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Bioelectrochemical control of neural cell development on conducting polymers

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ABSTRACT

Electrically conducting polymers hold promise for developing advanced neuroprostheses, bionic systems and neural repair devices. Among them, poly(3, 4-ethylenedioxythiophene) doped with polystyrene sulfonate (PEDOT:PSS) exhibits superior physicochemical properties but biocompatibility issues have limited its use. We describe combinations of electrochemical and molecule self-assembling methods to consistently control neural cell development on PEDOT:PSS while maintaining very low interfacial impedance. Electro-adsorbed polylysine enabled long-term neuronal survival and growth on the nanostructured polymer. Neurite extension was strongly inhibited by an additional layer of PSS or heparin, which in turn could be either removed electrically or further coated with spermine to activate cell growth. Binding basic fibroblast growth factor (bFGF) to the heparin layer inhibited neurons but promoted proliferation and migration of precursor cells. This methodology may orchestrate neural cell behavior on electroactive polymers, thus improving cell/electrode communication in prosthetic devices and providing a platform for tissue repair strategies.

1. Introduction

The success of neuroprostheses in restoring lost functions resides in the intimate contact between neural cells and electrodes [1] that must enable in the long-term safe cellular activation and high-quality electrophysiological recordings. Promoting neuronal growth onto implanted electrodes is a reasonable strategy to enhance and stabilize the electrical communication at the cell/electrode interface. Electrically conducting materials might also aid neural repair in the form of scaffolds implanted within lesion cavities to provide mechanical support and spatially arranged molecular cues for regenerating neurons [2]. In this case, electrical stimulation via the scaffold would activate the molecular machinery necessary for axon elongation either by inducing nerve action potentials [3] or multicellular healing responses [4]. Although attractive, this challenging strategy involves complex interactions between the electrode surface and several types of cells, ions and organic molecules, hence demanding a thoughtful electrode optimization and bio-functionalization. Conducting polymers (CPs) offer new possibilities to satisfy such requirements [5–7].

CPs deposited on metals enlarge the effective electrode area thus lowering the interfacial electrical impedance [7] which, added to their reversible redox activity and mixed electronic and ionic conductivity [8–10], increases electrical charge acceptance within safe stimulation conditions. Furthermore, CPs may present biochemical signals to cells. For instance polypyrrole (PPy), the CP most frequently investigated for biomedical applications [5,11], supports functionalization with cell adhesion molecules [12,13] and neurotrophic factors [14]. Pioneering work [15] showed that the application of electrical stimuli through PPy films enhanced neurite extension in neuron-like pheochromocytoma cells (PC12), probably being mediated by the adsorption of fibronectin from culture medium onto the PPy surface [16]. The superior thermal and electrochemical stability, charge capacity and ionic conductivity of PEDOT [7–10,17–19] make this CP even a more promising candidate for long-term implantation in the central nervous system (CNS). As PPy, PEDOT can be polymerized by electro-oxidation of the monomer from aqueous solutions containing anions such as PSS that are entrained in the polymer counterbalancing its positive charge [18]. PEDOT:PSS coating of microelectrodes decreases the impedance modulus by almost two orders of magnitude [7,19] facilitating the recording of neurophysiological signals [7,20] and the activation of neurons [21]. Recently, an electrophoretic ion pump was made of PEDOT:PSS to deliver ions (Ca^{2+} and K^{+}) or

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neurotransmitters [22,23] in quantities that induce biological signals. However, the application of PEDOT:PSS in neuroprostheses has been limited by its insufficient biocompatibility thought to arise from the acidic and surfactant behavior of PSS [24].

Although a few examples exist of electrical enhancement of neural growth on CPs using PPy to stimulate cochlear neurons [14] or PC12 cells [15] in short-term cultures (2–3 days), controlling the behavior of CNS neurons on CPs remains a challenge due to their particular requirements for adhesion and development. So far, it is not possible to regulate coexisting cell populations or to inhibit undesired growth, phenomena as crucial as the promotion of neurite elongation itself. The present work addressed these issues investigating PEDOT:PSS as substrate for primary cerebral cortex neurons.

2. Materials and methods

2.1. Polymerization of PEDOT:PSS

An Autolab PGstat30 galvanostat/potentiostat was used for PEDOT:PSS electropolymerization at constant anodic current (1 μA/mm^2) on gold-coated glass slides (Sigma–Aldrich, 10 nm gold layer on a 2 nm Ti layer to bind the gold to an aluminasilicate glass). Increasing polymerization charges of seven values (from 24 to 384 mC/cm^2) were used to control film thickness. A Pt foil served as counter-electrode and a saturated calomel electrode (SCE) as reference electrode. The solution for electrodeposition contained 15 mM EDOT (Sigma–Aldrich) and 20 mM poly (sodium-4-Styrene-sulfonate) (Sigma–Aldrich, 70000 MW) in aqueous 5 mM potassium phosphate-buffered saline (Milli-Q water purified to 18.2 MΩ cm^{-2} with 9 g NaCl, 0.8 g $Na_2HPO_4 2H_2O$ and 0.14 g KH_2PO_4 added per liter).

2.2. Polymer characterization

The morphology of PEDOT:PSS films was studied by scanning electron microscopy (SEM) with a Nova NanoSEM200 microscope (FEI company) at 2 kV in high-vacuum conditions, using a TLD detector at 2-mm working distance. The electrical conductivity of the polymer made it unnecessary to use metallic coatings and allowed the visualization of very small pores (~5 nm) in the surface. Film thickness was measured in SEM images perpendicular to the surface and the film density was estimated by:

$$d = n_{monomer}\, M/\rho A$$

where d is film thickness, $n_{monomer}$ are the moles of EDOT monomer deposited, M is the molar mass of the monomer, ρ is the density of the film, and A is the macroscopic film area [18]. It was assumed that the deposition of 1 EDOT monomer consumed 2.3 e^- [18] and entrained PSS in equimolar concentrations.

The atomic composition was determined utilizing energy dispersive X-ray (EDX, Oxford Instruments) integrated with the SEM and working at 5 kV. Fourier-Transform infrared spectroscopy (FTIR) was used to evaluate the relative oxidation state of the polymer. The spectra were collected with a Spotlight 400 FTIR imaging system equipped with a Spectrum-one spectrophotometer (PerkinElmer) operated in reflectance image mode, scanning from 4000 to 750 cm^{-1} with a resolution of 16 cm^{-1} wavenumbers and taking 16 scans/pixel at 50-μm pixel size. Nine spectra were averaged for each sample.

Electrochemical measurements were performed with the PGstat30 using a three-electrode cell configuration in PBS or supplemented Neurobasal™ culture medium. 1-cm^2 polymer coatings on gold functioned as the working electrode (WE) while a Pt foil and a SCE were used as the counter-electrode (CE) and reference electrode (RE), respectively. For electrochemical impedance spectroscopy (EIS) the potentiostat was configured to sequentially inject 5-mV sine waves at 36 frequencies logarithmically spaced from 0.1 Hz to 100 kHz. For cyclic voltammetry (CV), the Autolab was configured to sweep the voltage of the WE at 0.05, 0.5, 5 or 50 V/s from 0.7 to −1.0 V (the potential limits of water electrolysis) vs. SCE. The CV measurements were used to evaluate the electrochemical reactions in each solution and to calculate the cathodic or anodic charge storage capacity of the films by integrating the current enclosed by each phase of the CV and dividing by the sweep rate. Chronopotentiometry was performed in the same conditions as EIS and CV measurements. All redox reactions of PEDOT:PSS without cell cultures were performed using the Autolab in galvanostatic mode and the conditions specified in the text.

2.3. Measurement of conductivity of solutions

The electrical conductivity of liquid polyelectrolytes was determined directly from the electric resistance measured in a defined solution volume, using a squared chamber with two Pt electrodes (WE and CE/RE) of the same width as the chamber, placed on opposite sides and connected to the Autolab. Two different methods were used to get an exact measurement of the resistance. First, EIS (0.1 Hz–100 kHz) data were processed to obtain the equivalent circuit, the resistance of the solution corresponding to the value of the resistance in series with the double-layer capacitor in the equivalent circuit. This resistance equaled the impedance of the system at high frequencies. Second, potentiometry was performed while applying a squared-current pulse (0.1 s, 1 mA and 10 μA) and the resistance of the solution estimated by dividing the instantaneous voltage ohmic drop by the applied current.

2.4. Preparation of polyelectrolyte multilayers (PEMs)

For assembling PEMs, 5 mM PLL (30,000–70,000 MW), 10 mM heparin (3600, 4300, 5000 or 11,750 average MW) or 20 mM spermine were dissolved in PBS and applied for 2 min on the substrates at room temperature. bFGF (1 μg/ml in PBS) was applied for 1 h. The solutions were consecutively removed and the surface thoroughly washed with distilled water and let to dry before applying other molecules or being used for cell culture.

2.5. Quantification of the adsorption of PLL on PEDOT:PSS

PEDOT:PSS films were immersed in water or PBS solutions containing fluorescein isothiocyanate labeled-PLL (PLL-FITC) at 45 μg/ml or 650 μg/ml and different electrical charges (see Results) were applied to attempt binding the PLL to the polymer surface. After, the films were thoroughly rinsed with distilled water and let to dry, a small drop of PBS/Glycerol and a coverglass were placed on top and the surfaces were scanned with a laser confocal microscope (TCS SP2 SE, Leica Microsystems CMS GmbH) using a 63× - immersion objective, with excitation at 488 nm and collection at 500–590 nm. Six fields were scanned for sample, and at least three samples were used for each experimental paradigm. The ImageJ software (1.39u, National Institutes of Health, USA) was used to measure the average green intensity of the images obtained.

2.6. Cell culture and quantification

PEDOT:PSS substrates were used as the bottom of cell culture polystyrene chambers (BD Falcon) and sealed with silicone (MED-4210, NuSil). Control surfaces consisted of hydrolytic grade 1 borosilicate coverglasses (Marienfeld GmbH & Co. KG Lauda-Königshofen, Germany). All data reported were confirmed in three cell cultures from different animals, using a minimum of six material samples per analysis. In brief, neurons from the cerebral cortex of E18 Wistar rat embryos were cultured at 25,000 cells/cm^2 in Neurobasal™ supplemented with L-glutamine, B27 supplement, Penicillin-Streptomycin and Gentamicin. Cultures were kept at 37 °C in humid atmosphere with 5% CO_2, replacing half of the cell culture medium every 4 days. The cell cultures were fixed in Neurobasal containing 2% paraformaldehyde at room temperature for 12 min. Indirect double-immunofluorescent labeling combined with Hoechst 33342 nuclear staining (Molecular Probes, 2 μl/ml in PBS applied for 50 min after fixing the cells) was used for definition of cell phenotype and identification of condensed and fragmented nuclei from dying cells. Fixed cultures were incubated for 30 min in 0.1 M PBS pH 7.4 containing 0.2% Triton and 5% normal goat serum, rinsed with PBS and then incubated overnight at 4 °C with combinations of two of the following primary antibodies: rabbit polyclonal anti-TAU (Sigma T-6402, 1:500), mouse monoclonal anti-MAP2 (Sigma M1406, 1:500), rabbit polyclonal anti-NF (Affinity NA-1297, 1:750), mouse anti-GFAP (Dako Z-0334, 1:500), or mouse anti-vimentin (Neomarkers, MS-129, clone V9, 1:1000). Alexa-488 anti-rabbit (Molecular Probes, 1:500) and Alexa-594 anti-mouse (Molecular Probes, 1:1000) were used as secondary antibodies.

Precursor cells (Section 3.7) were characterized using the above-mentioned antibodies and also performing immunocytochemistry with rabbit polyclonal anti-NG2 (Chemicon AB5320, 1:500), mouse anti-Nestin (BD Biosciences 556309, 1:1500), mouse anti-fibronectin (Neomarkers clone FBN11, 1:500), and rabbit polyclonal anti-Von Willebrand Factor (Dako A0082, 1:300) antibodies.

Fibroblasts were cultured from the spinal meninges (dura mater and arachnoid mater) of adult rats. The spinal cord was dissected and the meninges were carefully peeled out, cleaned from blood and dissociated for 30 min at 37 °C in HBSS supplemented with pyruvate, albumin, trypsin, and DNAase (all from Sigma–Aldrich), followed by trituration with fire polished Pasteur pipettes. The cells were centrifuged, resuspended in Neurobasal™ or DMEM cell culture medium and plated on borosilicate coverglasses coated with PLL or PLL/Heparin/bFGF. The cells were seeded at 400 cells/cm^2 to investigate the optimal cell culture medium composition and at 5000 cells/cm^2 for studying the cellular effects of the different molecules used for coating the substrate. 10 ng/ml bFGF or 10% fetal calf serum (FCS) were added to the culture medium to stimulate cell proliferation. The fibroblasts were fixed at different survival times and processed for Hoechst 33342 nuclear staining combined with immunocytochemistry for the same markers used for neural precursor cells.

For cell quantification, pictures at high resolution (2776 × 2074 pixels) were taken using a digital microscope system (Olympus DP50) and the fluorescent images of both Hoechst and antibody staining were combined. Sixteen radial fields (563 × 401 μm each) were systematically photographed in each sample and cell counts were extrapolated to the total surface area. Confocal laser scanning