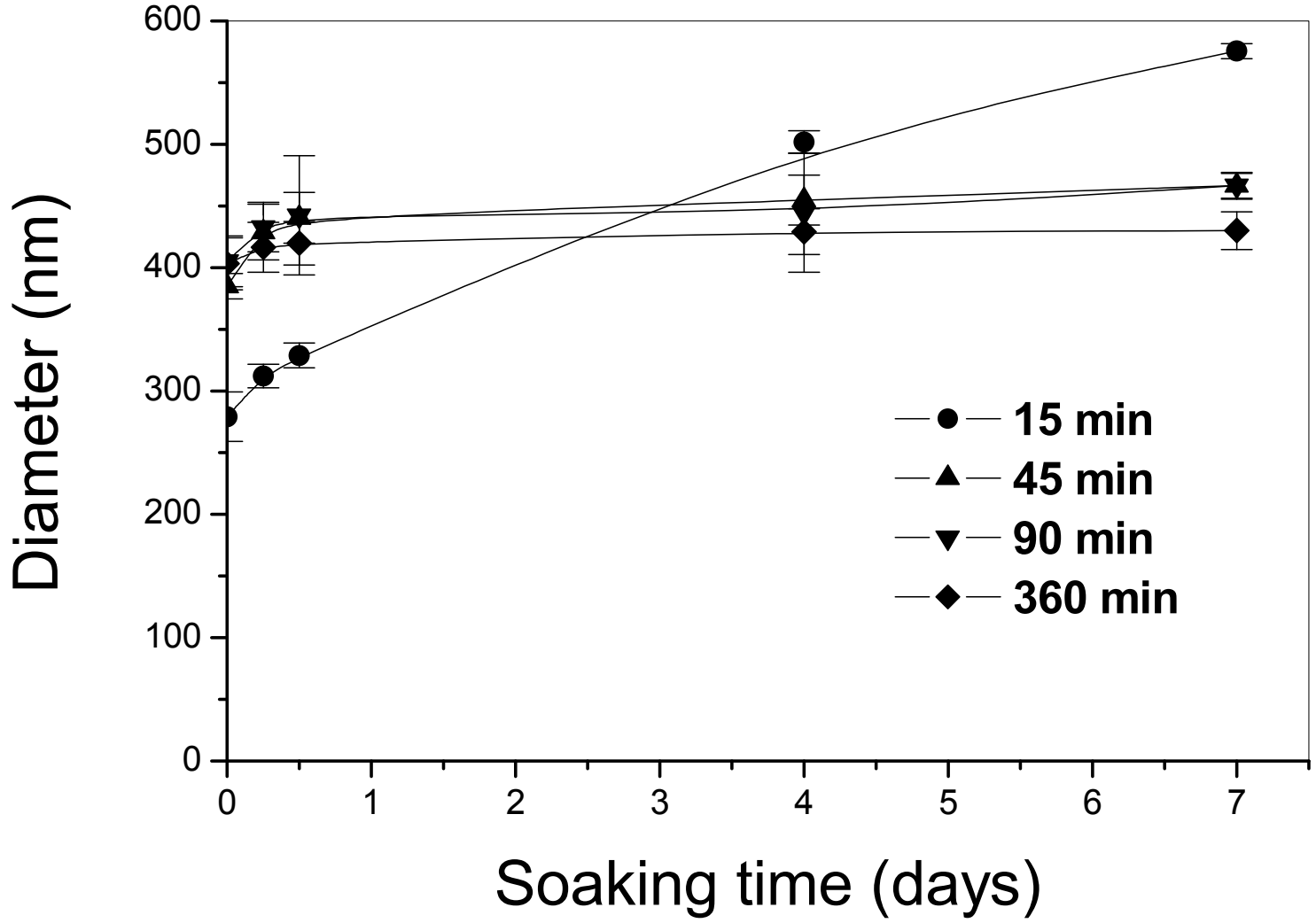
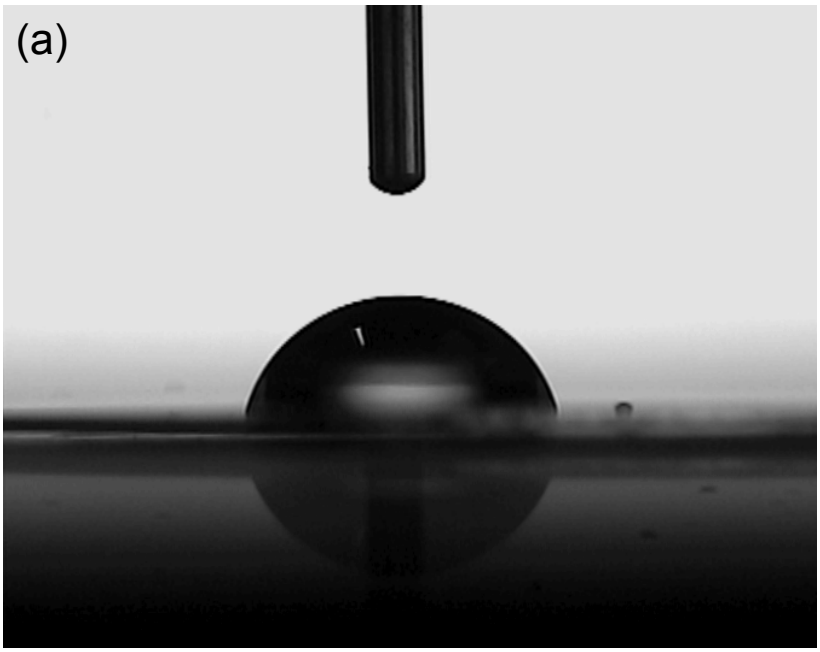

Diameter (nm)
600
500
400
300
200
100
0
0
1
2
3
4
5
6
7
Soaking time (days)
15 min
45 min
90 min
360 min

Figure 3.



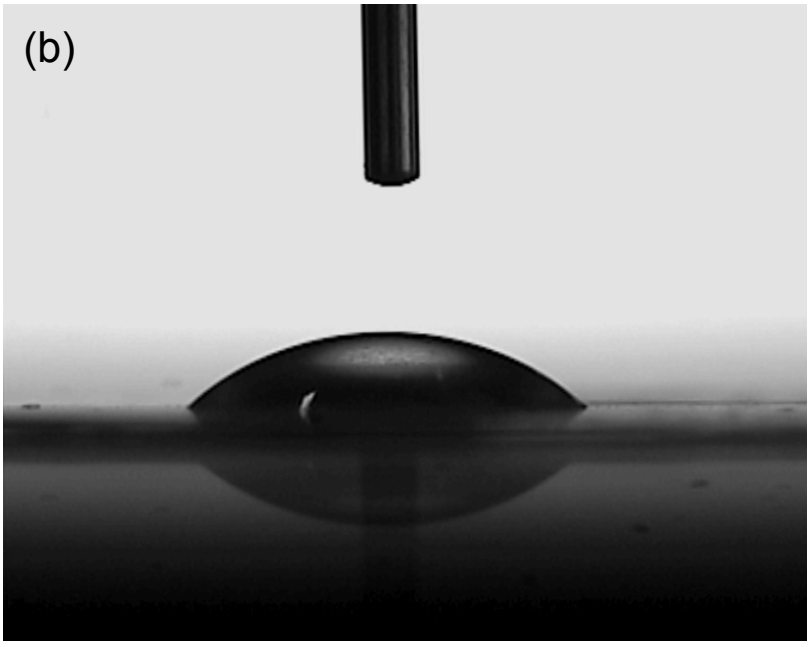


Figure 4.

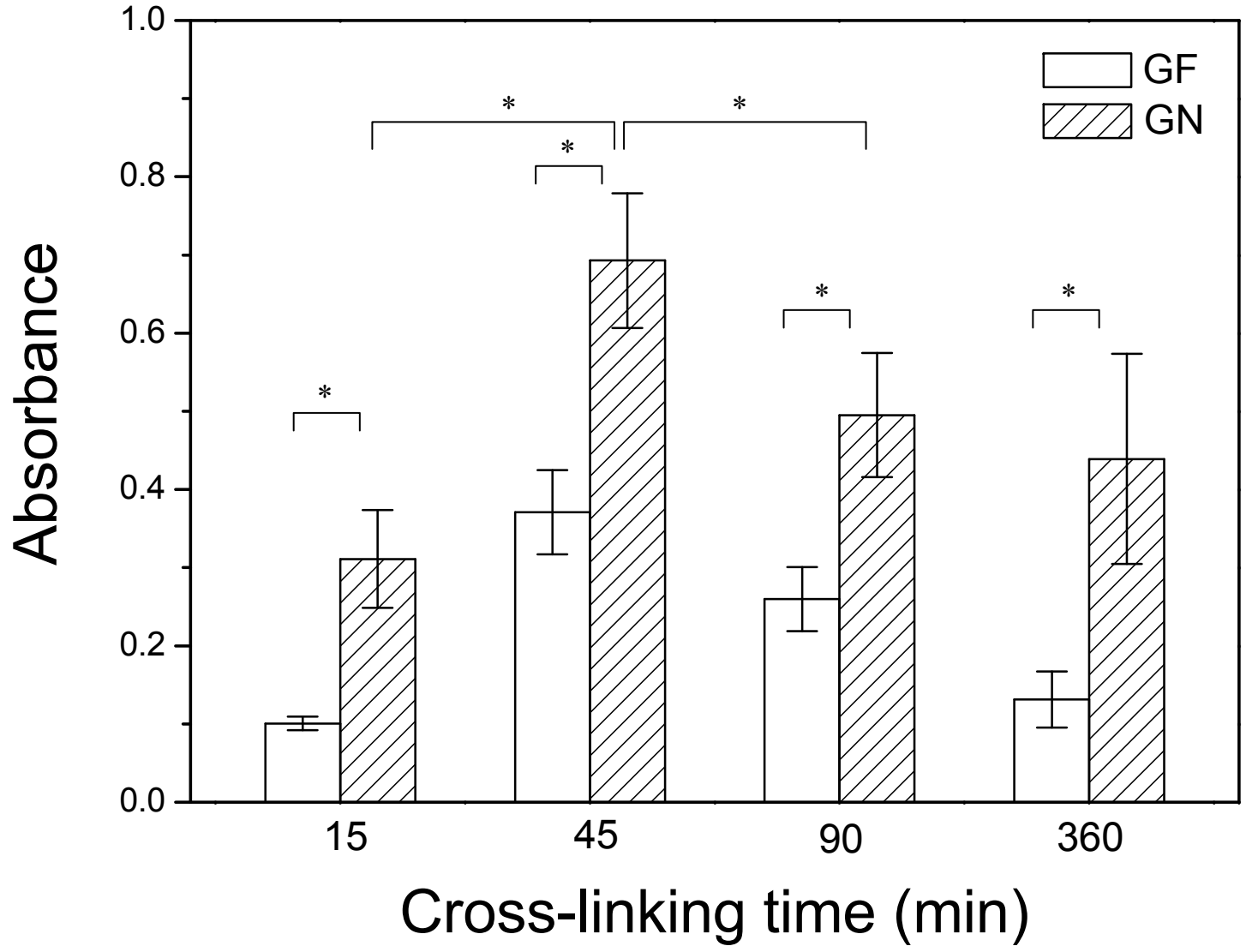

1.0
0.8
0.6
0.4
0.2
0.0
Absorbance
GF
GN
*
*
*
*
*
*
15
45
90
360
Cross-linking time (min)

Figure 5.

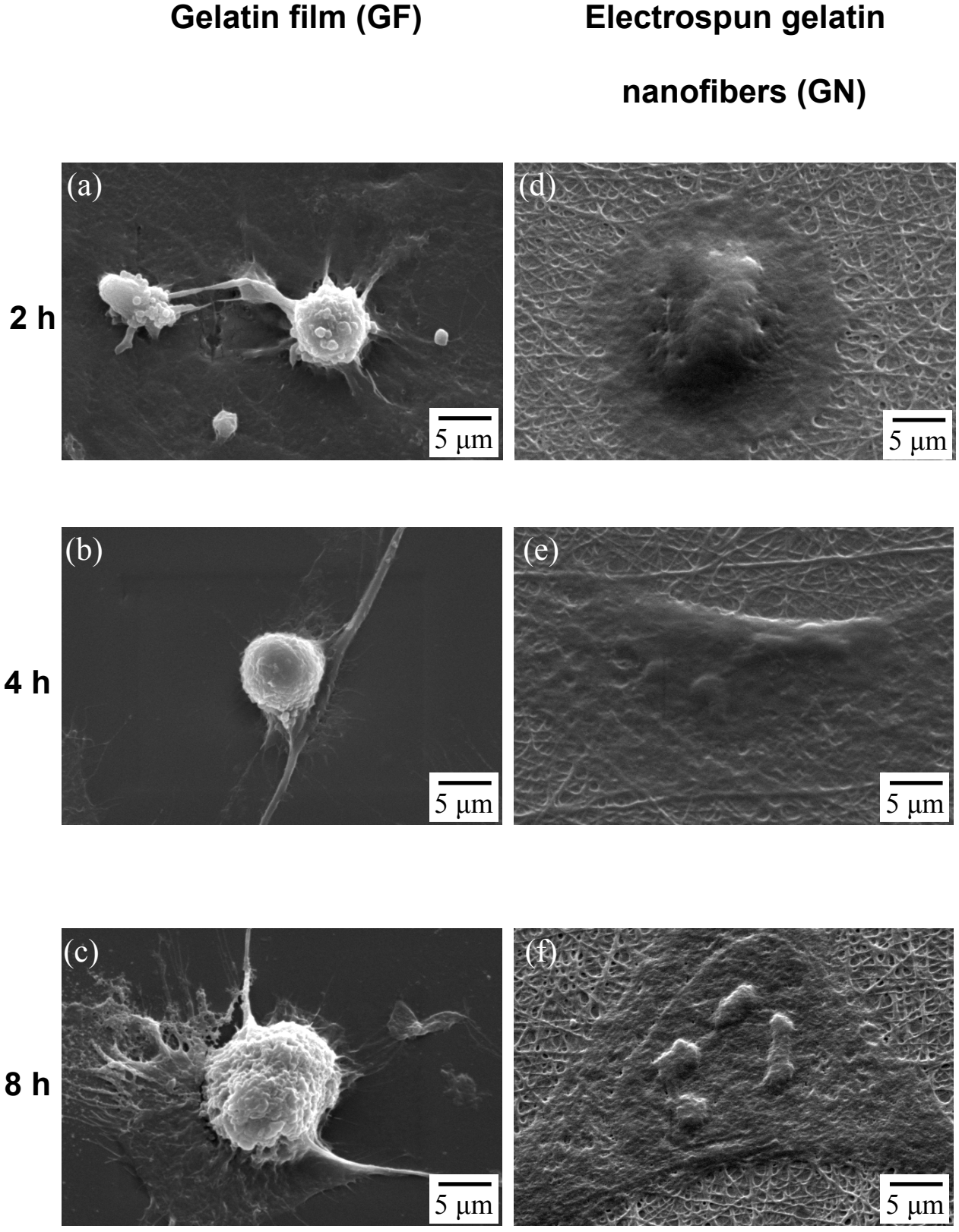
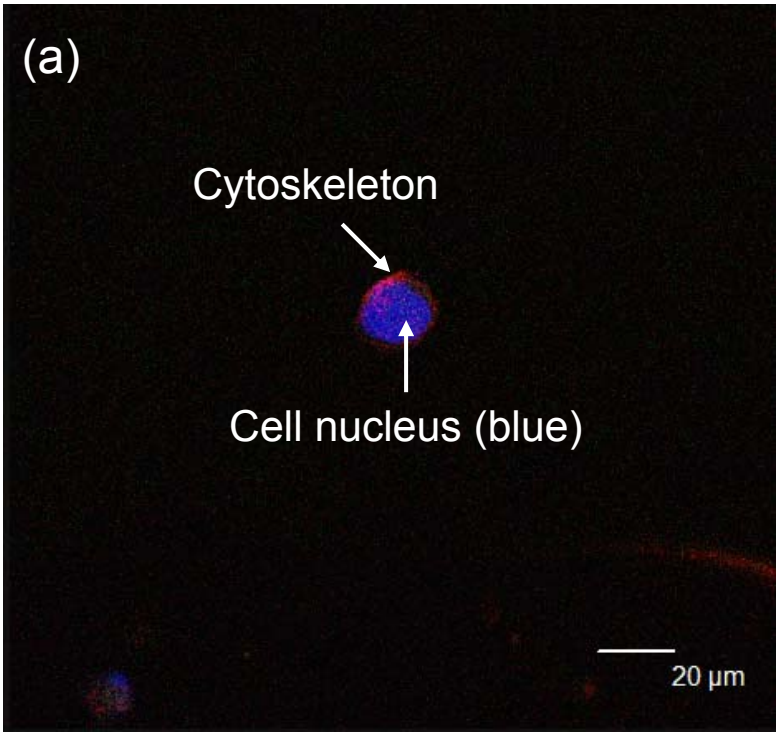

Gelatin film (GF)
Electrospun gelatin nanofibers (GN)
2 h
4 h
8 h
(a)
(b)
(c)
(d)
(e)
(f)
5 μm

Figure 6.


(a)
Cytoskeleton
Cell nucleus (blue)
20 μm

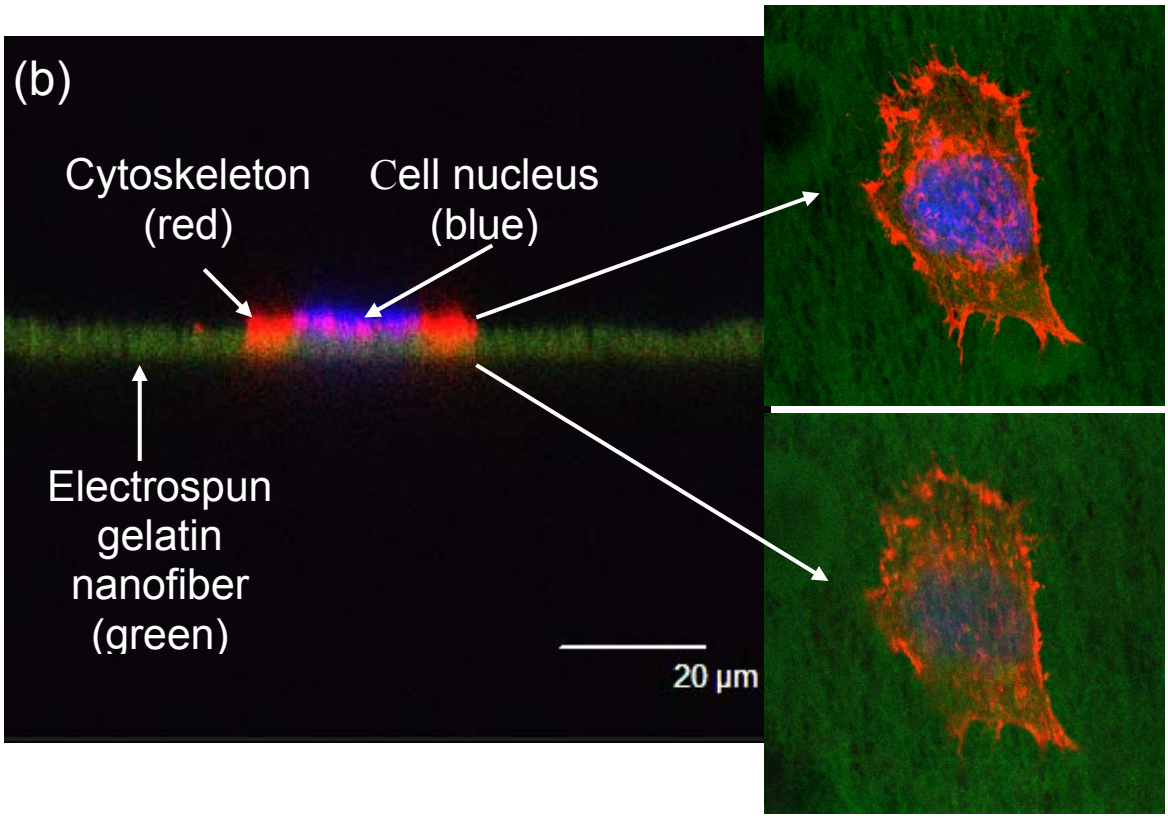

(b)
Cytoskeleton (red)
Cell nucleus (blue)
Electrospun gelatin nanofiber (green)
20 μm

Figure 7.

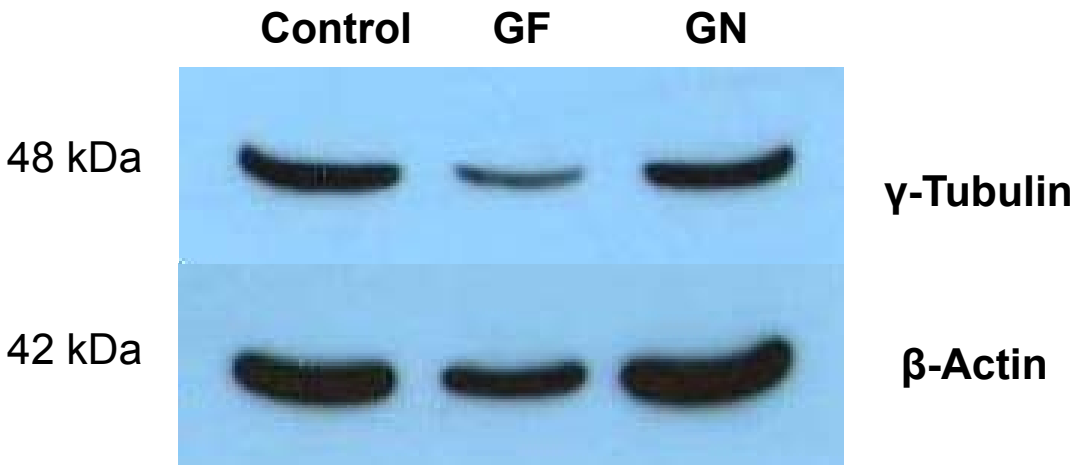


Figure 8.