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Fabrication and characterization of conductive poly (3,4-ethylenedioxythiophene) doped with hyaluronic acid/poly (L-lactic acid) composite film for biomedical application

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Poly 3,4-ethylenedioxythiophene (PEDOT), a polythiophene derivative, has been proved to be modified by chemical process as biocompatible conductive polymer for biomedical applications. In this study, novel hyaluronic acid (HA)-doped PEDOT nanoparticles were synthesized by the method of chemical oxidative polymerization, then conductive PEDOT-HA/poly(L-lactic acid) (PLLA) composite films were prepared. The physicochemical characteristics and biocompatibility of films were further investigated. FTIR, Raman and EDX analysis demonstrated that HA was successfully doped into PEDOT particles. Cyclic voltammograms indicated PEDOT-HA particles had favorable electrochemical stability. PEDOT-HA/PLLA films showed lower surface contact angle and faster degradation degree compared with PLLA films. Moreover, the cytotoxicity test of PEDOT-HA/PLLA films showed that neuron-like pheochromocytoma (PC12) cells adhered and spread well on the surface of PEDOT-HA/PLLA films and cell viability denoted by MTT assay had a significant increase. PEDOT-HA/PLLA films modified with laminin (LN) also exhibited an efficiently elongated cell morphology observed by fluorescent microscope and metallographic microscope. Furthermore, PEDOT-HA/PLLA films were subjected to different current intensity to elucidate the effect of electrical stimulation (ES) on neurite outgrowth of PC12 cells. ES (0.5 mA, 2 h) significantly promoted neurite outgrowth with an average value length of 122 ± 5 μm and enhanced the mRNA expression of growth-associated protein (GAP43) and synaptophysin (SYP) in PC12 cells when compared with other ES groups. These results suggest that PEDOT-HA/PLLA film combined with ES are conducive to cell growth and neurite outgrowth, indicating the conductive PEDOT-HA/PLLA film may be an attractive candidate with ES for enhancing nerve regeneration in nerve tissue engineering.

[**Key words:** Poly 3,4-ethylenedioxythiophene-hyaluronic acid/poly(L-lactic acid) composite film; Conducting polymer; PC12 cells; Neurite outgrowth; Electrical stimulation]

Recent advances have generated wide interest in engineering a biomaterial scaffold that is electrically conductive for the regeneration and functional recovery following injury or disease (1). Generally, engineered scaffolds are typically designed by imparting particular chemical and physical properties to promote the specific sequence of cellular events for the best functional recovery. Electroactive biomaterials are considered as the important part of the new generation of smart biomaterials. They allow the direct delivery of electrical, electrochemical and electromechanical stimulation to cells (2–5). It has recently been demonstrated that some conducting polymers (CPs) allow excellent control of the electrical stimulus, possess very good electrical and optical properties, have a high conductivity/weight ratio, and are biocompatible, biodegradable and porous (3–8). CPs exhibiting enormous potential could promote the transfer of electrical stimulation (ES) and provide a more conducive environment for cell integration (9,10). Studies on electroactive tissues (e.g., nerve, muscle or myocardium), also confirm that CPs may usefully regulate cellular response, including DNA synthesis, cell adhesion, migration, proliferation and differentiation (11–15). For ES biomedical application in tissue engineering, the ideal conductive scaffolds are essential to possess low resistance, high stability, desired dimensions, good biocompatibility, proper biodegradability and sterilizability (8,16,17). It is important for CPs modified with biological molecules to support cell growth and regulate cell activities (18). Several research groups have explored the functionalization of CPs by doping biological dopants or incorporating bioactive molecules into polymers, specifically growth factors (19–22), and extracellular matrices (ECMs) (23–26). However, it is still challenging in the preparation of clinically relevant CP-based tissue scaffolds with biomimetic chemical, mechanical and topological properties.

In the past few years, polyheterocycles family mainly including polyaniline, polypyrrole and polythiophene has been widely applied in biomedical engineering (8,17). In particular, 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 and high degree of processability (27–31). Compared to other CPs, PEDOT possesses higher electrical conductivity and stability. It has been successfully used in biosensor, neural electrodes, nerve grafts and heart muscle patches (3,32–34). For example, by using a

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multifaceted approach to PEDOT surface functionalization, the ultrasmall implantable composite microelectrodes with suitable size, stiffness, biocompatibility and flexibility have been developed to chronically optimize the implanted probes for brain recording (35). Recent studies have shown that biological and organic dopants can be added to PEDOT materials for enhancing their physicochemical and biological functionality. The involved dopants to PEDOT mainly include small molecule salt ions, macromolecule, and biological molecules such as amino acids, proteins, polysaccharide and neurotrophic substances. For instances, the adhesion of PC12 cells on PEDOT films was improved by doping with collagen (36), or low molecular weight peptides derived from laminin (LN) (37). Particularly, studies have noted that negatively charged glycosaminoglycans (GAGs), an important component of ECMs, can be incorporated into CPs by doping (38). Hyaluronic acid (HA) is one of the most important GAGs in vertebrate bodies. It has been used to improve the bioactivity of CPs because of its vital role in wound-healing, tissue regeneration and angiogenesis for the fabrication of synthetic matrices (39,40). Some studies have found that synthesized HA-doped polypyrrole composite could promote vascularization in vivo and trigger early osteogenic differentiation of human adipose stem cells (hASCs) (24,38). It also has been shown that electrical stimulation to nerve stem cells through conducting polymeric scaffolds (PANI/PG or PANI/PLLA) has important implication toward neurite outgrowth (41,42). Interestingly, one study has been established that pyrrole-conjugated hyaluronic acid (Py-HA) coating has the potential to mask conducting electrodes from adverse glial responses that occur upon implantation (43). Similar to other CPs, PEDOT also needs a balancing counterion as a dopant for its polymerization, although rare information about chemically doping with HA is available concerning.

In this study, novel HA-doped PEDOT conductive nanoparticles were firstly synthesized by chemical oxidative polymerization. Due to the inherent non-biodegradability of PEDOT, we chose biodegradable polymers poly (L-lactic acid) (PLLA) together with it to fabricate the biodegradable conductive PEDOT-HA/PLLA films as conductive matrix in tissue engineering. Chemical structure, electrochemical properties, surface morphology, degradability and biocompatibility of PEDOT-HA/PLLA films were evaluated, expecting to construct a promising electrically conductive and biodegradable scaffold for biomedical application. In addition, ES model in vitro was designed to further investigate the possible ES regulating effect on PC12 cells survival and neurite outgrowth.

MATERIALS AND METHODS

Materials 3,4-Ethylenedioxythiophene (EDOT, with 99.9% purity) was purchased from Shanghai Chemical Co. Ltd. (Shanghai, China). Hyaluronic acid (HA, sodium salt, MW 150,000 Da) was purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). Laminin (LN) was purchased from Invitrogen Inc. (Carlsbad, CA, USA). Poly (L-lactic acid) (PLLA, MW 180,000 Da) was provided by SAN Po Biological Material Co. Ltd. (Jilin, Changchun, China). Cell culture reagents were obtained from Invitrogen Inc. All other reagents and solvents are of analytical grade and were used as received.

Synthesis of PEDOT-HA particles In order to increase the plasticity of conductive polymer with PLLA, the nanoparticles of HA-doped PEDOT were synthesized firstly by chemical polymerization rather than film obtained by electrochemical polymerization (33). The chemical polymerization of EDOT monomers with dopant HA is represented schematically in Fig. 1. HA was firstly dissolved into double-distilled water by magnetically stirring. Subsequently, HA solution was poured into a 100 mL round bottom flask and mixed with 0.01 mol EDOT. The oxidative polymerization was initiated by adding ammonium persulfate (APS) dropwise into the liquid mixture, and the solution gradually turned dark blue under vigorously stirring at room temperature. After completion of the reaction, the mixture was poured into a centrifuge tube with an equal volume of acetone, and centrifuged at 1000 rpm for 10 min. To remove thoroughly any residual reactants, the sediment was washed centrifugally with ethanol and double-distilled water 3 times, respectively. Then the sediment was dried in vacuum at room temperature for a week to get blue-black PEDOT-HA powder. Considering the influence of various factors on the product performance, an orthogonal optimal experiment was designed as shown in Table S1. The conductivity, yield and morphology of resulting PEDOT-HA particles were considered as the evaluation indexes. By statistical analysis, reaction time was the main influencing factor and the amount of dopant was the weakest influencing factor on the conductivity of products. Meanwhile, the amount of oxidant was the main influencing factor and reaction time was the weakest influencing factor on the yield of products. According to comprehensive results, the optimal polymerization condition was achieved: EDOT (0.01 mol), HA (0.05 g), APS (0.015 mol) and reaction time (24 h). Under this condition, the yield of PEDOT-HA was 45.6%, and the conductivity of PEDOT-HA reached 0.36 S/cm. PEDOT-HA particles used in the next experiment were all polymerized under the optimal polymerization condition.

Preparation of PEDOT-HA/PLLA films For preparation conductive biodegradable PEDOT-HA/PLLA films, a certain mass of PEDOT-HA particles were added to PLLA solution in $CHCl_3$ with high-speed stirring for 1 h, ultrasonic dispersion for 30 min, and high-speed stirring for 1 h again. The mixture was casted onto glass plates and dried in air at room temperature for one day. Finally films were dried in vacuum oven for a week to remove residual solvent deeply. Four types of films with 0%, 10%, 30%, and 50% weight ratio of PEDOT-HA were obtained, and correspondingly named as PLLA (control), 10% PEDOT-HA/PLLA, 30% PEDOT-HA/PLLA, and 50% PEDOT-HA/PLLA.

Chemical structure analysis PEDOT-HA particles were analyzed 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, PEDOT-HA particles 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}.

Microtopography analysis The morphology of resulting PEDOT-HA particles and PEDOT-HA/PLLA films were measured by scanning electron microscope (SEM, S2520, Hitachi, Japan). Specimens were firstly sputter-coated with gold followed by the collection of images at various magnifications. Furthermore, energy dispersive X-ray (EDX) spectrometer as an accessory of SEM was performed to qualitatively and quantitatively analyze for the elements of materials.

Contact angle measurement instrument (OCA20, Dataphysics, Germany) was used to measure the surface contact angle of pure PLLA and PEDOT-HA/PLLA films. Drops of deionized water (1 μL) were placed on each film surface, and the contact angles were measured within 3 s in air at room temperature. Each sample was measured at least three times and each film was analyzed in triplicate.

Electrochemical performance characterization The electrical properties of resulting PEDOT-HA particles were assessed by cyclic voltammetry (CV). An eDAQ potentiostat and eCorder unit coupled with the supplied EChem software package (IviumStat, Holland) was used for CV measurement of PEDOT-HA particles. Both analyses were conducted in a three-electrode well system. CV was performed in 0.9% NaCl solution for 150 continuous cycles, with scanning potentials of −0.8 V

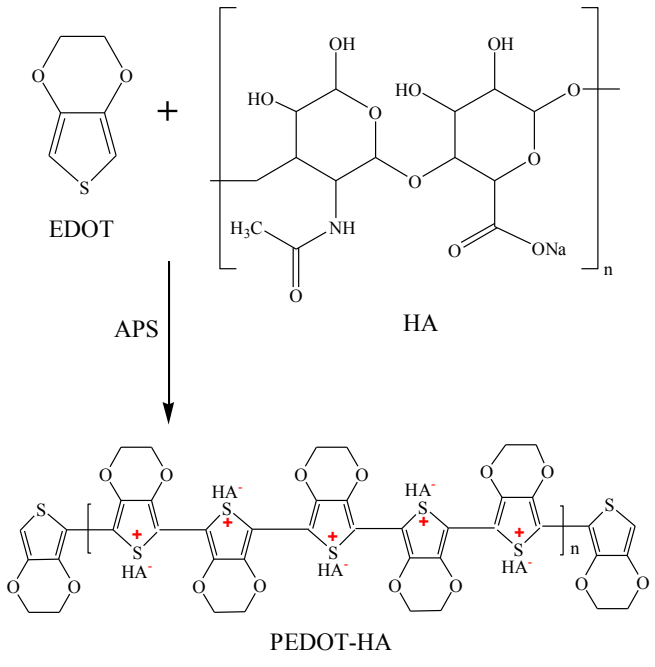

FIG. 1. Schematic diagram for chemical oxidative polymerization of EDOT with HA.