Sulfonated Polyaniline-Based Organic Electrodes for Controlled Electrical Stimulation of Human Osteosarcoma Cells

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ABSTRACT: Electrically conducting polymers (CPs) were found to stimulate various cell types such as neurons, osteoblasts, and fibroblasts in both in vitro and in vivo studies. However, to our knowledge, no studies have been reported on the utility of CPs in stimulation of cancer or tumor cells in the literature. Here we report a facile fabrication method of self-doped sulfonated polyaniline (SPAN)-based interdigitated electrodes (IDEs) for controlled electrical stimulation of human osteosarcoma (HOS) cells. Increased degree of sulfonation was found to increase the SPAN conductivity, which in turn improved the cell attachment and cell growth without electrical stimulation. However, an enhanced cell growth was observed under controlled electrical (AC) stimulation at low applied voltage and frequency (≤800 mV and ≤1 kHz). The cell growth reached a maximum threshold at an applied voltage or frequency and beyond which pronounced cell death was observed. We believe that these organic electrodes may find utility in electrical stimulation of cancer or tumor cells for therapy and research and may also provide an alternative to the conventional metal-based electrodes.

1. INTRODUCTION

Electric fields have been employed in several different types of cancer therapies.¹ Some of these involve radio frequency or microwave devices that can heat the tumor to greater than 43 °C to kill the cells via hyperthermia.² Others use pulsed electric fields to permeabilize the tumor cells to allow the introduction of toxic drugs or DNA.³ Cell death occurs by either necrosis or apoptosis.⁴ Necrosis is caused by irreversible plasma membrane rupturing as a result of severe cell damage and dysfunction, which further induces inflammatory reactions. On the contrary, in apoptosis the cell membrane integrity is maintained, and the cell is removed by phagocytosis without inducing an inflammatory response. Therefore, apoptosis is considered to be a better method for novel targeted anticancer therapy.⁵

Electrical charges play an important role in stimulating either the proliferation or differentiation of various cell types.⁶ Both in vivo and in vitro studies have demonstrated that electrical stimulation, either in AC, DC, or pulsed electromagnetic field, can stimulate “bone cell” growth or bone regeneration.⁷⁻⁹ Conducting polymers (CPs) combine the attractive properties of plastics such as easy processing and adjustable physical and chemical properties (adhesion, wettability, surface charge, etc.) with the electrical properties of metals.⁹⁻¹⁵ Therefore, CPs, including polypyrrole, polythiophene, polyaniline, and polyethylenedioxythiophene, have been explored for their applicability to tissue regeneration. ¹⁶⁻²⁴ In particular, oligoaniline-containing self-assembled monolayers stimulated spontaneous neuritogenesis in PC12 pheochromocytoma cells in the absence of neurotrophic growth factors, such as nerve growth factor (NGF).¹⁹ Polyaniline was found to be biocompatible, allowing cell attachment and proliferation of H9c2 cardiac myoblasts, albeit with a gradual decrease in conductivity by about 3 orders of magnitude after 100 h of exposure to physiological culture medium, apparently due to continual leakage of residual acid dopants, even after washing of the dopped polyaniline prior to its use.²⁰ Therefore, it is imperative that CPs utilized for effective electrical stimulation of various cell types (e.g., neurons, osteoblasts, fibroblasts) should exhibit a stable electrical conductivity and should have no toxic dopants that may leach out into the culture medium.²¹

Here, we report electrical stimulation of HOS cells utilizing a highly conductive and pH-stable,²² SPAN-based Interdigitated Electrodes (IDEs). The SPAN-based electrodes have several advantages, such as, high electrical conductivity and independent of the external protonation in a broad pH range, without the need of toxic dopants that may leach out into the cell culture medium and may cause undesired results. A series of SPANs were synthesized by copolymerization of aniline (AN) and metanilic acid (MA), with ammonium persulfate (APS) as an oxidant in 1 M HCl, as shown in Figure 1a. The amount of
2. MATERIALS AND METHODS

Aniline (AN) was purchased from Aldrich and purified by vacuum distillation at 70 °C. Metanilic acid (MA) was obtained from Eastman Kodak company, while ammonium persulfate (APS) and hydrochloric acid (HCl, 37.5 wt %) were purchased from Fisher Scientific. Demineralized (DI) water was provided by Ohio State University lab stores. All reagents except AN were directly used without further purification.

2.1. SPAN Synthesis. The bulk polymerization of SPAN was carried out in an ice bath with ammonium persulfate (APS) as an oxidant in 1 M HCl. The total amount of −NH₂ in aniline (AN) and metanilic acid (MA) was used to determine the amount of each monomer and oxidant. For instance, during synthesis of 50% degree of sulfonation (AN/MA = 5:5, 1/2 oxidant), 0.05 mol of AN was dropwise added into the solution of MA (0.05 mol) and APS (0.05 mol) dissolved in 1 M HCl. The reaction was kept in an ice bath and stirred for about 6 h. Precipitation was allowed to occur overnight. In the end, the product was collected by filtering, washing exhaustively with DI water, and later dried in a vacuum oven at 50 °C for about 24 h. The final dark green product was ground into powder for further use.

Yield was calculated as the weight of SPAN synthesized divided by the total amount of the monomers put into the reaction system. Conductivity was measured by a standard four-probe method using a Keithley 2400 source meter.

2.2. Fabrication of IDEs. A computer-designed micropattern was printed on a polylethylene teraphthalate (PET) film using a conventional laser inkjet printer. A thin layer of SPAN-40 (40% sulfonated) was applied through in situ polymerization. After washing with DI water and drying, a second layer of SPAN-65 (60% sulfonated) was applied through in situ polymerization. Again, after washing with DI water and drying, a third thin layer of SPAN-40 was similarly applied. Finally, the films were washed with DI water and dried, and then methanol/THF solvent mixture was applied to remove the ink and the residual SPAN that might short circuit from the printed PET film, which was confirmed by an optical microscope inspection. The thickness and the width of the SPAN-coated layers were measured using a surface profiler (ALPHA-STEP 500). Then, a thin layer of polyacrylic acid (PLA) was spin-coated on top of the SPAN electrodes to prevent SPAN's dissolution in the biomedium. Thus, the multilayered SPAN copolymers deposited on PET films were later used as IDEs for electrical stimulation of HOS cells.

2.3. Cell Culture. HOS cells were purchased from American Type Culture Collection (ATCC, cat# CRL-1543) and cultured in Minimum essential medium solution (Earle’s balanced salt solution, ATCC). A 10% fetal bovine serum (FBS, ATCC) and 1% antibiotics were added as supplements. SPAN samples, as well as controls of polyacryline and polystyrene, were washed by Dulbecco’s phosphate buffered saline (PBS, GIBCO) twice, and then underwent 30 min UV-radiation sterilization. After trypsinizing (trypsin-DETA: 0.25% trypsin, 1 × 10⁻³ M EDTA-4Na, GIBCO), HOS cells were evenly seeded into each well of a six well plate, where the samples were
placed. Samples of the same composition were cultured in triplicate under the same conditions and the experiments were repeated at least twice. Cell growth was checked after 48 h.

2.4. Alkaline Phosphatase (ALP) Activity. The ALP working solution was prepared by dissolving p-nitrophenyl phosphate tablet and tris-buffer tablet (1:1; Sigma-Fast, p-nitrophenyl phosphate tablet sets, N-1891) in DI water. A 20 μL supernatant was collected from each well and mixed with 400 μL working solution. After incubating at room temperature for 30 min, the reaction was stopped with 100 μL 3 N NaOH. Another 500 μL DI water was added then the whole solution was transferred to a cuvette. ALP activity was measured by a colorimetric method with a GENESYS 10UV scanning spectrophotometer. The amount of p-nitrophenyl formed, which represents the ALP activity, is in direct proportion to the absorbance at 405 nm.

2.5. Proliferation. For proliferation study, HOS cells after 48h incubation were first fixed by 70% ethanol, followed by staining with propidium iodide (PI/Rnase staining buffer, BD Pharmingen), then observed under fluorescence with laser scanning cytometry (LSC). Proliferation study was conducted by counting the number of cells in an area with a fixed radius of 1.8 mm. At least five areas were randomly chosen and counted. The average number of cells living on different substrates, both with or without electrical stimulation, as well as standard deviations were, hence, obtained and compared with each other.

3. RESULTS AND DISCUSSION

Cell viability was investigated by growing HOS cells on the SPAN-deposited polystyrene Petri dishes, without electrical stimulation. It can be seen from Figure 1b that the cells growth was clearly affected by the DS and reached a maximum at 50% DS, similar to the conductivity profile. However, the subsequent drop in the cells growth may also be due to the high solubility of the SPAN in aqueous medium at higher DS. In a previously reported cytocompatibility study, when HOS cells were grown on SPAN-deposited glass plates compared to cells grown on glass alone (controls) showed no abnormal cellular behavior or cell death, though the cells growth was less compared to that of the cells grown on poly(l-lactic acid). This has led us to investigate the effect of electrical stimulation (AC, voltage or frequency) on HOS cells proliferation using SPAN-based IDEs. The IDEs were fabricated by a layer-by-layer deposition of SPAN copolymers on a polyethylene terephthalate (PET) film utilizing a micropatterning technique, as shown in Figure 2. The two comb-like micropatterns are the two electrodes and are connected to a power source. Due to the increased solubility of SPAN in aqueous medium and decreased electrical conductivity, particularly at ≥50% DS, three layered SPAN-coated IDEs were fabricated. SPAN with a 40% DS was coated at the bottom and on the top of the middle layer (65% DS) in order to obtain high conductivity and optimal cell growth. The dimensions of the SPAN-coated layers were measured to be in the range of 10–20 μm in thickness, about 400 μm in width and about 600 μm separation distance between the electrodes as described in the Figure 2. Finally, a thin layer of PLA was spin-coated with a thickness in the range of 2–4 μm, to prevent the current leakage from the SPAN electrodes into the biological medium. The effective applied electrical field in the medium was about 2.0 V/mm at the peak, as discussed below.

Electrical stimulation of HOS cells seeded onto the IDEs was performed with a sine wave generator (BK Precision Instruments) initially at a fixed frequency, 1 kHz. The cells were subjected at a steady potential between 0 to 1600 mV. A seeding density of about 120 cells/mm² was maintained for all experiments. Cells were maintained in a CO₂ incubator for the duration of electrical stimulation. The applied electrical stimulus through SPAN-based IDEs was found to significantly enhance the cells growth, as shown in Figure 3a. Furthermore, the growth of the cells increased with the increased stimulus voltage and showed no abnormal cellular behavior or cell death up to 1000 mV. However, when the applied voltage was increased above 1200 mV, the cells death was more pronounced. This has led us to choose a safe stimulus voltage of 800 mV, which was well below the apparent threshold voltage of 1000 mV, for further investigation.

The effect of electrical stimulus frequency on the HOS cells proliferation at a fixed voltage of 800 mV and corresponding alkaline phosphatase (ALP) activity were plotted in Figure 3b. A rapid increase in the cells growth was observed at a very low applied frequency (≤1 kHz) and then it remained steady until 100 kHz. However, a significantly enhanced cells death was observed above the stimulus frequency of 200 kHz as shown in the Figure 3b, inset. The ALP activity profile was similar to the cells proliferation profile, which further confirmed the above results.

Microscopic images of the HOS cells grown under the electrical stimulation by utilizing the SPAN-based IDEs are shown in Figure 4. In the top-image, the green stripe is a microline of the SPAN-coated layer. The cells appeared to grow uniformly in the microline and the green stripe is a microline of the SPAN-coated layer.
uniformly across the surface of the IDEs. Fluorescence images of HOS cells stained with propidium iodide (PI/Rnase staining buffer) revealed more densely populated cells near and over the IDE’s microline surface, which also indicate that the cells were well adhered onto the surface of the SPAN-coated layers (Figure 5). These results clearly demonstrate that the SPAN- 

4. CONCLUSIONS

We have described the synthesis, characterization and fabrication of SPAN based IDEs for electrical stimulation of HOS cells in vitro. This electrode exhibited good cell attachment and proliferation under mild electrical stimulus conditions in vitro. Increased degree of sulfonation of the SPAN copolymer, DS from 0 to 50%, has increased the conductivity of the copolymer that in turn increased the number of cultured HOS cells. However, a further increase in DS above 50% has led to a marginal decrease in both the conductivity of the SPAN and the HOS cell count, perhaps due to increased solubility of SPAN in aqueous medium. When the applied voltage or the frequency was increased by keeping the other constant at 800 mV or 1 kHz, an increased HOS cell count was found. Furthermore, the observed cell growth reached a threshold at an applied voltage, 1200 mV, or frequency, 200 kHz. However, a pronounced cell death was observed when the applied electric field was increased beyond the threshold limits. It is believed that the cell death was possibly due to the cell membrane rupture and the disruption of normal functions of the cells that are stimulated above these threshold limits.

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