Preparation and Characterization of Conductive Chitosan/Polypyrrole Composites for Neural Repair

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Abstract. Electrical stimulation showed ability to promote the proliferation and differentiation of neurons. Thus, conducting polymers such as chitosan/polypyrrole (CS/PPY) composites can be a potential material for neural repair. To improve the preparation processes and biological activity of CS/PPY composites, a novel "one step" method was proposed in the present study. Polypyrrole (PPY) nanoparticles were synthesized in chitosan (CS) solution directly. FeCl3 was used as the oxidant and dopant with a mole ratio of 4:1 to pyrrole (PY). In the Fourier Transform infrared spectroscopy (FT-IR) spectrum of CS/PPY, the two bands at 1544 cm−1 and 1040 cm−1 are characteristic PPY peaks. The scanning electron microscope (SEM) results showed that the PPY nanoparticles were distributed uniformly in CS films. When the weight ratio of PY to CS was 20%, the conductivity of the composite films was 0.51 mS/cm. The CS/PPY film adsorbed more protein than the pure CS film. The results of cell viability assay showed that the composite had good biocompatibility with PC-12 cells. The "one step" method can improve the preparation processes of CS/PPY composites and provide a promising potential material for neural tissue engineering.

1. Introduction
Since neurons transmit signal through electrical activities, recent studies showed that electrical stimulus can improve cell viability and neural tissue repair, including dynamic changes of cytoskeleton [1], axonal growth [2] and nerve injury repair [3]. So conductive materials are used more widely in the field of neural tissue repair [4]. Compared with electrical stimulus itself, conductive materials benefit for cell growth and adhesion, especially for the guidance of axonal growth [5]. Conductive materials can build a microenvironment in the injured site [6], as well as combining growth factors or drugs together to control the release of them [7]. Therefore, conductive materials play crucial role on induction of stem cell differentiation and repair of nerve injury.

Among all kinds of conductive biomaterials, composites of polymers (poly-lactic acid, chitosan, gelatin, etc.) [8, 9] and conductive materials (polypyrrole, polyaniline, carbon nanotube, graphene, etc.) [10, 11] showed good biocompatibility and the ability of regulating cellular functions, such as adhesion, proliferation, differentiation, migration, DNA syntheses and protein secretion [12]. When manufacturing the composite, a traditional method is to prepare the conductive materials first, eg.
polypyrrole, then mix the polymer and conductive material together [3, 13]. The drawback of this method is that the conductive materials are hard to disperse evenly. Another convenient choice is to synthesize conductive material in the polymer solution [14], which takes only “one step” because the synthesis and the mixing are performed at the same time. However, since surfactants are usually used in this process to keep conductive nanoparticles stable, oil-soluble polymers such as poly lactic acid (PLA) are more suitable [14], few studies were reported using water-soluble polymers.

In the present study, the preparation of the composite of water-soluble polymer (chitosan) and conductive material (polypyrrole) using “one step” method was studied. Polypyrrole (PPY) nanoparticles were synthesized in chitosan (CS) solution directly. FeCl$_3$ was used as the oxidant and dopant. After the composite films were made, Fourier Transform infrared spectroscopy (FT-IR) and scanning electron microscope (SEM) were used to characterize the properties of the composite, 4-point probe method was adopted to test the conductivity of the films, protein adsorption and cell viability assays were performed to value the biocompatibility of different samples. It was assumed that the product from this simple “one step” method with no surfactant and no cross-linker would be a potential safe choice for neural tissue engineering.

2. Materials and Methods

2.1. Materials

Chitosan (CS), pyrrole (PY) and FeCl$_3$ were purchased from Aladdin (Shanghai, China). The PC-12 cell line was provided by the American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were procured from Gibco (Invitrogen, Grand Island, NY, USA). Penicillin-streptomycin and trypsin were bought from HyClone (Logan, UT, USA). Cell Counting Kit-8 (CCK-8) was acquired from Dojindo (Kumamoto, Japan). BCA protein assay kit was purchased from Beyotime (Shanghai, China). The other chemicals were overall supplied by Bopet Biotech Company (Chongqing, China).

2.2. Synthesis of Chitosan-Polypyrrole Composite

2% CS solution was prepared in 1% acetic acid at 40 °C. After cooling down to room temperature, 10% or 20% PY (PY/CS) was added to the CS solution stirring for 30 min. Pre-dissolved FeCl$_3$ solution was dropped to the above solution slowly with strong stirring. This mixed solution was stirred for 24 h at room temperature and then poured to a clean plate. The chitosan/polypyrrole (CS/PPY) films were collected after the samples were dried in a drying oven. The pure CS film was prepared using 2% CS solution. The same amount of PY as in CS/PPY solution was dissolved in deionized water to synthesize pure PPY nanoparticles.

2.3. Characterization Techniques

2.3.1. Fourier Transform Infrared Spectrum. Fourier transform infrared (FT-IR) spectra were adopted to determine the reactions between chitosan and polypyrrole (PPY). The powder of PPY was packed for FT-IR spectroscopy. The films of CS and CS/PPY were placed on the probe for FT-IR spectroscopy. The FT-IR spectra were examined in the range of 800-2000 cm$^{-1}$ under a Nicolet iS50 FT-IR spectrometer (Thermo Electron Corp., Madison, WI, USA).

2.3.2. Scanning Electron Microscope. Surface morphology was characterized by scanning electron microscope (SEM). Prior to observations, samples were gold-coated to inhibit electron entrapment surface charging. SEM images were recorded on a S-4800 analytical electron microscope (Hitachi, Tokyo, Japan).
2.3.3. Conductivity Test. The conductivity of CS, PPY and CS/PPY samples was tested by a RTS-8 4-point probe measurement system (4probes Tech, Guangzhou, China). The samples were placed on the sample stage and touched by the 4 point probes to test the conductivity.

2.3.4. Protein Adsorption Measurement. Films were cut into small round pieces of same area. Each piece was placed in one 0.65ml centrifuge tube. 0.1 mg/ml bovine serum albumin (BSA) solution were added to CS and CS/PPY samples and incubated at 37°C for 4 h. After the adsorption, the amount of the protein left in the solution was measured by the BCA protein assay kit (Beyotime).

2.3.5. Cell Culture. PC-12 cells were cultured in the cell-culture incubator with high glucose DMEM/F12 and 5% CO₂ at 37°C. The DMEM contained 10% FBS, 2 mmol/L L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Before cell seeding, the films of CS or CS/PPY were placed in the plates and then sterilized under ultraviolet light for 1.5 h. Cells at a predetermined density were added successively to the plates.

2.3.6. Cell Viability. The CCK-8 kit was adopted to assess the in vitro cytotoxicity of CS and CS/PPY films in PC-12 cells. After the sterilization of the films, the cells were placed in a 96-well plate at a density of 1000 cells/well and incubated for 24 h. CCK-8 solution (5 mg/ml in PBS solution) was added to the cells. After being treated for 2 h at 37°C, the absorbance of each well at 450 nm was screened with a microplate reader (Multiskan GO, Thermo Scientific, Waltham, MA, USA). Cell viability was calculated as $A_{450,\text{treated}}/A_{450,\text{control}} \times 100\%$, where $A_{450,\text{treated}}$ and $A_{450,\text{control}}$ denoted the absorbance values with or without addition of films. Five samples from each group were examined.

2.4. Statistical Analysis
The data were displayed as the mean ± standard deviation with no less than triplicate experiments for each group. Student’s t-test was performed for statistical analyses. P values less than 0.05 were considered statistically significant.

3. Results and Discussion

3.1. Synthesis of CS/PPY Composite
The chemical structures of CS, PPY and CS/PPY detected by the FT-IR spectra are presented in figure 1. The spectra of PPY and CS/PPY showed the characteristic PPY peaks at 1544 cm⁻¹ and 1040 cm⁻¹, which means PPY nanoparticles were synthesized successfully in CS solution. The spectra of CS/PPY also showed similar peaks to the spectra of CS at 940 cm⁻¹ and between 1200 cm⁻¹ and 1400 cm⁻¹, confirmed the composition of CS and PPY.

![Figure 1. The FT-IR spectra of CS, PPY and CS/PPY.](image-url)
3.2. Morphological Characterization
As shown in figure 2, the morphology of CS/PPY composite showed important differences with pure CS film. The pure CS film was flat and clean, with no pores on it (figures 2a and 2b). In contrast, CS/PPY films showed increased roughness, with layers and pores on them (figures 2c-2f). The sample with 20% PPY (CS/PPY20, figures 2e and 2f) exhibited more layers and pores than the sample with 10% PPY (CS/PPY10, figures 2c and 2d). The changes of morphology indicated that this rough surface may be more suitable for cell growth, because it can provide enough binding sites for protein adsorption and cell adhesion.

In addition to the roughness, small particles of PPY can be found in the 5000X images (figures 2d and 2f), which were dispersed evenly with uniform shape. This suggested PPY nanoparticles were successfully synthesized during the “one-step” process and well-distributed in CS.

![Figure 2](image)

**Figure 2.** Scanning electron microscopic (SEM) images of (a), (b) CS; (c) and (d) CS/PPY10; and (e) and (f) CS/PPY20 films. Left column: 1000X; right column: 5000X.

3.3. Conductivity Test
The conductivities of different samples are in table 1. The conductivity of CS film could not be detected, because CS is a kind of non-conducting material. The conductivities of CS/PPY10 and CS/PPY20 were 0.42 ± 0.08 mS/cm and 0.51 ± 0.09 mS/cm, respectively. But these two groups showed no significant difference (p > 0.05). The range of conductivity detected in this study meets the requirement of nerve tissue [14].
3.4. Protein Adsorption Measurement
To test whether the composite can provide a better environment for cell growth, the protein adsorption measurement was performed. As shown in figure 3, the amount of adsorbed protein was $0.029 \pm 0.009$ mg/ml, $0.037 \pm 0.010$ mg/ml and $0.041 \pm 0.008$ mg/ml, respectively. These results suggested that CS/PPY composite can adsorb more proteins than pure CS film ($p < 0.05$), which may subsequently induce more cells to attach. Combined the morphological result, the increased protein adsorption may cause by the increased roughness of CS/PPY composite.

Table 1. Conductivities of CS, CS/PPY10 and CS/PPY20.

| Sample   | Conductivity (mS/cm) |
|----------|----------------------|
| CS       | --                   |
| CS/PPY10 | $0.42 \pm 0.08$      |
| CS/PPY20 | $0.51 \pm 0.09$      |

3.5. Cell Viability
After the cultivation of PC-12 cells on control plate, CS film, CS/PPY10 film and CS/PPY20 film for 1 d, 3 d and 5 d, the in vitro cell viability of the three groups were assessed by CCK-8 kit. The statistical results are illustrated in figure 4. One day after seeding cells on the films, the cell proliferation was lower than blank plate ($p < 0.05$). However, at day 3, only cells on CS/PPY10 film had low proliferation compared to control ($p < 0.05$). Cell viability of CS and CS/PPY20 films had no significant difference with control ($p > 0.05$). At day 5, all the groups with films had no significant difference with control ($p > 0.05$). These results indicate that the CS/PPY composite had good biocompatibility after a long time cultivation.

A possible explanation for the declined cell viability at the first day is that the cell culture plate is treated specially for cell adhesion, which provides a fast adhesion and results in cell proliferation at the beginning. But after a long time cultivation, the advantage disappeared. These results indicate that the CS/PPY composite had good biocompatibility after a long time co-cultivation with cells.

Figure 3. Protein adsorption measurement of CS, CS/PPY10 and CS/PPY20. *: $p < 0.05$.

Figure 4. Cell viability of PC-12 cells seeded on control plate, CS film, CS/PPY10 film and CS/PPY20 film. n = 5; Error bar: standard deviation; *: $p < 0.05$.

4. Conclusion
As the passages had cleared, the CS/PPY composite with a PPY/CS ratio of 10-20% performed excellent PPY dispersibility, good conductivity and elevated protein adsorption. PC-12 cells co-cultured with CS/PPY films reflected negligibly low cytotoxicity. This study highlighted the novel fast
and safe “one step” manufacturing method for the composite with both water-soluble polymer and conductive material. The higher roughness of the product may provide sufficient binding sites for proteins and cells. Therefore, CS/PPY composite can be developed to a promising safe and bioactive material for neural tissue repair.

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References
[1] Rajnicek A M, Foubister L E and McCaig C D 2006 Growth cone steering by a physiological electric field requires dynamic microtubules, microfilaments and Rac-mediated filopodial asymmetry J. Cell Sci. 119 (Pt 9) 1736-45
[2] Kobelt L J, Wilkinson A E, McCormick AM, et al. 2014 Short duration electrical stimulation to enhance neurite outgrowth and maturation of adult neural stem progenitor cells. Ann Biomed Eng 42 (10) 2164-76
[3] Huang J, Zhang Y, Lu L, et al 2013 Electrical stimulation accelerates nerve regeneration and functional recovery in delayed peripheral nerve injury in rats Eur. J. Neurosci. 38 (12) 3691-701
[4] Gu X, Ding F and Williams D F 2014 Neural tissue engineering options for peripheral nerve regeneration Biomaterials 35 (24) 6143-56
[5] Ghasemi-Mobarakeh L, Prabhakaran M P, Morshed M, et al. 2011 Application of conductive polymers, scaffolds and electrical stimulation for nerve tissue engineering J. Tissue Eng. Regen. Med. 5 (4) e17-35
[6] Huang J, Lu L, Zhang J et al 2012 Electrical stimulation to conductive scaffold promotes axonal regeneration and remyelination in a rat model of large nerve defect PLoS One 7 (6) e39526
[7] Martino S, D'Angelo F, Armentano I, et al. 2012 Stem cell-biomaterial interactions for regenerative medicine Biotechnol. Adv. 30 (1) 338-51
[8] Cruz G J, Mondragónlozano R, Diazruiz A, et al. 2012 Plasma polypyrrole implants recover motor function in rats after spinal cord transection J. Mater. Sci. Mater. Med. 23 (10) 2583-92
[9] Nho Y, Kim J Y, Khang D, et al. 2010 Adsorption of mesenchymal stem cells and cortical neural stem cells on carbon nanotube/polycarbonate urethane Nanomedicine 5 (3) 409-17
[10] Defteral C, Verdejo R, Peponi L, et al. 2016 Thermally reduced graphene is a permissive material for neurons and astrocytes and de novo neurogenesis in the adult olfactory bulb in vivo Biomaterials 82 84-93
[11] Kumar A M, Suresh B, Das S, et al. 2017 Promising bio-composites of polypyrrole and chitosan: Surface protective and in vitro biocompatibility performance on 316L SS implants Carbohydr. Polym. 173 121-30
[12] Zhang Q, Yan Y, Li S, et al. 2010 The synthesis and characterization of a novel biodegradable and electroactive polypyrazolophene for nerve regeneration Mater. Sci. Eng. 30 (1) 160-66
[13] Thrivikraman G, Madras G and Basu B 2014 Intermittent electrical stimuli for guidance of human mesenchymal stem cell lineage commitment towards neural-like cells on electroconductive substrates Biomaterials 35 (24) 6219-35
[14] Xu H, Holzwarth JM, Yan Y, et al. 2014 Conductive PPY/PDLLA conduit for peripheral nerve regeneration Biomaterials 35 (1) 225-35