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Multi-porous electroactive poly(L-lactic acid)/ polypyrrole composite micro/nano fibrous scaffolds promote neurite outgrowth in PC12 cells☆

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Abstract
In this study, poly(L-lactic acid)/ammonium persulfate doped-polypyrrole composite fibrous scaffolds with moderate conductivity were produced by combining electrospinning with *in situ* polymerization. PC12 cells were cultured on these fibrous scaffolds and their growth following electrical stimulation (0–20.0 μA stimulus intensity, for 1–4 days) was observed using inverted light microscopy, and scanning electron microscopy coupled with the MTT cell viability test. The results demonstrated that the poly(L-lactic acid)/ammonium persulfate doped-polypyrrole fibrous scaffold was a dual multi-porous micro/nano fibrous scaffold. An electrical stimulation with a current intensity 5.0–10.0 μA for about 2 days enhanced neuronal growth and neurite outgrowth, while a high current intensity (over 15.0 μA) suppressed them. These results indicate that electrical stimulation with a moderate current intensity for an optimum time frame can promote neuronal growth and neurite outgrowth in an intensity- and time-dependent manner.

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Key Words
neural regeneration; tissue engineering; poly(L-lactic acid)/polypyrrole composite; multi-porous fibrous scaffold; electrical stimulation; PC12 cell lines; axon; electric spinning; grants-supported paper; photographs-containing paper; neuroregeneration

Research Highlights
(1) Poly(L-lactic acid)/ammonium persulfate doped-polypyrrole composite fibrous scaffolds with moderate conductivities were fabricated by combining electrospinning with *in situ* polymerization.
(2) The poly(L-lactic acid)/ammonium persulfate doped-polypyrrole composite fibrous scaffold had good biocompatibility and had no impact on the viability of PC12 cells.
(3) Electrical stimulation on poly(L-lactic acid)/ammonium persulfate doped-polypyrrole composite fibrous scaffolds can promote axonal growth in PC12 cells in a strength- and time-dependent manner.

Abbreviations
PLLA, poly(L-lactic acid); PPy, polypyrrole; APS, ammonium persulfate

INTRODUCTION

Electrical stimulation is an effective cue to enhance neurite and axonal outgrowth[1-4], and consequently makes the use of electrically conductive polymers very attractive in the construction of nerve guidance channels. Polypyrrole (PPy), an inherently conductive polymer, is shown to support neuronal growth, as the electrical stimulation applied through the PPy scaffolds enhances neurite outgrowth[5-6]. However, because of its highly conjugated molecular backbone, PPy is brittle, rigid, and nonbiodegradable, and is difficult to use alone to make a nerve guidance tube. Thus, an increasing number of studies have focused on the fabrication of PPy/biodegradable polymer composites. Zhang *et al* [7] synthesized the polystyrenesulfonate or butane sulfonic acid PPy-coated PDLLA/CL scaffolds by combining the salt leaching method and *in situ* polymerization. Wang and Wen[8] produced the porous PDLA/PPy composite scaffolds by combining phase separation methods and *in situ* polymerization. By coating PPy onto the surface of the biodegradable polymer substrate, the composite scaffold was electrically conductive, mechanically flexible, and largely biodegradable, with PPy anchored to its surface. The *in vivo* biocompatibilities of these conductive composites have already been demonstrated[9]. These polymer composites can be made into various forms of electrically conductive scaffolds to promote tissue regeneration following electrical stimulation. However, many studies have used protonic acids, such as, butane sulfonic acid[7], camphorsulfonic acid[10], and para-toluene sulfonic acid[11], to dope PPy, and found that these conductive polymers gradually lose their conductivity because of deprotonation. Moreover, there is conflicting evidence as to whether the electrical stimulation can promote neuronal growth and neurite outgrowth. Some studies report increased growth on conductive PPy substrates[12-14] and others reveal reduced growth, no effect[15-16], or an increase in growth only when the stimulation parameters are within an appropriate range[1, 5]. Given that most studies do not indicate the resistance of the substrate[17], it is hard to compare results. Moreover, studies with conductive substrates have mainly investigated how electric fields applied through the substrate affect neuronal growth. However, few studies have investigated how the passive property of conductivity in the substrate can affect neuronal growth. It is important to systematically study the reaction of the neural cells under different stimulation parameters and optimize the stimulation parameters.

Recently, electrospinning has been used as an alternative scaffold fabrication technique in soft-tissue cell transplantation and hard-tissue regeneration. The electrospun fibers provide an interconnected porous scaffold, which is desirable for drug gene/cell delivery and biomedical substrates for tissue regeneration, immobilization enzymes and catalyst systems, as well as artificial blood vessels[18-20].

In this study, the poly(L-lactic acid) (PLLA)/ammonium persulfate (APS) doped-PPy composite fibrous scaffolds with a three-dimensional multi-porous structure were fabricated by combining electrospinning with *in situ* polymerization. This study was aimed at demonstrating that PLLA/APS doped-PPy composite fibrous multi-porous scaffolds can be used as the scaffold on which electrical stimulation can promote tissue regeneration; the current intensity and continuous stimulation time can influence the neurite and axonal outgrowth of nerve cells.

RESULTS

Unique microstructure of PLLA/APS doped-PPy composite fibrous scaffolds

Figure 1 shows scanning electron microscopic images of PLLA fibrous scaffolds and PLLA/APS doped-PPy composite fibrous scaffolds.

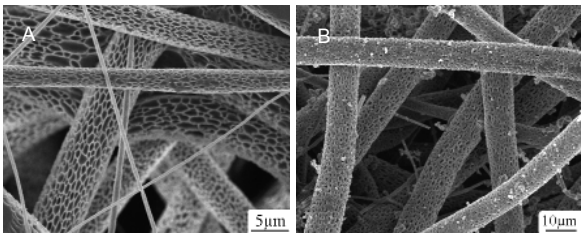

Figure 1 PLLA fibrous scaffold (A) and PLLA/APS doped-polypyrrole composite fibrous scaffold (B) (scanning electron microscopy; scale bars: 5 μm in A and 10 μm in B).

Both PLLA fibers and PLLA/APS doped-PPy composite fibrous scaffolds had a multi-porous structure.

PLLA: Poly(L-lactic acid); APS: ammonium persulfate.

As shown in Figure 1, both the PLLA fibrous scaffolds and PLLA/APS doped-PPy composite fibrous scaffolds were dual multi-porous scaffolds. PLLA fibers were multi-porous fibers with diameters ranging from 80 nm to 6.2 μm. PLLA/APS doped-PPy composite micro/nano fibers were also multi-porous fibers. Although all the pores in the PLLA fibers were filled with PPy

nanoparticles, the pores in the fibers were still clearly visible. The three-dimensional interstices formed through the fibers intercrossing were clear and visible, showing an irregular shape with sizes of 30–150 μm. Moreover, the PPys around PLLA fibers formed a compact and smooth layer.

Conductivity of PLLA/APS doped-PPy composite micro/nano fibrous scaffolds

The conductivities of the PLLA/APS doped-PPy micro/nano fibrous scaffolds were 1.08–2.56 × 10^{-1} s/cm. X-ray photon spectroscopy analysis showed that the PPy was doped by the oxidant (APS). Table 1 summarizes the elemental compositions of the sample surfaces. For the PLLA/APS doped-PPy composite, sulfur atoms were detected. Doping levels of the PLLA/PPy components were calculated from the atomic ratio of S to N from the high-resolution X-ray photon spectra. From Table 1, it can be found that doping levels of the PLLA/PPy components were affected by the polymerization temperature.

Table 1 Elemental compositions and doping levels of polypyrrole-coated poly(L-lactic acid)

Polymerization temperature	Elements (atomic %)		Doping level (S/N, %)
	S	N	
0°C deionized water	10.33	89.67	11.52
20°C deionized water	7.48	92.52	8.08

Cell viability on the PLLA/APS doped-PPy micro/nano fibrous scaffolds

Figure 2A shows the ratios of viable PC12 cells on the tested PLLA/APS doped-PPy micro/nano fibrous scaffolds and PLLA micro/nano fibrous scaffolds following 4 days of culture. It can be seen that the ratios of viable PC12 cells on both PLLA/APS doped-PPy and PLLA micro/nano fibrous scaffolds were not statistically different ($P > 0.05$). Both scaffolds had high ratios of viable PC12 cells.

Cell proliferation on PLLA/APS doped-PPy micro/nano fibrous scaffolds

Figures 2B, C present the absorbance at 490 nm and relative growth rate of PC12 cells on different micro/nano fibrous scaffolds recorded each day. The results presented in Figures 2B, C demonstrate that the adhesion of PC12 cells was a little better on PLLA micro/nano fibrous scaffolds than on PLLA/APS doped-PPy micro/nano fibrous scaffolds, resulting in slightly lower absorbance values at 490 nm and relative growth rates each day. However, with the time extended, the variation trends in absorbance at 490 nm in the two scaffolds were quite similar to that of the control, and the relative growth rates were all above 88% every day. The results showed no significant differences, verifying that the dual porous PLLA/APS doped-PPy micro/nano fibrous scaffolds had no cytotoxic effects on the *in vitro* proliferation of PC12 cells, but increased the cell proliferation rate.

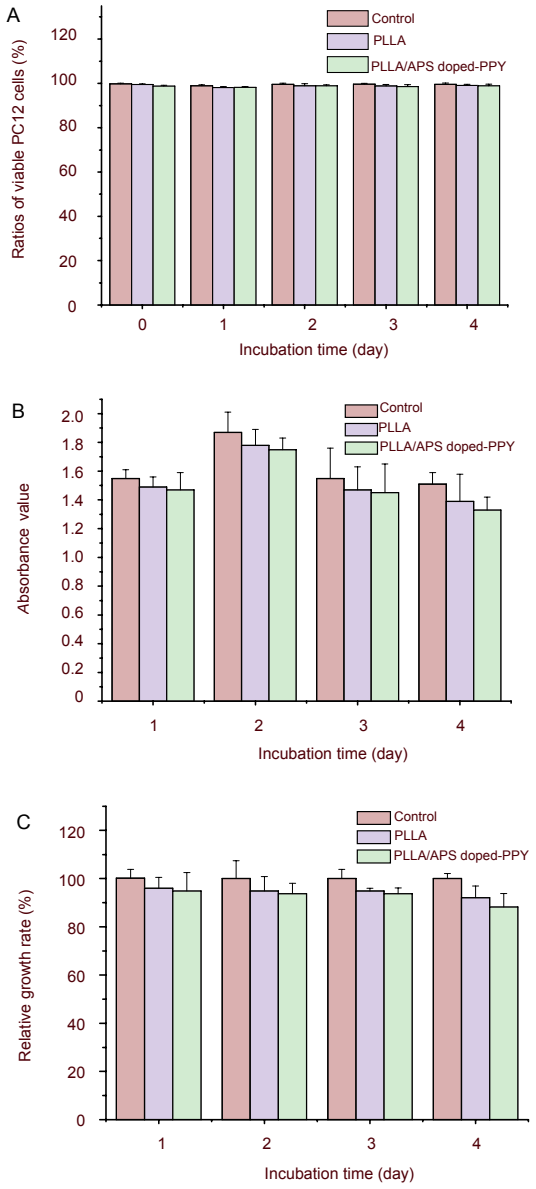

Figure 2 Changes in the ratios of viable PC-12 cells (A), absorbance at 490 nm (B) and relative growth rate (C) after they were cultured in different extraction fluids for 0, 1, 2, 3 or 4 days.

Data are expressed as mean ± SD from four independent experiments. There was no significant difference among groups using one-way analysis of variance. PC12 cells in the control group were cultured in RPMI 1640 media containing 10% (v/v) fetal calf serum.

PLLA: Poly(L-lactic acid); PPy: polypyrrole; APS: ammonium persulfate.

Proliferation of PC12 cells and neurite outgrowth after electrical stimulation

Following fixation of the electrical stimulation time, the relationship between the current intensity and absorbance at 490 nm was determined, as shown in Figure 3A. From Figure 3A, it can be seen that the absorbance value first increased with an increase in current intensity when it was lower than 10 μA, and decreased when the current intensity was above 10 μA. A constant current of 10 μA was applied through the PLLA/APS doped-PPy composite fibrous scaffolds, and the absorbance values at 490 nm in PC12 stimulated under different duration times were recorded, as shown in Figure 3B. From Figure 3B, it can be seen that the absorbance increased with an increase in stimulation time when the time of duration was within 2 days, and reversed as the stimulation time was over 2 days.

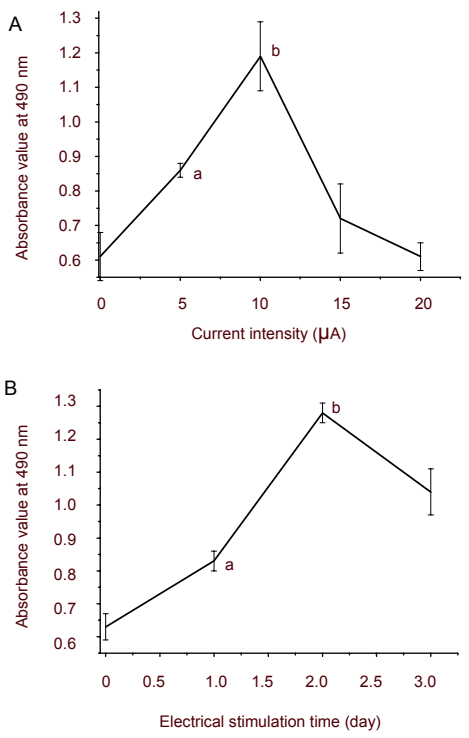

Figure 3 Proliferation of PC12 cells stimulated under different current intensities (A) and electrical stimulation times (B).

Data are expressed as mean ± SD from four independent experiments. [a]$P < 0.05$, [b]$P < 0.01$, *vs*. control (0 μA or 0 day group) using one-way analysis of variance.

Neurites were observed in all the samples under electrical stimulation. Semiquantitative analysis was performed by counting cells with and without neurites following stimulation with different current intensities. More neurite-bearing PC12 cells and longer neurites were observed with electrical stimulation compared with unstimulated control cells ($P < 0.01$; Figure 4A). Cells simulated with a current intensity of 5 μA (Figure 4C), revealed obvious neurite outgrowth, but the lengths of neurites were shorter than those of cells stimulated with a current intensity of 10 μA (Figure 4D). Surprisingly, cells simulated with a current intensity of 15 μA appeared to have suppressed neurite outgrowth, and a few short neurites were observed (Figure 4E). Moreover, no neurites were observed in cells simulated with a current intensity of 20 μA (Figure 4F).

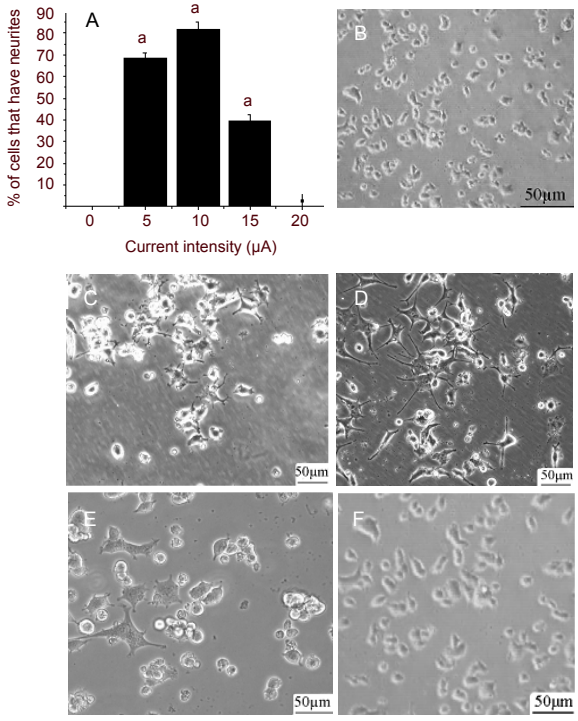

Figure 4 Neurite outgrowth of PC12 cells under electrical stimulation.

(A) Percentage of PC12 cells with neurites. Data are expressed as mean ± SD from four independent experiments. [a]$P < 0.01$, *vs*. 0 μA group using one-way analysis of variance.

(B–F) Microscope images of electrically stimulated PC12 cells with 0 μA (B), 5 μA (C), 10 μA (D), 15 μA (E), 20 μA (F) current intensity for 2 days (inverted light microscope, scale bars: 50 μm). Cells stimulated with a current intensity of 10 μA recorded the highest number of neurites and longest axons.

Under an inverted light microscope, neurites were observed every day. When the stimulation time was 1 day, neurite outgrowth was apparent (Figure 5A), but the neurites were very short compared with those stimulated for 2 days (Figure 5B). PC12 cells stimulated for 2 days had the highest number of neurites with the longest axon length of about 500 μm (Figure 5C), which was 33.3 times longer than the median axon length of cells stimulated for 1 day (about 15 μm). However, as

stimulation time was extended to 3 days, axon growth became apparently slow; the longest axon length was only about 550 μm (Figure 5D).

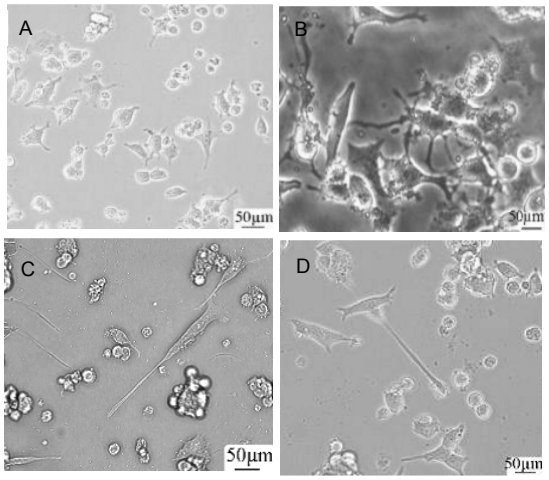

Figure 5 Representative microscope images of electrically stimulated PC12 cells under different electrical stimulation times when the current intensity was 10 μA (scale bars: 50 μm).

(A) At 1 day, neurites were very short.

(B) At 2 days, neurites were longer than those at 1 day.

(C) At 2 days, the longest axon length was about 500 μm.

(D) At 3 days, the longest axon length was about 550 μm.

Under scanning electron microscopy, PC12 cells could be seen to attach and grow on the PLLA/APS doped-PPy micro/nano fibrous scaffolds. PC12 cells grew not only on the surface of the composite fibers, but also in the pores or among the fibers (Figure 6A). It is obvious that electrical stimulation stimulated neurite outgrowth. Under electrical stimulation, the shape of PC12 cells changed from an ellipse (Figure 6B) to a tadpole shape (Figure 6C). Moreover, we found that PC12 cells aligned along the fiber axial following electrical stimulation (Figure 6C).

DISCUSSION

Protonic acid dope PPy gradually loses its conductivity in the culture medium because of deprotonation[10-11]. In this study, the dual multi-porous electrically conductive PLLA/APS doped-PPy micro/nano fibrous scaffolds with conductivities between (1.08–2.56) × 10^{-1} s/cm were generated by combing electrospinning with *in situ* polymerization.

Controversy exists as to whether electrical stimulation can promote neuronal growth and neurite outgrowth[10-16]. However, our study indicates that electrical stimulation affects the proliferation of PC12 cells. A moderate current intensity increased the proliferation of PC12 cells and stimulated neurite formation and growth; however, higher intensities had the opposite effect. The cells stimulated with a current intensity of 10 μA recorded the highest number of neurites and longest axon. This is in agreement with results reported by Zhang *et al* [7]. The exact mechanism of action of electrical stimulation is not fully understood, however, the level of electrical potential has an impact on the degree of stimulation, with a lower potential more favorable for promoting neurite outgrowth in PC12 cells[11]. Meanwhile, there is a range of biologically effective current for the electrophysiological behavior of biological tissue or organs[8].

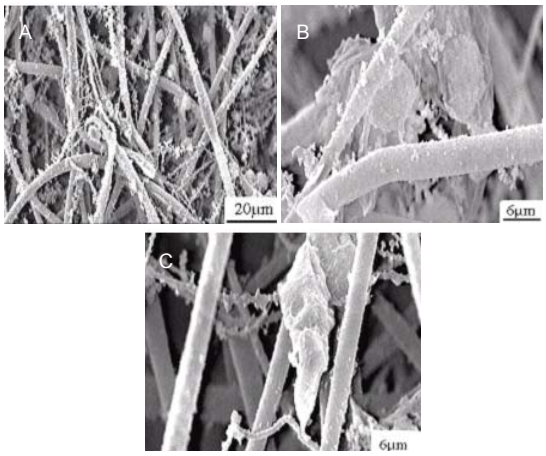

Figure 6 PC12 cells grew on the poly(L-lactic acid)/ammonium persulfate doped-polypyrrole scaffolds for 2 days (scanning electron microscopy, scale bars: 20 μm in A, and 6 μm in B, C).

(A, B) No electrical stimulation was given.

(C) After 10 μA electrical stimulation, the shape of PC12 cells changed from an ellipse to a tadpole shape, and aligned along the fiber axial.

In addition, our results show that the stimulation time influenced the proliferation of PC12 cells. There was an optimum stimulation time. The axon length increased when the stimulation time was extended, but growth rate slowed down. Under our experimental conditions, the fastest increase period was from 1 to 2 days under the current intensity of 10 μA. The main reason behind this phenomenon may be explained by the lack of nutrients because PC12 cells were seeded onto test scaffolds and cultured in RPMI 1640 medium containing 5% (v/v) fetal bovine serum without nerve growth factor.

Overall, our study indicates that the PLLA/APS doped-PPy micro/nano fibrous scaffolds with a dual pore structure have well-proportioned pores for the

attachment and growth of PC12 cells, and that electrical stimulation can be used to promote neurite outgrowth of PC12 cells.

MATERIALS AND METHODS

Design

An *in vitro* contrast observational experiment regarding nerve tissue engineering.

Time and setting

Experiments were performed at the Laboratory of Electrospinning, Materials and Textile Engineering College and Laboratory of Cell Culture, Medical College of Jiaxing University, China between March 2010 and July 2011.

Materials

Pyrrole monomer (Guoyao Chemical Reagent Co., Ltd., Shanghai, China) was distilled under reduced pressure and stored below 0°C. PLLA granules were purchased from Jiaxing Haobang Science and Technology Development Corporation, China.

Methods

Preparation of PLLA micro/nano fibers by electrospinning

The preparation of PLLA micro/nano fibers by electrospinning was as described previously[21]. In brief, PLLA granules were dissolved in dichloromethane using a magnetic stirrer for at least 5 hours to prepare a 12% (w/v) PLLA solution. The solution was held in a syringe for electrospinning. A piece of flat aluminum foil was placed 13 cm below the tip of the needle, and used to collect the PLLA micro/nano fibers. The voltage for electrospinning was 12 kV. The spun micro/nano fibers were left in the aluminum foil for 24 hours to allow the volatilization of dichloromethane to complete. The fibers were then peeled off from the aluminum foil.

Preparation of PLLA/APS-doped PPy composite micro/nano fibrous scaffolds

The thickness of the PLLA fibrous scaffold was approximately 0.45 mm. PPy was introduced using *in situ* polymerization on the surface of the PLLA multi-porous fibrous scaffold. Typically, 0.5 g of pyrrole was dissolved in 40 mL of deionized water while stirring at 0°C. A dimension of 45 mm × 35 mm PLLA multi-porous fibrous scaffold was immersed into the above solution followed by ultrasonication for 30 minutes to allow the scaffold to be saturated with the pyrrole solution. APS (1.22 g) in 40 mL of deionized water at 0°C was added dropwise to the above mixture with ultrasonic aided dispersion. The polymerization process proceeded for 5 hours without any dispersion. A thin, black PPy coating thus formed on the porous surface and was anchored to the polymer substrate. The pyrrole monomer was oxidized and doped with APS. The scaffold was then washed three times with 80 mL of deionized water and acetone (in sequence) and incubated in 40 mL deionized water for 24 hours to remove any unreacted pyrrole monomers. Finally, the fibrous scaffold was dried under vacuum and stored for usage.

Characterization of fibrous scaffolds

The morphologies of PLLA and PLLA/APS doped-PPy composite fibrous scaffolds were observed using the Hitachi JSM-5510 scanning electron microscope (Tokyo, Japan). Conductivities of PLLA/PPy composite fibrous scaffolds were measured using the four-probe method (ST512-SZT-2A; Zhongxiyuanda Science and Technology Co., Ltd., Beijing, China). X-ray photon spectroscopy was used to characterize the surface compositions of PLLA/PPy composite fibrous scaffolds. High resolution spectra of elements were obtained using a Kratos AXIS Ultra XPS system (Shimadzu, Tokyo, Japan).

PC12 cell culture

Rat PC12 cells (Cell Bank of Chinese Academy of Science in Shanghai, China) were cultured with RPMI 1640 culture solution (Promega) containing 10% (v/v) fetal calf serum and 100 U/mL penicillin and streptomycin, in an incubator containing 5% CO_2 at 37°C for 24 hours. Using 0.05% (w/v) trypsin-0.01% (v/v) ethylenediaminetetraacetic acid solution, the PC12 cells were detached from the culture flasks. Following two washes, PC12 cells were resuspended in RPMI 1640 culture solution at 1.6 × 10^6 cells/mL and were seeded onto the surface of the test scaffolds at a density of 2.4 × 10^4 cells/cm^2.

PC12 cell viability and proliferation on selected scaffolds

Following a 4-day culture, PC12 cells were detached from the test scaffolds after treatment with a 0.05% (w/v) trypsin-0.01% (v/v) ethylenediaminetetraacetic acid solution for 10 minutes at 37°C. Following enzyme treatment, the cells were washed twice with RPMI 1640 culture solution and centrifuged for 10 minutes at 3 000 r/min. The obtained centrifuged pellet was resuspended in 1 mL of culture solution and was used to determine cell viability using the MTT test. Briefly, 50 μL

MTT (5 mg/mL in PBS; Promega) was added to each well of the plate and incubated at 37 ± 1°C for 4 hours, followed by the addition of 150 μL of dimethyl sulfoxide. The plate was gently agitated until the formazan precipitate was dissolved, and the absorbance was measured at 490 nm with an EIX-800 Microelisa reader (Bio-Tek Inc., Winooski, VT, USA). Data were reported as mean ± SD of four independent experiments. Noncoated PLLA micro/nano fibrous scaffolds were used as references. Four independent experiments were performed. The relative growth rate was calculated as follows:

Relative growth rate = (average absorbance value at 490 nm of the sample/absorbance value at 490 nm of the control) × 100%.

Electrical stimulation of PLLA/APS doped-PPy micro/nano fibrous scaffolds

The PLLA/APS doped-PPy micro/nano fibrous scaffolds with conductivities between 1.08–2.56 × 10^{-1} s/cm were used in this experiment. A sample scaffold 2.5 cm × 3 cm in size was fixed at the bottom of a homemade electrical cell culture plate. Two opposite edges of the scaffold were in tight contact with two flat platinum electrodes connected to a low voltage electrical power source as well as to a computerized monitoring system (self-made). To avoid direct contact between the electrodes and the culture medium, medical grade silicone grease (Promega) was used to isolate electrodes from the culture medium. Under this configuration, there was no measurable current between the two electrodes when a PLLA/APS doped-PPy micro/nano fibrous scaffold was installed. The surface area of the test scaffold exposed to the cell culture medium was 2 cm × 2 cm. Following their assembly into the electrical cell culture plates, the scaffolds were sterilized by extensive ultraviolet irradiation and alcohol prewashing, followed by extensive washing in sterilized culture medium. The PC12 cells were seeded onto the test scaffolds and cultured in RPMI 1640 medium containing 5% (v/v) fetal bovine serum without nerve growth factor, in 100 U/mL penicillin and 25 μg/mL streptomycin. The culture medium was changed daily. Before electrical stimulation, the PC12 cells (2.4 × 10^4 cells/cm^2) were grown for 24 hours to allow settling and adhesion. Electrical stimulation was carried out in two phases. In the first phase, twenty culture plates were divided into five groups. Fixing the electrical stimulation time at 2 days, the plates of each group were applied with currents set at 0, 5, 10, 15, and 20 μA. In the second phase, sixteen culture plates were divided into four groups; the plates of each group were applied with the same current of 10 μA, and the electrical stimulation time was 1, 2, 3, 4 days. The temperature in the culture medium was measured and found to remain at 37 ± 1°C.

Assessment of cellular morphology

After several days of culture, half of the tested scaffolds were removed from the culture wells carefully. The morphological changes of PC12 cells detached from the fibrous scaffolds were observed under an inverted light microscope (Olympus, Tokyo, Japan) and photographed. Axon/neurite length was measured as the linear distance between the cell junction and the tip of an axon/neurite[22]. In addition, the percentage of PC12 cells with neurites and the numbers of neurites per cell (for cells that expressed at least one neurite) were calculated. More than 600 PC12 cells were analyzed for each condition. For PC12 cells, data was collected for neurite lengths greater than 5 μm. More than 100 PC12 cells were analyzed for each condition. Cellular morphologies on another half of the tested fibrous scaffolds were visualized using scanning electron microscopy. The samples were treated as previously described[11]. In brief, the fixed cells were dehydrated using increasing ethanol/water concentrations. Samples were dehydrated with hexamethyl disilazane (Sigma, St. Louis, MO, USA) and dried in air overnight. Gold was coated on the sample using a sputter coater. Scanning electron microscopy images were obtained with the Stereo Scan 260 Scanning Electron Microscope (Hitachi).

Statistical analysis

All data presented were expressed as mean ± SD. SPSS 17.0 statistical software (SPSS, Chicago, IL, USA) was used for statistical analysis of experimental data. One-way analysis of variance was carried out to compare the means of different data sets, and a value of $P < 0.05$ was considered statistically significant.

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Author contributions: Qiaozhen Yu was responsible for study design and implementation, performed data analysis and drafted the manuscript. All authors contributed to study implementation and data collection, and approved the final version of the paper.

Conflicts of interest: None declared.

Author statements: Manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

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