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Neuroactive conducting scaffolds: nerve growth factor conjugation on active ester-functionalized polypyrrole

Jae Young Lee[1], Joo-Woon Lee[2] and Christine E. Schmidt[1,3,4,5,*]

[1]*Department of Chemical Engineering,* [3]*Department of Biomedical Engineering,*
[4]*Texas Materials Institute, and* [5]*Center for Nano- and Molecular Science and Technology,*
University of Texas at Austin, Austin, TX 78712, USA
[2]*Chemistry-Division of Liberal Arts and Sciences, Chungju National University,*
Chungju, Chungbuk 380702, Republic of Korea

Electrically conductive and biologically active scaffolds are desirable for enhancing adhesion, proliferation and differentiation of a number of cell types such as neurons. Hence, the incorporation of neuroactive molecules into electroconductive polymers via a specific and stable method is essential for neuronal tissue engineering applications. Traditional conjugation approaches dramatically impair conductivities and/or stabilities of the scaffolds and ligands. In this study, we developed copolymers (PPy-NSE) of *N*-hydroxyl succinimidyl ester pyrrole and regular pyrrole, which can be immobilized with nerve growth factor (NGF) without significantly hindering electroconductivity. The presence of active ester groups was confirmed using reflectance infrared spectroscopy and X-ray photoelectron spectroscopy (XPS) from the copolymers prepared from different monomer compositions. We selected PPy-NSE_{50} (polymerized from a 50 : 50 monomer ratio of pyrrole : pyrrole-NSE) for further modification with NGF because this copolymer retains good conductivity (approx. 8 S cm^{-1}) and presents active ester groups for NGF immobilization. We tethered NGF on the PPy-NSE_{50} surface, and found that PC12 cells extended neurites similarly to cells cultured in NGF-containing medium. XPS and enzyme-linked immunosorbent assay confirmed that NGF immobilized via the active ester on the PPy-NSE_{50} film was stable for up to 5 days in phosphate-buffered saline solution. Also, application of an external electrical potential to NGF-immobilized PPy films did not cause a significant release of NGF nor reduce their neurotrophic activity. This novel scaffold, providing electroconductive and neurotrophic activities, has potential for neural applications, such as tissue engineering scaffolds and biosensors.

Keywords: polypyrrole; nerve growth factor; PC12 cells; nerve tissue engineering

1. INTRODUCTION

Neurons are highly affected by the components of their environment including bioactive molecules such as neurotrophic factors, electrical signals, extracellular matrix molecules and cell-to-cell interactions (Lutolf & Hubbell 2005; Chalfoun *et al.* 2006; Li & Hoffman-Kim 2008). As a consequence, neuronal tissue engineering has focused on how to produce optimal scaffolds by integrating multiple cues to mimic natural nerve tissue (Serini & Bussolino 2005; Zhang *et al.* 2005; Silva 2006). In particular, electrical stimulation has been shown to play an important role in inducing cell adhesion and differentiation and reducing inflammation (Schmidt *et al.* 1997; McCaig *et al.* 2002; Guimard *et al.* 2007). Therefore, incorporating neuroactive molecules, such as neurotrophic factors (George *et al.* 2006; Gomez & Schmidt 2007; Richardson *et al.* 2007), extracellular matrix components (Collier *et al.* 2000; Sanghvi *et al.* 2005; Song *et al.* 2006) and topographical features for contact guidance (Gomez *et al.* 2007), into electroconductive materials is a key goal in nerve tissue engineering.

Polypyrrole (PPy) is a conductive polymer that has been widely studied for biomedical applications such as biosensors, drug delivering vehicles and nerve tissue engineering scaffolds. Beneficial properties of PPy, such as biocompatibility, good conductivity and ease of synthesis, make it a promising material to interface with neurons for neuron signal recording or electrical stimulation (Ateh *et al.* 2006; Guimard *et al.* 2007). However, the lack of functional groups on PPy has limited the ability to tailor PPy with various biochemical properties (Lee *et al.* 2006; Song *et al.* 2006; Guimard *et al.* 2007). A number of non-covalent modification techniques have been studied, which involve doping and entrapping different molecules, such as hyaluronic acid (Collier *et al.* 2000), heparin (Li *et al.* 2005), laminin fragments (Cui *et al.* 2003; Stauffer & Cui 2006) and nerve

*Author and address for correspondence: Department of Biomedical Engineering, University of Texas at Austin, Austin, TX 78712, USA (schmidt@che.utexas.edu).

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growth factor (NGF; Hodgson *et al.* 1996; Kim *et al.* 2007) during PPy polymerization. Also, affinity peptides that have specific binding to chloride-doped PPy were used to tether RGD peptides and to promote PC12 adhesion (Sanghvi *et al.* 2005). These non-covalent coupling techniques, however, require a high concentration of molecules during the synthesis and may not support stable retention of the ligand activity for a sufficient time period for tissue regeneration. Thus, creating covalent links of bioactive molecules to conductive polymers can be considered as an alternative to designing tissue engineering scaffolds (George *et al.* 2006; Lee *et al.* 2006; Song *et al.* 2006; Gomez & Schmidt 2007). However, covalent modification often faces practical hurdles. For example, non-specific covalent reactions such as photocrosslinking may degrade the polymer structure and biomolecules (Scriven 1984; Hermanson 1996; Kapur & Shoichet 2003). Functionalized pyrroles (e.g. amino, carboxylic acid, active ester) have been used to specifically couple chemical compounds, peptides and proteins; however, changes in the pyrrole structure result in a decrease in conductivity by approximately four orders of magnitude (Skotheim *et al.* 1998; Lee *et al.* 2006; Guimard *et al.* 2007). Consequently, incorporation of bioactive molecules should be performed in a controllable and specific way to minimize the reduction in bioactivity and conductivity of the biomaterial.

Neurotrophins modulate neuron survival, differentiation and extracellular signalling (Ebadi *et al.* 1997; Chao 2003). NGF is the best characterized of the neurotrophins. It plays critical roles in preventing apoptosis, inducing morphogenesis, and maintaining synaptic activities of neurons (Levi-Montalcini 1987; Shooter 2001). In addition, NGF has been reported to be involved in various functions of other cell types, such as angiogenesis in endothelial cells (Nico *et al.* 2008) and differentiation of immune cells (Aloe *et al.* 1997). Neurotrophins including NGF hold significant promise in therapeutic use for the treatment of degenerative diseases and injured nerve tissue (Ebadi *et al.* 1997; Ramer *et al.* 2000; O'Neill *et al.* 2005). Injured tissues do not produce sufficient levels of neurotrophins (Boyd & Gordon 2003) so that scaffolds bearing long-term neurotrophic activities are of importance for nerve tissue regeneration. In addition, immobilized NGF has been shown to trigger intracellular cascades and to stimulate neurons without a classical internalization process (Zhang *et al.* 2000; Kapur & Shoichet 2003; Gomez & Schmidt 2007). Hence, stable and specific chemical conjugation of neurotrophins on conducting polymers is desirable to integrate electrical cues and biochemical cues for improved nerve tissue applications.

In an effort to combine electrical cues with neurotrophic signals, we present a strategy to tailor conductive PPy films with NGF. We synthesized conductive and functionalized electroconductive PPy copolymers with active ester groups for functionalization; these materials show reasonably good conductivity with only one order of magnitude decrease compared with pristine PPy films. The cellular effects of NGF-coupled PPy films were evaluated for neurite formation of PC12 cells in comparison with NGF-containing culture medium. Stabilities of immobilized NGF on the PPy copolymer were studied under physiological conditions and with the application of an external electrical potential.

2. MATERIALS AND METHODS

2.1. Synthesis of pyrrole-NSE

Pyrrole-NSE (**3**) was synthesized in two steps, based on previously published protocols (Azioune *et al.* 2004; Bousalem *et al.* 2004): conversion of 1-(2-cyanoethyl) pyrrole (**1**) to 1-(2-carboxyethyl)pyrrole (**2**), followed by the addition of *N*-hydroxy succinimide (NHS; figure 1*a*). First, a solution of 16.5 g 1-(2-cyanoethyl)pyrrole (**1**; Aldrich) in 50 ml of 6.7 M KOH (Fisher) was refluxed for 20 hours under argon atmosphere until NH_3 gas no longer evolved. The reaction mixture was neutralized with 5 N HCl (Fisher) and recrystallized with cold heptane (Fisher). The product, 1-(2-carboxyethyl)pyrrole (**2**), was characterized by NMR. The ^{1}H NMR ($CClD_3$, ppm) spectrum of (**2**) showed peaks at 2.81 (t, 2H, CH_2COO), 4.15 (t, 2H, CH_2CH_2), 6.11 (dd, 2H, $CH_{\alpha\text{-pyrrole}}$) and 6.62 (dd, 2H, $CH_{\beta\text{-pyrrole}}$), which were in agreement with other published data (Azioune *et al.* 2004; Lee *et al.* 2006).

Next, pyrrole-NSE (**3**) was synthesized by the addition of 1.73 g NHS (Sigma) and 3.8 g 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Sigma) to a solution of 1.4 g 1-(2-caryboxyethyl)pyrrole (**2**) in 100 ml double deionized (ddI) water. The reaction was carried out for 30 min at room temperature. Insoluble product precipitated from the reaction mixture and was purified by filtration and washing with excess ddI water. The product was dried in a vacuum chamber overnight. The final product was a white powder and was obtained in 80 per cent yield. The structure and purity were confirmed using NMR and IR spectroscopies. ^{1}H NMR ($CClD_3$, ppm) spectrum of (**3**): 2.87 (m, 4H, CH_2CON); 3.11 (t, 2H, CH_2COO); 4.35 (t, 2H, CH_2N); 6.18 (dd, 2H, $CH_{\alpha\text{-pyrrole}}$); and 6.71 (dd, 2H, $CH_{\beta\text{-pyrrole}}$). The IR spectra of (**3**) showed peaks of the succinimidyl ester groups and pyrrolidinone groups at 1740, 1782 and 1814 cm^{-1}. The results were in good agreement with the published literature (Azioune *et al.* 2004; Bousalem *et al.* 2004; Khan *et al.* 2006).

2.2. Polymerization of polypyrrole copolymer films

Polypyrrole copolymers of unmodified regular pyrrole and pyrrole-NSE (**3**) were electrochemically synthesized on 10×20 mm pieces of either gold-coated glass slides or indium tin oxide-coated glass slides (Delta Technologies). Gold-coated glass slides were prepared by the deposition of 3 nm chromium and 30 nm gold onto glass slides (25×75×1 mm; Fisher) with a thermal evaporator (Denton). Various mixtures of pyrrole (Aldrich) and pyrrole-NSE (**3**) were prepared with 0.1 M tetrabutylammonium perchlorate (Aldrich) as the dopant in acetonitrile (Fisher). The total concentration was maintained at 50 mM and molar ratios were varied as follows: 100 : 0 (pyrrole : pyrrole-NSE) for PPy-NSE_0; 75 : 25 for PPy-NSE_{25}; 50 : 50 for

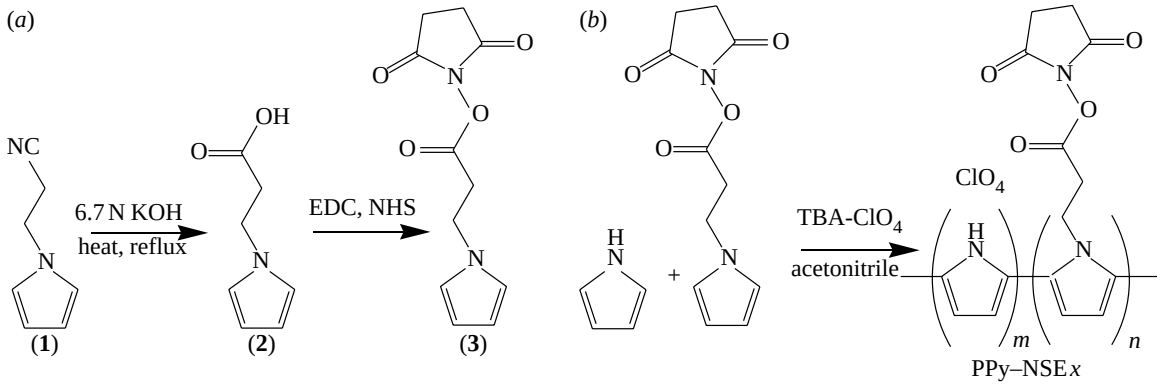

Figure 1. (*a*) Schematic of the pyrrole-NSE synthesis (**3**). 1-(2-Cyanoethyl)pyrrole (**1**) was converted to 1-(2-carboxyethyl) pyrrole (**2**), followed by the NHS substitution at the carboxyl group. (*b*) Electrochemical synthesis of the copolymers of pyrrole and pyrrole-NSE in acetonitrile with tetrabutylammonium perchlorate (TBA-ClO_4) as the dopant. PPy-NSE*x* indicates the copolymer which is synthesized with *x* mol% of pyrrole-NSE from a 50 mM total monomer concentration in the polymerizing solution. For example, PPy-NSE_{75} is prepared in a solution of 12.5 mM pyrrole and 37.5 mM pyrrole-NSE.

PPy-NSE_{50}; 25 : 75 for PPy-NSE_{75}; and 0 : 100 for PPy-NSE_{100} (table 1). A constant potential of 1.2 V, versus a saturated calomel electrode (SCE) reference (Fisher), was applied using a Pine Instrument AFRDE5 bipotentiostat for 20 s to synthesize the PPy-NSE films in a three-electrode configuration.

2.3. Conductivity measurements

Conductivities of the PPy copolymer films were measured by the four-point probe method (Runyan 1975). Film thickness was measured by a Veeco profilometer (Dektak 6M Stylus). PPy copolymer films were peeled off and transferred to non-conductive tape (3M). Three different films were prepared on different days, and the thickness and sheet resistivity (R_s) were quantified, from which the conductivity was calculated using the following equation:

$$\sigma = \frac{1}{t \times R_s} = \frac{1}{t} \times \left(\frac{I}{k \times V} \right),$$

where σ is the conductivity (S cm^{-1}, 1 S = 1Ω^{-1}); t is the film thickness; R_s is the sheet resistance (ohm per square); V is the voltage (V) between the two outer probes; I is the current (A) passing across the two inner probes; and k is a geometric factor for a thin sheet (here, $k = \pi/\ln 2 = 4.532$).

2.4. Infrared spectroscopy

Fourier transform infrared spectra were collected using a VeeMax II (Pike Technologies) variable-angle grazing accessory in a dry-air purged Nicolet Magna-IR 860 spectrometer (Welltech) equipped with a liquid-nitrogen-cooled mercury cadmium telluride detector (Krapchetov *et al.* 2006). An angle of incidence of 78° relative to the substrate normal was used. PPy sample films on gold substrates were characterized by reflectance IR using *p*-polarized light at a resolution of 2 cm^{-1}. Sixty-four scans at room temperature were performed to obtain the average IR spectra for all samples. Clean gold substrates were used as the background. Baseline was corrected with GRAMS/AI software (Thermo Scientific) in all sample spectra.

Table 1. Copolymers synthesized with different initial feed ratios and their conductivities.

	concentration in the solution (mM)		
copolymer	pyrrole	pyrrole-NSE	conductivity (S cm^{-1})
PPy-NSE_0	50.0	0.0 (0%)	$6.9\times10^{1}\pm7.4$
PPy-NSE_{25}	37.5	12.5 (25%)	$3.0\times10^{1}\pm1.3\times10^{1}$
PPy-NSE_{50}	25.0	25.0 (50%)	8.1 ± 2.5
PPy-NSE_{75}	12.5	37.5 (75%)	$5.6\times10^{-1}\pm2.4\times10^{-1}$
PPy-NSE_{100}	0.0	50.0 (100%)	$8.5\times10^{-3}\pm6.0\times10^{-3}$

2.5. X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS) was used to confirm active ester groups present on the surface of the PPy copolymers. The spectra were obtained using a physical electronic (PHI model 5700) spectrometer employing a monochromatic Al K$\alpha_{1,2}$ source. Calibration of the binding energy was performed by setting *C*–C/*C*–H component in the C_{1s} peak at 284.6 eV. Typical operating conditions were 1×10^{-9} torr in the chamber, and 14 kV and 250 W for the Al X-ray source. High-resolution elemental scans were collected with a pass energy of 11.75 eV at a take-off angle of 45° between the sample and the analyser. Peak deconvolution was performed for high-resolution C_{1s} spectra using XPSPEAK software (v. 4.1) to characterize the carbon atoms. Also, conjugation of NGF on the copolymer films was assessed by comparing the spectra and elemental compositions of the copolymer films with those of the control films not treated with NGF.

2.6. Nerve growth factor conjugation

NGF conjugation was accomplished via active ester groups present on the PPy-NSE_{50} films. First, the synthesized films were incubated in phosphate-buffered saline (PBS) buffer (0.1 M, pH 7.2) overnight to exchange dopants as described in other literature

(Azioune *et al.* 2004; Bousalem *et al.* 2004). Fifty microlitres of NGF solution (20 μg ml^{-1} in 0.1 M PBS, pH 7.2) were loaded on the 1 cm^2 sample piece. NGF molecules were immobilized on the PPy-NSE$_{50}$ films by incubation at room temperature for 4 hours. After conjugation, the samples were washed five times to remove unbound NGF using a sterile PBS buffer with 5 min incubation per washing step. For PC12 cell culture experiments, all samples were UV sterilized for 1 hour prior to NGF immobilization, and washed with sterile PBS buffers in a laminar flow hood. Then, the samples were treated with polyallylamine (Sigma) solution (1 mg ml^{-1} in ddI water) for 2 hours at room temperature to improve cell adhesion and washed two times with sterile ddI water. For XPS experiments, the samples were washed with deionized water two additional times to remove extra phosphate and chloride ions.

2.7. Cell culture

PC12 cells were maintained at 37°C in a humid, 5 per cent CO_2 incubator in F-12K culture medium (Sigma) supplemented with 15 per cent heat-inactivated horse serum (Hyclone), 2.5 per cent foetal bovine serum (Hyclone) and 1 per cent penicillin–streptomycin solution (Sigma), and passaged with a 0.25 per cent trypsin–EDTA solution (Sigma) every week. Cell priming was accomplished by culturing in medium containing 50 ng ml^{-1} NGF one week prior to an experiment. For *in vitro* assays of neurotrophic activity, the NGF-coupled PPy samples and unmodified PPy samples were transferred to a 24-well culture plate. Then, 2×10^4 primed cells were inoculated into each well and cultured for 5 days in RPMI-1640 (Gibco) medium containing 10 per cent foetal bovine serum, 5 per cent heat-inactivated horse serum and 1 per cent penicillin–streptomycin solution. Controls consisted of: (i) PPy-NSE$_0$ (non-functionalized PPy) in 50 ng ml^{-1} NGF medium (positive control), (ii) PPy-NSE$_{50}$ films in 50 ng ml^{-1} NGF medium (positive controls), (iii) PPy-NSE$_0$ in NGF-free medium (negative control), and (iv) PPy-NSE$_{50}$ in NGF-free medium (negative control). In addition, to study the non-specific adsorption of NGF on the PPy films, the PPy-NSE$_0$ films were prepared and treated with NGF in the same way as NGF-conjugated PPy-NSE$_{50}$ samples for PC12 cell culture designed above.

2.8. Fluorescence microscopy and image analysis

After 5 days in culture, PC12 cells were fixed with 4 per cent paraformaldehyde (Sigma) and 4 per cent sucrose (Sigma) in PBS buffer for 15 min, permeabilized with 0.1 per cent Triton X-100 (Fluka) and 2 per cent bovine serum albumin (BSA; Jackson ImmunoResearch) in PBS buffer for 5 min, and blocked with 2 per cent BSA in PBS buffer for 30 min at room temperature. The PC12 cells were stained with phalloidin–TRITC (Sigma) for 30 min and DAPI (Invitrogen) for 5 min for actin filaments and cell nuclei, respectively. The PC12 cells were then washed three times with PBS buffer, and stored at 4°C until fluorescence images were acquired.

Fluorescence images of cells and neurites were captured using a colour CCD camera (Optronics MagnaFire) attached to a fluorescence microscope (IX-70; Olympus), and analysed using ImageJ (NIH) software. Neurite length was measured from the tip of a neurite to the cell junction. The longest neurite from each cell was measured and counted only when the length was greater than that of the cell body. The number of DAPI-stained nuclei was quantified to obtain the total cell number in each image. The percentage of neurite-bearing PC12 cells was calculated as the number of neurite-bearing cells divided by the total number of cells. Five separate experiments were performed on different days. The averages and standard deviations were obtained and the statistical significance was assessed using a Student's *t*-test with Origin software.

2.9. Stability of immobilized NGF on the PPy-NSE$_{50}$ films

To study the stability of NGF immobilized on the PPy-NSE$_{50}$ films, we incubated the samples in PBS buffer at 37°C for 5 days. NGF stability was measured using two techniques. First, an enzyme-linked immunosorbent assay (ELISA) was used to quantify the released NGF in the medium. A commercial NGF-ELISA kit (Immuno-Max; Promega), with a detection limit of 5 pg ml^{-1}, was used. According to the manufacturer's protocol, the 98-well ELISA plates, hydrochloric acid and sodium carbonate were purchased from Corning, Fisher and Sigma, respectively. Spectroscopic results were measured at 450 nm using a plate reader (ELx808; BioTek). These assays were performed in triplicate. As a second technique to assess NGF stability, XPS analysis was performed to compare the sample spectra and elements on the sample surface after incubation. The ratio of the N_{1s} and C_{1s} peak areas was also calculated to monitor the NGF level on the PPy films. Three samples were analysed for each condition.

Electrical potentials were applied to NGF-immobilized PPy-NSE$_{50}$ samples to study the stabilities of NGF links and neurotrophic activities of the films according to previous literature (Hodgson *et al.* 1996; George *et al.* 2006). A constant reducing potential, −1 V versus SCE, was applied to the NGF-PPy-NSE$_{50}$ films in PBS buffer for 5 min with a Pt counter electrode. The samples taken at 2 and 5 min were quantified using a previous ELISA method. Also, after washing electrically stimulated NGF-PPy-NSE$_{50}$ films with PBS buffer (three times), *in vitro* PC12 cell culture was performed to evaluate neurotrophic activities with unstimulated NGF-PPy-NSE$_{50}$ (positive), PPy-NSE$_{50}$ with exogenous NGF (50 ng ml^{-1}, positive) and PPy-NSE$_{50}$ without exogenous NGF (negative). The PC12 cells were cultured and analysed in terms of neurite formation in an analogous manner as previously described. Three samples per condition were employed ($n=3$).

3. RESULTS

3.1. Synthesis and characterization of PPy-NSE copolymers

To prepare PPy-NSE copolymer films, regular pyrrole and pyrrole-NSE were polymerized in 0.1 M tetrabutylammonium perchlorate in acetonitrile solution at a potential of 1.2 V (figure 1). We synthesized various PPy copolymers (PPy-NSEx), where x represents the molar per cent of pyrrole-NSE in the total monomer, by varying the monomer feed ratio while keeping the total monomer concentration constant (0.05 M; table 1).

The existence of active ester groups in PPy-NSE copolymers was confirmed using reflectance IR. As shown in figure 2, the characteristic peaks, attributed to C=O stretching vibrations of the succinimidyl ester groups and pyrrolidinone groups, were observed at 1740, 1782 and 1814 cm^{-1} in PPy-NSE_{50}, PPy-NSE_{75} and PPy-NSE_{100}, but not in PPy-NSE_0 as predicted. Copolymers synthesized with higher pyrrole-NSE ratios in the reaction solution led to the stronger peak at 1740 cm^{-1} (active ester groups) while showing similar transmittance at 1550 cm^{-1} (attributed to the C=C stretch in pyrrole rings). These spectra imply that pyrrole-NSE was not favoured in the polymerization reaction compared with unmodified pyrrole, because the incorporation of pyrrole-NSE, which has a branch at the nitrogen atom in the pyrrole ring, may limit the access of the monomer to the growing polymer chain.

To characterize the surface elements on the PPy copolymers, XPS analysis was performed (figure 3). The peaks at 288.8 and 288.1 eV of the high-resolution C_{1s} spectra are attributed to N–O–*C*=O and N–*C*=O, respectively, which resulted from monomeric pyrrole-NSE incorporation into the copolymer films. Similarly, the XPS spectra of N_{1s} indicate distinguishable increases at 402 eV of *N*–O as the portion of pyrrole-NSE increases. These XPS results indicate that *N*-succinimidyl ester, which has the capability of forming covalent bonds with nucleophiles such as amines, was present on the PPy-NSE_{50} film surface.

We measured the conductivities of each PPy copolymer film using a four-point probe. As the pyrrole-NSE portion in the copolymer increased, the conductivities drastically decreased from 6.9×10^{1} S cm^{-1} (PPy-NSE_0) to below 1×10^{-2} S cm^{-1} (PPy-NSE_{100}; table 1). Low conductivity would be a major challenge when using the functionalized pyrrole for biomedical applications such as biosensors or electroconductive scaffolds. Therefore, we selected the PPy-NSE_{50} films for further protein conjugation because a film of this composition presents significant active functional groups on the surface, and shows a reasonably good conductivity of 8.1 ± 2.5 S cm^{-1} in the semiconductor range.

3.2. PC12 cell culture for neurite outgrowth studies

PC12 cells, extending neurites in response to NGF, were used as the cell system to assess immobilized NGF activity. NGF immobilization was accomplished simply by loading NGF solution onto the functionalized PPy (PPy-NSE_{50}) presenting *N*-hydroxy succinimide-activated carboxyl groups to form amide bonds.

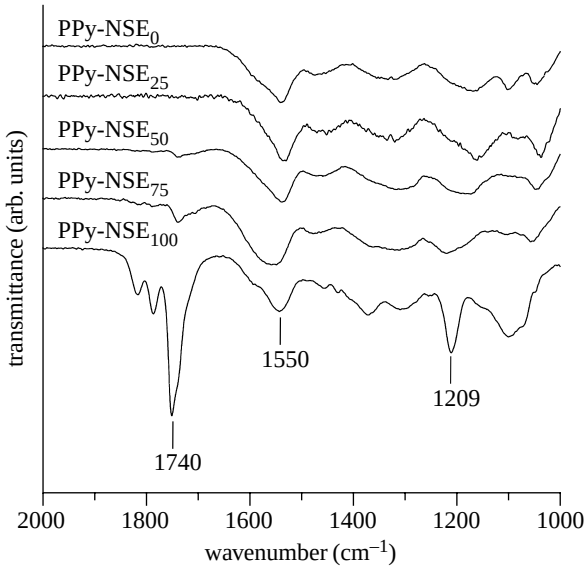

Figure 2. Reflectance IR spectra. PPy-NSE copolymers were synthesized from different pyrrole monomer ratios of pyrrole and pyrrole-NSE. Peaks at 1740, 1782 and 1814 cm^{-1} represent *N*-succinimidyl ester and pyrrolidinone, and those at 1550 cm^{-1} are typical for PPy rings. The peak at 1209 cm^{-1} is attributed to N–O stretching.

NGF-conjugated PPy-NSE_0 (non-functionalized PPy) films, treated with 20 μg ml^{-1} NGF in the same manner as PPy-NSE_{50}-NGF, were used to assess the effects of non-specific physical adsorption of NGF. Figure 4 shows a significant increase in the number of neurites for cells grown on NGF-conjugated PPy-NSE_{50} films ($20\pm5\%$, $p<0.001$) compared with cells grown on PPy-NSE_{50} films without NGF ($2\pm2\%$) and PPy-NSE_0 films (0%). Also, the effects were significantly different from the NGF-treated PPy-NSE_0 films ($5\pm2\%$), indicating minimal non-specific binding of NGF. Neurite outgrowth from cells grown on immobilized NGF on PPy-NSE_{50} was similar to positive controls in which cells were cultured on either PPy-NSE_0 ($25\pm5\%$) or PPy-NSE_{50} ($27\pm5\%$) films with the exogenous addition of 50 ng ml^{-1} of NGF in the culture medium. Figure 5 depicts representative fluorescence images of PC12 cells stained with phalloidin–TRITC after 5 days on various samples. The average neurite length for PC12 cells cultured on NGF-immobilized PPy-NSE_{50} was not significantly different from those in NGF-containing medium (positive controls).

3.3. Stability of immobilized NGF on PPy-NSE_{50}

To assess the stability of NGF coupled via active ester groups, we performed an ELISA assay with a detection limit of 4 pg ml^{-1} to analyse any NGF released into solution. XPS analysis was also performed to determine the elemental compositions of the substrate surfaces to monitor for loss of NGF. The samples were incubated in sterile PBS solution at 37°C for 5 days and compared with freshly prepared PPy-NSE_{50} films. NGF bound to the PPy-NSE_{50} films may be partly attributed

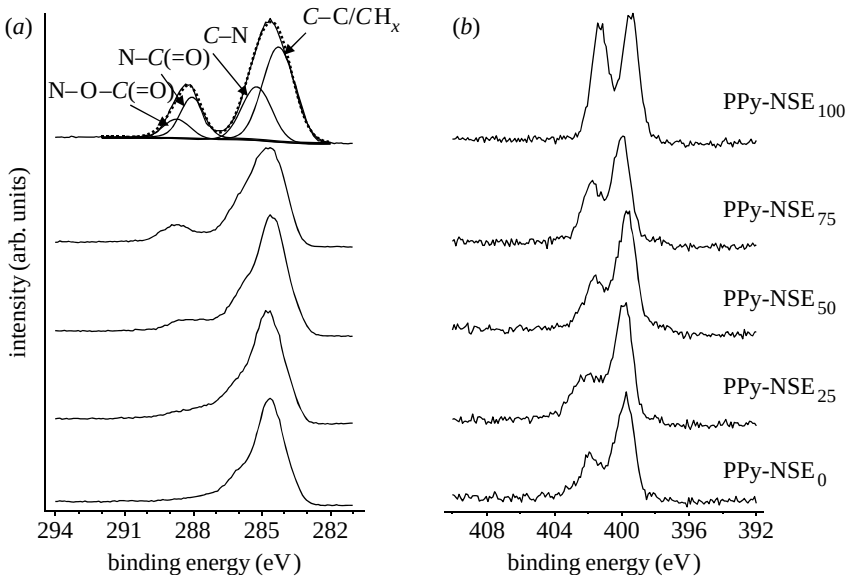

Figure 3. High-resolution (*a*) C_{1s} and (*b*) N_{1s} XPS spectra obtained from PPy-NSE copolymer films. The *N*-succinimidyl ester group in PPy (PPy-NSE*x*) copolymers was recognized by noticeable peaks at 288.8 eV (N–O–*C*(=O)) and 288.1 eV (N–*C*(=O)).

to physical adsorption that could be leached into solution. However, there was no detectable NGF leached from NGF-immobilized PPy-NSE_{50} films after a 5-day incubation.

Figure 6*a* displays high-resolution C_{1s} XPS spectra of PPy-NSE_{50} and NGF-PPy-NSE_{50} samples that were either freshly prepared or incubated for 5 days in PBS buffer. The C_{1s} spectra of the incubated samples were very similar to those prior to incubation. In particular, the characteristic peaks at 288.1 eV, attributed to NGF or links with PPy-NSE_{50}, were retained after the 5-day incubation, suggesting that the NGF moiety was chemically conjugated and stable on the surface. On the other hand, unmodified samples displayed substantially different C_{1s} profiles from NGF-immobilized samples. These findings are further evidence of the NGF-immobilization on PPy-NSE_{50}. Since NGF on films alters the elemental compositions of the surfaces, the atomic ratios of C_{1s} and N_{1s} from the XPS analysis were analysed to quantify the stability of the immobilized NGF (figure 6*b*). NGF-immobilized films were found to have significantly lower C_{1s}/N_{1s} values (approx. 5.0) compared with non-treated samples (approx. 6.6). Also, a 5-day incubation of NGF-immobilized samples did not cause substantial change in the C_{1s}/N_{1s} ratio (5.0 ± 0.5) compared with the freshly prepared samples (5.0 ± 0.4).

In addition, external electrical potentials were applied to the NGF-PPy-NSE_{50} films, conditions which were used to release NGF from NGF-doped PPy films as described by others (Hodgson *et al.* 1996; George *et al.* 2006). Minimal amount of NGF (less than 15 pg cm^{-2} film) was detected after applying -1 V for 5 min. Also, as shown in figure 7, the NGF-immobilized films (stimulated) that had a history of exposure to electrical potential induced neurite outgrowth ($23 \pm 6\%$) as much as other positive controls, 21 ± 2 per cent and 22 ± 7 per cent for unstimulated NGF-PPy-NSE_{50} (unstimulated) and PPy-NSE_{50} with exogenous NGF in medium (NGF in solution), respectively.

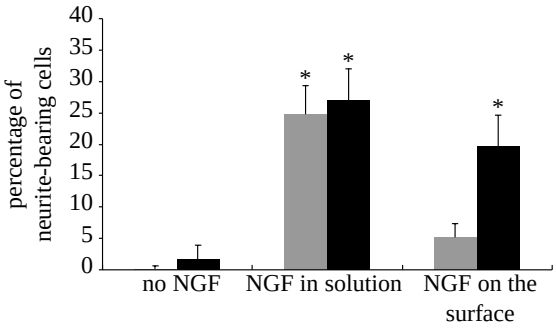

Figure 4. Neurite outgrowth of PC12 cells cultured on regular PPy films (grey bars) and on PPy-NSE_{50} (black bars). The PC12 cells were cultured for 5 days, fixed with 4% paraformaldehyde and stained with phalloidin–TRITC. Without NGF treatment either in the medium or on the surface, there were few cells bearing neurites. For the positive controls, approximately 25–27% of the PC12 cells cultured in NGF-containing medium (50 ng ml^{-1}) extended neurites. NGF-conjugated PPy-NSE_{50} films (black bars) promoted neurite extension similarly to positive controls, but significantly higher than NGF-treated PPy-NSE_0 films (grey bars). Five independent experiments were performed. $^*p < 0.005$.

4. DISCUSSION AND CONCLUSION

Conductive films bearing neurotrophic activity would be a desirable platform for neural tissue engineering scaffolds and neural electrode surfaces; combined electrical and biochemical stimulation should yield better regeneration and interfacing with neurons (Zhang *et al.* 2005; George *et al.* 2006; Gomez & Schmidt 2007; Guimard *et al.* 2007). Hence, neurotrophins are required to be stable in/on conducting scaffolds and with electrical potentials. Also, reasonable conductivities of the materials are essential to provide electrical stimulation through the scaffolds. Table 2 summarizes various techniques reported for the production of neurotrophin-modified conducting polymers with their important characteristics including electrical properties. Mostly, non-covalent methods have been developed, which include doping NGF/NT-3 during the

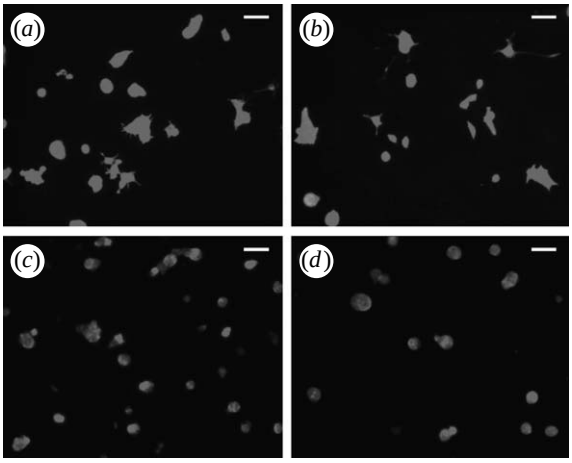

Figure 5. Immunostaining of PC12 cells cultured in different conditions. After 5 days in culture, phalloidin–TRITC was used to stain the fixed PC12 cells on the PPy films. The PC12 cells cultured (*a*) in NGF-containing media on the PPy-NSE_{50} films and (*b*) on NGF-conjugated PPy-NSE_{50} films extended neurites, while those cultured (*c*) without NGF either in solution or on the PPy-NSE_{50} films and (*d*) on NGF-treated regular PPy films did not form as many neurites as (*a*) or (*b*). Scale bar, 50 µm.

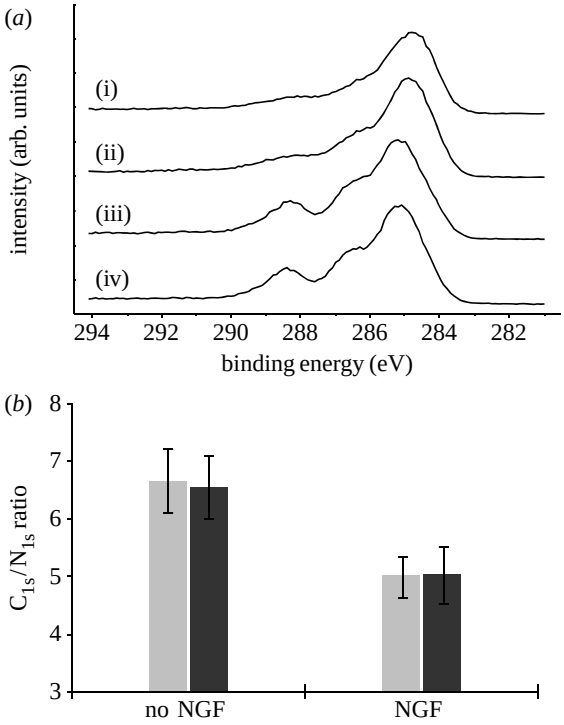

Figure 6. XPS analysis was used to assess the stability of NGF-immobilized PPy-NSE_{50} films. (*a*) High-resolution C_{1s} spectra of the PPy-NSE_{50} samples (i) freshly prepared and (ii) incubated, and of the NGF-immobilized PPy-NSE_{50} samples (iii) freshly prepared and (iv) incubated. Incubation was carried out for 5 days in PBS buffer at 37°C in a cell culture incubator. (*b*) C_{1s} and N_{1s} ratios of NGF-immobilized PPy-NSE_{50} films (NGF) and non-treated PPy-NSE_{50} films (no NGF). After the incubation, the ratios of both incubated films (black bars) were compared with those of freshly prepared films (grey bars). NGF on the film surfaces affects the ratios of C_{1s} and N_{1s}.

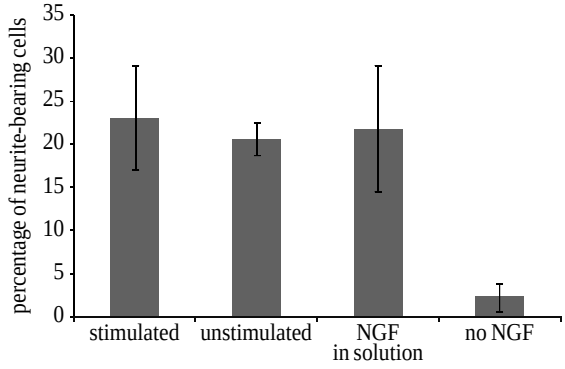

Figure 7. *In vitro* PC12 cell culture on NGF-immobilized PPy-NSE_{50} films after applying an external electrical potential. NGF-PPy-NSE_{50} films were stimulated with an electrical potential of −1.0 V (versus SCE) for 5 min, followed by washing and PC12 cell culture. Neurotrophic activity (percentage of neurite-bearing cells) of the stimulated NGF-PPy-NSE_{50} was similar to the two positive controls, (i) freshly prepared unstimulated NGF-PPy-NSE_{50} and (ii) PPy-NSE_{50} with exogenous NGF (50 ng ml^{-1} in medium), but significantly different from the negative control of PPy-NSE_{50} without NGF ($p<0.01$). Experiments were performed in triplicate.

electrochemical synthesis of PPy (Hodgson *et al.* 1996; Kim *et al.* 2007; Richardson *et al.* 2007) and coupling biotinylated NGF on avidin-treated biotin-doped PPy films (George *et al.* 2006). These non-conjugation methods showed good conductivities and controllable NGF release profiles in response to an external electrical stimulation to induce neurite formation from PC12 cells. However, the NGF is released and consumed gradually after successive electrical stimulations.

Therefore, for long-term applications, chemical conjugation approaches appear reasonable because chemically immobilized NGF exhibited neurotrophic activities on various materials, such as polystyrene sulphonate (PSS)-doped PPy films and poly(2-hydroxyethylmethacrylate) (PHEMA) gels coupled with NGF using the arylazido-polyallylamine (azido-PAA) photocrosslinkers (Kapur & Shoichet 2003; Gomez & Schmidt 2007) and glutaraldehyde-activated beads (Naka *et al.* 2004). Although non-specific photocrosslinkers can be employed to immobilize most organic substrates, this technique can disrupt neurotrophin activities and conductive polymer surfaces as well (Kapur & Shoichet 2003). Thus, the functionalized PPy described here can be a good option to specifically conjugate NGF under relatively simple and mild conditions. One obstacle is that pyrrole derivatives, particularly *N*-substituted pyrrole derivatives, hinder the structural planarity of π-electron conjugation in PPy, which results in a severe decrease in conductivity (Nalwa 1997; Skotheim *et al.* 1998; Lee *et al.* 2006; Guimard *et al.* 2007). This drastic drop in conductivity of pyrrole derivatives can be mitigated to some extent by synthesizing copolymers of pristine pyrrole and pyrrole derivatives that exhibit a reasonable conductivity and significant reactive functional groups (i.e. *N*-succinimidyl ester groups) on the surface. In this study, the PPy-NSE_{50} copolymer still had a reasonably good conductivity comparable with other techniques (table 2) and better than common functionalized PPy and large molecule-doped PPy. Also, bulk conductivities of the PPy can be further improved when

Table 2. Various techniques and properties of neurotrophin-incorporated conducting polymers. (Other modification techniques for peptides/proteins could be theoretically used to immobilize neurotrophin(s), such as affinity peptide-mediated modification (Sanghvi *et al.* 2005), covalent coupling on dopants (Song *et al.* 2006) and plasma treatment (Kang *et al.* 1997).)

techniques	advantages	disadvantages	conductivity[a]	neurotrophin	reference(s)
non-covalent techniques					
doping/entrapping	simple electrically controllable release of neurotrophin(s) may be desired for certain applications	requires high concentration of neurotrophin for synthesis	dependent on doping levels of neurotrophin[b]	NGF	Hodgson *et al.* (1996) and Kim *et al.* (2007)
		biomolecule can be released spontaneously and with electrical stimulation, so that material may not be suitable for long-term use		NT-3	Richardson *et al.* (2007)
affinity binding to a dopant (biotin)	simple specific conjugation electrically controllable release of neurotrophin(s)	multiple steps requires neurotrophin modification biomolecule is consumed with electrical stimulation (not suitable for long-term use)	dependent on doping levels of biotin[c]	biotinylated NGF	George *et al.* (2006)
covalent techniques					
conjugation using radical linkers[d]	applicable to most organic polymers minimized loss of bulk conductivity stable and suitable for long-term applications	complicated, multiple steps non-specific reaction impairing ligand activities and surface properties of conducting substrates	9.3 S cm^{-1}	NGF	Gomez & Schmidt (2007)
conjugation on functionalized polypyrrole(s)	simple, specific conjugation of ligand various controllable properties by copolymerization stable links for long-term applications	decrease in conductivity depending on polymer composition requires synthesis of pyrrole derivatives	8.1 S cm^{-1}[e]	NGF	this study

[a]Conductivities of regular PPy are in a range of 5–200 S cm^{-1} depending on dopant types and polymerization conditions (Guimard *et al.* 2007; Fonner *et al.* 2008).
[b]Conductivity was not directly measured. Impedances of NGF-doped PPy varied with polymerization time and showed higher impedances compared with regular PPy (Kim *et al.* 2007). High-level doping of macromolecules is reported to result in a severe conductivity drop (Guimard *et al.* 2007). For example, hyaluronic acid-doped PPy displayed a conductivity of 3.1×10^{-3} S cm^{-1} (Collier *et al.* 2000).
[c]Electrical property of the materials was not measured.
[d]Azido compound (azido-modified poly(allylamine)) was used as a photocrosslinker to immobilize NGF on PPy doped with PSS.
[e]The value (8.1 S cm^{-1}) was measured from the copolymer of PPy-NSE_{50}, which was doped with perchlorate (ClO_4^-).

synthesizing layer-by-layer structures, where the PPy-NSE copolymer is deposited on top of a more conductive regular PPy layer (i.e. PPy-NSE_0).

A copolymer of *N*-succinimidyl ester pyrrole can be used to simply immobilize biomolecules so that amine-bearing compounds could be directly conjugated onto the PPy surface in physiological conditions without an additional activation step. PPy-NSE polymers and copolymers were reported to bind human serum albumin, BSA and drug molecules (Azioune *et al.* 2004; Bousalem *et al.* 2004; Khan *et al.* 2006).

Our preliminary studies showed that PC12 cells exhibited maximum neurite outgrowth at a concentration of 50 ng ml^{-1} soluble NGF in medium; this condition was used as a positive control to compare with the effects of NGF-immobilized PPy-NSE_{50} and PPy-NSE_0 sample films (figure 5). The results demonstrate that neurotrophic activity on PPy-NSE_{50} resulted from NGF immobilized via active ester groups, not as a result of physically adsorbed NGF. NGF-immobilized substrates showed neurotrophic activity similar to exogenous NGF in medium.

Immobilized NGF on PPy-NSE_{50} showed good stability at physiological conditions and even to electrical potential. We assumed that some of the NGF on the PPy-NSE_{50} films would be attributed to physical adsorption that might be leached into the medium. However, ELISA analysis indicated that NGF was not detected after 5 days of incubation of NGF-conjugated PPy-NSE_{50} films in PBS buffer at 37°C. Furthermore, the XPS analysis indicated no significant change in the ratio of C_{1s} and N_{1s} after incubation. These results suggest a negligible release of NGF from the PPy-NSE_{50} films. Besides leaching and elemental analysis in this paper, cell culture on the incubated sample will be required in future work to confirm the long-term stability of NGF activity on the surface.

Stable activity of immobilized NGF to electrical potentials is also critical to provide neurotrophic activities with electrical cues (George *et al.* 2006). Non-covalently incorporated neurotrophins were released not only in the manner of passive diffusion into medium but also responding to changes in an external electrical potential (Maddison & Jenden 1992; Guimard *et al.* 2007; Richardson *et al.* 2007). Hodgson *et al.* were able to release NGF in the medium by applying −0.7 or −0.85 V for 3 min, resulting in neurite outgrowth of PC12 cells. Similarly, reduction of NGF-incorporated PPy films caused a quick release of NGF up to 10 μg ml^{-1} into medium within 150 s. On the other hand, our results show that NGF-PPy-NSE_{50} films did not release a significant level of NGF into the medium in response to a constant potential (−1 V) for 5 min. More importantly, these electrically stimulated NGF-PPy-NSE_{50} films maintained the activities to induce neurite formation of PC12 cells as much as non-stimulated films, suggesting that the immobilized NGF on the PPy-NSE_{50} films was stable on the substrates to electrical potentials and thus useful to provide long-term neurotrophic activities with simultaneous electrical stimulation. Together, these data suggest that chemical coupling with amide bond formation is stable and suitable for long-term applications with electrical stimulation as multifunctional tissue engineering scaffolds.

In conclusion, we successfully introduced neurotrophic factors onto active ester-functionalized PPy polymers and maintained good electrical conductivity. We have shown that polypyrrole films tailored with NGF are stable and thus have the potential to integrate electrical and biochemical cues for nerve tissue engineering scaffolds.

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