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In Situ Synthesis of Conductive Polypyrrole on Electrospun Cellulose Nanofibers: Scaffold for Neural Tissue Engineering

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Abstract

This study reports the synthesis of conductive polypyrrole (PPy) on electrospun cellulose nanofibers. The cellulose nanofibers were electrospun via cellulose acetate and surface modified using in situ pyrrole polymerization. PPy adhered to the cellulose nanofiber surface as small particles and caused a 10^5 fold increase in conductivity compared to unmodified cellulose nanofibers. In addition, tests revealed no cytotoxic potential for the PPy coated cellulose nanofiber materials. In vitro culturing using SH-SY5Y human neuroblastoma cells indicated enhanced cell adhesion on the PPy coated cellulose material. SHSY5Y cell viability was evident up to 15 days of differentiation and cells adhered to the PPy coated cellulose nanofibers and altered their morphology to a more neuron like phenotype.

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Keywords Cellulose, Fiber, Polypyrrole, Electrospinning, Tissue Engineering

Introduction

In the research field of tissue engineering the role of the scaffold is to mimic the extra cellular matrix (ECM) until cells can repopulate and synthesize a new cell specific ECM. An ideal scaffold must therefore be biocompatible and preferably degradable, while degradation products must be non-toxic for the cells. The micro structure of the tissue engineering scaffold should have a porosity that favors cell survival and that also allows for the diffusion of growth factors to cells (Yang et al. 2001). Electrospun nanofiber materials have been used to successfully mimic ECM in tissue engineering scaffolds (Li et al. 2002). A wide range of natural and synthetic polymers can be electrospun, and since electrospinning is a simple method for generating ultrathin nanofibers, it has become a useful technique for tissue engineering scaffold production (Pham et al. 2006). For instance, aligned electrospun fibers have recently been shown to guide tumor cells (Jain et al. 2014).

Cellulose is a natural linear polysaccharide of β (1→4)-D-glucopyranose units and the load-bearing component of plant cell walls. Native cellulose is a biocompatible polymer (Miyamoto et al. 1989; Klemm et al. 2001; Helenius et al. 2006) and can be electrospun directly from ionic liquid solvents (Härdelin et al. 2012) or LiCl/Dimethylacetamide (He et al. 2014). A more efficient manufacturing process is to electrospin cellulose from cellulose acetate with subsequent deacetylation (Liu and Hsieh 2002). Recently, tissue engineering scaffolds of electrospun cellulose fiber networks have been used to culture cells, both on unmodified cellulose (Jia et al. 2013; He et al. 2014) and on surface modified cellulose (Rodríguez et al. 2011). In addition to the micro structure, cells are influenced by the surface chemistry of the scaffold. Therefore, surface modified and composite fibers have been electrospun to enhance cell attachment and proliferation on scaffolds (Grafahrend et al. 2011; Wang et al. 2013).

Polypyrrole (PPy) is a conductive polymer that has been thoroughly investigated for biological applications. PPy has the advantage of being highly electrically conductive, biocompatible and easily synthesized from pyrrole monomer (Bendrea et al. 2011). The driving force for making the surface of tissue engineering scaffolds conductive using PPy is that neural cells can respond to electrical stimulation, which can promote cell differentiation and neurite growth (Schmidt et al. 1997; Guimard et al. 2007; Liu et al. 2009). Electric stimulation thereby can promote nerve growth in the scaffold which has made PPy very interesting for the manufacture of tissue engineering scaffolds. For neural tissue engineering purposes, PPy has been previously synthesized on the surface of electrospun nanofiber of poly lactic-co-glycolic acid (Lee et al. 2009). Furthermore, PPy has been synthesized on the surface of porous celluloses stemming from algae and wood. These nanofibrillated cellulose PPy composites were created mainly for application in energy storage devices (Nyström et al. 2010; Razaq et al. 2012; Carlsson et al. 2012; Wang et al. 2014). However, very recent studies have reported the use of porous cellulose/PPy materials as neural tissue engineering scaffolds (Muller et al. 2013; Shi et al. 2014). Muller et al. (2013) synthesized PPy

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particles on the surface of bacterial nanocellulose scaffolds and reported an increase in cell adhesion and growth of PC12 neural cells for their PPy loaded scaffold. However, they suggest a more rigorous evaluation of the effect on neural cells by the PPy. Shi et al. (2014) produced tissue engineering scaffolds from porous cellulose precipitated from the NaOH/Urea aqueous solvent system. Pyrrole monomer was then *in situ* synthesized as nanoparticles on the cellulose surface, which made the scaffolds conductive and stiff but also caused a severe loss of bulk porosity. Furthermore, Shi et al. cultured PC12 cells on the scaffold surface with electrical stimulation and evaluated after 6 days. The apparent increase in neurite growth was attributed to electrical stimulation. This study reports the use of *in situ* synthesized PPy on electrospun cellulose nanofibers for neural tissue engineering. Electrospun cellulose scaffolds were used since their structure mimic an ECM and -SY5Y human neuroblastoma cells were used to evaluate the scaffolds for neural tissue engineering.

Materials and Methods

Chemicals

N,N-Dimethylacetamide (DMAc) (99.8%), pyrrole (98%), iron(III) chloride, sodium hydroxide and cellulose acetate with an acetyl content of 39.8 %wt and M_n of 30,000 was obtained from Sigma-Aldrich Co. and pyrrole was distilled before use. Acetone (Fischer Scientific) was 99.98 % and ethanol (Solveco) was 99.7%.

Electrospinning

Cellulose nanofibers were fabricated at constant temperature of 20 °C and constant relative humidity of 65%. The electrospinning equipment consisted of a high voltage power supply, a NE-1000 syringe pump and a 10 ml syringe connected to a blunt-nozzle stainless steel needle. For collection of the fibermats a 2.5 cm wide cylindrical (10 cm diameter) grounded collector rotating at 25 rpm was used. The distance between the needle and the collector was 15 cm. In a typical electrospinning procedure, 18 %wt. cellulose acetate was dissolved in a solvent mixture of DMAc:acetone, with a volume fraction of 11:14. The cellulose acetate solution was then electrospun for 3 h with a feed rate of 0.350 mL/hour and an applied voltage of 18-20 kV. The resulting cellulose acetate nanofiber mats were dried in 80 °C and then immersed in 0.05 M NaOH in ethanol over night to hydrolyze the acetyl groups in order to generate cellulose. After deacetylation the cellulose nanofiber mats were washed thoroughly with deionized water to remove sodium and acetate ions.

Polypyrrole synthesis

Three cellulose/PPy electrospun nanofiber materials were created with different loadings of PPy. The electrospun cellulose fiber mats were immersed for 3 h in a 0.1 M HCl solution containing 0.05 M, 0.15 M or 0.45 M pyrrole. Then each of the pyrrole soaked cellulose fiber mats were

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transferred to a 0.1 M HCl solution solution containing 0.120 M, 0.360 M or 1.08 M $FeCl_3$. Pyrrole was allowed to polymerize for 2 h at 5°C and then washed thoroughly with deionized water after polymerization to remove excess ions. Washing of the nanofibers mats was stopped when the conductivity of the wash water reached below 0.25 mS/cm. The color of the nanofiber mats changed from white to black during PPy synthesis. The fiber mats were then punched out to 8 mm diameter round scaffolds.

SH-SY5Y Cells

Human neuroblastoma cells (SH-SY5Y) obtained from Health Protections Agency Culture Collections (HPACC) was used in this study. The culture medium was composed of equal volumes of minimum essential medium (MEM, Life Technologies) and F-12 Nutrient Mixture (Life Technologies), supplemented with 1% non-essential amino acids (NEA, PAA Laboratories), 1% L-glutamine (PAA Laboratories), 1% antibiotic - antimiotic (Life Technologies) as well as 15% heat inactivated fetal bovine serum (Hyclone). For differentiation purposes, the same medium was used except for the following modifications: The fetal bovine serum concentration was lowered to 1% while 10 µM retinoic acid (Sigma Aldrich) were added to the mixture. The cells were cultured in 48-well culture plates with a seeding density of 100.000 cells per well (95 000 cells/cm^2). The cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. After 48 h, the differentiation was initiated. Three batches of cells were used during this study and passaging of cells was never beyond passage 27. The cells were differentiated for 5, 10 and 15 days. Medium was changed 3 times per week. Prior to SEM imaging, the medium was removed from the samples which were thereafter rinsed twice with PBS and then fixed in 2% glutaraldehyde for 1 h. The samples were then rinsed in PBS and dehydrated by passing the scaffolds with cells through increasing ethanol concentrations 60%, 70%, 80%, 90% and 100% with 30 minutes in each step. Samples were then dried in ambient conditions.

Characterization

Water contact angles were measured using sessile droplet technique on an Attension Theta contact angle meter (Biolin Scientific). Contact angles were calculated using the software integrated in the instrumentation

The electrical conductivity measurements were performed using a two-point probe system (Parameter Analyzer-Keithley 4200-SCS). The scaffolds were not thicker than 0.25 mm and therefore regarded as two-dimensional structures.

X-ray photoelectron spectroscopy (XPS) was performed with the Quantum 2000 scanning XPS microprobe from Physical Electronics. An Al Ka (1486.6 eV) X-ray source was used and the beam size was 100 µm. The analyzed area was approximately 500×500 μm^2 with a depth of 4-5 nm. Results were evaluated using MultiPAK 6.0 software.

Micro structural investigations were performed using Scanning Electron Microscopy (SEM) with a LEO Ultra 55 FEG SEM. The SEM was operated at an acceleration voltage of 1.5- 3.0 kV and all samples were gold sputtered in a vacuum for 80 seconds at 10 mA

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Atomic force microscopy (AFM) was used to determine fiber surface morphology. The microscope used was a Digital Instrument Nanoscope IIIa with a type G scanner (Digital Instrument Inc.) The cantilever used was a Micro Masch silicon cantilever NSC 15. The measurements were performed in air using tapping mode.

A test was performed according to ISO standard 10993-5:2009(E) Annex C to analyze the cytotoxicity of the electrospun cellulose/PPy nanofiber materials. A detailed description of the experimental procedure is available in the Electronic Supplementary Material (ESM).

Results

Surface chemistry of cellulose/PPy nanofibers

Table 1 Summary of results of the characterization of electrospun scaffold materials

Material Sample	PPy conc.[a] [M]	Elemental composition (XPS)				Electrical Conductivity [S/cm]	Water Contact angle[b] [º]
		C%	N%	O%	Cl%		
Cellulose	-	54.53	-	45.47	-	7.8×10^{-7}	14.6
Cellulose/PPy 0.05	0.05	62.67	7.31	29.00	1.01	5.7×10^{-2}	10.6
Cellulose/PPy 0.15	0.15	62.69	7.82	28.95	0.54	2.6×10^{-4}	15.5
Cellulose/PPy 0.45	0.45	66.31	14.45	18.70	0.55	5.2×10^{-4}	22.6

[a] Pyrrole concentration used in the *in situ* polymerization. [b] Advancing contact angle.

Three cellulose/PPy electrospun nanofiber materials were created with different loadings of PPy. These materials were analyzed with XPS, conductivity measurements, water contact angle and the results are summarized in Table 1. The element compositions are extracted from XPS measurements. Hydrogen is not detectable by XPS and in Table 1, the entry for pure cellulose nanofiber material have only values for carbon and oxygen. The carbon to oxygen ratio corresponds to anhydrous glucose, which is the smallest repeating unit in cellulose. The effect of pyrrole polymerization can be seen in the appearance of nitrogen and chlorine peaks in the XPS spectra. PPy is a nitrogen and carbon containing polymer and as anticipated, both the nitrogen and carbon content increased as the concentration of reactants was increased. Full XPS spectra and resolved C1s peaks are available in the ESM. In Figure S1 four peaks are resolved C-C (284.4 eV), C-O and C-N (285.8 eV), O-C-O (287.2 eV) and O-C=O (288.6 eV) (Beamson and Briggs 1992). For the cellulose sample the C-O peak is dominant and the C-C peak is clearly present. The cellulose/PPy samples show a clear increase in the C-C peak compared to cellulose, indicating a change in surface composition.

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In order to be conductive, PPy must be in its oxidized state and have anionic counter ions present. In this study chloride ions were present at the polymerization and served as anionic counter ions. A rise in electrical conductivity can be seen in cellulose/PPy nanofiber materials compared to the unmodified cellulose nanofiber material (Table 1). The increase in conductivity for the cellulose/PPy nanofiber materials confirmed the presence of conductive PPy at the fiber surface. The material synthesized with the lowest concentration of pyrrole, 0.05 M, displayed a 10^5 fold increase in conductivity compared to unmodified cellulose nanofibers. This material also had the highest conductivity and its chlorine content was also the highest with 1%, which indicate that this material had the highest amount of oxidized conductive PPy present at the nanofiber surface, consequently giving this material the highest conductivity. Both the cellulose nanofiber material and the cellulose/PPy nanofiber materials were very hydrophilic and wettable by water. The porous structure of electrospun cellulosic materials together with the hydrophilic surface property made materials instantly wettable by water. The water contact angles presented in Table 1 are therefore advancing contact angles measured immediately after the droplet was placed on the material. Advancing contact angles for PPy modified nanofibers ranged from 10.6º to 22.6º and the surfaces can therefore be considered to be highly hydrophilic.

Microstructure of cellulose/PPy nanofibers

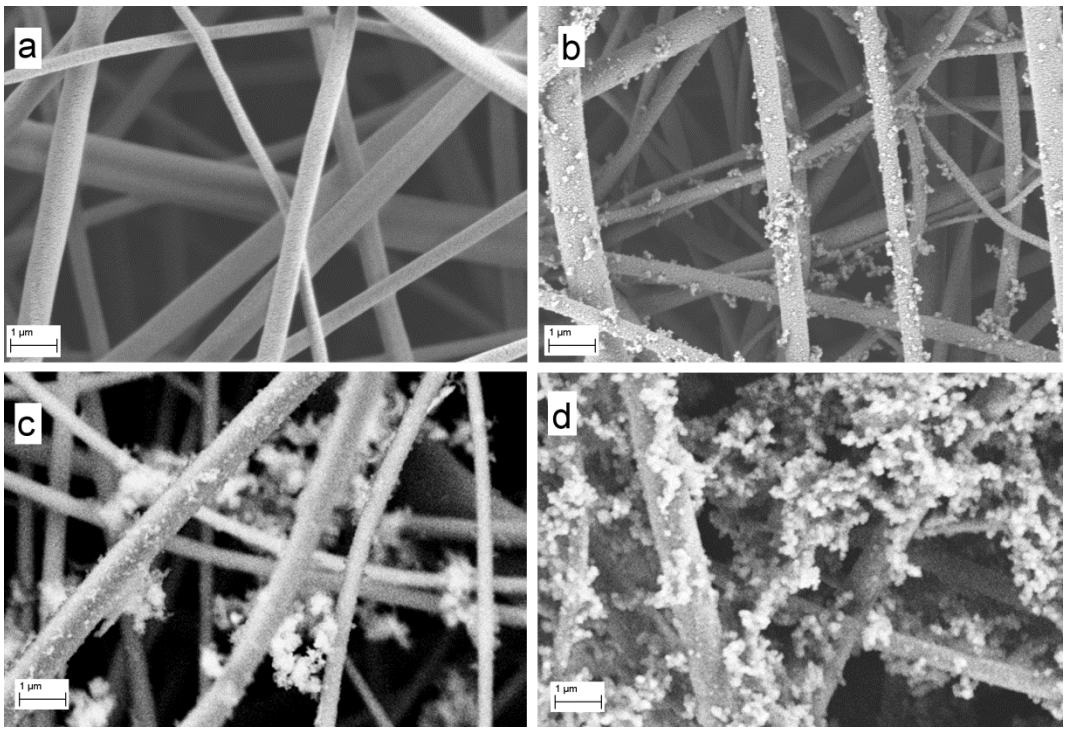

Fig. 1 SEM images of (a) electrospun cellulose nanofiber, (b) cellulose/PPy 0.05 nanofiber, (c) cellulose/PPy 0.15 nanofiber and (d) cellulose/PPy 0.45 nanofiber. Scale bars are 1 µm

SEM images of the electrospun cellulose nanofibers revealed that their diameter ranged between 300 nm and 1500 nm. Furthermore, SEM images of the cellulose/PPy scaffolds show PPy particles adhering to the fiber surface (Figure 1). The Cellulose/PPy 0.05 and the Cellulose/ PPy 0.15

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nanofiber materials are similar and have mostly small PPy particles adhering to the nanofiber surface. The cellulose/PPy 0.45 material on the other hand, has much more PPy particles visible in the fiber network. These PPy particles seem to cluster together and reduce the porosity of the overall nanofiber material. High resolution AFM images of single electrospun nanofibers of cellulose and cellulose/PPy 0.05 are available in the ESM, Figure S2. The unmodified cellulose nanofiber has a very smooth surface, while nano sized PPy particles seem to cover the whole Cellulose/PPy 0.05 fiber surface, consequently making the surface rougher. In addition, PPy particles seem to be evenly distributed on the nanofiber surface, providing continuous conductive material.

Cytotoxicity of cellulose/PPy nanofiber scaffolds

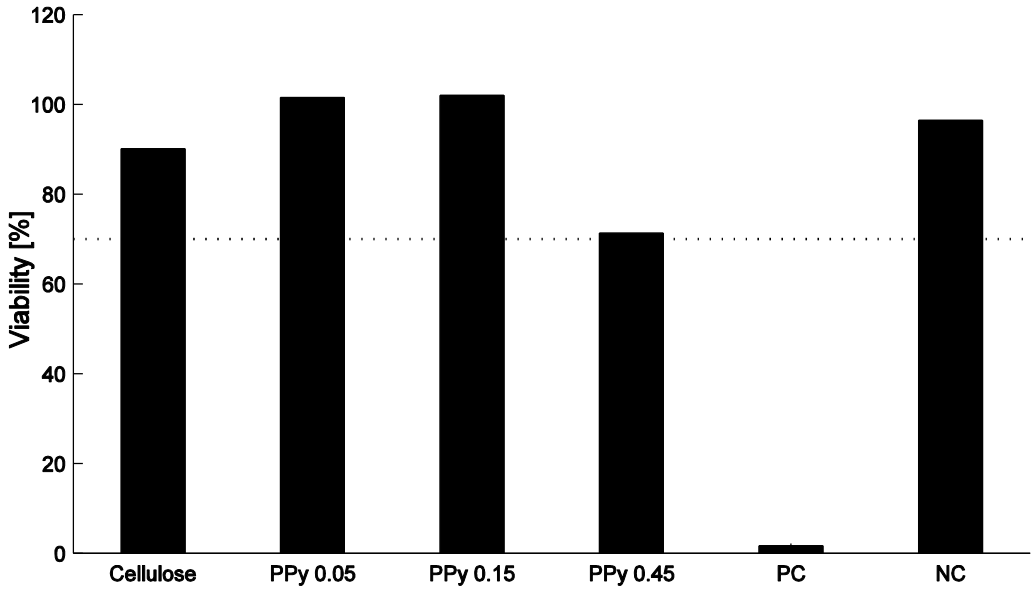

Fig. 2 Cytotoxicity analysis of PPy modification of electrospun cellulose. None of the three cellulose/PPy materials were below the threshold of the analysis (<70% viability) although 0.45 were just above the threshold (71.3%), showing that PPy modification of cellulose does not cause cytotoxicity. Positive control (PC) known to cause cytotoxicity and cell death and negative control (NC) with no harmful effects were used as quality control of the assay.

Prior to the neural cell study, a cytotoxicity analysis was performed in accordance with ISO standard to investigate potential cytotoxicity of the cellulose/PPy nanofiber materials. This analysis answers the question if the material is harmful or release compounds harmful to cells, i.e. has a cytotoxic potential. The viability of cells exposed to the material is compared to unexposed cells and presented as viability in percentage. A value below 70 % means that the material has cytotoxic potential, according to the ISO standard. To ensure quality control of the analysis, positive (a cytotoxic compound that will decrease cell viability, in this case DMSO) and negative (a safe compound, in this case the lid of a test tube) controls are added. The results of the cytotoxicity test are shown in Figure 2. None of the cellulose/PPy nanofiber materials developed in this study showed cytotoxic potential although the cellulose/PPy 0.45 was close to the threshold.

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Neither cellulose/PPy 0.05 or 0.15 had any effect on cell viability. In light of the cell viability analysis, and due to the similarity of the cellulose/PPy 0.05 and cellulose/PPy 0.15 nanofiber scaffolds, the cellulose/PPy 0.05 nanofiber scaffold was chosen as the most suitable material for cell culture experiments.

SH-SY5Y Cell Study

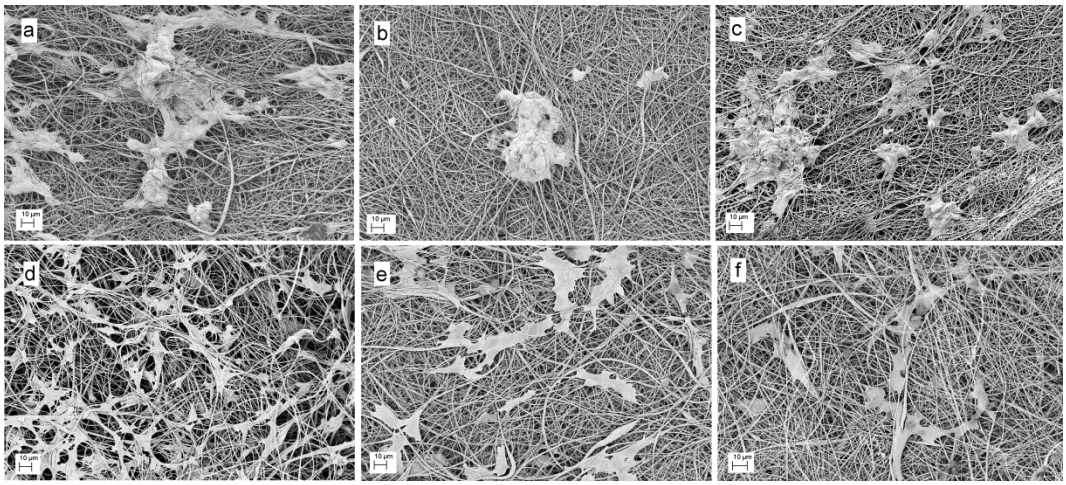

Fig. 3 SEM images of SH-SY5Y cells differentiated on cellulose nanofibers (a) day 5, (b) day 10, (c) day 15; cellulose/PPy 0.05 nanofibers (d) day 5, (e) day 10, (f) day 15. Scale bars are 10µm

The cellulose/PPy 0.05 scaffold material was deemed best suited for neural cell culture study since it had the highest conductivity and no cytotoxic potential. SH-SY5Y cells were seeded on the scaffolds and after 48 hours of culture, differentiation was initiated by medium change. SEM images of SH-SY5Y cell morphology at the three time points are shown in Figure 4. SEM images of higher magnification are available in the ESM (Figure S3 and S4). After 5 days of differentiation, cells on the unmodified cellulose nanofiber scaffold had a tendency to form aggregates and to attach to each other. The same phenomenon was observed on day 10 and day 15. On the cellulose/PPy scaffolds, SH-SY5Y cells have a more even distribution on the nanofibers scaffold, which could indicate improved cell adhesion. On day 10 and 15 the cells continue to exhibit integration on the cellulose/PPy scaffolds. The morphological characteristics of SH-SY5Y also differ on the two different materials. Cells on the unmodified cellulose nanofiber scaffold had a spherical shape, while the morphological characteristics point towards a more neuron-like phenotype on the cellulose/PPy scaffolds.

Discussion

The cellulose/PPy 0.05 scaffold material had the highest conductivity. This material was synthesized with the lowest concentration of pyrrole (0.05 M) and $FeCl_3$ (0.12 M), the concentration ratio of pyrrole and $FeCl_3$ was kept constant to avoid effects from polymerization efficiency in the synthesis of the cellulose/PPy materials. A $FeCl_3$ concentration above 0.1 M has been shown to cause over-oxidation of PPy, which reduce conductivity (Kaynak and Beltran

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2003). The cellulose/PPy materials polymerized with high pyrrole and $FeCl_3$ concentrations may suffer the drop in conductivity due to over-oxidation. Also, the cellulose/PPy 0.05 material had the highest content of chlorine, which indicates that this material contained the highest content of oxidized conductive PPy. It has been shown that chloride ions can diffuse out of PPy in water (Fonner et al. 2008). It is possible that the washing step after PPy synthesis on cellulose nanofibers might have influenced the chlorine content.

Scaffolds designed for neural tissue engineering are expected to mimic the electrical properties of nerves and the primary reason for growing neural cells on conductive substrates is that electrical stimulation can be used to enhance neurite growth in neural cells (Schmidt et al. 1997; Guimard et al. 2007; Liu et al. 2009). The SH-SY5Y cell line (Biedler et al. 1973) is widely used in neuroscience research and can be differentiated into several neural cell phenotypes (Pahlman et al. 1995; Das et al. 2012). It is also characterized with a high proliferation capacity and a homogeneous cell population (Selinummi et al. 2006). In order to evaluate the applicability of electrospun cellulose/PPy nanofibers as neural tissue engineering scaffolds, we used an incubation time of 15 days and evaluated samples at three different differentiation time points. Cell viability was evident up to 15 days of differentiation and the cells seemed to adhere to the PPy coated nanofibers and differentiated to a more neuron-like phenotype, while the cells on unmodified cellulose nanofiber scaffold had a tendency to form aggregates and to adhere to each other. The mechanism behind the differences in cell adhesion and cell morphology in the PPy coated scaffolds is however unclear.

The cytotoxicity analysis revealed high cell viability for both the cellulose/PPy scaffolds and the unmodified cellulose, which indicates that both materials were non-toxic to cells. Also, the apparent water wettability of the two nanofiber materials was very similar and high, which indicate that hydrophilic nature of the two materials are the same.

Pyrrole was evenly polymerized on the cellulose nanofibers resulting into a continuous conductive material. The nano-scale surface roughness induced by the PPy coating could favor cell attachment to the nanofibers since surface roughness favors cell adhesion (Fonner et al. 2008). Recently, it has also been shown that tumor cells have adhesion preference to nano rough surfaces compared to smooth surfaces (Chen et al. 2013).

Amine containing polysaccharides like chitosan have been show to be suitable for neural tissue engineering (Prabhakaran et al. 2008, Cooper et al. 2011). The synthesis of PPy introduced amine groups to the nanofibers surface, which could have influenced the neural cell performance.

The passive conductive property of substrates for neural tissue engineering have been studied by Malarkey et al. (2009), who compared neural cell growth on films with similar roughness but with different conductivity. Films substrates of certain conductivity (0.3 S/cm) slightly promoted neurite outgrowth. The conductivity of cellulose/PPy 0.05 scaffold material (0.057 S/cm) is unlikely to have an effect on neural cell performance.

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Conclusions

PPy was successfully in situ synthesized on the surface of electrospun cellulose nanofibers which rendered a conductive material mimicking an ECM. The conductivity and the microstructure were affected by the synthesis parameters used. SEM and AFM showed that PPy adhered to the nanofiber surface as small particles, which increased the surface roughness of the nanofibers. The non-toxic property of electrospun cellulose was retained after PPy synthesis. Neural cell culture experiment indicated that PPy enhanced SH-SY5Y cell adhesion and on electrospun cellulose/PPy nanofiber scaffolds. SHSY5Y cell viability was evident up to 15 days of differentiation in the cellulose/PPy scaffolds, which opens up possibilities for this cellulose based material to be utilized as neural tissue engineering scaffolds.

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