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Electrical stimulation to promote osteogenesis using conductive polypyrrole films

Wei-Wen Hu [a,*], Yi-Ting Hsu [a], Yu-Che Cheng [b,c], Chuan Li [d], Ruoh-Chyu Ruaan [a,b], Chih-Cheng Chien [c,e,f], Chih-Ang Chung [d], Chia-Wen Tsao [d]

[a] Department of Chemical and Materials Engineering, National Central University, Jhongli City, Taiwan
[b] Institute of Biomedical Engineering, National Central University, Jhongli, Taiwan
[c] Department of Medical Research, Cathay General Hospital, Taipei, Taiwan
[d] Department of Mechanical Engineering, National Central University, Jhongli, Taiwan
[e] School of Medicine, Fu Jen Catholic University, Taipei, Taiwan
[f] Department of Anesthesiology, Sijhih Cathay General Hospital, Sijhih City, Taipei, Taiwan

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ABSTRACT

In this study, we developed an electrical cell culture and monitoring device. Polypyrrole (PPy) films with different resistances were fabricated as conductive surfaces to investigate the effect of substrate-mediated electrical stimulation. The physical and chemical properties of the devices, as well as their biocompatibilities, were thoroughly evaluated. These PPy films had a dark but transparent appearance, on which the surface cells could be easily observed. After treating with the osteogenic medium, rat bone marrow stromal cells cultured on the PPy films differentiated into osteoblasts. The cells grown on the PPy films had up-regulated osteogenic markers, and an alkaline phosphatase activity assay showed that the PPy films accelerated cell differentiation. Alizarin red staining and calcium analysis suggested that the PPy films promoted osteogenesis. Finally, PPy films were subjected to a constant electric field to elucidate the effect of electrical stimulation on osteogenesis. Compared with the untreated group, electrical stimulation improved calcium deposition in the extracellular matrix. Furthermore, PPy films with lower resistances allowed larger currents to stimulate the surface cells, which resulted in higher levels of mineralization. Overall, these results indicated that this system exhibited superior electroactivity with controllable electrical resistance and that it can be coated directly to produce medical devices with a transparent appearance, which should be beneficial for research on electrical stimulation for tissue regeneration.

1. Introduction

Various cells have been proven to be susceptible to electrical stimulation and electrically assisted therapy has been applied to different tissues, such as nerves and muscles, to promote their regeneration [1–4]. Direct current field (DCF), capacitive coupling electrical field (CCEF), and electromagnetic field (EMF) are three frequently used forms of electric stimulation [5]. Of these, CCEF and EMF are noninvasive and have been studied widely. However, these treatments are not spatially specific so both normal and pathological tissues would be stimulated [6]. By contrast, DCF can be applied directly to a wound site and the range of stimulation can be confined to avoid unwanted side-effects [7]. Stimulation using electrodes mediated by a fluid is the simplest method for DCF treatment [8–13]. However, electrolysis may occur to change the local pH values [14]. In addition, the electric current may trigger a gradient ion distribution, which could affect the cell physiology and increase the difficulty of clarifying the effects of DCF on cells [15]. These drawbacks all limit the mechanistic studies of the effects of DCF on cell differentiation.

Substrate-mediated DCF is an alternative approach where cells are cultured on conductive material surfaces for *in situ* treatment. The electric current passes through the cell substrate; thus, electrolysis and ion movement can be avoided. To simulate an *in vivo* environment, the electric resistance of conductive materials should be similar to those of real tissues. Different materials have been used for substrate-mediated DCF treatments, but metal is the most popular because of its good conductivity [16–18]. However, compared with normal tissues, the extremely low resistance of metallic materials leads to the passage of a high electric current during stimulation, which may negatively affect cells. The corrosion of metal may also occur during DCF treatment, which can release cytotoxic debris [19]. To overcome these difficulties, composite materials have been developed to produce biocompatible surfaces with appropriate resistivity. Multiwalled carbon nanotubes have been embedded in a polylactide matrix to electrospin conductive nanofibers [20]. Polypyrrole (PPy) has also been doped into biodegradable polymers to prepare conductive films [21,22]. These materials have good conductivity and can be used for substrate-mediated DCF treatment,

* Corresponding author at: Department of Chemical and Materials Engineering, National Central University, No.300, Jhongda Rd., Jhongli City, Taoyuan County, Taiwan. Tel.: +886 3 422 7151x34243; fax: +886 3 422 5258.
E-mail address: huweiwen@cc.ncu.edu.tw (W.-W. Hu).

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but they are all opaque so the surface cells cannot be observed directly. Thus, fluorescent staining and scanning electron microscopy are required for visualization, whereas the cell morphology is probably affected during fixation. In addition, end-point observations of these systems are impossible to continuously monitor surface cells. These problems suggest that the preparation of transparent conductive materials with adjustable resistivity would be beneficial for the research on the effects of DCF on cell differentiation.

Therefore, we aimed to develop a convenient device that can be used easily to investigate the effect of electrical stimulation on surface cells. Conductive PPy films were polymerized *via* chemical oxidation to deposit them on substrate surfaces. The electrical resistance of PPy films was regulated by controlling the monomer concentrations. These films were biocompatible and had a transparent appearance so the surface cells could be observed easily. Rat bone marrow stromal cells (rBMSCs) were used as a model system to test our devices. The differentiation of rBMSCs was accelerated on these electroactive surfaces. Next, the PPy films were applied to a homemade device before DCF treatment. The mineralization of rBMSCs was improved greatly after subjecting them to electric fields, which suggests that this device can promote osteogenesis. These results support that this substrate-mediated DCF system is suitable for performing osteogenesis and it has potential applications in other areas of tissue differentiation research.

2. Materials and methods

2.1. Materials

Pyrrole and ammonium persulfate were purchased from Acros (Geel, Belgium) and Showa (Tokyo, Japan), respectively. Fetal bovine serum (FBS), Dulbecco's modified Eagle medium (D-MEM), and trypsin-EDTA were obtained from Biowest (Nuaillé, France). Dexamethasone, 2-phospho-L-ascorbic acid trisodium salt, β-glycerophosphate disodium salt hydrate, Triton X-100, and glutaraldehyde were purchased from Sigma-Aldrich (St Louis, MO, USA).

2.2. The preparation of PPy films

Polystyrene (PS) Petri dishes with a diameter of 35 mm (Nunc, Penfield, NY, USA) were used as the substrate for PPy film deposition. Pyrrole was dissolved in water at concentrations of 0.1, 0.3, and 0.5 M. Aqueous solutions of oxidant ammonium persulfate were prepared using the corresponding pyrrole solutions so the molar ratio of pyrrole relative to ammonium persulfate was always 5:1 (*i.e.* 0.02, 0.06, and 0.1 M). Next, 2 ml of pyrrole and ammonium persulfate solutions were added to Petri dishes, with gently mixing for 15 min at room temperature. Finally, these films were washed with deionized (DI) water and dried in an oven.

2.3. Scanning electron microscopy (SEM)

The morphologies of the PPy films were visualized by SEM (3500N, Hitachi, Japan) after gold sputtered-coating. To determine the film thickness, the films were cryofractured in liquid nitrogen to produce cross-sections.

2.4. Characterization of PPy films

To characterize the deposited PPy films, Fourier transform infrared (ATR-FTIR) spectroscopy (Spectrum 100, PerkinElmer, Waltham, MA, USA) was used to obtain the IR spectra at a resolution of 1 cm^{-1}. In addition, elemental analysis of the PPy films was performed using X-ray photon-electron spectrometry (XPS, K-alpha, Thermo) where the binding energy was measured from 0 to 1100 eV.

2.5. Four-point probe analysis

Four-point probe (EverBeing, Hsinchu, Taiwan) analysis was used to determine the sheet resistance of PPy films. Twenty points in different regions were examined in each film to confirm the spatial stability of the sheet resistance. Furthermore, the resistivities of PPy films were converted using the thickness measurements derived from the SEM results.

2.6. In vitro cell culture of rBMSCs

Rat bone marrow stromal cells (rBMSCs) harvested from the 8-week-old Sprague–Dawley rats were used in this study to simulate osteogenesis. These cells were maintained in regular medium of DMEM with 10% FBS. Osteogenic supplements (100 μm ascorbic-2-phosphate, 10 mM β-glycerophosphate, and 100 nM dexamethasone) were added to the regular medium during osteogenesis experiments.

2.7. Biocompatibility of PPy films

Polypropylene rings were glued onto PPy films to create wells. To evaluate the biocompatibility of PPy films, rBMSCs were seeded onto PPy films at a density of 17,000 cells/cm^2, and an (4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed to quantify cell viability. After culture for 2 days, 20 μl of MTT solution (5 mg/ml in phosphate-buffered saline (PBS)) and 180 μl of medium were added to each well for 3 h at 37 °C. The supernatant was removed and 200 μl of dimethyl sulfoxide (DMSO) was added to dissolve the formazan, which was analyzed spectrophotometrically at a wavelength of 550 nm.

A lactate dehydrogenase (LDH) assay was used to quantify the cell numbers with CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega). Briefly, 100 μl of fresh medium was replaced in each well before the assay and 15 μl of lysis buffer was added to sample for 1 h at 37 °C to release LDH from live cells. After transferring 50 μl of the LDH-released medium to 96-well multiplates, 50 μl of LDH reagent was added, which was followed by incubation for 30 min at room temperature. Finally, 50 μl of stop solution was added to each well and they were analyzed spectrophotometrically at a wavelength of 490 nm. A standard curve was also produced by lysing known cell amounts, which was used to convert the absorbance into a total cell number.

2.8. Quantitative and qualitative analyses of calcium deposition in the extracellular matrix (ECM)

To quantify the level of calcium deposition, a colorimetric assay was performed using the calcium-(*o*-cresolphthalein complexone) (Ca-OCPC) complex method [23]. Before the assay, the osteogenic medium was removed from the well using two washes of PBS. Next, 100 μl per well of 0.5 N acetic acid was used to release calcium. Ten microliters of calcium-released sample was added to 200 μl of calcium-binding reagent (0.1 g/l of *o*-cresolphthalein complexone and 1 g/l of 8-hydroxyquinoline) and 200 μl of buffer reagent (1.6 M of 2-amino-2-methyl-1-propanol, pH 10.7). After 15 min incubation at room temperature, 100 μl of purple-colored Ca-OCPC complex was transferred to 96-well multiplates, which was quantified based on the absorbance at a wavelength of 575 nm. The amount of calcium in cell lysate was converted based on the linear calibration results obtained using calcium chloride standard solutions.

Before alizarin red S staining, the cultures were rinsed with PBS and fixed (1% glutaraldehyde in PBS) for 30 min at 37 °C, then stained with 2% alizarin red S solution for 20 min at room temperature. After washing with PBS, the stained samples were observed using an inverted microscope (Eclipse Ti-U, Nikon, Japan).