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Electrospun PLGA–silk fibroin–collagen nanofibrous scaffolds for nerve tissue engineering

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Abstract Electrospun nanofibrous scaffolds varying different materials are fabricated for tissue engineering. PLGA, silk fibroin, and collagen-derived scaffolds have been proved on good biocompatibility with neurons. However, no systematic studies have been performed to examine the PLGA–silk fibroin–collagen (PLGA-SF-COL) biocomposite fiber matrices for nerve tissue engineering. In this study, different weight ratio PLGA-SF-COL (50:25:25, 30:35:35) scaffolds were produced via electrospinning. The physical and mechanical properties were tested. The average fiber diameter ranged from 280+26 to 168+21 nm with high porosity and hydrophilicity; the tensile strength was 1.76±0.32 and 1.25±0.20 Mpa, respectively. The results demonstrated that electrospinning polymer blending is a simple and effective approach for fabricating novel biocomposite nanofibrous scaffolds. The properties of the scaffolds can be strongly influenced by the concentration of collagen and silk fibroin in the biocomposite. To assay the cytocompatibility, Schwann cells were seeded on the scaffolds; cell attachment, growth morphology, and proliferation were studied. SEM and MTT results confirmed that PLGA-SF-COL scaffolds particularly the one that contains 50% PLGA, 25% silk fibroin, and 25% collagen is more suitable for nerve tissue engineering compared to PLGA nanofibrous scaffolds.

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Introduction

The regeneration of the nervous system is extremely limited. Trauma, tumor resection, congenital malformation, and other factors cause nerve damage can lead to chronic disability. Nerve autograft is considered to be the "golden standard" for bridging nerve gaps (Cao et al. 2009). However, the shortage of the donor sites, secondary deformity, and the potential different size limited its progress (Ducker et al. 1970; Beris et al. 2007). With the development of tissue engineering technology, it is popular to fabricate implantable scaffolds for bridging long nerve gaps that will produce similar results of autografting (Cao et al. 2009; de Ruiter et al. 2009; Agnew et al. 2010; Jiang et al. 2010). Nanoscaled scaffolds can mimic the structure of extracellular matrix (ECM), which can provide a suitable environment to support cell regeneration (Zhang et al. 2007; Sill et al. 2008; Ashammakhi et al. 2009). Nanofibrous scaffolds can be fabricated through many ways, such as template synthesis (Bechara et al. 2010), solvent casting (Ahmed et al. 2010), phase separation (Liu et al. 2010), self-assembly (Shahmoon et al. 2010), and electrospinning (Zhang et al. 2007; Kumbar et al. 2008; Sill et al. 2008). However, electrospinning is the most popular method as it can produce the scaffolds rapidly and efficiently. In addition, the morphology, porosity, pore size, and fiber diameter of the scaffolds can be changed by varying parameters, such as solution concentration, spinneret diameter, injecting viscosity, applied electric field strength, and the distance between the target substrate and the spinneret (Murugan et al. 2007). Electrospun nano-

fibrous scaffolds have interconnected pores, high porosity, and large surface area that can enhance the contact between the cells and scaffolds, improve the exchange of nutrition, and production of metabolism.

Material choice plays a crucial role in ensuring the success of tissue engineering. The materials for nerve tissue engineering should have appropriate degradation rate and mechanical properties to minimize inflammatory response, while it can provide the required support and guidance axons regenerating (Cao et al. 2009). Different synthetic and natural materials have been used for the fabrication of nanofibrous scaffolds for nerve tissue engineering (Yang et al. 2004a, b; Yang et al. 2005; Bini et al. 2006; Li et al. 2006a, b; Patel et al. 2008; Gupta et al. 2009), such as PCL, PLGA, PLLA, collagen, silk fibroin, chitosan and gelatin. Both of these two kinds of materials have their own merits and deficits. Synthetic materials are attractive for fabricating scaffolds because of their mechanical robust, but they are hydrophilic and lack binding sites for cell adhesion, while natural materials are hydrophilic and suit cell adhesion but they degrade too fast and lose the mechanical strength early. Recent studies confirmed that biocomposite material scaffolds can combine the virtues of synthetic and natural materials with enhanced mechanical properties over natural materials and improved hydrophilicity over synthetic ones; these scaffolds have proved to be the better ones for nerve tissue engineering (Schnell et al. 2007; Ghasemi-Mobarakeh et al. 2008).

The Food and Drug Administration has approved PLGA for fabrication of a variety of biomedical applications. Electrospun PLGA nanofibrous scaffolds have been proved suitable for PC12 nerve stem cells to adhere, migrate, differentiate, and proliferate (Bini et al. 2006). Collagen is one of the main components of ECM proteins (Park et al. 2006) and collagen-based conduits promoted Schwann cells regeneration and axonal enhancement in nerve tissue engineering (Kemp et al. 2009). Silk fibroin (SF) derives from natural silk; many studies confirmed that SF has broad applications in biomedical fields, especially in tissue engineering for the regeneration of ligaments, artificial bones, and nerves (Karageorgiou et al. 2006; Li et al. 2006a, b; Yang et al. 2007a, b). In order to combine merits of these three materials, we fabricated PLGA–silk fibroin–collagen (PLGA-SF-COL) biocomposite nanofibrous scaffolds by electrospinning. The properties such as morphology, fiber diameter, porosity, hydrophilicity, and tensile strength were tested; Schwann cells growth and proliferation were also studied for understanding the cytocompatibility and cytotoxicity of the scaffolds for nerve tissue engineering, and our final objective is to fabricate an ideal PLGA-SF-COL conduit that contains Schwann cells for bridging nerve gap.

Materials and Methods

Materials. PLGA (10:90, Mw 100,000) was provided by Life Science Institution, Sichuan University, Chengdu, China; collagen and silk fibroin were purchased from Sigma–Aldrich (St. Louis, MO) and HFIP was obtained from Fluka (Buchs, Germany); Dulbecco's modified Eagle's medium (DMEM) was obtained from Sigma (Singapore); fetal bovine serum (FBS), collagenase, trypsin, and MTT were purchased from GIBCO Invitrogen (Carlsbad, CA). Hexamethyldisilazane (HMDS) was bought from Sigma.

Fabrication of nanofibrous scaffolds. The polymer solution with concentration of 5 wt.% was prepared by dissolving PLGA, SF, and COL with a weight ratio of 50:25:25 and 30:35:35 in HFIP, stirred for 6 h at the room temperature and pressure. For electrospun, the solution was fed into a 10-ml syringe with a needle diameter of 0.4 mm, a high voltage (10 kv) was attached to the tip of the needle to generate electric field when a fluid jet was ejected at the speed of 4 ml/h. The resulting PLGA-SF-COL fibers were collected on a substrate that was placed at a distance of 10 cm from the syringe tip. The PLGA scaffolds were fabricated at the same way. The electrospun progress was carried out at the normal temperature and pressure.

Characterization of nanofibrous scaffolds. The morphology of the nanofibrous scaffolds were studied by using a scanning electron microscopy (SEM; JSM 5,600, JEOL, Tokyo, Japan) at an accelerating voltage of 10 kv. Before observation, the scaffolds were coated with gold using a sputter coater (JEOL, JFC-1,200 fine coater). The diameter and diameter distribution of the fibers were measured based on SEM micrographs using image analysis software (Image J, National Institutes of Health, Bethesda, MA).

The porosity of the scaffolds was tested by liquid displacement method: the PLGA scaffold was placed in a certain volume (V1) of ethanol, then removed the air bubbles and vacuumed, the ethanol and scaffold total volume was tested (V2), the scaffold was taken out, the remaining ethanol volume was (V3) examined. Scaffold porosity $p=(V1-V2)/(V2-V3)$. The two PLGA-SF-COL scaffolds were tested in the same way.

For determination of hydrophilicity of the scaffolds, the contact angle of the nanofibrous scaffolds membrane was measured by a contact angle system (JC2000C2, Zhongchen, Shanghai, China). The droplet size was set at 1.0 ml. Five samples were used for each test. The average value was reported with standard deviation (SD).

The tensile strength of different nanofibrous scaffolds were tested on a tabletop testing machine (Reger 3,050, Beijing, China). All samples were sheared in the form of rectangular shape with dimensions of 50×50 mm^2.

The stretching rate was 100 mm/min. Five samples were used for each test. The average value was reported with SD.

Schwann cell isolation, purification, and identification. Bilateral sciatic nerves were harvested from 3-d old Sprague Dawley rats (Animal Center of Sichuan University). The experimental procedures and the animal use and care protocols were approved by the Institutional Animal Care and Treatment Committee of Sichuan University. The epineurium of the sciatic nerve was stripped under a dissecting microscope. The nerves were cut into small pieces and enzymatically dissociated using 0.2% collagenase and 0.25% trypsin at 37°C for 20 min. The solution was centrifuged (1,000 rpm, 8 min), and supernatant was discarded. Cells were then incubated in DMEM supplemented with 10% FBS. After culturing for 30 min, the unattached cells were transferred to another flask to remove the fibroblasts by the different attachment rate of the two kinds of cells. After 24 h, the medium was replaced by G418 for 48 h. At the fourth day, the SCs were washed with PBS three times prior to adding fresh DMEM with 20% FBS, after that the DMEM was changed for every 2 d until the bottom of the flask was filled with cells. Trypsin-EDTA was used for subculture. After these steps, immunohistochemistry using mouse anti-rat S-100 monoclonal antibody (1:100, Sigma) was performed to assess the purity of Schwann cells.

Schwann cell culture. SCs were seeded onto the three aforementioned nanofibrous scaffolds. The scaffolds were cut to fit into the wells of a 6-well plate and sterilized by soaking in 75% ethanol for 30 min followed by exposure to ozone and ultraviolet radiation for every 30 min. Then the scaffolds were rinsed with PBS three times and incubated with DMEM for 24 h. SCs seeding density were 6×10^5 per scaffold. Each well was filled with 2 ml of DMEM with 20% FBS. The medium was changed every 2 d.

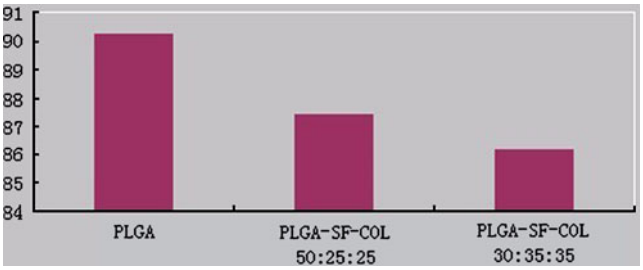

Fig. 2. Porosity distribution rang of the three scaffolds ($p<0.05$).

Morphological evaluation. The morphology of Schwann cells on the three nanofibrous scaffolds were observed by SEM. After seeding 3 and 7 d, the cell-cultured scaffolds were processed for SEM studies. The scaffolds were rinsed three times with PBS and fixed in 2.5% glutaraldehyde for 2 h. Thereafter, the scaffolds were washed repeatedly with PBS to remove glutaraldehyde and dehydrated with upgrading concentrations of ethanol (50%, 70%, 90%, 100%) for 15 min each. Final washing with 100% ethanol was followed by treating the specimens with HMDS. The HMDS was air-dried by keeping the samples in fume hood. Finally, the scaffolds were sputter coated with gold and then observed under SEM at an accelerating voltage of 20 kv.

Cell proliferation. The MTT assay was utilized for testing the proliferation of SCs on the three different scaffolds. The testing comprised of four groups: Three groups of nanofibrous scaffolds comprise PLGA, PLGA-SF-COL 50:25:25, and PLGA-SF-COL 30:35:35 and the control group. Briefly, the scaffolds were cut to fit into the wells of a 96-well plate and SCs were seeding on them in the density of 5×10^4. In the control group, the cells were seeding onto the 96-well plate directly. All groups were incubated with DMEM plus 20% FBS, and the medium was changed every 2 d. Cell activity was quantified after culturing 1, 3, 5, 7, 9, and 11 d. Each time, three wells were

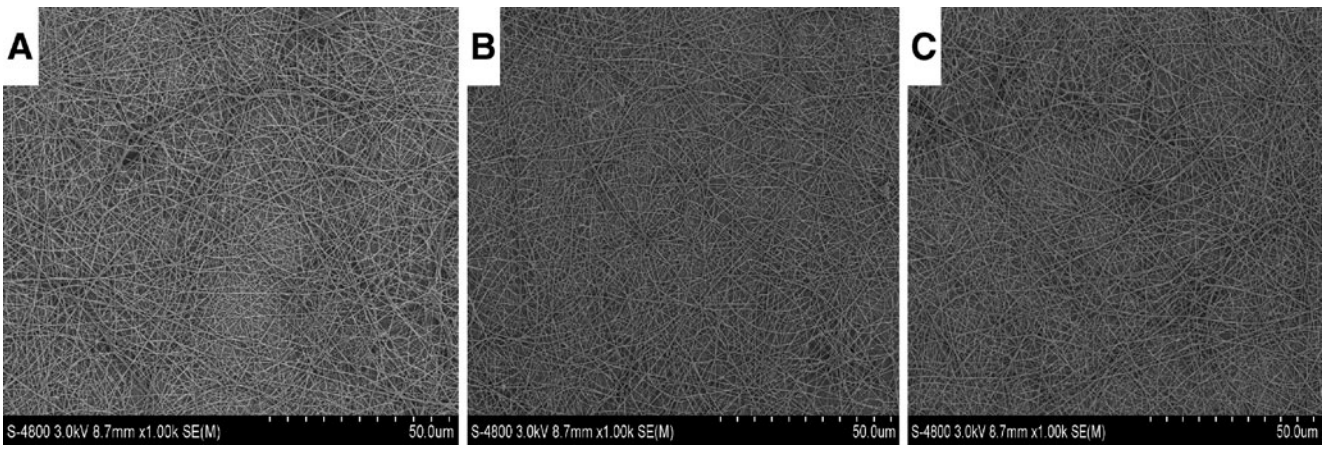

Fig. 1. SEM micrographs of nanofibrous scaffolds. (*A*) PLGA. (*B*) PLGA-SF-COL 50:25:25. (*C*) PLGA-SF-COL 30:35:35.

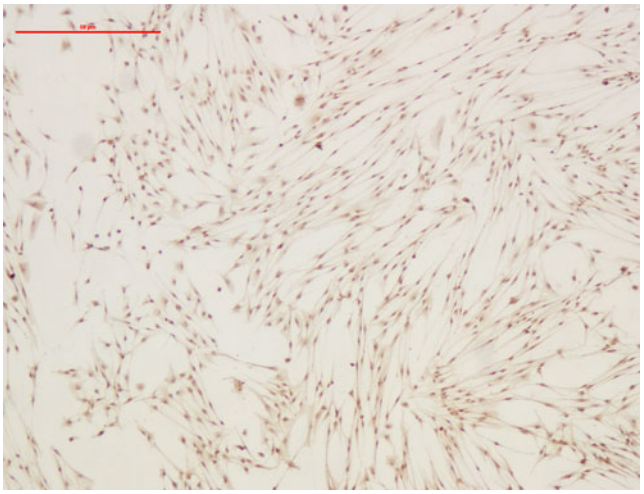

Fig. 3. Immunohistochemistry staining observation of Schwann (S-100); the SC purity is more than 95%.

chosen, and the medium was replaced of serum-free DMEM, adding MTT 5 mg/ml solution 20 µ1. Four h after incubation at 37°C, MTT solution was removed and 200 µl dimethyl sulfoxide was added. After oscillation in 10 min, the solution was moved to another 96-well plate to measure the absorbency at 490 nm using a microplate reader.

Statistical analysis. All data presented were expressed as mean ± SD. Statistical analysis was carried out using single-factor analysis of variance. A value of $p<0.05$ was considered statistically significant.

Results

Fabrication of electrospun nanofibrous scaffolds. The SEM was employed to study the morphology of the scaffolds. Figure 1*A–C* shows the micrographs of electrospun PLGA and PLGA-SF-COL 50:25:25 and 30:35:35 biocomposite nanofiber scaffolds. All of the three scaffolds are defect-free and porous; the fibers are mutually cross-linked. The fiber diameters is 168±21, 280±26, and 320±32 nm for PLGA-SF-COL 30:35:35, 50:25:25, and PLGA. Fiber diameter is found to decrease with increasing the weight ratio of silk and collagen. Significant differences ($p<0.05$) of the fiber diameter exist between every two scaffolds.

Porosity of the scaffolds. Structural properties assessed by liquid displacement method. Figure 2 shows the pore rate distribution of the three scaffolds. The PLGA scaffolds have the highest pore rate reached to 90.3±1.8%, while with reducing PLGA, the pore rate decreased. This can be attributed to the reduction of the fiber diameter, more layers of fibers might overlap with each other when the diameter is small resulting to less porosity. However, all of them are higher than 85%, which indicates that the nanofibrous scaffolds are highly porous structures.

Hydrophilicity characteristics. Contact angle studies of PLGA and PLGA-SF-COL nanofibrous scaffolds revealed the surface hydrophilicity nature. The two PLGA-SF-COL nanofibrous scaffolds are extremely hydrophilic with their water contact angle being zero showing 100% wettability. The contact angle significantly increases in PLGA scaffolds than the two PLGA-SF-COL scaffolds, as it reaches to 106.20±2.82°, which means it is hydrophobic.

Mechanical properties of electrospun nanofibers. For determining the mechanical properties of electrospun nanoscaffolds, the tensile strength was tested. The tensile strength is 2.05±0.36, 1.76±0.32, and 1.25±0.20 Mpa,

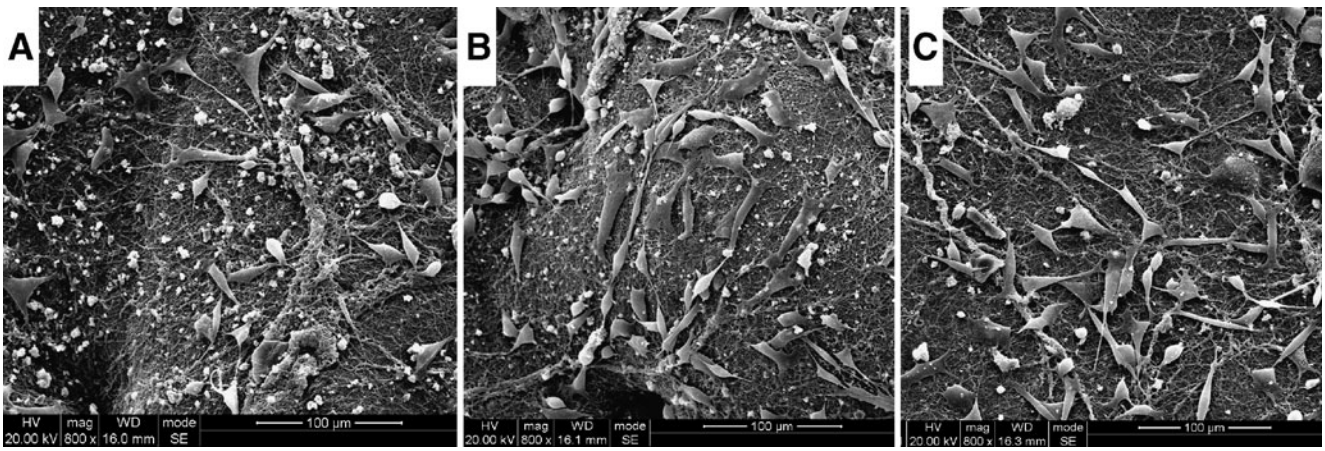

Fig. 4. SEM micrographs of Schwann cells cultured for 3 d on the scaffolds. (*A*) PLGA. (*B*) PLGA-SF-COL 50:25:25. (*C*) PLGA-SF-COL 30:35:35. Schwann cells exhibit the normal phenotype and axon outgrowth as immunostaining on the three scaffolds.

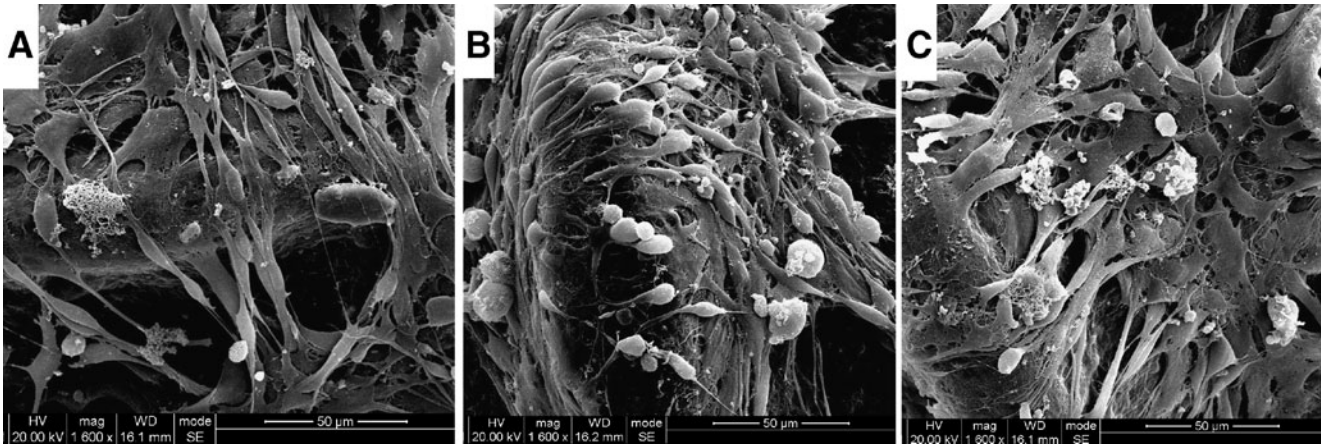

Fig. 5. SEM micrographs of Schwann cells cultured for 7 d on the scaffolds. (*A*) PLGA. (*B*) PLGA-SF-COL 50:25:25. (*C*) PLGA-SF-COL 30:35:35. The Schwann cell density increases compared to day 3 on the same scaffolds; the axons are connected together.

respectively, for the three scaffolds. This indicates with increasing collagen and silk fibroin mass ratio caused a reduction in mechanical strength. This phenomenon is likely due to the weak physical properties of collagen. Significant differences ($p<0.05$) are found between every two scaffolds.

Cell morphological studies. As Schwann cells play an important role in peripheral regeneration and function recover, Schwann cells should reach to high purity for nerve tissue engineering. In this study, primary Schwann cells were employed to test the cytobiocompatibility of the scaffolds. Figure 3 shows the finally purified SCs with bipolar or tripolar morphologies of S-100 immunohistochemistry staining. The SC purity is more than 95%. Cell morphology on the scaffolds was evaluated by SEM. Figure 4*A–C* represents micrographs of Schwann cells cultured for 3 d on different scaffolds, the overall results indicate that Schwann cells attach well and exhibit the normal phenotype and axon outgrowth as immunostaining. Figure 5*A–C* shows cells cultured for 7 d, the body of the cells are much bigger, cell density increases compared to day 3 on the same scaffolds, and the axons have been connected together already.

Cell proliferation. MTT test was carried out to evaluate the Schwann cells adhesion and proliferation on the three scaffolds. As shown in Fig. 6, the adhesion on PLGA-SF-COL scaffolds was significantly ($p<0.05$) higher than on PLGA scaffold. PLGA-SF-COL 30:35:35 scaffold is better for Schwann cells to adhere compared with PLGA-SF-COL 50:25:25 ($p<0.05$). On the first 7 d, PLGA-SF-COL 30:35:35 scaffolds showed a cell proliferation higher than PLGA and PLGA-SF-COL 50:25:25, while the cell proliferation on PLGA-SF-COL 50:25:25 scaffolds was found higher than PLGA-SF-COL 30:35:35 from day 9 ($p<0.05$).

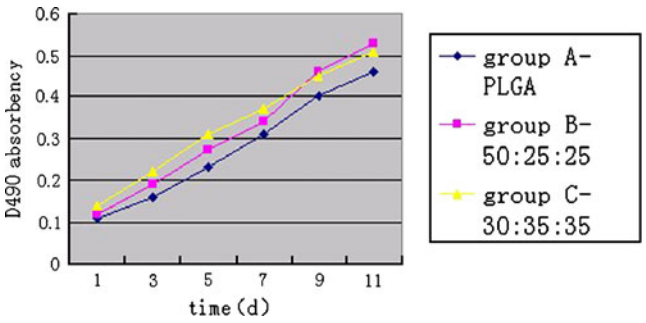

Fig. 6. MTT shows Schwann cell adhesion and proliferation on the three scaffolds. The adhesion of Schwann cell on PLGA-SF-COL scaffolds was higher than on PLGA scaffolds at the first day ($p<0.05$). At the first 7 d, PLGA-SF-COL 30:35:35 scaffolds showed a cell proliferation higher than on PLGA and PLGA-SF-COL 50:25:25, while the cell proliferation on PLGA-SF-COL 50:25:25 scaffolds was higher than on PLGA-SF-COL 30:35:35 from day 9.

Discussion

Numerous synthetic materials have been introduced to tissue engineering; PLGA as the representative has been widely used due to its nontoxic nature, biodegradability, and high tensile strength. SEM micrograph shows electrospinning is an efficient way to fabricating nanoscale PLGA scaffold. Synthetic materials are hydrophobic, and according to previous studies, hydrophobic scaffolds lead to lower cell adhesion in the initial step of cell culture, while incorporating in natural materials to the scaffolds, the hydrophilicity can be enhanced. This could be attributed to the carboxylic and amine functional groups structure in natural materials while such functional groups do not exist in synthetic structure. Collagen is an important material in

tissue engineering. When bridging peripheral nerve gap by collagen-based conduit, partial nerve function can be recovered (Kemp et al. 2009). Previous studies proved that the pure collagen I substrate is good for Schwann cells to adhere, but it is not good for cells to migrate and proliferate due to its high degradation rate and lower mechanical property (Milner et al. 1997; Vleggeert-Lankamp et al. 2004). Silk fibroin is flexible and has better mechanical property than other natural materials. Yang et al. (2007a, b) cultured rat dorsal root ganglia and Schwann cells on the substrate made from silk fibroin to examine the biocompatibility and cytotoxicity; the result showed that silk fibroin supports the cell growth and there are no cytotoxic effects on the cell phenotype. In order to join the merits of synthetic and natural materials and to get an almost ideal scaffolds for nerve tissue engineering, we combined PLGA, silk fibroin, and collagen to fabricate a biocomposite scaffolds, and the SEM morphology of the PLGA-SF-COL scaffolds show it is possible to fabricate such kind of scaffolds by electrospinning. The contact angle of PLGA scaffold is 106.20±2.82°, it is hydrophobic; when collagen and silk fibroin are mixed, in contrast, the contact angle of the two PLGA-SF-COL scaffolds are both 0°, they are highly hydrophilic. This phenomena proves that when natural materials are added to synthetic scaffolds the hydrophilicity can be improved. Ghasemi-Mobarakeh et al. (2008) compared the contact angle of PCL and PCL/gelatin scaffolds; the latter one is hydrophilic while PCL is hydrophobic. Gupta et al. (2009) obtained the same results about these two kinds of scaffolds. Scaffolds for tissue engineering should have proper diameter and porosity. The PLGA-SF-COL diameter ranged from 100 to 300 nm, they are nanoscaled. The porosity of the biocomposite PLGA-SF-COL scaffolds are less than the PLGA scaffolds in this study, but they are both higher than 85% and meet the request for nerve tissue engineering. Scaffolds for nerve tissue engineering should have suitable mechanical properties for the bearing of stresses during the surgical procedure and implantation time (Schnell et al. 2007). Therefore, PLGA-SF-COL 30:35:35 nanofibrous scaffolds are not favorable for nerve tissue engineering due to its high decreasing strength tensile compared with PLGA scaffold, while PLGA-SF-COL 50:25:25 might be suitable although it also decreased, but the range is small.

Schwann cells play a vital role in peripheral nerve regeneration; they are the primary structural and functional cells. Therefore, it is very important to have a thorough insight into the biocompatibility between PLGA-SF-COL scaffolds and Schwann cells. For this reason, we focused on testing whether or not the scaffolds had cytotoxic effects on Schwann cells. In this study, cell morphology was evaluated by SEM; it can be observed that Schwann cells can adhere, migrate, and spread out neurites on the surface of the scaffolds. Schwann cells show normal bipolar morphology on the biocomposite scaffolds, which is the same as S-100 immunostaining. This means that these scaffolds have good biocompatibility and no cytotoxicity. The surface hydrophilicity plays a fundamental role in cell adhesion; PLGA-SF-COL biocomposite nanofibrous scaffolds show better hydrophilicity than PLGA. MTT result indicates that PLGA-SF-COL scaffolds are more suitable for Schwann cell to adhere especially the 30:35:35 one compared with PLGA scaffolds; the cell number on the front one is higher on the first 24 h. Cell proliferation on PLGA-SF-COL scaffolds is much higher than on PLGA scaffolds all the time, while PLGA-SF-COL 30:35:35 is higher than 50:25:25 on the first 7 d but lower from day 9; this can be attributed to too high natural materials weight ratio in PLGA-SF-COL 30:35:35 scaffolds and the natural materials degrade fast, which influences the cell proliferation. Ghasemi-Mobarakeh et al. (2008) compared PCL/gelatin 70:30 and 50:50 scaffolds; they found PCL/gelatin 50:50 is not favorable for nerve tissue engineering due to their high degradation rate and weak mechanical properties while PCL/gelatin 70:30 is much suitable. Combining the property and cell behavior on the scaffolds, we conclude that when fabricating PLGA-SF-COL biocomposite scaffolds, the synthetic and natural material weight ratio should reach a balance; PLGA-SF-COL biocomposite nanofibrous scaffolds show better hydrophilicity than PLGA. Both of the two PLGA-SF-COL scaffolds have proper diameter and porosity, while PLGA-SF-COL 50:25:25 scaffolds show better strength tensile and cell proliferation than PLGA-30:35:35 scaffolds, so PLGA-SF-COL 50:25:25 nanofibrous scaffolds are potential substrates for nerve tissue engineering.

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