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# Hyaluronic acid doped-poly(3,4-ethylenedioxythiophene)/chitosan/gelatin (PEDOT-HA/Cs/Gel) porous conductive scaffold for nerve regeneration

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ABSTRACT

Conducting polymer, as a "smart" biomaterial, has been increasingly used to construct tissue engineered scaffold for nerve tissue regeneration. In this study, a novel porous conductive scaffold was prepared by incorporating conductive hyaluronic acid (HA) doped-poly(3,4-ethylenedioxythiophene) (PEDOT-HA) nanoparticles into a chitosan/gelatin (Cs/Gel) matrix. The physicochemical characteristics of Cs/Gel scaffold with 0–10 wt% PEDOT-HA were analyzed and the results indicated that the incorporation of PEDOT-HA into scaffold increased the electrical and mechanical properties while decreasing the porosity and water absorption. Moreover, *in vitro* biodegradation of scaffold displayed a declining trend with the PEDOT-HA content increased. About the biocompatibility of conductive scaffold, neuron-like rat phaeochromocytoma (PC12) cells were cultured in scaffold to evaluate cell adhesion and growth. 8% PEDOT-HA/Cs/Gel scaffold had a higher cell adhesive efficiency and cell viability than the other conductive scaffolds. Furthermore, cells in the scaffold with 8 wt% PEDOT-HA expressed higher synapse growth gene of GAP43 and SYP compared with Cs/Gel control group. These results suggest that 8%PEDOT-HA/Cs/Gel scaffold is an attractive cell culture conductive substrate which could support cell adhesion, survival, proliferation, and synapse growth for the application in nerve tissue regeneration.



## 1. Introduction

The nervous system is an important part of human body. However, neuronal repair for injured nervous tissue is very difficult partially because of the complexity of the nervous anatomical system, its functioning and the inefficiency of conventional repair approaches [1]. Nowadays, tissue engineering provides a new medical therapy instead of implantation of autografts, allografts and xenografts. Tissue engineering is an interdisciplinary field aimed at regenerating artificial tissues in order to repair or replace damaged organs. In the tissue engineering, biomaterial scaffold is one of the most important factors to promote cell differentiation and proliferation toward the formation of the desired new tissue. The tissue engineered scaffolds should have several functions including appropriate porosity, optimal mechanical strength, controlled biodegradability, sterilization, and biocompatibility with host tissue [2,3]. Recently, biomimetic natural biopolymers have attracted great interest as scaffolding biomaterial because of their superior biological response compared with synthetic scaffolds. Chitosan (Cs) is a linear polysaccharide derived from partial deacetylation of chitin and has the ability to interact with negatively charged molecules. Chitosan has been widely applied in nerve tissue engineering as scaffold [4–6]. Gelatin (Gel) is a soluble protein derived from collagen which possesses low antigenicity, and could promote cell adhesion and migration. It has been reported that Cs could be blended with Gel polymers for improving the mechanical and biological properties of Cs [7–10].

Successful nerve regeneration requires tissue engineered scaffolds that provide not only mechanical support for growing neurites and prevention of in growth of fibrous scar tissue, but also biological signals to direct the axonal growth cone to the distal stump [11]. Living cells, especially nerve cells possess many of the properties of electrical systems. Recent interest in electrical stimulation arises from a growing knowledge of the electrical properties of tissues and cells. Presently, electroactive biomaterials are considered as the important part of the new generation "smart" biomaterials. They allow the direct delivery of electrical, electrochemical and electromechanical stimulation to cells [12–14]. Conducting polymer, as an electroactive biomaterial, has been increasingly used to construct engineered scaffold for nerve tissue regeneration and its chemical, electrical and physical properties can be tailored to meet the specific application by incorporating antibodies, enzymes and other biological moieties into it [15–17].

Poly(3,4-ethylenedioxythiophene) (PEDOT), a polythiophene derivative, is proved to be one of the most promising conductive polymers responsible for its tunable electro-optical properties. PEDOT has been widely used in biosensing and bioengineering applications [13]. For example, a neural electrode was interfaced with the surrounding brain tissue through the *in situ* polymerization of PEDOT [18]. This formed PEDOT filaments extending far enough away from the electrode to

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breach the glial scar around it and forming sensitive contacts with the plasma membrane of the neurons. PEDOT has also been polymerized within acellular muscle tissue, where it formed a network of elongated tubular structures throughout the tissue, in essence converting it into an extensive conductive three-dimensional substrate [19]. In another study, an eight-channel chronically implantable optrode array was fabricated and modified with poly(3,4-ethylenedioxythiophene)/poly(styrenesulfonate)-poly(vinyl alcohol)/poly(acrylic acid) interpenetrating polymer networks (PEDOT/PSS-PVA/PAA IPNs) for improving the optrode-neural tissue interface. The electrical recording results suggested that the modified optrode arrays showed significantly reduced impedance and RMS noise and an improved SNR as compared to unmodified sites, which may have benefited from the improved electrochemical performance and biocompatibility of the deposited IPN films [20]. On the other hand, to overcome the drawbacks such as poor mechanical properties, poor processability, hydrophobicity and non-degradability of conducting polymers, polymer blends or composites based on conducting polymers and degradable polymers such as poly lactide (PLA), chitosan, gelatin, collagen, and hyaluronic acid (HA) have been explored and extensively investigated [17]. In our previous study, conductive HA-doped PEDOT (PEDOT-HA) was synthesized to enhance the biocompatibility of PEDOT by introducing the bioactive molecule HA. Poly (L-lactic acid) (PLLA), as a biodegradable polymer material, was used to combine with conductive PEDOT-HA nanoparticles. The results indicated that the prepared PEDOT-HA/PLLA films could support PC12 cells proliferation and synapse growth as a conductive matrix [21]. However, porous conductive scaffold for nerve tissue engineering would be a better matrix because of its biomimetic structure for cell infiltration and growth.

In this study, a novel porous conductive scaffold was prepared by incorporating conductive PEDOT-HA nanoparticles into a chitosan/gelatin (Cs/Gel) matrix for its potential application in nerve regeneration. The chemical structure, porosity, water absorption, conductivity, and mechanical properties of PEDOT-HA/Cs/Gel scaffold were evaluated, as well as *in vitro* biodegradation. Neuron-like rat phaeochromocytoma (PC12) cells were used to evaluate the biocompatibility of conductive scaffold including cell adhesion, viability, proliferation, and synapse growth gene expression.

## 2. Materials and methods

### 2.1. Materials

3,4-Ethylenedioxythiophene (EDOT, with 99.9% purity) was purchased from Shanghai Chemical Con. Ltd. Hyaluronic acid (HA, sodium salt, MW 150,000 Da) was purchased from Sigma-Aldrich Inc. Chitosan was purchased from Haidebei Marine Bioengineering Co. Ltd., the molecular weight of which is <5000 and the degree of deacetylation is around 85.6%. Gelatin, the analytical agent, was purchased from Sinopharm Co. Ltd., the purity of which is >99.5%. The cross-linkers 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and N-hydroxy-succinamide (NHS) were purchased from the Shanghai Medpep Co., Ltd. Cell culture reagents were obtained from Invitrogen Inc. (Carlsbad, CA, USA). The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). Calcein-AM, Hoechst 33,258, and propidium iodide (PI) were purchased from Sigma–Aldrich Inc. (St. Louis, Mo, USA). The reagents of Real-time qPCR were purchased from Takara Bio Inc. (Otsu, Shiga, Japan). All other reagents and solvents are of analytical grade and were used as received.

### 2.2. Preparation of PEDOT-HA nanoparticles and PEDOT-HA/Cs/Gel scaffolds

A nanocomposite hyaluronic acid (HA) doped-poly-3,4-ethylenedioxythiophene (PEDOT-HA) with diameter of about 200 nm was synthesized according to the method in our previous work [21]. Briefly, HA solution was poured into a 100 mL round bottom flask and mixed with EDOT. The oxidative polymerization was initiated by adding ammonium persulfate (APS) into the mixture. After completion of reaction, the mixture was poured into a centrifuge tube with an equal volume of acetone and centrifuged. To remove thoroughly residual reactants, the sediment was washed centrifugally with ethanol and deionized water for 3 times, respectively. Then sediment was dried in vacuum at room temperature for one week to obtain PEDOT-HA nanoparticles.

Chitosan was dissolved (2%, w/v) into the aqueous acetic acid (2%, v/v) for 6 h under stirring and gelatin was dissolved (2%, w/v) into deionized water at 40 °C for 3 h under gentle stirring. Then the above two solutions were mixed with volume ratio of 1:1. A homogeneous mixture was obtained after stirring 3 h. PEDOT-HA nanoparticles were added to the mixture in the amount of 2%, 5%, 8%, 10% of the total mass of chitosan and gelatin. The mixture was homogenized and degassed by stirring for 2 h, ultrasonic dispersing for 30 min, stirring for 1 h. And mixture was poured into 24-well cell culture plates and frozen for 1 day at −80 °C. Then the samples were lyophilized for 1 day in a FreeZone Freeze Dry Systems (Labconco, USA). After freeze-drying, the scaffolds were put into an ethanol solution (40%, v/v) containing 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (50 mM)/N-hydroxy-succinimide (50 mM) for crosslinking 6 h. Then the scaffolds were deacidified with $Na_2HPO_4$ (0.1 M, pH = 7.4) solution, rinsed with deionized water, and lyophilized after 1 day of freeze at −80 °C. The diagram of preparation PEDOT-HA/chitosan/gelatin scaffold was shown in Fig. 1. Scaffolds were cut into small pieces (5 mm × 5 mm × 2 mm) to sterilize for the experiment. Five types of scaffolds were obtained, namely 0%PEDOT-HA/Cs/Gel or Cs/Gel (control), 2%PEDOT-HA/Cs/Gel, 5%PEDOT-HA/Cs/Gel, 8% PEDOT-HA/Cs/Gel, and 10% PEDOT-HA/Cs/Gel.

### 2.3. Characterization of PEDOT-HA/Cs/Gel scaffolds

#### 2.3.1. Fourier transform infrared spectroscopy and Raman spectroscopy analysis

To analyze the chemical bonding and functional groups, samples were investigated by Fourier transform infrared spectrometer (FTIR, Nicolet6700, Thermo Fisher, USA). Specimens were grinded to tablet with KBr prior to assay. Test wavelength was from 400 $cm^{-1}$ to 4000 $cm^{-1}$, scanning accuracy was 4 $cm^{-1}$, and scanning frequency was 16 times at room temperature. In addition, conductive scaffolds were analyzed by a Raman100 spectrometer (Chinese Academy of Sciences, Dalian, China) with excitation wavelength of 532 nm, spectral resolution of 1 $cm^{-1}$, and frequency range of 400 $cm^{-1}$-4000 $cm^{-1}$.

#### 2.3.2. Scanning electron microscope observation

PEDOT-HA/Cs/Gel scaffolds were measured by scanning electron microscope (SEM, S2520, Hitachi, Tokyo, Japan). Specimens were firstly sputter-coated with gold followed by the collection of images at various magnifications. For cell-scaffold constructs morphology, the constructs were fixed with 2.5% glutaraldehyde at 4 °C for 3 h. After washed with phosphate buffer saline (PBS) three times, the constructs were sequentially treated in a series of graded ethanol solution (60%, 70%, 80%, 90%, and 100%) for dehydration, each for 15 min at room temperature. Then constructs were dried in a vacuum oven at 50 °C overnight. Finally, specimens were observed by SEM after coating with a thin gold layer.

#### 2.3.3. Porosity and swelling ratio analysis

The porosity of the scaffold was calculated by an ethanol replacement method. The weight of dry scaffold was record as $W_d$. The dry scaffold was immersed in ethanol and the mixture was subjected to vacuum to force the ethanol completely penetrate into the scaffold pores. After 5 min, the scaffold was transferred immediately into a weighing bottle containing a known initial volume ($V_1$) and weight ($W_1$) of ethanol.