Self-doped polyaniline-based interdigitated electrodes for electrical stimulation of osteoblast cell lines

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We report a facile method of synthesis and fabrication of self-doped sulfonated polyaniline (SPAN)-based interdigitated electrodes (IDEs) for cell stimulation. In this method, a layer-by-layer of SPAN copolymers was deposited in situ polymerization on a micropattern printed polyethyleneterephthalate (PET) film. Bone marrow stromal cells (BMSCs) and pre-osteoblast cells (MC3T3-E1) were utilized for the cell compatibility and proliferation studies. Increased degree of sulfonation was found to increase the SPAN conductivity, which in turn improved the cell attachment and cell growth. The in vitro osteogenesis of the BMSCs or the MC3T3-E1 cells grown on the SPAN based IDEs under the electrical stimulation was investigated by utilizing an alkaline phosphatase (ALP) activity assay. The Von Kossa staining of the cells revealed significantly increased mineralization by both the cells compared to their respective controls (without electrical stimulation). The high electrical conductivity and stability of the SPAN in a broad pH range of physiological culture medium in addition to the excellent cyocompatibility make the SPAN based IDEs as potential scaffold materials for in vitro cell culture and tissue engineering applications. © 2014 Published by Elsevier B.V.

1. Introduction

The next generation of implantable biomaterials, materials that incorporate stimulatory cues such as electrical signals, will be interactive and programmable, and thus capable of seamless communication with surrounding tissues, and can regulate cell attachment, proliferation, and differentiation [1]. Electrical charges play an important role in stimulating either the proliferation or differentiation of various cell types [2–4]. Both in vivo and in vitro studies have demonstrated that electrical stimulation, either in AC, DC, or pulsed electromagnetic field can stimulate cell growth or bone regeneration [5–7]. Conducting polymers (CPs) combine the attractive properties of plastics such as easy processing, adjustable physical and chemical properties (adhesion, wettability, surface charge, etc.) with the electrical properties of metals [8–15]. Therefore, CPs including polypyrrole, polythiophene, polyaniline, and polyethylenedioxythiophene, have been explored for their applicability to tissue regeneration [16]. 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) [17–19]. Polyaniline was also 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 doped polyaniline prior to its use [20]. Therefore, it is essential 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 in to the culture medium. Moreover, the materials utilized for tissue regeneration must be biocompatible, non-cytotoxic, non-carcinogenic, non-immunogenic and non-mutagenic [21].

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Here, we report electrical stimulation of bone marrow stromal cells (BMSCs) and pre-osteoblast cells (MC3T3-E1) utilizing a highly conductive and pH-stable sulfonated polyaniline (SPAN) based interdigitated electrodes (IDEs). Our interest in SPAN and its properties is attributed to the fact that the functional groups (−SO$_2$H) in the backbone chain improve solubility without substantially sacrificing conductivity and without the need for external dopants. For example, the conductivity of HCl doped polyaniline was found to decrease significantly when exposed to a cell culture medium [20]. In contrast, the conductivity of SPAN is independent of the external protonation in a broad pH range [22]. Additionally, the covalently bonded dopant (−SO$_2$H) will not be released into the biomedium due to its strong electron-withdrawing property, which makes the SPAN much more stable than polyaniline in aqueous medium [23].

In the present investigation, the cell compatibility was assessed by growing cells on SPAN-deposited polystyrene petri dishes without the electrical stimulation. In addition, the cells were grown on the IDEs under electrical stimulation and the cell growth was observed under a microscope. The time dependent osteogenesis was assessed by utilizing an alkaline phosphatase (ALP) activity assay [24], and the mineralization was assessed by the Von Kossa staining method [25]. Our aim was to introduce SPAN as a potential organic electrode, with good cyocompatibility, easy processing, and without the need of toxic dopants, and to replace traditional metal electrodes such as platinum for cell stimulation and tissue engineering applications.

2. Experimental

2.1. Chemicals and materials

All reagents and solvents were purchased from Sigma–Aldrich and used as received unless noted.

2.2. SPAN synthesis [8]

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 −NH$_2$ 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 sulfonated SPAN (AN: MA = 5:5, 1/2 oxidant), AN (4.6 g, 0.05 mol) was added dropwise to the stirred solution of MA (8.6 g, 0.05 mol) and APS (11.4 g, 0.05 mol) in HCl (1 M, 500 ml) at 0 °C. After 2 h stirring at 0 °C, 2-amino-benzene sulfonic acid (0.05 g, 0.29 mmol) was added to terminate the growing polymer chain and control the molecular weight of the SPAN at ~10,000 g mol$^{-1}$ [26]. The reaction was kept in ice bath, and stirred for another 6 h. Precipitation was allowed to occur overnight. The product was collected by filtering, washed exhaustively with DI water, and then dried in a vacuum oven at 50 °C for 24 h. The final dark green product was ground into powder stored in a sealed glass vial for future 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 the standard four-probe method using a Keithley® 2400 source meter [27].

2.3. Gel permeation chromatography (GPC) [28,29]

Molecular weights of the various polymers were determined by GPC using a Shimadzu LC-20AT pump, a RID–10A refractive index detector, and a Phenogel GPC 300 × 7.8 mm × 5 μm column. NMP/LiCl was used as the eluent at a flow rate of 1.0 mL/min. Sample concentrations of 5–10 mg/mL and injection volumes of 50–100 μL were used. Narrow polystyrene standards with molecular weights ranging from 550 to 480,000 g mol$^{-1}$ (Millipore, Waters Chromatography Division, MA, 1,757) were used to calibrate the system. The system calibration data were acquired and relative molecular weight calculations were processed using Shimadzu Class-VP software. All samples and vials must be scrupulously free of any particulate matter and all solvents and solutions filtered through 0.45 μm Teflon$^{{	ext{TM}}}$ filters before use.

2.4. Fabrication of SPAN based electrodes and electrode dish

A computer-designed micropattern was printed on a PET film using a conventional laser 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 (65% 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 from the printed PET film. The electrode dish devices were handmade by authors. The PDMS silicon based glue was used to connect with the micropatterned PET substrate with pre-ordered PC based frameworks. After the completion of the PDMS curing, the device was tested with DI-water to make sure there is no leaking. If a small leaking was found, the device can be easily re-bonded by utilizing the PDMS glue.

The entire electrode dish was irradiated by UV (λ = ~360 nm) in a biologically sterile hood for at least 1 h. The inside of the dish was rinsed by PBS before loading the cells and the medium.

2.5. BMSC and MC3T3-E1 cell culture

The mouse bone marrow stromal cells were obtained from healthy experimental mice, following the procedure described by Maniatis et al. [30]. Isolation and expansion of murine bone marrow stromal cells (BMSC) – femora, tibiae, and humeri – were dissected from an adult C57 Black-6 mouse, which was euthanized by CO$_2$ inhalation for reasons unrelated to this study. The epiphyses were removed and bone marrow flushed with a standard growth medium, which consisted of an alpha-modified minimum essential medium (α-MEM) with GlutaMAX$^{{	ext{TM}}}$ (Invitrogen), 10% FBS, 250 U/mL penicillin, and 250 μg/mL streptomycin (standard growth medium). The cells were incubated with 5% CO$_2$ in cell culture flasks at 37 °C. The media were changed every 3–4 days for 14 days, at which time BMSC were identified as the adherent monolayer. The animal surgery was carried out in the animal facility in the OSU veterinary labs, and the cell cultures were maintained in Dr. Rosol’s lab.

The MC3T3-E1 cell line was obtained from commercially available source (ATCC). Generally, MC3T3-E1 was cultured in polystyrene culture flasks in GlutaMax$^{{	ext{TM}}}$ (Gibco, Invitrogen) containing 10% fetal bovine serum in a 37 °C humidified incubator with a 5% CO$_2$ supply. The cells were subcultured every 3 days at the split ratio of 1/3 to 1/5, using 0.1% trypsin (Invitrogen). For electrical stimulation experiments, MC3T3-E1 cells were plated in the dishes with electrodes with 3 mL GlutaMax$^{{	ext{TM}}}$ and supplemented with 10% fetal bovine serum.

2.6. Osteogenic differentiation and mineralization of BMSC and MC3T3-E1 cells

Pre-osteoblastic MC3T3-E1 cells were plated at an initial density of 200,000 cells per well and BMSC at 350,000 cells per well in custom-made 6-well plates (Corning) in 2.5 mL of standard growth medium. At confluence, the cells were treated with 2.5 mL of osteogenic medium, consisting of the standard growth medium, 250 μM ascorbic acid (Fisher), and 10 mM β-glycerophosphate (MP
biomedicals). The osteogenic medium for BMSC consisted of the previous reagents plus 10 mM dexamethasone. The osteogenic medium was collected and frozen at −80 °C every 3 days for 15 days, with a fresh osteogenic medium applied after each collection.

2.7. Alkaline phosphatase (ALP) activity

ALP activity was measured in the medium of each well at days 3, 6, 9, and 12 using a p-nitrophenyl phosphate (pNPP) colorimetric assay (Anaspec). A 50 μL of pNPP reaction mixture was added to a 50 μL of medium in a 96-well microplate, and then incubated the mixture for 30 min. The pNPP absorbance was measured at 405 nm using a plate reader (molecular devices).

2.8. Von Kossa staining for mineral deposition

The cell culture flasks were rinsed with PBS. After 15 days, the wells were fixed with 95% ethanol for 15 min, rinsed, and dried. The wells were treated with 5% silver nitrate in the dark for 20 min, rinsed, and treated with 0.5% hydroquinone (Acros Organics) under ultraviolet light for 5 min. The wells were then rinsed, treated with 5% sodium thiosulfate (Fisher), rinsed again, and dried. The plates were imaged with a 12.5 megapixel resolution 16-bit digital camera (Nikon).

2.9. Statistical analyses

Numerical data were analyzed using standard analysis of variance (ANOVA) techniques and statistical difference was considered at p < 0.05. All cell experiments were completed three times and two replicates were used for each experiment.

3. Results and discussions

3.1. Synthesis and properties

A series of SPAN was synthesized by copolymerization of aniline (AN) and metanilic acid (MA), with ammonium persulfate (APS) as an oxidant in 1 M HCl, as shown in Fig. 1(a). The amount of functional group (−SO₃H) incorporated into the back bone chain was controlled by adjusting the ratio of the two monomers. The growing polymer chain was terminated by addition of 2-amino-5-benzene sulfonic acid end group. A key control factor for the electroactive phenomena, processing, and potential applications is the degree of sulfonation, that is, the sulfur-to-nitrogen (S/N) ratio [8,31]. Hence, the S/N ratio in the synthesis of SPAN is an important parameter, which can be precisely controlled by simply changing the monomer ratio.

The conductivity of the synthesized SPAN series was measured with the standard four-probe method. Prior to the conductivity

![Fig. 1. The synthetic scheme of SPAN (a) and conductivity and molecular weight table (b) at pH 1 and pH 7.4. Where PANI is polyaniline and SP-xx is SPAN with %xx of sulfonation.](image-url)

<table>
<thead>
<tr>
<th></th>
<th>Pani</th>
<th>SP-10</th>
<th>SP-15</th>
<th>SP-25</th>
<th>SP-30</th>
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<td>7.0</td>
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<tr>
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<td>11.4</td>
<td>11.4</td>
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<tr>
<td>Yield (%)</td>
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<td>74.9</td>
<td>68.4</td>
<td>61.8</td>
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<td>23.5</td>
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<td>0.5</td>
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<td>0.08</td>
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<tr>
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<td>3x10⁻⁵</td>
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<td>7x10⁻⁴</td>
<td>2x10⁻³</td>
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measurement, powder samples of SPAN were dissolved in a phosphate buffered saline (PBS, pH 7.4) solution and subsequently cast into thin films and dried in a vacuum oven at 50 °C for 2 days. The conductivity of the undoped polyaniline (0%, sulfonation) was found to be, \( \sigma_{\text{dc}} = 5 \times 10^{-10} \text{ s/cm} \). It increased with the degree of sulfonation (DS) and reached a maximum conductivity, \( \sigma_{\text{dc}} = 5 \times 10^{-2} \text{ s/cm} \) at 50% of sulfonation, and then it decreased upon further increase in DS as expected due to the strong intramolecular interactions [31–33]. The conductivity of the synthesized SPAN series was measured at pH 1 and at pH 7.4 along the polymer yield and the results are presented in Fig. 1(b).

The molecular weight of selected SPAN series was measured with GPC. 0.05% weight percent of LiCl was added to reduce the ionic effect [28,29]. The GPC spectra, as shown in Figs. S1–S5, gave the weight average molecular weight, number average molecular weight and polydispersity index (PDI) which are presented in Fig. 1(b). Generally an increased DS led to a decreased molecular weight and increased PDI.

### 3.2. Cell compatibility

Cell compatibility was investigated by growing BMSC or MC3T3-E1 cells on the SPAN-deposited polystyrene petri dishes without electrical stimulation. No abnormal cellular behavior or cell death was observed. However, the cell growth (relative amount of cells at a particular time) was clearly affected by the extent of sulfonation of SPAN. The BMSC cell growth reached a maximum at 60% of DS, as shown in Fig. 2(a), while the MC3T3-E1 cells reached a maximum at 50% of DS, as shown in Fig. 2(b), respectively after 5 days, similar to the SPAN’s conductivity profile as shown in Fig. 1(b). Both cell lines exhibited similar cell growth on the SPAN films and the cell growth was comparable to their respective

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**Fig. 2.** Assessment of SPAN biocompatibility utilizing (a), BMSCs and (b), MC3T3-E1 cells, without electrical stimulation, after 5 days. The controls were cells grown on polystyrene petri dishes. *Denotes a statistically significant difference when compared to control; compared with a 1-way RM-ANOVA and a Newman–Keuls post-hoc analysis with \( p < 0.05, n = 6 \). (c) Schematic fabrication method of interdigitated electrodes (IDEs) by layer-by-layer deposition of SPAN copolymers by in situ polymerization on a polyethylene terephthalate (PET) film utilizing a micropatterning technique.
controls. However, further increase in DS has led to a decrease in the cell growth due to the increased solubility of the SPAN in aqueous medium [31], which may have resulted into cell detachment. In a previously reported cytocompatibility study, when human osteosarcoma (HOS) cells were grown on SPAN deposited glass plates compared to cells grown on glass alone showed no abnormal cellular behavior or cells death, our previous research showed that in proliferation studies more than 70% of cells were found viable on SPAN compared to 88% for poly(ε-lactic acid) with the number of cells growing on glass as a control, indicating generally good biocompatibility [8].

3.3. SPAN based IDEs for cell stimulation

For electrical stimulation of osteoblast cell lines, we have fabricated the IDEs by utilizing a facile micropatterning technique [34]. In this method, a layer-by-layer of SPAN copolymers were deposited by in situ polymerization on a micropattern printed polyethylene teraphthalate (PET) film. The two comb–like micro-patterns, as shown in Fig. 2(c), are the two electrodes and are connected to a power source. Due to the increased solubility of SPAN in aqueous medium, particularly at ≥50% DS, three layered SPAN coated IDEs were fabricated. SPAN with a 40% DS was coated at the bottom and on top of the middle layer (65% DS) in order to obtain high electrical conductivity.

3.4. Electrical stimulation of osteoblasts

Electrical stimulation of the BMSCs or MC3T3-E1 cells seeded onto the IDEs was performed with a sine wave generator (BK Precision Instruments) at a fixed frequency, 1 kHz and at a steady potential, 500 mV. These conditions were chosen based on our preliminary experimental results (data not shown). It was found that the effect of the stimulation on the cell growth was untenable at frequency ≥50 kHz and voltage ≥800 mV. In addition, an increased cell death was observed. For example, at 50 kHz and voltage of 800 mV, the MC3T3-E1 cells did not proliferate and began to die and disappear completely after 9 days.

Fig. 3. Electric field effect on the secretion of the osteogenic biomarker alkaline phosphatase (ALP) by (a) BMSC cells, and (b) MC3T3-E1 cells grown on the SPAN based IDEs. *Denotes a statistically significant difference when compared to control: a, when compared to day 3; and b, when compared to day 6. Bars represent mean ± 1 S.D of 6 culture wells. The images show BMSCs and MC3T3-E1 cells grown on the SPAN based IDEs, after 15 days without (left, control) or with (right) electric stimulation under a controlled electric field (500 mV + 1 kHz), after Von Kossa staining, which stains osteoblast deposited mineral black. The images were recorded with a digital camera (Nikon coolpix 950) directly from the eyepiece of the microscope at magnification ×200.

Similar to the MC3T3-E1 cells, the BMSC cells too had very good response at 1 kHz and voltage of 500 mV. These cells proliferated well with an increase in cell numbers. Under the microscope, many granules of mineral deposition between the BMSC cells were clearly visible. However, the BMSC cells too did not appear healthy and gradually died when these cells were stimulated at ≥50 kHz and voltage of ≥800 mV. From the cells (MC3T3-E1 and BMSC) responses, it was clear that the stimulation conditions at frequency ≥50 kHz and at voltage ≥800 mV were not suitable for cell culture.

A special morphology of the MC3T3-E1 cells, which were aligned in a specific direction within Day 3, was also observed. These aligned thin and long MC3T3-E1 cells were found to be dying. However, from our preliminary results (data not shown) we found that both the cells (MC3T3-E1 and BMSC) tend to align in the applied electric field direction. Tight coupling between cell shape and growth under electrical stimulation also been reported in the literature [35,36]. For example, rounding of bovine aortic endothelial cells plated on oxidized fibronectin–polypyrrole films under electric potential (−0.5 V) was reported [2]. Although, several possible mechanisms were proposed for rounding of cells, it was speculated that electrically conducting polymers can be utilized for both small- and large-scale cell cultures since they provide a non-invasive way to regulate cell form and function.

3.5. Osteogenesis and mineralization

Alkaline phosphatase (ALP) is an important biomarker of the osteogenesis process, and there are many commercial kits for the
alkaline phosphatase test. The most common method is to test alkaline phosphatase using p-nitrophenylphosphate (p-NPP) as the enzyme substrate [24]. The product of the hydrolytic reaction catalyzed by alkaline phosphatase is p-nitrophenol, which has high molar absorption at the wavelength of 405 nm. The in vitro osteogenesis of the BMSCs or the MC3T3-E1 cells grown on the SPAN based IDEs under the electrical stimulation was investigated by staining the culture medium for ALP activity for a period of up to 12 days and compared with the controls [37] (without electrical stimulation) as shown in Fig. 3. The applied electrical stimulation was found to affect the cell growth for both cell lines. However, the stimulation effect on cell growth was observed to be more pronounced on the MC3T3-E1 cells when compared to the BMSC cells. In fact the stimulus effect on the BMSC cell growth was reduced when compared to its control after 12 days, also shown in Fig. 3(a), and this was evidenced in the ALP activity assay as well. Nevertheless, both the cell lines grown on the SPAN based IDEs under the electrical stimulation exhibited an enhanced cell growth and osteogenesis.

The Von Kossa staining, which stains osteoblasts deposited mineral black, for samples after 15 days are shown in Fig. 3, indicates a significant increase in the mineralization by both the cells compared to their respective controls (without electrical stimulation). In particular, the BMSC cells have more cells that are differentiated into osteoblasts and more cells have expressed the bone feature. These results are very encouraging because it reflects the real bone-formation feature of normal cells grown on SPAN based IDEs under the electrical stimulation. Also, these results indicate the real potential of the SPAN based electrodes for bone cell stimulation and mineralization for bone constructs, which ultimately may find applications in tissue engineering.

It is well-known that electrical stimulation can enhance healing of bone fractures [38]. Electrical stimulation can induce the level of transforming growth factor-beta 1 (TGF-B1) in osteoblastic cells by a mechanism involving calcium/calmodulin pathway. [6] TGF-B is one of the important growth factors involved in formation of bone and cartilage. The voltage-gated calcium ion channel plays a crucial role in the cellular signal transduction. This special calcium ion channel on the surface of cells can detect the membrane potential and change the opening of channels, which can change the cellular calcium ion concentration. The alteration of cellular calcium ion concentration can in turn induce multiple physiological pathways. However the precise mechanism is unknown, the calcium ion channel on the surface of cells may have played a significant role in the observed electrical stimulus effect on the enhanced cell growth and mineralization by SPAN based IDE, and it is the subject of our future investigation.

4. Conclusions

We have described the synthesis, characterization and fabrication of electrically conducting self-doped sulfonated polyaniline based interdigitated electrodes for osteoblast cell proliferation and mineralization under electrical stimulation in vitro. Tests showed 40–60% sulfonation gave significant better conductivity from pH 1 to 7.4. The SPAN based electrodes have several advantages such as high electrical conductivity and independent of the external protonation in a broad pH range [23], without the need of toxic dopants that may leach out into the cell culture medium and may cause undesired results. Under the electric field of 50 kHz and 800 mV, both BMSC and MC3T3-E1 cells grow faster than control. Furthermore, SPAN based electrodes may find utility in tissue engineering applications as a temporary scaffold for bone cell attachment and proliferation and as a carrier for electrical signals to stimulate the cells for tissue regeneration.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.synthet.2014.10.035.

References

[26] Each 2-aminobenzene sulfonic acid stopped one growing polymer chain. The molecular weight (MW) of 2-aminobenzene sulfonic acid is 173, 0.05 g of that is 0.29 mmol. Ideally, 0.29 mmol of SPAN could be got after the polymerization reaction. The MW of aniline (AN) and metallic acid (MA) are 93 and 173 respectively, and the ideal average MW of SPAN is [(93 + 173) * 0.05/0.29] = 1000 ÷ 46 K.

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