Assessing the use of conjugated polymers and electric fields in cell culture

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Abstract

Neural stem cell-based tissue engineering therapies are one of the most attractive therapeutic strategies for treating neurodegenerative disorders. *In vivo*, neural stem cells (NSCs) are adherent to extracellular matrix, which provides chemical, mechanical and topographical cues, controlling in this way cell behavior and functionality. In this study, different substrates were designed and tested to control fetal NSCs alignment and elongation through specific control of the topography and electric stimulation. RenCell VM cell line was used to evaluate the efficacy of PEDOT:PSS substrates to promote cell adhesion, proliferation and differentiation. Our findings suggest that PEDOT:PSS exhibits adequate physicochemical properties and a good biocompatibility that promote cells attachment and proliferation. Our results suggest that the application of an AC electric field (EF) of 1 V/cm elongates and stretches more the cells as compared to control surfaces that not were exposed to EF. The differentiation potential of conductive PEDOT:PSS/glass substrate was evaluated by immunostaining of key neuronal and glial markers, expressed during NSCs differentiation. The conductive PEDOT:PSS/glass substrates promoted differentiation into neuronal and glial lineage and the NSCs cultured in their surface increased β-III tubulin expression and the neurite length as compared with control. Findings from this study suggest that combining EF and PEDOT:PSS provides a promising strategy to modulate NSCs elongation and differentiation, having a great potential to be used in regenerative medicine and disease therapies.

Introduction

Stem cells are distinguished from the other cells by their ability to divide producing copies of themselves (self-renewal) and/or to differentiate into mature cells that have characteristic shapes and specialized functions, such as skin cells or nerve cells [1].

NSCs are multipotent cells that can be isolated from *in vivo* sources, the fetal and adult nervous systems, or *in vitro* derived from embryonic stem cells (ESCs), derived from blastocyst stage, or from induced-pluripotent stem cells (iPSCs), derived from reprogrammed cells[2,3,4]. NSCs are able to self-renew and generate neurons, astrocytes, and oligodendrocytes[5]. After embryonic development, cells are kept in a niche to produce new cells of the nervous system. The niche provides microenvironmental cues essential to the balance between stem cell quiescence and proliferation and to direct neurogenesis versus gliogenesis lineage decisions[6]. In the adult brain, the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus of the hippocampus are well-characterized germinal regions that support neurogenesis and the place where NSCs reside. The occurrence of neurogenesis and the activation of endogenous [7] and exogenous NSCs are very important once these offer possible sources for neural repair and NSC-based treatment of neurodegenerative disorders [2]

Biomaterial scaffolds combined with bioactive factors and multipotent cells have been used in tissue engineering strategies to, after transplantation into damaged tissue, promote tissue development and function improvement [8](Figure 1).

Researchers have tested several natural and synthetic polymers and have modeled topography, surface chemistry, electricity and stiffness of the material in order to get substrates with similar properties to those of extracellular matrix (ECM).

Conjugated polymers (CP) are a new class of polymers that have gain attention in the sense that they respond to light and electrical stimuli and their

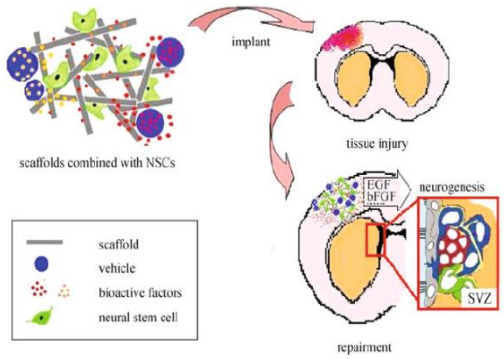

Figure 1. Neural repair and regeneration strategy based on immobilization of growth factors on biomaterial scaffold. The NSC are seeded on scaffold and transplanted to injury tissue to promote neurogenesis (taken from [8]).

molecular structure can be tuned to minimize toxic effects. CP are molecular materials whose backbone is made of alternating double- and single-bonds. These polymers are able to transfer charge from a biochemical reaction, they are biocompatible, allow the control over the level and duration of electrical stimulation for tissue engineering applications and possess the ability to entrap and controllably release biological molecules [9]

Poly(3,4-ethylenedioxythiophene) doped with polystyrenesulfonate (PSS) (PEDOT:PSS) is, probably, the most promising candidate for long-term implantation in the central nervous system because, when compared to other conductive polymers such as polypyrrole (PPy), possesses several advantageous properties: it combines a moderate bandgap located at the transition between the visible and near-IR regions of the spectrum and a low oxidation potential with good stability in the oxidized state[10] and has superior thermal and electrochemical stability[11]. PEDOT has been used as coating of neural electrodes and nano-fiber electrodes due to its high charge delivery capacity leading to very low impedance and highly effective charge transfer, which results in higher signal-to-noise ratios for improved electrophysiological recordings, a more effective interface for constant current stimulation[12] and an excellent substrate to regulate adhesion, proliferation and signaling of neuronal cells[13].

The ability of PEDOT:PSS to sustain growth and differentiation of ReNcell VM progenitor cells was assessed during this work.

The first task of this work was to assess the toxicity of all selected conjugated polymers using L929 cell line for material ISO cytotoxicity tests. The second task was to design conjugated polymer-based structures to provide electrical and patterned stimuli to cell culture. Replica molding and microcontact printing were the methods used for fabricating topographically patterned substrates for use as engineered microenvironments for cells. The patterns were obtained using a vinyl record disc as mold. Therefore, cells were grown on substrates with topographic patterns with micron-scale features.

The final task was to test with ReNcell VM cells the effect of material constructs on cell behavior such as cell adhesion, expansion and differentiation, confirming the effect of topography in cell morphology and organization.

The improvement of the platforms for cell culture is essential not only for a better understand of the cell behavior *in vivo*, but also to contribute to the design of scaffolds for tissue replacements.

Materials and Methods

Preparation of conducting polymer films

Different conjugated polymer solutions were prepared and used to make thin films by spin coating. To prepare polymeric films by spin coating, the following dispersions were prepared.

Sample 1: Cross-linked PEDOT:PSS

The ethylene glycol (Sigma-Aldrich) was added in a volume ratio of 1:4 to PEDOT:PSS (Heraeus, CLEVIOS P AI 4083, solids content 1.3-1.7%). Dodecylbenzenesulfonic acid (DBSA) (0.5µL/mL) and 3-glycidoxypropyltrimethoxysilane (GOPS) (10 mg/mL) were added to the solution to improve film formation and stability.

Sample 2: Neutralized PEDOT:PSS

The ethylene glycol was added in a volume ratio of 1:4 to PEDOT:PSS. Then, the crosslinker GOPS (20mg/mL) was added to improve film stability. The sodium hydrogen carbonate ($NaHCO_3$) was added until achieve a neutral pH.

Sample 3, 4, 5: P3HT, MEH-PPV, F8T2

Poly (3-hexylthiophene) (P3HT) and poly-(2-methoxy-,-5-(2'-ethyl-hexyloxy)-*p*-phenylenevinylene) (MEH-PPV) were dissolved in chloroform and poly(9,9-dioctylfluorene-*alt*-bithiophene) (F8T2) was dissolved in xylene in order to be used in the preparation of thin films.

The aqueous dispersions of PEDOT:PSS was spin coated on lamellas at 1800 rpm (60s) and post annealing (150°, 2 min) films with ~ 90µm thickness was obtained. The others polymers were spin coated on lamellas at 1500 rpm suffering the same annealing process.

Measurement of conductivity of films

PEDOT:PSS dispersions were coated on square glass slides. Film resistance was measured using the two- and four point probe method. The thickness of the films was measured using a Dektak 3.21 Profilometer.

The conductivity was then calculated from Equation 1::

$$\sigma(S.cm^{-1}) = \frac{1}{film\ resistivity\ (ohm.cm)}$$

Cytotoxic tests

Cytotoxicity assays *in vitro* were performed according to the ISO 10993-5:2009(E) guidelines in order to assess the biocompatibility of materials. Materials were exposed directly and indirectly in contact to the cell culture system (L929 mouse fibroblast cell line). Triplicates for each material were placed on 6-well plates containing 2 mL of IMDM 10% with 10% (v/v) of FBS and kept in an incubator (37ºC, 5% CO_2, fully humidified) for 72 hours. The L929 fibroblasts were seeding in 24-well plate, at an initial density of 8×10^4 cells/cm^2, and were incubated with liquid extracts of materials for 24h. The cell proliferation was analyzed using the cell proliferation reagent WST-1 (Roche). In order to evaluate the effect of the direct interaction between materials and L929 fibroblasts, the materials were placed in contact over the cells in 12-well plates ($11{,}7\times10^4$ cells per well) with IMDM 10% (v/v) of FBS and kept in an incubator (37 ºC, 5% CO_2, fully humidified) for 48h. After the incubation period, cells were observed under an inverted fluorescence microscope in

order to qualitatively evaluate if they are confluent or if a halo of inhibition is formed.

Conductive substrates

Perpendicular electric field setup

The scheme of the electric field setup developed to apply perpendicular pulsed electric field during *in vitro* cell culture is shown on Figure 2. The PEDOT:PSS coated glass substrate that supports the cells was used as an electrode and the gold plate positioned above of substrate was used as another electrode. Both the electrodes were kept parallel to each other and a perpendicular electric field was generated between the electrodes. An external AC power supply, connected to the electrodes, was used to apply electric field.

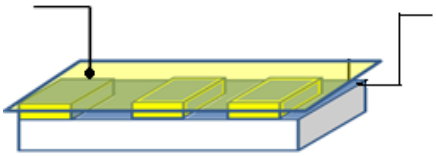

Figure 2. Schematic illustration of the perpendicular electric field setup used in the present study to stimulate cells.

Longitudinal electric field setup

Square glass slides were cleaned with isopropanol, dried with nitrogen and exposed to oxygen plasma (3min) previously to the spin coating of PEDOT:PSS at 1300 rpm (60s). Ten layers of PEDOT:PSS were deposited making an annealing of the 130º during 15 min after each deposition. A hollow cylinder was glued on the substrate with a biocompatible glue FDA approved (Silastic® medical adhesive silicone, type A) in order to stanch the culture system. In order to enhance the electrical contact, strips of gold with 40 nm of thickness were deposited on each end of the central ring which contains the cell culture. Platinum wires (Sigma-Aldrich®) were placed in these strips and an external AC power supply, connected to the electrodes, was used to apply an electric field vector that runs horizontally.

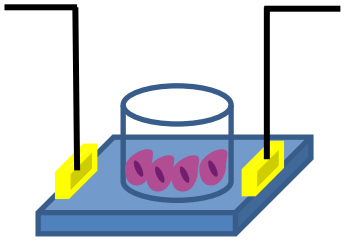

Figure 3. Schematic illustration of the longitudinal electric field setup.

Anisotropic and conductive substrates

Polyacrylamide gels

First, amino-silanated coverslips were prepared. Different elastic moduli substrates were prepared varying the relative concentration of acrylamide and bis-acrylamide The acrylamide (Sigma-Aldrich®) (40%) and bys-acrylamide (Sigma-Aldrich®) (2%) were mixed in the desired concentrations in distilled H_2O. A volume of 1/100 (v/v) of ammonium persulfate (APS, Sigma-Aldrich®) and 1/1000 (v/v) of tetramethylethylenediamine (TEMED, Sigma-Aldrich®) was added to the solution. To obtain PA hydrogels with grooves, the hydrogels were polymerized on the top of a piece of vinyl. A two-step zero-length crosslinking procedure was used to functionalize the PolyA hydrogels[14]. Laminin (Sigma-Aldrich®)(20µg/ml in PBS) was incubated, at room temperature, for 15 minutes with 2 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC; Sigma-Aldrich®) in the presence of 5 mM *N*-hydroxysuccinimide (sulfo-NHS; Sigma-Aldrich®). The reaction is stopped by the addition of β-mercaptoethanol and 1mM NaOH was added to raise the pH . Hydrogels were put in 24 wells ultra-low attachment cell culture plates (Dow Corning®) and were sterilized with a solution of Antibiotic-Antimitotic (Gibco®) for 2 hours. The hydrogels were twice washed with PBS to remove any traces of the antibiotic-antimitotic solution and the cells were plated at an initial density of 140 000 cell/cm^2.

Transparent vinyl substrates

The transparent vinyl disc was cut into circles with 9cm^2 and these were washed with isopropanol and dried with a nitrogen stream. The circles of vinyl were exposed to an oxygen plasma (3min) and ten layers of PEDOT:PSS were deposited making an annealing of the 130º during 15 min after each deposition. A hollow cylinder was made using a biocompatible glue in order to stanch the culture system and strips of gold with 40 nm of thickness were deposited on each end of the central ring which contains the cell culture. Platinum wires were placed in these strips and an external AC power supply, connected to the electrodes, was used to apply an electric field vector that runs parallel to the grooves. These substrates were immerse in isopropanol during three days and then in ethanol for 1 day. The circles of vinyl disc were put on 6-wells cell culture plate (Dow Corning®) and sterilized with UV during 1h30 min and with a solution of antibiotic-antimitotic during 2h.

Cell line

ReNcell VM (Millipore®) is an immortalized human neural progenitor cell line that was derived from the ventral mesencephalon region of a human fetal brain tissue and that was immortalized by retroviral transduction with the v-myc oncogene. This cell line has the ability to differentiate into neurons and glial cells[15]. In order to expand cells, RenCell VM were platted on T-flasks (Falcon®, BD Biosciences). The T-flasks with cells were incubated at 37ºC and 5% CO_2 in a humidified atmosphere using DMEM/F12 medium supplemented with 20 ng/mL EGF (Prepotech), 20 ng/mL FGF-2, 1% N2 supplement (Life Tecnologies), 20 µl/mL B27 supplement (Life Tecnologies), 20 µg/mL additional insulin (Sigma), 1,-6 g/L additional glucose (Sigma) and 1% penicillin/streptomycin (Life Technologies). The medium was changed every 2 days until cells reached an 80-90% confluence.

Cell culture in the various substrates

Prior to the seeding of cells, PEDOT:PSS/substrates were sterilized with UV for 1h 30 min and with a solution of antibiotic-antimitotic (Life Tecnologies) for 2 hours. The

substrates were incubated with poly-L-Ornithine (Sigma) for 30 minutes, at 37°C and 5% CO_2 humidified environment, washed once with PBS and incubated for 3 hours with laminin (20µg/mL, Sigma-Aldrich®)) diluted in PBS. Cells were plated at an initial cell density of 140 000 cells/cm^2 in DMEM medium supplemented with EGF (20ng/mL), FGF (20ng/mL) and B27 (20µg/mL).

Morphology of NSCs on substrates

The NSCs nuclei and cytoskeleton were labeled with 4',6-diamino-2-phenylindole (DAPI, Sigma) and rhodamine phalloidin probe (Sigma), respectively, in order to analyze the adhesion, morphology and spreading of these cells on the polymeric substrates. NSCs were fixed with 4% paraformaldehyde (PFA, Sigma) for 15 min, washed with PBS, and permeabilized with staining solution (0,1% Triton-X-100 (Sigma) in PBS with 5% Normal Goat Serum (NGS, Sigma)) for 15 min. Secondly, cells were stained with 300 µL of rhodamine phalloidin probe (1 µL.mL-1 in PBS) for 45 min at room temperature (RT). After washing once in PBS, cells were incubated in 300 µL of DAPI (1.5 µg/mL in PBS) for 5 min at 37ºC under a 5% CO_2 humidified atmosphere. Finally, cells were twice washed in PBS, kept in PBS, and protected from light. Blue-stained nuclei and red-stained cytoskeleton were visualized under a fluorescence optical microscope (DMI 3000B, Leica) and digital images were taken with a digital camera (DXM 1200F, Nikon).

Elongation of NSCs on PEDOT:PSS substrates

The elongation of adherent NSCs on conductive PEDOT:PSS was investigated after 4 days of culture using SEM imaging. Elongation of cells was assessed by measuring the length of the long axis and short axis. A least minimum of 30 cells were evaluated. The factor *E* equals the long axis divided by the short axis, shown on Equation 2:

$$Elongation\ (E) = \frac{Length\ of\ major\ axis}{Length\ of\ minor\ axis}$$

The cells were fixed with glutaraldehyde 1,5% (v/v) in PBS at 37° for 1h. The samples were washed three times with PBS and after that, the samples were immersed in ethanol solutions at different concentrations, 25%, 50%, 75% and 99% for 30 min each at 37°. Before the observation of the substrates they were coated with a 30 nm Au/Pd layer using a Polaron model E5100 coater (Quorum Technologies). Images were obtained using a Field Emission Gun Scanning Electron Microscope (FEG-SEM) (JEOL, JSM-7001F model).

Differentiation of NSCs on PEDOT:PSS substrate

When the EGF and FGF are omitted from the medium, the NSCs undergo differentiation in neuronal and glial direction. The differentiation process was carried out for 7 days, changing the DMEM/F12 medium every 3 days.

In order to evaluate the differentiation of NSCs into different neural phenotypes, the cells were immunostained for neuronal marker (Tuj1) and for astrocyte marker (GFAP). Cells were fixed with PFA 4% (2mL per well of six well-plate) for 30 minutes at room temperature, and then washed twice with PBS. Cells were incubated for 30 minutes at room temperature with blocking solution (90%PBS, 10% NGS and 0,1% Triton X) and, after this, the primary antibodies diluted in staining solution were incubated at 4°C overnight. After the incubation with the primary antibody, cells were washed once with PBS and incubated with the appropriate secondary antibody for 1 hour at room temperature in a dark container. Finally, cells were washed once with PBS and incubated with DAPI nucleic acid stain for 2 minutes at room temperature and washed twice with PBS. The stained cells were visualized under a fluorescence microscope (Leica DMI 3000B).

Results & Discussion

Electrical characterization of PEDOT:PSS films

The conductivity of PEDOT:PSS films (Aldrich and Clevios) prepared from the as-received are compared with films prepared with PEDOT:PSS:GOPS and PEDOT:PSS:GOPS:DBSA.

Table 1 shows the results obtained for each type of film, using the four-probe technique.

The Aldrich PEDOT seems to be more conducting comparatively to Clevios PEDOT. The weight ratio of PEDOT:PSS purchased from Sigma and Clevios are 1:1.6 and 1:6 , respectively. PEDOT Clevios has a highly excess of PSS in solution which lowers the conductivity of the films.

Similar results were obtained using the two-point probe method (data not shown). The four-point probe method is superior due to the use of two additional probes that measure the voltage potential of the material. These probes do not carry any current, thus eliminating the contact resistance (Rc) and the resistance caused by current flowing into the sample surface (spreading resistance, Rsp) measured in the two-point probe method.

Table 1. Conductivity values obtained for different PEDOT:PSS films

Four-point probe method	
PEDOT:PSS Aldrich	4.3 $\Omega^{-1}m^{-1}$
PEDOT:PSS:GOPS Aldrich	3.07×10^3 $\Omega^{-1}m^{-1}$
PEDOT:PSS:GOPS:DBSA Aldrich	3.0×10^3 $\Omega^{-1}m^{-1}$
PEDOT:PSS Clevios	8.28×10^{-1} $\Omega^{-1}m^{-1}$
PEDOT:PSS:GOPS Clevios	4.92×10^{-1} $\Omega^{-1}m^{-1}$
PEDOT:PSS:GOPS:DBSA Clevios	5.78 $\Omega^{-1}m^{-1}$

Making use of the secondary doping process, the PEDOT:PSS films were exposed to dichloroacetic acid in order to improve their conductivity[16]. Exposing PEDOT:PSS films to a simple

postdeposition solvent annealing treatment does not improve the electrical conductivity (data not shown).

Cytotoxic tests with fibroblasts

Indirect and direct cytotoxic tests were performed following the ISO 10993-5 guidelines for medical devices in order to assess the cytotoxicity of each polymer. The values of cell metabolic activity obtained in cytotoxicity assays showed that none of the polymers has cytotoxic effects when compared to commonly used tissue culture polystyrene (negative control) (Figure 4).

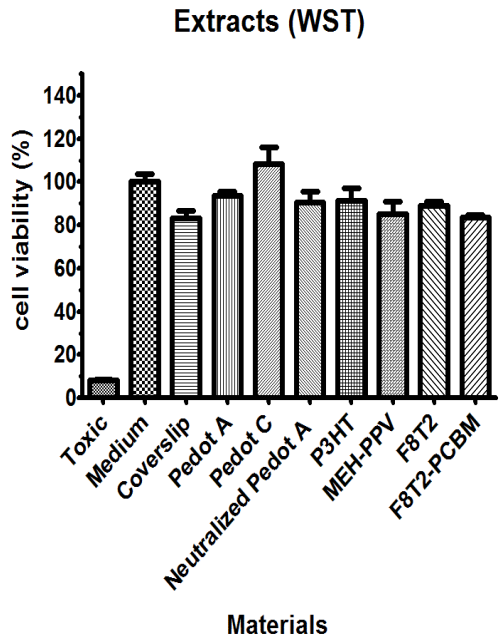

Figure 4. Cytotoxicity assays for P3HT, F8T2, F8T2:PCBM, MEH-PPV, Neutralized PEDOT Aldrich, PEDOT Aldrich and PEDOT Clevios, following the ISO standards for biomaterials with tissue culture plate (polystyrene) as negative control and a piece of latex glove (toxic) as positive control. Triplicates were performed for each condition.

When direct assays (Figure 5) where performed to evaluate polymers cytotoxicity no inhibition halo was observed. Therefore, these polymers are suitable to promote stem cell adhesion and proliferation.

Perpendicular electric field effect in NSCs

Besides chemotactic signals and topography, electric fields can be a source of directional cues *in vitro* to control cellular processes including microfilament reorganization, proliferation, differentiation and migration of NSCs towards specific targets.

Electric fields of 1-2V/cm occur during morphogenesis and wound healing and have been shown to orient the movements of a wide variety of cells *in vitro*[17].

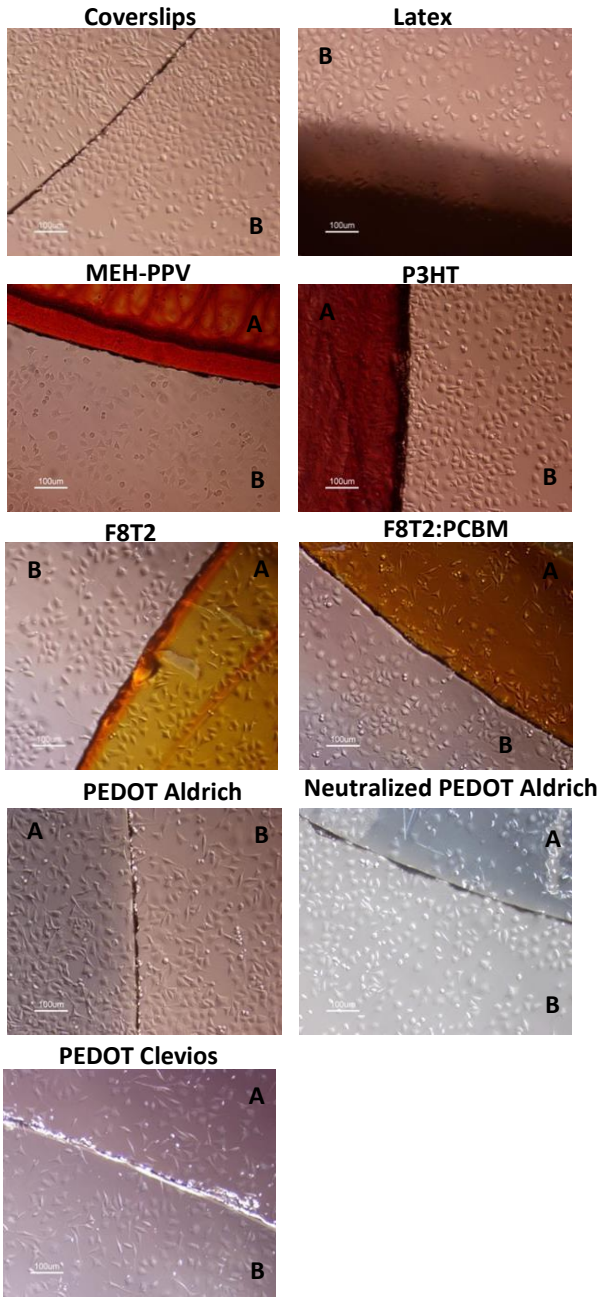

Figure 5 . Cytotoxicity assay for the direct contact assay. The positive control is a piece of latex glove and the negative control is the culture plate of the polystyrene. Conjugated polymer-coverslip (A) were put in contact with cells cultured on polystyrene plate (B).

Taking this into account and also in order to avoid the electrolysis of water (occurs at 1,2V) that would change pH of medium due to the ions produced or consumed, the 1V/cm was the magnitude chosen to be applied.

The proliferation assays and the NSCs culture were compromised due to some characteristics of substrates namely the leakage of cells (because they were not stanched) and also due to its opacity, making impossible to observe the cells under the

optical microscope. After 7 days, cells were re-plate in a 24-well-plate in order to analyze their viability (Figure 6).

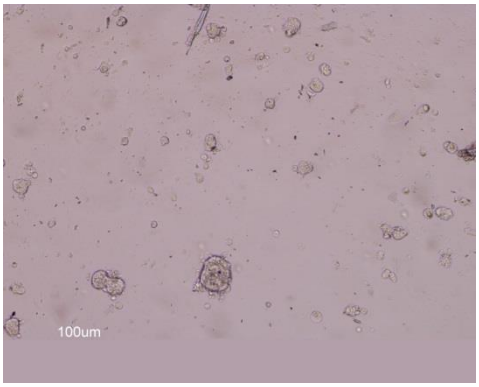

Figure 6. Replating of NSCs after 7 days *in vitro* under the influence of vertical electric field.

The cells did not adhere, meaning that they were not viable. The surface topography and the vertical electric field may have negatively influenced the laminin adsorption and the interactions that retain the proteins at the surface of substrates. The absence of strong interactions between proteins and the surface of substrate causes desorption of biomolecules and, consequently, the failure of cell adhesion.

Longitudinal electrical field and *in vitro* culture system

We engineered a substrate (Figure 7) that modulates electrical fields on living cells to investigate the effects of square AC electric field on fetal NSCs proliferation and differentiation processes.

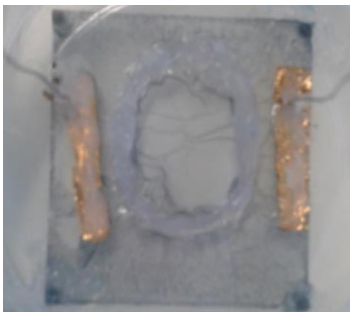

Figure 7. Longitudinal electric field setup.

We exposed the cells to a 100 Hz electrical stimulation with a magnitude of 1V/cm with 10 ms pulse duration in a continuous manner.
NSCs adhered and proliferated under applied AC electric field and in control experiment with no applied electric field. When cells were placed in a low conducting medium under a non-uniform electric field they suffered changes in their shape. The AC electric field introduced a mechanical stress in cells, elongating and stretching them more than control (Figure 8).

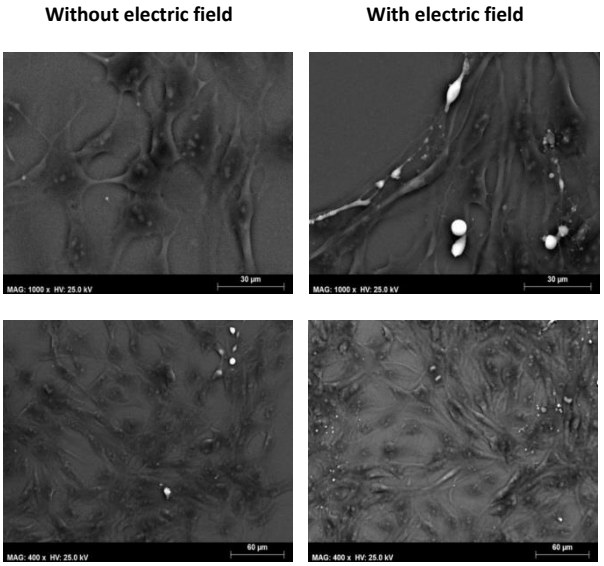

Figure 8. SEM images of NSCs on control (no electric field applied) and on PEDOT:PSS:glass substrates where AC electric field was applied.

Table 2. NSCs elongation under AC electric field and in control condition (no electric field applied).

Cell Elongation	
Electric field	5.02 ± 2.4
No electric field	1.9 ± 0.78

Cells exposed to AC stimulation appear larger and have more lamellipodia, as opposed to their nonpolarized morphology under normal culture conditions. The implications of AC electric field in cellular morphology change need further investigations. The charged surface of this conductive substrate can have affected adjacent ECM or can have attracted or repelled ions or proteins in the media influencing cell behavior.

Differentiation of NSCs on electrically conductive substrate

The generation of functional nerve tissue from stem cells requires differentiation down both neuronal and glial lines.
NSC differentiation was studied under applied AC electric fields and compared to control experiments with no applied electric field. We exposed the cells to a 100 Hz electrical stimulation with a magnitude of 1V/cm with 10 ms pulse durations in a continuous manner during expansion time and over 12h per day during differentiation time.
Cells were fixed with 4% PFA after 4 expansion days and 7 days of exposure to AC electric field for

12h/day in the absence of growth factors. An immunofluorescence study was performed using specific antibodies against Tuj1, which labels neurons in early development as well as mature neurons, and GFAP to identify the differentiated level of astrocytic populations. The fetal NSCs on PEDOT:PSS/glass substrates under and without electric stimulation, differentiated into neurons and into astrocytes confirmed by Tuj1 and GFAP immunoreactivity, respectively (Figure 9)

The results from ImageJ software and from immunocytochemistry seem to indicate that there is a higher number of Tuj1-positive cells in the electrically conductive PEDOT:PSS/glass substrates compared to control (Figure 9).

Several studies suggest that conductivity of substrate can be an essential factor for differentiation and determination of cell fate[18]. The electrical stimulation influences the neuronal differentiation, so, when the electrically PEDOT:PSS/glass substrate was placed into an electrolyte solution, it was able to sustain local electrochemical currents between the substrate and cell monolayer.

The electric field between the conductive surface and cell monolayer may have changed the intracellular distribution of redox couples and, consequently, the intracellular redox potential, regulating in this way the cell differentiation.

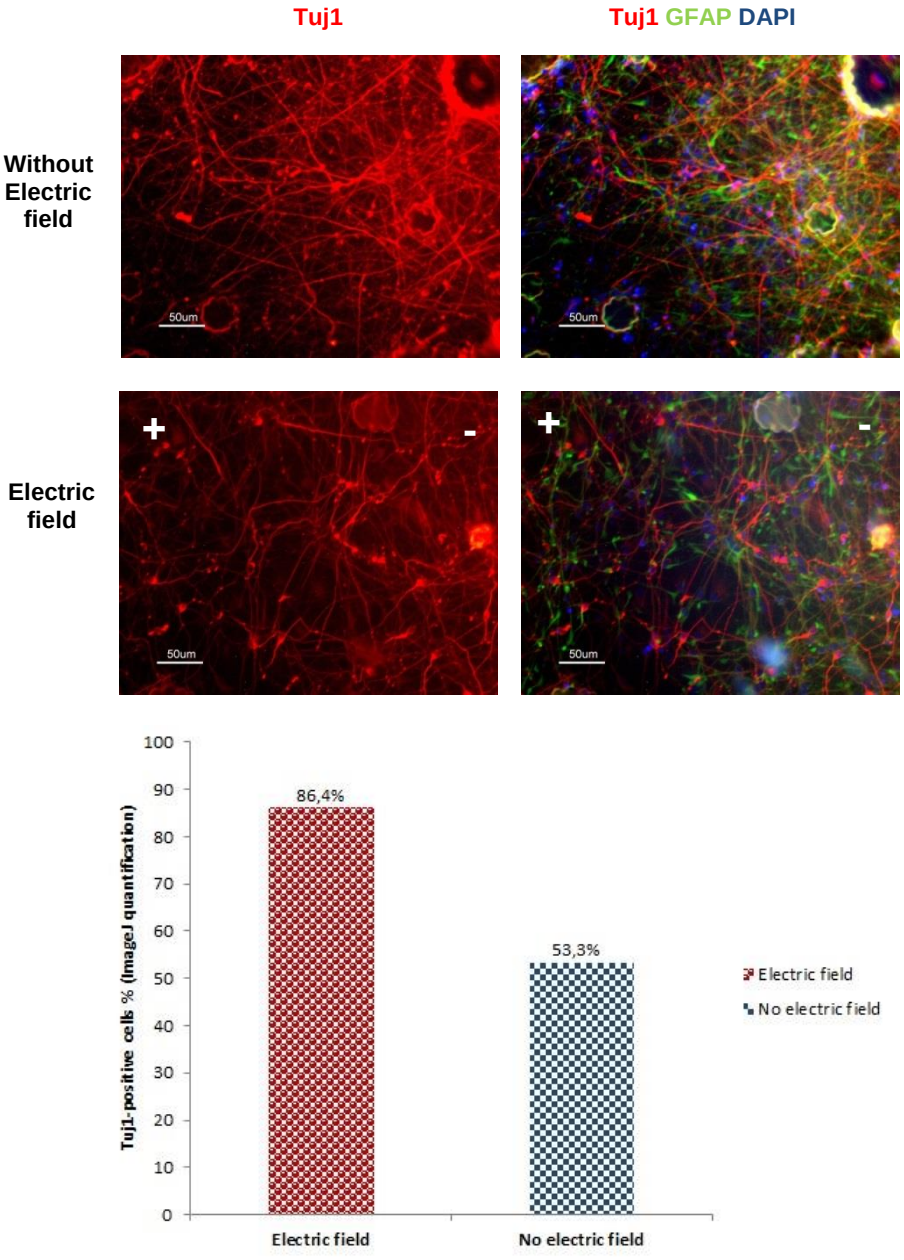

Figure 9. Electrically conductive PEDOT:PSS coated glass enable NSCs differentiation and neuronal growth. NSCs differentiation on PEDOT:PSS coated glass without electric stimulation are shown for comparison. A) Immunofluorescence images of anti-Tuj1 (red) and anti-GFAP (green) followed by DAPI (blue) staining for the nuclei of the fetal NSCs on PEDOT:PSS coated glass. Magnification is 200X. B) ImageJ software quantification of cells showing positive immunostaining for Tuj1 in NSCs at day 7 of differentiation.

Changes in the effective redox potential may alter the conformation of proteins or may stimulate signaling molecules that make cells more oxidized, which is a prerequisite for neural differentiation. However, deeper investigation is needed to understand the effects of electrically conducting polymers on cell differentiation.

There was no directional growth of neurites at 1V/cm (Figure 9) as the neurites emerged from many directions. A qualitative assessment of the AC electric field on the neurite length measured from differentiated fetal NSCs was performed (Figure 10.). The neurite length was ~ 64 µm under control culture condition (no electric field applied), but a longer neurite length of ~ 96 µm was measured at field strength of 1 V/cm.

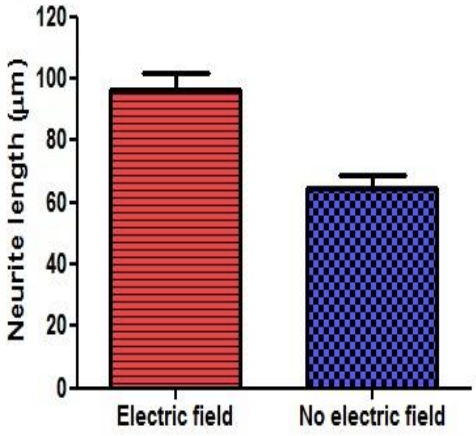

Figure 10. Qualitative assessment of the AC electric field on the neurite length measured from differentiated fetal NSCs.

Several studies suggest that electrical stimulation regulates neurite outgrowth through regulating intracellular signaling pathways and gene expression and interacting with cation-dependent cytoskeletal components that modulate the morphology and motility of the growth cone[19].

Alignment of NSCs on grooved- PolyA gels

In this study, we tested the hypothesis that NSCs accept mechanical cues for growth and spread from the substrate by culturing them on flat and grooved PolyA gels of varying stiffness. PolyA was the polymer chosen due to facility in varying its modulus of elasticity by changing the concentrations of acrylamide and bis-acrylamide and due to their porous structure that prevents cells entering to the substrate. After PolyA surface covalent functionalization with laminin using Sulfo-NHS, it was found that NSCs could spread fully on all flat and grooved substrates tested (Figure III.11.).

The substrates used in this study had grooves with the following dimensions: around 60 µm width, 70 µm spacing and 0,3 µm depth.

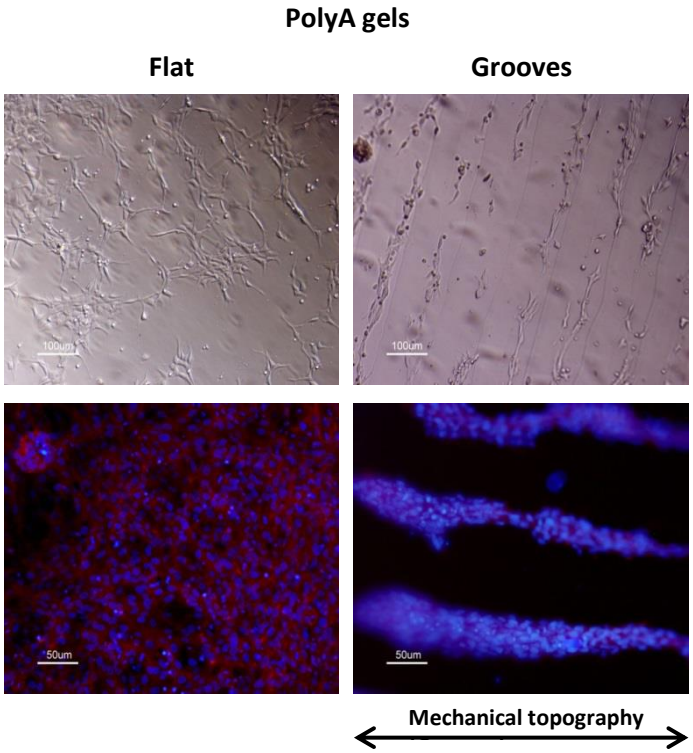

Figure 11. Influence of substrate stiffness on the spreading of NSCs. NSCs adhere fully on flat and grooved polyacrylamide (PolyA) substrates with stiffness equals to 10 kPa. Cells seem to align on grooved PolyA gels in contrast to the cells cultured on flat hydrogels that seems randomly oriented.

Analyzing Figure 11. and Table 3 it appears that nuclei on grooved PolyA hydrogels are more elongated that on flat PolyA hydrogels. The cell bodies and extending branches of NSCs exhibited random distribution and showed no preference of direction on the flat PolyA hydrogel, while cell nucleus elongated along the axis of the grooves and cell extended branches that were guided by topological directionality on grooved substrates.

Table 3. Elongation of NSCS and NSCs nuclei cultured on flat and grooved PolyA hydrogels

	Elongation (E)	
	Flat hydrogels	Grooved hydrogels
NSCs	2.5 ± 0.77	3.56 ± 0.9
NSCs nuclei	1.4 ± 0.48	1.6 ± 0.4

NSCs proliferation on conductive PEDOT:PSS/ vinyl disc substrate

Our previous studies showed that, after 24h of culture, the cells adhere and elongate along the grooves of

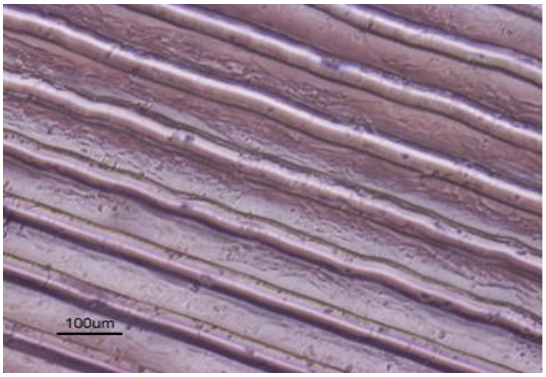

Figure 12. NSCs spreading on PEDOT:PSS spin-coated-vinyl disc.

PEDOT:PSS vinyl disc, although they die within 48 hours (Figure 12).

We related this cell death with a possible weak sterility of the vinyl disc or a possible toxicity of the glue used. To solve this problem, new substrates were immersed in organic solvents such as ethanol and isopropanol to release some lixiviates and we used a biocompatible glue to make a hollow cylinder that was necessary to confine the culture system.

We exposed the cells to a 100 Hz electrical stimulation with a magnitude of 1V/cm in a continuous manner. The AlamarBlue indirect method was used to quantitatively measure the cell proliferation on the substrate that was exposed to AC electric fields and on control substrate that was not exposed to electric field.

The results for the 2^{nd} and 4^{th} day of culture, depicted in Figure 13, show that the cell number in electrically treated culture is significantly higher than it in the controls. This data suggest that rapidly proliferating cells are sensitive to electric field stimulation.

Cells were fixed with 4% PFA after 4 expansion days and 7 days of exposure to AC electric field for 12h/day in the absence of growth factors.

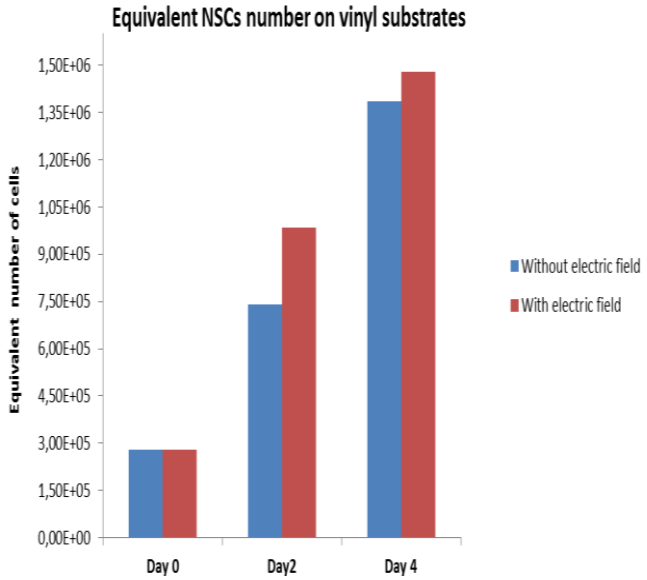

Figure 13. Effect of electric field stimulation on cell number in proliferating cultures.

Immunofluorescence study was performed using specific antibodies against Tuj1, which labels neurons in early development as well as mature neurons and GFAP to identify the differentiated level of astrocytic populations. However, this study was affected by PEDOT:PSS and vinyl disc. PEDOT: PSS and vinyl disc removed some fluorescence which conditioned the acquisition of fluorescence images for specific differentiation markers. Only images with immunostained cells with DAPI was possible to acquire (Figure 14). Immunostained images suggested that cells were confined to the grooves. To confirm this supposition, we visualized these cells using SEM. Figure 14, shows that a high number of cells adhered to the vinyl although it is unclear if they were differentiated or not.

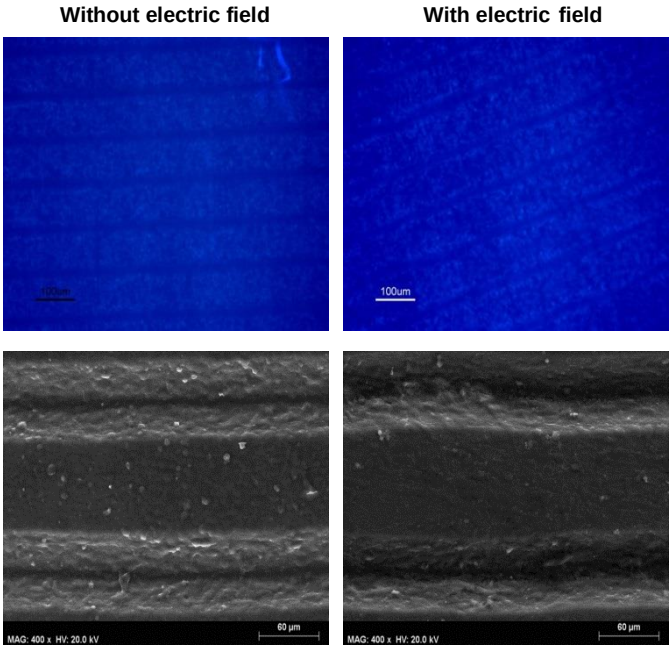

Figure 12. PEDOT:PSS vinyl substrates supported NSCs adhesion. NSCs nuclei counterstained with DAPI and SEM images of NSCs on control (no electric field applied) and on PEDOT:PSS:glass substrates where AC electric field was applied.

Conclusions

The development of new scaffolds, focusing on the use of NSCs and conjugated polymers able to sustain proliferation and differentiation of NSCs and neurite outgrowth, represents a promising strategy, for instance to test drugs targeted against neurodegenerative disorders such as Alzheimer´s and Parkinson´s diseases.

In their *in vivo* microenvironment, cells grow in a complex 3D environment and are exposed to various endogenous electric fields, different

stiffnesses and biomolecules that modulate their behavior.

In this study, we explore the strategy of combining conjugated polymers and soft lithography techniques in order to design a different and innovative scaffold for NSCs adhesion, proliferation and differentiation.

When NSCs were cultured on grooved-PolyA hydrogels they aligned and elongated their cell bodies along the direction of the groove axis, while NSCs exhibited random distribution and showed no preference of direction on flat PolyA hydrogels. In this study, the microcontact printing, an easy and cheap technique, was used to obtain the grooves and the results indicated that the micropatterned PolyA substrates provided a 3D support for spatial guidance and cell growth.

After performing the cytotoxicity tests, we found that all conjugated polymers tested are not toxic, offering new possible coatings for scaffolds for tissue engineering. As the PEDOT:PSS was the more conductive and transparent polymer and offered a higher cell viability and cell growth, its potential to sustain NSCs growth and differentiation was tested with and without AC electrical stimulation. The AC electric field introduces a mechanical stress in cells, elongating and stretching more the cells comparatively to control. Cells cultured on the PEDOT:PSS/glass substrates with and without electric stimulation, remained capable of acquiring a neuronal or glial phenotype. AC electric fields might benefit cell survival and appear to increase the number of Tuj1-positive cells and the neurite length in the electrically conductive PEDOT:PSS/glass substrates compared to controls. These studies demonstrated that laminin-fuctionalized PEDOT:PSS substrates may be used to control complicated cellular functions of fetal NSCs such as cell morphology, proliferation and differentiation.

Several challenges have been identified and should be the starting focus point for the progress of future research. The application of electrical stimulation to cells is complicated because cells are highly conductive, allowing for large current flow, which generates heat and changes in pH, significantly increasing the detrimental effects in stimulated cell culture. A study to optimize parameters such as intensity, frequency, direction and duration of the applied electric field must be performed. In addition, quantitative studies, such as Alamar blue assay, PCR, flow cytometry and LDH assays, must be done to correlate the alterations on signaling pathways and gene expression with electrical stimulation.

A future study could examine the combined influence of substrate conductivity and other adhesion ligands (fibronectin, RGD) on cell behavior with topographically patterned substrates.

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