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Electrospun nanofibers immobilized with collagen for neural stem cells culture

Wensheng Li · Ying Guo · Hui Wang · Dejin Shi · Chaofeng Liang · Zhuopeng Ye · Feng Qing · Jin Gong

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Abstract Fibrous mats via electrospinning have been widely applied in tissue engineering. In this work, nanofibers were prepared via electrospinning from polymer with different content of carboxyl groups. A natural material, collagen, was then immobilized onto the nanofiber surface by N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC)/N-Hydroxysuccinimide (NHS) activation process. It was found that the immobilization degree of collagen could be facilely modulated. The obtained collagen-modified nanofibers were used for neural stem cells culture, and unmodified nanofibers were used as a control. Results indicated that the modification of collagen could enhance the attachment and viability of the cultured neural stem cells.

Introduction

Neural stem cells have the ability for extensive self-renewal and multilineage differentiation into neurons, astrocytes, and oligodendrocytes. They play an important role in nerve disease treatment and nerve injury repair. Furthermore, the neural stem cells can form nerve tissue [1–14]. However, the shortage of the source hindered their clinic application. Therefore, growth of implanted cells must be controlled in order to guide differentiation and neurite outgrowth. Development of suitable scaffolding materials to support cells on implantation is required [15–17].

It has been generally accepted that extracellular matrix mimics may improve the attachment, proliferation, and viability of the cultured cells [18]. Hydrogels are attractive polymer scaffolds because of their highly porous and hydrated structure that allows cells to assemble spontaneously and become organized into a recognized tissue, and permit the infusion of nutrients and oxygen and transfer of waste products and CO_2 out of the cells [19]. However, not every polymer is suitable to be cross linked. Electrospinning has been rapidly developed into a technique to prepare nanofibers with the diameter ranging from tens of nanometers to several microns [20–22]. The electrospun fibrous mats also show extremely high surface area and large porosity. Besides, the fibrous structure of the electrospun mats may mimic the extracellular matrix. It is well known that collagen is a natural extracellular matrix component of nearly every tissue such as bone, skin, tendon, ligament, and so forth. Fiber diameters of the electrospun nanofibrous mats even approach that of collagen fiber bundles, between 50 and 500 nm [22]. Therefore, during the past several years, many works considering the tissue engineering of electrospun nanofibers have been reported. Most recently, electrospun nanofibers were prepared by Yang et al. and applied in neural tissue engineering [23].

Although the presented nanofibers may mimic the morphologies of extracellular matrix to some extent, some modifications are still required to create a friendly environment for the cells' attachment, proliferation, and functions such as communications. Some natural materials such as collagen, fibronectin and some peptides have been reported as scaffold modifiers [24, 25]. In this work, nanofibers with excellent mechanical strength were

W. Li · Y. Guo (✉) · H. Wang · D. Shi · C. Liang · Z. Ye · F. Qing · J. Gong
Department of Neurosurgery, 3rd Affiliated Hospital of SUN YAT-SEN University, Guangzhou 510630, P.R. China
e-mail: sumsgy@21cn.com

prepared using electrospinning technique and then collagen was immobilized onto the nanofiber surfaces to create a biocompatible environment for neural stem cells culture.

Experimental section

Electrospinning

In order to produce nanofibers via electrospinning technique, the copolymer of methyl methacrylate (MMA) and acrylic acid (AA) (PMMAAA) with different contents of carbonyl group were dissolved in acetone at room temperature with gentle stirring for 6 h to prepare a polymer solution with a concentration of 8 wt.%. The copolymers used were synthesized by common solution polymerization method with changing the molar ratios of MMA and AA in feed. Electrospinning was carried out under ambient temperature in a vertical setup using a 1.2 mm inner diameter blunt needle according to a common procedure [26–28]. The applied voltages were about 16 kV, driven by a high voltage power supply (MGD-1A, Tianjin University, China). The solution flow rate was controlled at 2 mL/h via a syringe pump (WZ-500C2, Zhejiang, China). Fibers were collected on an electrically grounded aluminum foil placed at 15 cm below the needle tip. All collected fibrous mats were dried under vacuum oven at room temperature to remove the residual solvents.

Determination of carboxyl contents

The nanofibers were rinsed with 1 M HCl followed by ultrapure water and then thoroughly dried before determination. Carboxyl contents of the nanofibers were determined according to the method reported by Gupta et al. [29] The nanofibers were placed in 0.5 M KCl solution for 18 h at ambient temperature. The solution was then titrated against 0.01 M NaOH solution using phenolphthalein indicator. The carboxyl content was represented as the mmol/g of the dry nanofibers.

Collagen immobilization

Collagen (type I, from bovine achilles tendon, purchased from Sigma) was immobilized onto the nanofiber surface according to the following protocol. A weighted amount of the unmodified nanofibrous mat was rinsed with acetic acid buffer solution (50 mM, pH 5.0). After this the mat was submerged into the collagen solution (15 mg/mL in acetic acid buffer solution, 50 mM, pH 5.0) in the presence of EDC/NHS (10 mg/mL, the molar ratio of EDC to NHS is 1:1) and shook gently for 24 h at room temperature. Finally, the modified mat was taken out, washed several times with acetic acid buffer solution to remove the physically adhered collagen and then dried. Following this process, the collagen-immobilized nanofibrous mat was obtained. The immobilization degree of collagen were determined by gravimeter according to the following equation:

$$\mathrm{IDC} = (w - w_0)/w_0 \times 100\%$$

where w_0 is the mass of nascent nanofibrous mat and w is the mass of collagen-immobilized mat.

Cell culture

Cortical neural stem cells were prepared from pregnant rat embryos on days 14–16 according to a reported protocol [30]. Briefly, rat embryonic cerebral cortices were dissected, cut into small pieces and mechanically triturated in cold Hank's balanced salt solution (5.4 mM KCl, 0.3 mM $Na_2HPO_4{\cdot}7H_2O$, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 0.5 mM $MgCl_2{\cdot}6H_2O$, 0.6 mM $MgSO_4{\cdot}7H_2O$, 137 mM NaCl, and 5.6 mM D-glucose). The dissociated cells were collected by centrifugation and were resuspended in a serum-free medium containing DMEM-F12, 8 mM glucose, glutamine, 20 mM sodium bicarbonate, 15 mM HEPES and N_2 supplement (25 mg/mL insulin, 100 mg/mL human apotransferrin, 20 nM progesterone, 30 nM sodium selenite, pH 7.2). The number of live cells was counted by trypan blue exclusion assay in a hemocytometer.

Cerebral cortical neural stem cells were purified and cultured in culture flasks at a density of 50,000 cells/cm^2 in the above culture medium in the presence of bFGF at a concentration of 20 ng/mL. The incubator were maintained at 37 °C in 5% CO_2. After 1–3 days in vitro, cells were undergoing cell division and the proliferating cells formed the neurospheres, which were suspended in the medium. Subsequently, the adherent cells were discarded and the suspended neurospheres were collected by centrifugation, mechanically dissociated and subcultured as single cells in a new culture flask at a density of 50,000 cells/cm^2 in the fresh culture medium containing the same concentration of bFGF. These cells grew into new spheres in the subsequent 2–3 days, that is, single cells proliferated and formed new neurospheres. The procedure of subculture was repeated again to achieve the purified cortical neural stem cells and proliferating neurospheres.

Nascent nanofibrous mat and collagen-immobilized mats were affixed to the bottom of wells in 24 well polystyrene tissue culture plates. Following placement of the samples in the tissue culture plates, the entire plate was exposed to UV light under a laminar flow hood for 30 min to sterilize the samples. The culture wells containing the samples were washed 3 times with sterile PBS before cell

cultures. Subsequently, neurospheres were seeded on the sample-affixed wells at 400 neurospheres/cm^2 in the serum-free medium after bFGF withdrawal for investigating the effects of substrates on neural stem cells. The wells with unmodified-mat were used as controls. At indicated time points, morphologies of cultured neurospheres were observed.

MTT assay

The cell viability was measured by the methylthiazoletetrazolium (MTT) assay. After 3 days of incubation, 20 μL of MTT (5 mg/mL in PBS) was added into each well, followed by another 4 h incubation at 37 °C. Then, the supernatants were removed and 100 μL of DMSO added to dissolve the tiny crystals. The absorbance at 490 nm was recorded and four parallel measurements were averaged for each sample. MTT conversion data were expressed as fold growth relative to seeding neurospheres.

Characterization

The morphology of nanofibrous mats and cells were observed using field emission scanning electron microscopy (JSM-6330F) after gold sputtering. Digital photomicrographs were taken of random fields of neurospheres at indicated experimental points. Quantification of process growth was evaluated by the end-to-end distance of extensive processes. The lengths of 10–15 longest processes (neurites from neurons or processes from glial cells) per neurosphere were estimated from the edge of the neurospheres to the tip of the processes in linear form. Process lengths were measured by tracing the processes using ImageTool software (version 2.0). The length of processes of 20 independent neurospheres was calculated at each experiment, and the means and standard error of mean (SEM) were also calculated.

Results and discussion

Preparation of nanofibrous mats via electrospinning

To study the effects of immobilization amount of collagen on the cells culture and their behaviors, in this work, three kinds of polymers were used, which contain different contents of carboxyl groups, i.e. 5, 10, and 15 wt.%. Therefore, these polymers were denoted as PMMAAA05, PMMAAA10, and PMMAAA15, respectively.

Electrospinning has been widely used as a method to prepare nanofibers. Many kinds of materials including biomacromolecules, organic polymers, and even inorganic particles can be electrospun into nanofibers. It has been reported that many factors may influence the diameters and morphologies of the resultant nanofibers, which include the concentration, the molecular weight of the polymer, the applied voltage, the flow rate, etc. We explored the effects of such parameters and then optimized for nanofibrous mats preparation for cell cultures. PMMAAA10 solutions with different concentration (4, 6, 8, and 10 wt.%) were prepared for electrospinning by fixing other parameters such as applied voltage (16 kV), collection distance (15 cm), and flow rate (2 mL/h). It was found that at extremely low concentration (4 wt.%) only beads were formed and some very thin fibers were connected between the beads. With the increase of concentration, uniform nanofibers were formed and the diameters of the obtained nanofibers increased, which varied from 200 nm to around 900 nm (Fig. 1). The effects of applied voltage and flow rate on the fiber diameters are shown in Figs. 2 and 3, respectively. Results showed that the diameters slightly decreased with the applied voltage while increased with the flow rate. However, it should be noted that the changes induced by the applied voltage and flow rate were far smaller that those caused by the polymer solution concentration.

Consequently, polymers with different contents of carboxyl group were electrospun into nanofibrous mats with the optimized parameters: the concentration is 8 wt.%, the applied voltage is 16 kV, the collection distance is 15 cm, and the flow rate is 2 mL/h. As shown in Fig. 4, uniform nanofibers with diameters around 450 ± 88 nm were prepared for all the three samples. The diameters of the nanofibers were shown in Fig. 5. Because the molecular weight was almost the same (about 1,80,000 g/mol) for each polymer, the similar diameters indicated that the introduction of carboxyl groups into the polymers has only slight effect on the electrospinning. It can also be observed that the diameters decreased with the increase of carboxyl groups, which may be attributed to the increase of electrical conductivity induced by the carboxyl groups. Therefore, the as-spun mats were dried under vacuum oven to remove residual solvent and then used for further studies.

Immobilization of collagen on the nanofibrous mats

Collagen is a naturally derived polymer that is most commonly used in tissue engineering [31–33]. It has been well known for its application in neural tissue engineering. Most recently, collagen has been electrospun into nanofibers and used in tissue engineering. For example, Matthews et al. [34] carefully studied the effects of parameters on the electrospinning of collagen. Cell culture on collagen-containing nanofibers were reported and proven to be a promising scaffold material [34–40]. However, it was also

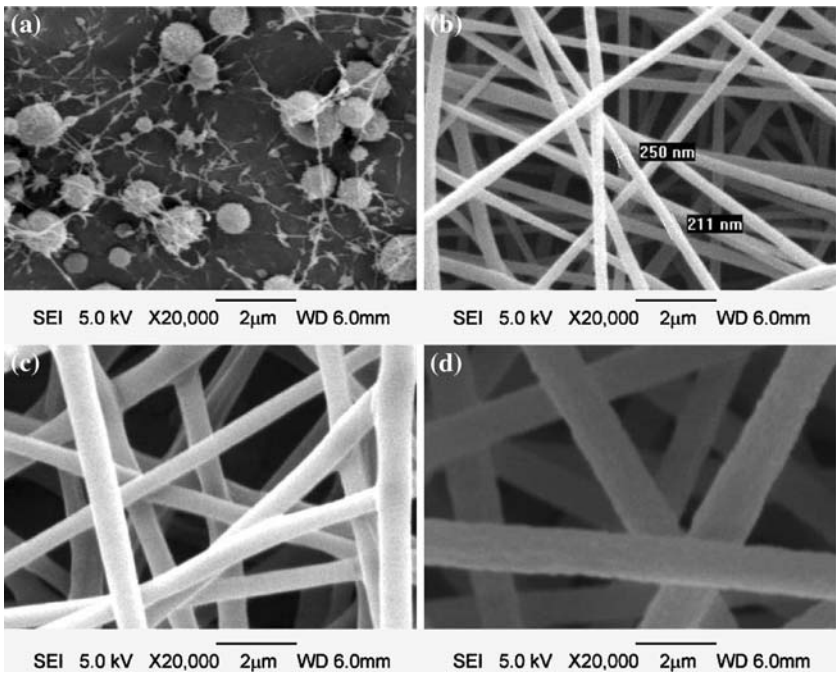

Fig. 1 SEM images of nanofibers electrospun from solutions with different concentration: (**a**) 4 wt.%, (**b**) 6 wt.%, (**c**) 8 wt.%, (**d**) 10 wt.%

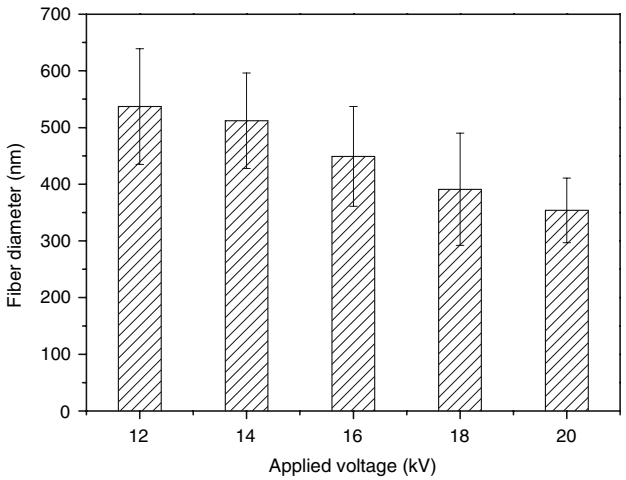

Fig. 2 Effects of electrospinning voltage on the diameters of nanofibers. The bars represent standard deviations

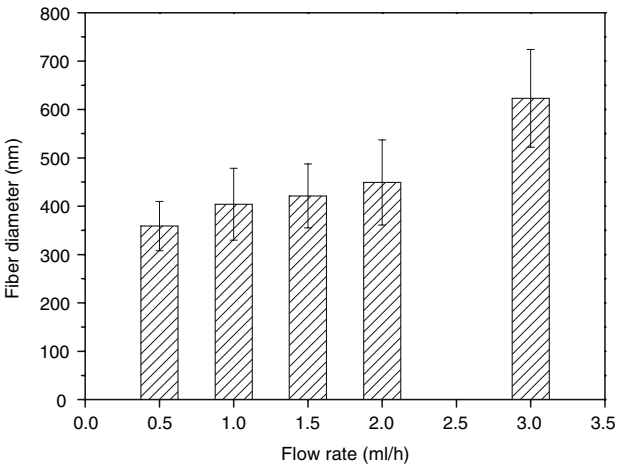

Fig. 3 Effects of electrospinning flow rate on the diameters of nanofibers. The bars represent standard deviations

reported and generally accepted that collagen fibers is of low mechanical strength [37]. Considering its poor mechanical strength, in this study, collagen was immobilized onto the electrospun PMMAAA nanofiber surface.

It has been reported that collagen could be facilely grafted to polymer surface because it can afford many amino groups. In this study, collagen was grafted onto the PMMAAA nanofiber surface via EDC/NHS activation. Three kinds of polymer matrix with different carboxyl groups were used for collagen immobilization according to the same protocol. The contents of carboxyl groups were measured. The corresponding immobilization degree of collagen is showed in Fig. 6. It was found that, with the increase of contents of carboxyl group in the nanofibers, the immobilization degree of collagen increased in our cases from 2.4% to 5.7%. In this condition, all the three samples remained their morphologies and therefore are suitable for the further use.

Morphologies and viability of the cultured neural stem cells

During the culture process, the cell morphology observed via FESEM was shown in Fig. 7. It was clear that the cells on collagen-modified nanofiber surfaces could proliferate and grow better than those on unmodified surface.

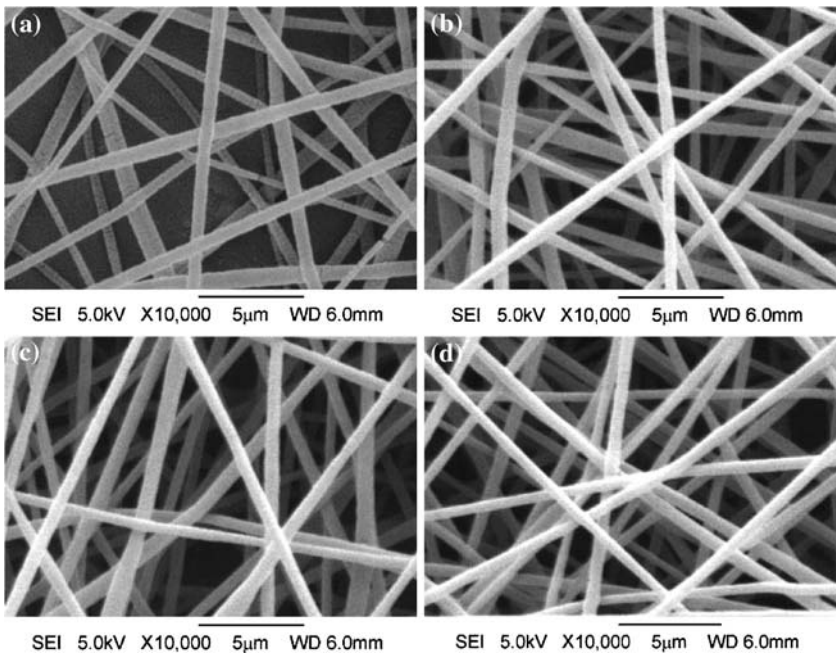

Fig. 4 SEM images of nanofibers electrospun from polymers with different contents of carboxyl groups: (**a**) 0, (**b**) 5%, (**c**) 10%, (**d**) 15%

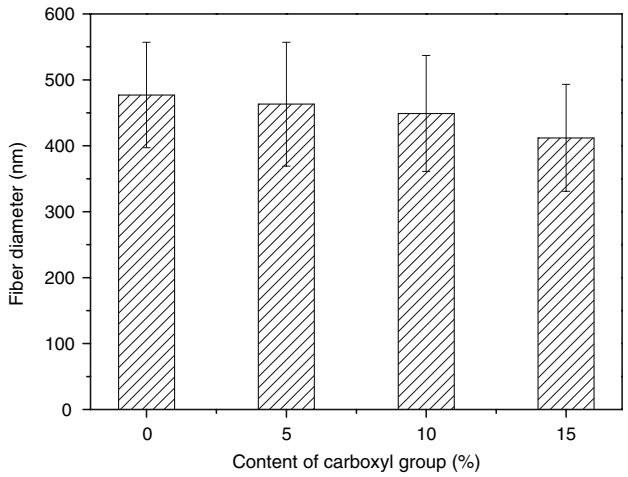

Fig. 5 Diameters of nanofibers electrospun from polymers with different contents of carboxyl groups. The bars represent standard deviations

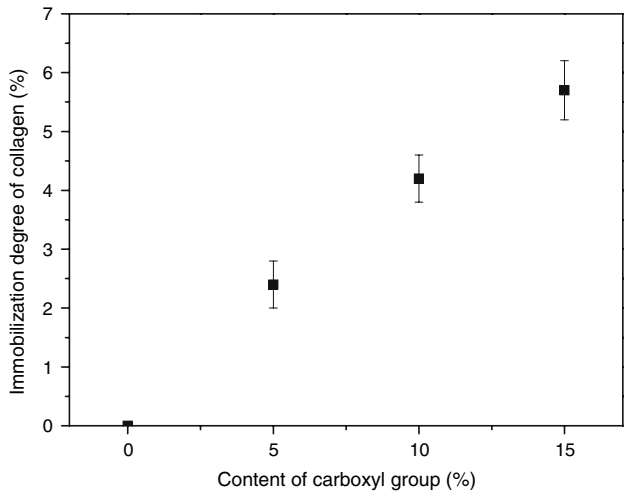

Fig. 6 Immobilization degree of collagen on nanofibers with different content of carboxyl groups. The bars represent standard deviations

Moreover, an interesting phenomenon was observed in our cases. It is well known that neural stem cells usually grow in neurospheres in vitro. During the culture on nanofiber surface in our study, however, lots of "neuroparticles" instead of neurospheres existed on the nanofiber surface. Besides, the "neuroparticles" connected with each other, as shown in Fig. 7. This phenomenon can be explained by the fact that, when the neural stem cells proliferate, they need to grow together and the shapes of the underlying nanofibers may guide or limit them to expand only along the fibers. This observation may be useful in the design of scaffolds for neural tissue engineering. In fact, Yang et al. [23] prepared aligned poly(L-lactic acid) (PLLA) fibers and neural stem cells were cultured. They reported that the direction of neural stem cells elongation and its neurite outgrowth is parallel to the direction of PLLA fibers for aligned scaffolds. Kwon et al. [37] also reported that the human umbilical vein endothelial cells cultured on nanofibers were highly elongated and well spread on the fibrous surfaces. In other words, the nanofibers is capable to guide or limit the growth of neural stem cells.

Figure 8 shows quantitative analysis of process growth of the differentiated cells on nanofiber surfaces with or

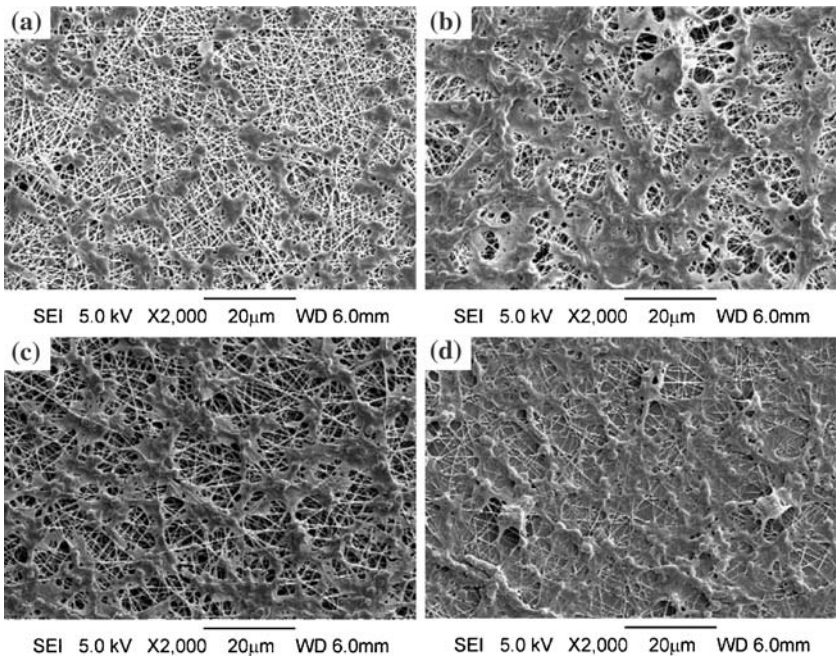

Fig. 7 Morphologies of neural stem cells on nanofiber surface. (**a**) unmodified PMMAAA10, and collagen-modified (**b**) PMMAAA5, (**c**) PMMAAA10, (**d**) PMMAAA15

without modification by collagen after 2 and 4 days of culture under serum-free conditions. The lengths of 10–15 longest processes per neurospheres were measured from the edge of the "neuroparticles" to the tip of these processes. It can be found that the average process length on collagen-modified nanofiber surfaces was larger than that on unmodified surface. Furthermore, the length increased with the immobilization degree of collagen, which indicates the existence of collagen promotes the culture of neural stem cells in vitro.

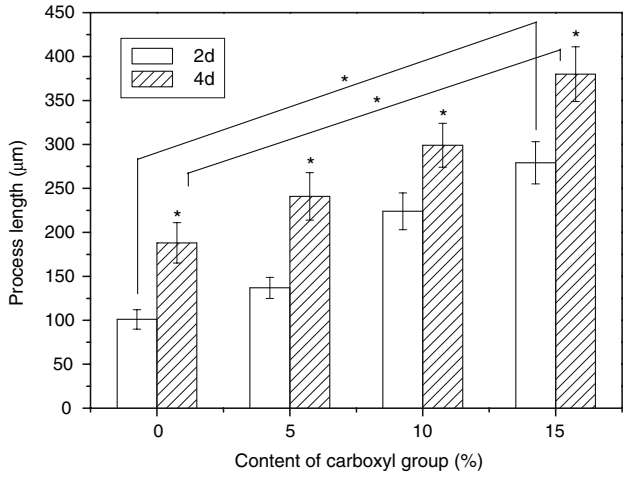

Fig. 8 Quantification of process growth of neurospheres on different nanofiber surfaces. The bars represent standard deviations. Asterisk denotes significant differences of process length compared to that after two days or on mats with low content of collagen ($p<0.05$) as determined by Student's *t*-test

The cell viability was studied by MTT assay. Only the mitochondria in living cells can reduce MTT to form products that can be detected at 490 nm. The cell viability of neural stem cells on different surfaces is presented in Fig. 9. After 4 days of incubation, the cell viability on collagen-modified nanofiber surfaces is clearly higher than that on unmodified surface. Collagen is the major class of insoluble fibrous protein in the mammalian extracellular matrix. Collagen has been electrospun into nanofibers for tissue engineering, such as human umbilical vein endothelial cells culture [37]. Zhang et al. [36] studied the behaviors of fibroblasts on collagen-containing nanofiber

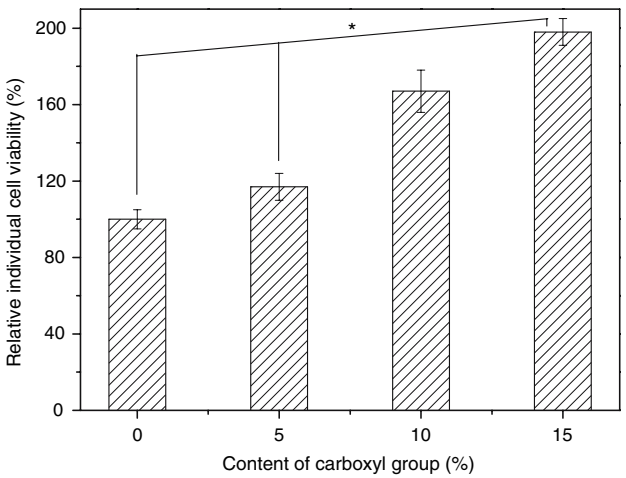

Fig. 9 Relative individual cell viability from MTT assay. The bars represent standard deviations. Asterisk denotes significant differences ($p<0.05$) as determined by Student's *t*-test

surface and suggested that the nanofibers could be used as novel functional biomimetic nanofibers toward achieving excellent integration between cells and scaffolds for tissue engineering. Recently, Rho et al. [38] also prepared collagen nanofibers. Behaviors of normal human keratinocytes indicated that this kind of nanofibers were a good candidate for biomedical applications such as wound dressing. He et al. [40] prepared nanofibers via electrospinning method and then coated collagen on the nanofiber surface. They proposed that the collagen-coated nanofibers enhanced the spreading and attachment as well as the viability of human coronary artery endothelial cells. Furthermore, collagen has also been recognized an effective materials for neural tissue engineering. Although it is generally recognized that nanofibers may mimic extracellular matrix, the increase of viability of neural stem cells may be mainly due to the existence of collagen. In conclusion, this kind of collagen-modified nanofibers may be a promising material for neural tissue engineering applications.

The representative SEM micrograph of neural stem cells cultured for 2 days on the PMMAAA15 nanofibers is shown in Fig. 10. The SEM image of two neural stem cells indicates that the cell body had an apparent bipolar elongated morphology. The interactions between the cells and nanofibrous mat can also be observed, which is very important for a scaffold and has been further studying in our lab.

Conclusions

Collagen-modified nanofibers were prepared via electrospinning and subsequent immobilization process for neural stem cells culture. Nanofibers with different contents of carboxyl groups were successfully fabricated from copolymer PMMAAA. It was found that the main factor influenced the morphologies and diameter of nanofibers was the polymer concentration. Collagen was immobilized onto these nanofibers and the immobilization degree increased with the content of carboxyl group. Cultured neural stem cells attached and spread well on the collagen-modified nanofibers. Cell viability evaluated by MTT assay also indicated that collagen-modified nanofibers were a potential substrate for neural stem cells culture. The special structure of nanofibers as well as the biocompatibility of collagen may be responsible for the attachment, proliferation, and viability of the cultured cells.

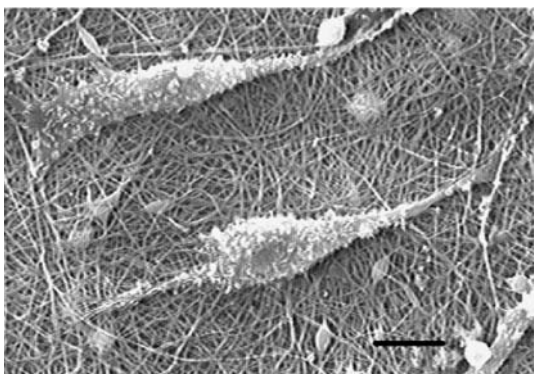

Fig. 10 Typical SEM images of neural stem cells on PMMAAA15 nanofiber surface after cultured for 2 days. The bar is 5 μm

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