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Nerve Growth Factor-Immobilized Electrically Conducting Fibrous Scaffolds for Potential Use in Neural Engineering Applications

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Abstract

Engineered scaffolds simultaneously exhibiting multiple cues are highly desirable for neural tissue regeneration. To this end, we developed a neural tissue engineering scaffold that displays submicrometer-scale features, electrical conductivity, and neurotrophic activity. Specifically, electrospun poly(lactic acid-co-glycolic acid) (PLGA) nanofibers were layered with a nanometer thick coating of electrically conducting polypyrrole (PPy) presenting carboxylic groups. Then, nerve growth factor (NGF) was chemically immobilized onto the surface of the fibers. These NGF-immobilized PPy-coated PLGA (NGF-PPyPLGA) fibers supported PC12 neurite formation ($28.0 \pm 3.0\%$ of the cells) and neurite outgrowth ($14.2 \mu\text{m}$ median length), which were comparable to that observed with NGF (50 ng/mL) in culture medium ($29.0 \pm 1.3\%$, $14.4 \mu\text{m}$). Electrical stimulation of PC12 cells on NGF-immobilized PPyPLGA fiber scaffolds was found to further improve neurite development and neurite length by 18% and 17%, respectively, compared to

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unstimulated cells on the NGF-immobilized fibers. Hence, submicrometer-scale fibrous scaffolds that incorporate neurotrophic and electroconducting activities may serve as promising neural tissue engineering scaffolds such as nerve guidance conduits.

Index Terms

Conducting fibers; electrical stimulation; neural tissue engineering; nerve growth factor

I. Introduction

Peripheral nerve regeneration remains a medical challenge. Current clinical treatments, especially for large nerve gaps, involve the utilization of autografts and suturing of the gaps. However, this approach is often ineffective because of the mismatch of sizes between the donor and recipient tissues, formation of neuromas, and loss of function at the donor sites [1]. Therefore, development of artificial nerve guidance conduits (NGCs) is desirable as an alternative. Since nerve regeneration is a complicated process that requires multiple cues for neuronal survival and neurite regrowth, scaffolds that simultaneously present multiple cues are promising for functional recovery of injured nerve tissue [2]. Thus, materials that provide multiple stimuli, such as contact guidance (topography), neurotrophic activity, porosity, and electrical activity, are attractive [1], [2].

Electrospun fibers with diameters smaller than a micrometer have been widely produced for neural tissue engineering scaffolds because of their interconnecting pores, high surface area-to-volume ratio, and topography which mimics the natural extracellular matrix [3], [4]. Submicrometer-scale fibers have been shown to support attachment and differentiation of various nerve tissue-associated cells, such as dorsal root ganglion neurons (DRG), Schwann cells, hippocampal neurons, and PC12 cells [5]–[7].

Electrically conducting polymers have garnered great attention for tissue engineering materials because they are capable of delivering electrical cues to target sites and can simultaneously provide physical support for cell growth [8]. In particular, polypyrrole (PPy) and its derivatives have been explored for use as neural tissue engineering materials because of their biocompatibility and ease of synthesis [9]. Electrical stimulation of PC12 cells through smooth PPy films has resulted in improved neurite outgrowth compared to unstimulated controls [10]. In addition, we previously showed more neurite formation and increased neurite lengths on electrical stimulated PPy-coated nanofibers, suggesting their potential use in NGC applications [7].

Concurrently, biomaterials possessing neurotrophic activity are promising for neural engineering scaffolds because neurotrophic activity is essential for regeneration of injured nerve tissues [1], [12], and plays a critical role in neuron survival, differentiation, and maintenance of synaptic activities [13]. Recently, to fabricate biologically active scaffolds, different neurotrophins (e.g., nerve growth factor (NGF), neurotrophin-3) have been immobilized onto various substrates [14]. For example, chemically-immobilized NGF supported sprouting, neurite outgrowth, and axon establishment in PC12 cells, hippocampal neurons, and DRG neurons [15], [16]. Furthermore, to produce electrically active and

neurotrophic materials, NGF was immobilized onto smooth PPy films. These NGF-immobilized conducting films also supported neurite outgrowth of DRG neurons and PC12 cells [15], [17].

NGF molecules were incorporated into an electrically active submicrometer-scale fibrous scaffold. Specifically, PLGA nanofibers were electrospun (denoted as PLGA), and coated with PPy to obtain electrically conducting fibers (denoted as PPyPLGA). Next, a second conducting layer—containing carboxyethyl pyrrole—was made (denoted as PPy(COOH)PLGA), to which NGF was chemically tethered (denoted as NGF-PPy-PLGA) [Fig. 1(b)]. PC12 cells were cultured on the NGF-PPy-PLGA. Effects of immobilized NGF and electrical stimulation through conducting fiber meshes on neurite outgrowth were examined.

II. Materials and Methods

A. Material Synthesis

Fabrication of NGF-immobilized electrically conducting fibers consisted of four sequential steps: 1) electrospinning PLGA fibers (PLGA); 2) pre-coating of PLGA fibers with PPy (PPyPLGA); 3) carboxyl functionalization of the pre-coated PPyPLGA by copolymerization with pristine pyrrole and 1-(2-carboxyethyl)pyrrole (PPy(COOH)PLGA); and 4) chemical NGF immobilization (NGF-PPyPLGA).

Electrospinning PLGA—The 75/25 poly(lactic-co-glycolic acid) (PLGA) (inherent viscosity 0.55–0.75 dL/g, Lactel Biodegradable Polymers) was used for electrospinning as described previously [18]. The polymer solution was prepared as 6.5 wt% in hexafluoro-2-propanol (HFIP, Sigma). Electrospinning was performed on aluminum foil wrapped around the 7.6 cm drum with a syringe equipped with a 22 gauge steel needle at a 15 kV potential, a throw distance of 15 cm, and a syringe flow rate of 3 mL/h. After electrospinning, PLGA meshes were air dried for 2 days to remove residual HFIP. Meshes were cut into 1.5×1.5 cm squares, and then carefully removed from the aluminum foil.

Polypyrrole Coating of PLGA Fibers—Pyrrole (Aldrich) was first purified by passing it through a column of activated basic alumina (Aldrich). Next, a PLGA mesh (1.5×1.5 cm) was placed in a 1 mL aqueous solution of 14.4 mM pyrrole and 14.4 mM sodium para-toluene sulfonate (pTS) (Sigma) in a 15 mL polypropylene tube, followed by ultrasonication for 30 s. The mesh was pre-incubated at 4 °C for 1 h, followed by addition of 1 mL of 38 mM ferric chloride (Aldrich) solution and further incubation with shaking at 4 °C for 20–24 h. The PPy-coated mesh was washed with copious amounts of deionized water. This PPy coating step was repeated once more to create reproducible conducting PPyPLGA fibers.

Carboxyl Functionalization of the PPyPLGA—First, 1-(2-carboxyethyl) pyrrole was synthesized from 1-(2-cyanoethyl)pyrrole as described previously [17]. The 1-(2-carboxyethyl)pyrrole product was characterized by NMR. To introduce carboxyl groups onto the pre-coated fibers (PPyPLGA), a copolymer of pyrrole and PyCOOH was deposited on top of PPy layers on PPyPLGA. The PPyPLGA mesh was incubated in fresh solution of 14.4 mM pyrrole, 14.4 mM 1-(2-carboxyethyl)pyrrole, 14.4 mM, pTS, and 38mMFeCl₃

with shaking at 4 °C for 20–24 h. The product, PPy(COOH)PLGA, was transferred onto a clean glass slide and dried in a vacuum oven at room temperature for 2 days.

NGF Immobilization Onto the Functionalized PPyPLGA—For NGF immobilization, the PPy(COOH)PLGA mesh (1 cm × 1 cm) was incubated in EDC (Sigma) and NHS (Sigma) solution (8 mg/mL NHS and 4 mg EDC in 0.1 M MES (2-(N-morpholino)ethanesulfonic acid hydrate; Sigma) solution (pH 5.5)). After washing the sample twice with MES, 50 µL of NGF (2.5S NGF, Promega) solution (20 µg/mL in 0.1 M MES) was loaded on the 1 cm² of exposed sample and incubated at room temperature for 4 h. After conjugation, unbound NGF was removed from the samples by washing five times using sterile phosphate-buffered saline (PBS) to remove unbound NGF with 5-minute incubations for each washing step. Fibers with nonspecifically bound NGF, NGF(nsp)-PPyPLGA, were also prepared as controls using PPy(COOH)PLGA in an analogous manner as for NGF-PPyPLGA preparation, except without the use of EDC and NHS reagents.

B. Material Characterization

Fiber Mesh Size and Morphology—Fiber diameter and morphologies of PLGA meshes and PPy-coated PLGA meshes were examined using a Zeiss SUPRA 40 VP Scanning Electron Microscope (Carl Zeiss SMT). Samples were sputter-coated with 5 nm of platinum/palladium (Ted Pella) using a Cressington Scientific Instruments Model 208HR (Cranberry). The acquired SEM images were analyzed using ImageJ (NIH) for fiber diameter. At least 200 fibers were analyzed per sample.

Surface Carboxylic Groups—Carboxylic groups on the meshes were quantified using the Toluidine Blue O assay [19]. Samples of 1.5 × 1.5 cm were incubated in 5 mL of Toluidine Blue O solution (0.5 mM, pH 10) at 30 °C for 6 h. The samples were then thoroughly washed with NaOH solution (10 mM, pH 9) to remove unbound dye. The samples were transferred to a new tube containing 50% acetic acid (Fisher) solution and incubated for 20 min to extract the dye from the samples. Absorbance of the sample solutions and standard Toluidine Blue O solutions were measured at 633 nm by a spectrophotometer. The number of carboxylic groups was calculated assuming that Toluidine Blue O dye and surface carboxylic groups on the samples form a 1:1 complex; results were reported as the number of carboxylic groups per cm² of mesh. Measurements for each condition were performed in triplicate ($n = 3$).

Electrical Resistivity of Fiber Surfaces—Surface resistance of PPyPLGA meshes was measured as previously described [7]. In brief, two silver wires separated by 1 cm were placed onto the sample. Resistance was measured between the two silver electrodes using a digital multimeter (DM-8A, Sperry Instrument). Surface resistance was then calculated by dividing the measured resistance by the distance between two wires and multiplying by the width of the sample. Measurements were performed in triplicate ($n = 3$).

Chemical Composition of Fiber Surfaces—Surface compositions of the samples were analyzed using X-ray Photoelectron Spectroscopy (XPS). A monochromatic Al K α_1 source was employed. Typical operating conditions were 1×10^{-9} Torr chamber pressure, 15 kV

and 150 W for the Al X-ray source. Elemental scans were collected with a pass energy of 20 eV and a takeoff angle of 90° between the sample and analyzer.

C. Cell Culture

Sample Preparation—Each fiber sample was assembled into a cell culture chamber as described previously [7]. The assembly was sterilized by exposure to UV for 1 h. The substrates were incubated overnight in solution of rat tail type I collagen (0.1 mg/mL in deionized water, BD), washed twice with sterile deionized water and incubated in sterile PBS solution for 2 days.

PC12 Cell Culture and Electrical Stimulation—PC12 cells were maintained at 37 °C in a humid, 5% CO₂ incubator in F-12K culture medium (ATCC) containing 15% heat-inactivated horse serum (Hyclone), 2.5% fetal bovine serum (Hyclone), and 1% penicillin-streptomycin solution (Sigma). Cells were passaged weekly and primed by culturing them in medium containing 50 ng/mL soluble NGF one week prior to an experiment. Cell culture and electrical stimulation of PC12 cells was performed according to the experimental conditions as previously reported [7], [10], [20]. PC12 cells were seeded at 2×10^4 cells/cm² of a substrate, as described previously [7]. After 24 h in culture, a constant electrical potential of 10 mV/cm was applied across two electrodes for 2 h in the incubator using a potentiostat (Electrochemical Analyzer, CH Instruments). Cells were further incubated for 24 h after electrical stimulation. To investigate the effects of immobilized NGF onto conducting fibers and electrical stimulation of PC12 cells on neurite outgrowth, cells were cultured on unmodified PPy(COOH)PLGA in 50 ng/mL NGF medium either 1) with electrical stimulation or 2) without electrical stimulation, and on NGF-PPyPLGA without exogenous soluble NGF either 3) with electrical stimulation or 4) without electrical stimulation. Four substrates were employed for each condition ($n = 4$).

D. Fluorescence Microscopy and Image Analysis

For immunostaining of the immobilized NGF, the substrates were incubated in blocking buffer (3% goat serum (Sigma) in PBS) for 1 h. The substrates were then incubated with rabbit anti-NGF antibody (1:500 dilution in blocking buffer) (Sigma) at 4 °C overnight and washed with PBS buffer three times (10 min each), followed by treatment with Alexa-Fluor 568-labeled goat anti-rabbit antibody (1:500 in blocking buffer) (Invitrogen) at room temperature for 1 h. The substrates were washed carefully three times using PBS (10 min each).

For PC12 cell staining, the cells were fixed using 4% paraformaldehyde and 4% sucrose in PBS buffer for 15 min. The cells were permeabilized in 0.1% Triton X-100 (Fluka) and 2% bovine serum albumin (BSA, Sigma) in PBS for 15 min, followed by blocking with 2% BSA in PBS for 30 min at room temperature. PC12 cells were incubated with Alexa-Fluor 488-labeled phalloidin (Invitrogen) for 30 min to stain actin filaments, and with 4',6-diamidino-2-phenylindole diacetate (DAPI, Invitrogen) for 5 min to stain nuclei, then washed twice with PBS, and stored at 4 °C until analysis.

Fluorescence images were captured using a color CCD camera (Optronics MagnaFire, Olympus) attached to a fluorescence microscope (IX-70, Olympus), and analyzed using Image J software. Neurite length was measured as a linear distance between the cell junction and the tip of the neurite and collected only when neurite length was greater than 5 μm [7], [21]. The numbers of DAPI-stained nuclei were counted using the same image to obtain the total cell number. The percentages of neurite-bearing PC12 cells were calculated as the number of cells (bearing at least one neurite) divided by the total number of cells, where more than 500 PC12 cells were analyzed for each substrate. For neurite formation, the averages and standard deviations were obtained and statistical significance was assessed using a Student's *t*-test ($p < 0.05$). For the evaluation of neurite extension, median lengths were calculated and reported for each condition because the measured neurite lengths were not normally distributed [7], [10], [20]. For this neurite length analysis, more than 900 neurites were analyzed for each experimental condition. Statistical differences between neurite median lengths were calculated with a Mann-Whitney U test [22] ($p < 0.05$).

III. Results

A. Synthesis and Characterization of Fibers

NGF-immobilized conducting fibers were synthesized for in vitro studies. First, electrospun PLGA fiber meshes were deposited with PPy to form electrically conducting shells on the PLGA nanofiber cores under previously reported conditions [7]. Then, equal molar ratios of 1-(2-carboxyethyl)pyrrole (Py-COOH) and pristine pyrrole monomers were copolymerized (PPy(COOH)PLGA) to introduce carboxylic groups for chemical immobilization of NGF. SEM images of fibers were used to analyze fiber feature sizes (Fig. 2). Analysis indicated that the PPy(COOH)PLGA still presented submicrometer-scaled features with an average diameter of $0.73 \pm 0.30 \mu\text{m}$ and displayed highly porous structures (Table I). The Toluidine Blue O assay indicated that the PPy(COOH)PLGA scaffolds contained $8.1 \pm 1.1 \mu\text{mol}$ of carboxylic groups per cm^2 of mesh, whereas PLGA and PPyPLGA had significantly lower values of 0.3 ± 0.2 and $1.1 \pm 0.5 \mu\text{mol}/\text{cm}^2$, respectively. Surface resistances of the PPyPLGA and PPy(COOH)PLGA were 190 ± 170 and $64 \pm 50 \text{ k}\Omega/\text{square}$, respectively, which suggests that the additional conducting layer of the PPy(COOH) copolymer on the PPyPLGA further decreased the surface resistance of the meshes. After NGF immobilization, surface resistance was found to increase by approximately $290 \pm 54\%$. This increased surface resistance was a result of the chemical conjugation procedure rather than the presence of NGF because PPy(COOH)PLGA meshes treated the same as meshes undergoing NGF conjugation, but without addition of NGF, resulted in $430 \pm 99\%$ increase in surface resistance. Most likely, activation and washing steps for NGF immobilization caused dedoping and/or overoxidation of PPy and thus lowered PPy conductivity [23].

XPS analysis (Table II) indicates that the PPy coating and the incorporation of PyCOOH altered the elemental composition of the surfaces, as demonstrated by the appearance of nitrogen and decrease in the oxygen content from $47.0 \pm 7.2\%$ to $15.1 \pm 2.7\%$, $p = 0.01$. With chemical immobilization of NGF, more nitrogen atoms ($17.9 \pm 7.2\%$) were detected from the NGF-PPyPLGA compared to unmodified PPy(COOH)PLGA ($14.2 \pm 2.1\%$), $p = 0.06$. The carbon-to-nitrogen ratio also decreased from 5.1 ± 0.8 to 3.6 ± 0.5 ($p = 0.03$), which

presumably resulted from the presence of protein on the surfaces [17]. Nonspecific adsorption of NGF on the PPy(COOH)PLGA (denoted as NGF(nsp)-PPyPLGA) was also assessed for comparison with chemically immobilized NGF. With non-specific NGF immobilization on PPy(COOH)PLGA, nitrogen content increased from $14.2 \pm 2.1\%$ to $15.1 \pm 1.5\%$ ($p = 0.56$), which indicates that some NGF was nonspecifically adsorbed. However, this nitrogen content ($15.1 \pm 1.5\%$) was lower than that for NGF-PPyPLGA ($17.9 \pm 2.5\%$, $p = 0.12$). These results indicate that the immobilization of NGF on NGF-PPyPLGA was attributed to both chemical attachment and non-specific adsorption and that more NGF was immobilized via chemical conjugation. In addition to elemental composition analysis, immunostaining was performed to verify NGF immobilization on conducting fibers prepared by both chemical and nonspecific procedures. Fluorescence imaging confirmed the presence of NGF on the NGF-PPyPLGA fibers, whereas weak fluorescence was detected from the NGF(nsp)-PPyPLGA (Fig. 3). These results suggest that NGF was predominantly bound to the PPy(COOH)PLGA fibers via chemical attachment during the production of NGF-PPyPLGA.

B. In Vitro PC12 Cell Culture and Electrical Stimulation

Since electrical, topographical, and biological cues have been shown to have a positive impact on neuronal regeneration, electrically conducting fibers immobilized with NGF may improve neurite outgrowth. In this study, the individual and combined effects of immobilized NGF and electrical stimulation on neurite outgrowth of PC12 cells were studied (Fig. 4). First, the activity of the immobilized-NGF on electrically conducting submicrometer fibers was studied in terms of neurite outgrowth and compared with positive control, unmodified PPy(COOH)PLGA with exogenous NGF ($50 \mu\text{g/mL}$) in medium in the absence of electrical stimulation. No statistical difference was found in percentages of neurite-bearing cells on the NGF-PPyPLGA ($28.0 \pm 3.0\%$) compared to the positive control ($29.0 \pm 1.3\%$) [Fig. 5(a)]. However, few cells formed neurites ($1.2 \pm 2.1\%$) on unmodified PPy(COOH)PLGA fibers in NGF-free medium (negative control). Also, median neurite lengths of $14.7 \mu\text{m}$ and $14.2 \mu\text{m}$ for the cells cultured on the NGF-PPyPLGA and the control, respectively, were not statistically different [Fig. 5(b)]. These results indicated that immobilized NGF on the conducting fibers was as effective as exogenous NGF for inducing neurite formation and extension.

Second, the effect of electrical stimulation in combination with immobilized NGF was evaluated. With electrical stimulation, the number of neurite-bearing cells and neurite lengths were both increased for cells cultured on the NGF-PPyPLGA meshes. The percent of neurite-bearing cells significantly increased from $28.0 \pm 3.0\%$ to 32.9 ± 1.6 with electrical stimulation, whereas neurite length was $16.5 \mu\text{m}$ with electrical stimulation as compared to $14.2 \mu\text{m}$ without electrical stimulation ($p < 0.05$). These differences were similar for the cases in which cells were cultured on unmodified PPyPLGA with soluble NGF. Electrical stimulation on PPy(COOH)PLGA with soluble NGF significantly enhanced both neurite development (from $29.0 \pm 1.3\%$ to $35.5 \pm 3.4\%$) and neurite extension (from $14.7 \mu\text{m}$ to $19.1 \mu\text{m}$) compared to unstimulated controls. When comparing effects of soluble NGF and immobilized NGF under electrical stimulation, the percentage of neurite-bearing cells was

not significantly different; however, median neurite length was significantly shorter on the NGF-PPyPLGA. Key findings in this study are summarized in Table III.

IV. Discussion

Neural tissue regeneration requires multiple cues for axonal regrowth and functional recovery of injured nerve tissue [1], [2]. An engineered scaffold simultaneously exhibiting multiple positive cues can be a desirable platform for neural tissue engineering applications [2], [24]. In particular, electrically conducting substrates immobilized with NGF can deliver combined benefits of neurotrophic activity and electrical stimuli. Several studies have successfully demonstrated NGF incorporation into PPy films [15], [17], [25], [26].

However, most PPy substrates were smooth films without fibrous features and thus are not practical for tissue scaffold applications. Recent tissue engineering strategies have focused on designing tissue engineering scaffolds with submicrometer-scale fibers or fibrous architectures to better mimic the ECM and to create an interconnecting pore network with high surface area [27].

Functional activity of NGF immobilized on the conducting mesh, NGF-PPyPLGA, was found to exhibit a similar level of activity to NGF added to the medium. Evidence suggests that immobilized NGF can enhance neuronal viability and induce differentiation by interacting with receptors, such as TrkA and p75, and to enhance neuronal viability and induce differentiation without the internalization of the growth factor [29], [30]. This non-internalization mechanism enables long-term efficacy of NGF without inhibitory receptor down-regulation [31]. In this present study, NGF was conjugated by coupling NGF amine groups to active ester groups on the functionalized PPy fibers. Previously, NGF was immobilized onto functionalized PPy films bearing active ester groups to form amide links [17]. These NGF-immobilized PPy films supported neurite formation and were stable under physiological conditions for 5 days and with an application of a reducing potential. Thus, the NGF immobilized in this study is expected to exhibit similar stability and functional activity.

Finally, the combined effects of electrical stimulation and immobilized NGF were observed. When applying electrical potential (10 mV/cm) through the conducting fibers, similar proportions of neurite-bearing cells were found on the NGF-immobilized fibers and unmodified fibers with soluble NGF (control); however, a shorter median length was found with the immobilized NGF compared to soluble NGF. This difference may be attributed to higher surface resistance of the NGF-PPyPLGA. This increased resistance possibly attenuates the impact of electrical stimulus on neurite extension. Nevertheless, electrical stimulation through the NGF-PPyPLGA significantly improved neurite formation and extension compared to non-stimulation with NGF-PPyPLGA and unmodified PPy(COOH)PLGA with soluble NGF. These results indicate that the immobilized NGF can act together with electrical stimuli for neurite outgrowth. The mechanisms of electrical stimulation are not completely understood. However, the effects of electrical stimulation are thought to be related to alteration of protein adsorption onto materials [20], redistribution of membrane proteins [32], and decrease in membrane potentials [33].

V. Conclusion

With an aim at simultaneous presentation of electrical stimuli with submicrometer-scale fibrous topography and neurotrophic activity, NGF-immobilized PPy-coated PLGA submicrometer-scale fibers were successfully fabricated for potential use as neural tissue engineering scaffolds. These multifunctioning scaffolds supported PC12 cell growth and neuritogenesis without exogenous NGF in culture medium. Electrical stimulation of PC12 cells through NGF-PPyPLGA increased neurite formation (17%) and median neurite length (18%), indicating that the effects of immobilized-NGF and electrical stimulation could be combined together into a scaffold bearing submicrometer-scale fibrous features. As a result, the potential of neuroactive and electrically conductive nanofiber scaffolds could be used for neural tissue engineering applications.

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Fig. 1.

A. NGF-PPy-PLGA provides multiple cues for neurite outgrowth as the scaffolds deliver submicrometer-scale fibrous features, electrical conductivity, and neurotrophic activity. B. Schematic for fabricating NGF-immobilized PPyPLGA fibers (NGF-PPy-PLGA). i) Electrospun PLGA nanofibers were coated with PPy and PPy(COOH), producing PPy(COOH)-PLGA. ii) NGF (represented as circles) was chemically immobilized onto PPy(COOH)-PLGA.

Fig. 2.
Representative scanning electron micrographs of template PLGA nanofibers and PPy(COOH)PLGA fibers. Scale bars are 1 μm .

Fig. 3. Immunofluorescence images for NGF deposited by (a) chemical immobilization using NHS and EDC and (b) nonspecific adsorption to fibers. Scale bars are 10 μm .

Fig. 4.

Representative immunofluorescence images of PC12 cells cultured on various substrates: (a) PPy(COOH)PLGA without any NGF either on the surface or in medium (negative control); (b) PPy(COOH)PLGA with exogenous NGF (50 ng/mL in medium) (positive control); (c), (d) NGF-PPyPLGA without additional NGF in medium both without electrical stimulation (c) and with electrical stimulation (10 mV/cm for 2 h) (d). Immobilized NGF on PPy fibers supports neurite formation and outgrowth, which were comparable to soluble NGF (positive control). In addition, 10 mV/cm was applied for 2 h through NGF-PPy-PLGA. Scale bars are 50 μm .

Fig. 5.

Analysis of PC12 cells cultured on PPy(COOH)PLGA and NGF-PPyPLGA meshes either with or without electrical stimulation. (a) Percentages of neurite-bearing PC12 cells. Four substrates were used for each condition ($n = 4$) and at least 300 cells were analyzed for each substrate. Student's t-test was employed for statistical analysis with a criteria of $p < 0.05$. (b) Median neurite lengths. Median lengths were calculated and reported for each condition

because the measured neurite lengths were not normally distributed. Statistical differences between medians were calculated with a Mann-Whitney U test ($p < 0.05$).

TABLE I

Characteristics of Template PLGA Fibers and Carboxylic Functionalized PPyPLGA Fibers

Fibers	Fiber diameter (nm)	Surface carboxyl group ($\mu\text{mol}/\text{cm}^2$)	Surface resistance ($\text{k}\Omega/\text{square}$)
PLGA	382 (± 191)	0.3 (± 0.2)	N/A
PPyPLGA	601 (± 298)	1.1 (± 0.5)	190 (± 170)
PPy(COOH)PLGA	732 (± 304)	8.1 (± 1.1)	64 (± 50)

TABLE II

Elemental Compositions of PLGA, Functionalized PPyPLGA, and NGF-Immobilized PPyPLGA Using XPS Analysis

Fibers	Elements (atomic %)			
	C	O	N	S
PLGA	53.0 (± 7.2)	47.0 (± 7.2)	0.0	0.0
PPy(COOH)PLGA	70.6 (± 1.0)	15.1 (± 2.7)	14.2 (± 2.1)	0.0 (± 0.1)
NGF-PPyPLGA	63.2 (± 4.0)	18.6 (± 4.8)	17.9 (± 2.5)	0.3 (± 0.1)
NGF(nsp)-PPyPLGA *	65.8 (± 4.9)	18.9 (± 6.1)	15.1 (± 1.5)	0.1 (± 0.1)

* Nonspecifically immobilized NGF(nsp)-PPyPLGA was prepared in an analogous manner for NGF-PPyPLGA except no use of EDC/NHS reagents.

TABLE III

Summarized Results

Characteristics of the multifunctional scaffolds	
<ul style="list-style-type: none">Fibrous with 732 ($\pm$304) nm average fiber diameterElectrically conductive ($R_s < 500 \text{ k}\Omega/\text{square}$)Chemically immobilized with NGF	
Cellular effects with the multifunctional NGF-PPyPLGA scaffolds	
<ul style="list-style-type: none">Support neurite outgrowth (neurite formation and elongation) as effectively as soluble NGF.Further increase neurite formation and median length, by 18% and 17%, respectively, with electrical stimulation (10 mV/cm), compared to unstimulated controls.With electrical stimulation, neurite formation on NGF-PPyPLGA was comparable to that with soluble NGF; however, neurite length was shorter on NGF-PPyPLGA.	