Biomaterials 34 (2013) 3603–3617

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

N-Cadherin- and L1-functionalised conducting polymers for synergistic stimulation and guidance of neural cell growth

Jorge E. Collazos-Castro [a,*], Gabriel R. Hernández-Labrado [b], José L. Polo [b], Concepción García-Rama [a]

[a] Neural Repair Laboratory, Hospital Nacional de Parapléjicos (SESCAM), Finca La Peraleda s/n, 45071 Toledo, Spain
[b] Escuela de Ingeniería Industrial, Universidad de Castilla-La Mancha, Avda, Carlos III s/n, 45071 Toledo, Spain

ARTICLE INFO

Article history:
Received 10 January 2013
Accepted 26 January 2013
Available online 16 February 2013

Keywords:
Conducting polymers
PEDOT
N-Cadherin
L1
Microfibre
Neural

ABSTRACT

Conducting polymers are promising materials for advanced neuroprostheses and neural repair devices. However, these challenging technologies demand stable presentation of multiple biomolecules on the polymer surface and fabrication of scaffolds suitable for implantation. We electrosynthesised poly(3,4-ethylenedioxythiophene) doped with poly[(4-styrenesulfonic acid)-*co*-(maleic acid)] (PEDOT:PSS-*co*-MA) on gold-coated surfaces or carbon microfibres, functionalised the polymer by covalent immobilisation of anti-IgG antibodies and subsequent binding of N-Cadherin and L1 recombinant proteins, and used these materials as substrates for culturing cerebral cortex neurons. N-Cadherin and L1 were much more effective than polylysine in promoting axonal elongation and collateralisation on the polymer. However, N-Cadherin also induced cell migration and dendritic extension and branching, whereas L1 inhibited dendrites. Dual functionalisation with N-Cadherin and L1 produced synergistic effects on neuronal growth that could not be achieved with either of the proteins when used alone. PEDOT:PSS-*co*-MA electrosynthesised on carbon microfibres showed good electrochemical properties and, when biofunctionalised with N-Cadherin or L1, stimulated very long and guided axonal elongation. Finally, electrochemical impedance spectroscopy, cyclic voltammetry and chronoamperometry showed that the good electrical properties of PEDOT:PSS-*co*-MA were not degraded by covalent peptide attachment, indicating that this polymer is suitable for multiple biofunctionalisation of electroactive surfaces in neuroprosthetic and lesion-bridging applications.

1. Introduction

Electrically conducting polymers (CPs) exhibit electronic and ionic conductivity [1–3] and are promising materials for developing advanced neuroprostheses and neural repair devices [4–10]. Besides the electrical advantages of coating electrodes with CPs [3–5,7,11,12], binding biomolecules to the polymer surface can promote neural growth onto the electrode itself thus improving the electrical communication at the cell/electrode interface. This technology also has potential for use in the treatment of neurological lesions, in which case implantable three-dimensional scaffolds made of biofunctionalised CPs may aid neural repair by providing mechanical support and spatially arranged molecular cues to regenerating cells [7,13]. In this application, electrical stimuli administered via the scaffold would provide a powerful tool to potentiate reparative responses, either by releasing growth factors or modifying the molecular interactions at the polymer/cell interface [7,14–16], or by directly activating the molecular machinery necessary for axonal elongation and myelination [17–19]. For this challenging enterprise, the CPs must present multiple biomolecules appropriately oriented to neural cells, and those mediating cell adhesion must resist detachment under electrostimulation.

Compared to other CPs, poly(3,4-ethylenedioxythiophene) doped with polystyrene sulfonate (PEDOT:PSS) has superior thermal and electrochemical stability, charge capacity and ionic conductivity [1–4,20]. PEDOT:PSS can be functionalised by electrochemical and molecule self-assembling methods that adsorb polylysine (PLL) on the polymer surface, with the possibility of subsequently assembling heparin and heparin-binding molecules (for instance bFGF or spermine) to promote neuronal growth or glial precursor cell proliferation and migration [7]. However, if the first molecular layer is electrostatically adsorbed to the polymer, it will be prone to detach from the surface under applied electrical stimulation. A convenient approach to overcome this limitation would be to attach the first molecular layer covalently to the polymer. This can be achieved by incorporating to the CP dopants

* Corresponding author. Tel.: +34 925247758; fax: +34 925247745.
E-mail address: jcollazos@sescam.org (J.E. Collazos-Castro).

0142-9612/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2013.01.097

bearing carboxylic groups for subsequent covalent bonding of the amine groups of biomolecules, as has been performed for poly-glutamic acid [21] or chondroitin sulphate-doped [22] polypyrrole. Nevertheless, the dopant must be carefully selected to avoid deteriorating the electrochemical properties of the CP. In this regard, doping PEDOT with polyanions that have both sulfonate and carboxylic groups, for instance poly[(4-styrenesulfonic acid)-*co*-(maleic acid)] (PSS-*co*-MA), is expected to produce CPs that have good electrical performance and sufficient carboxylic groups available for surface functionalisation.

Depending on the neuroprosthetic application, it may be desirable that either or both axons and dendrites grow on the electroactive surface. In this regard, L1 and N-Cadherin (NCad) are two cell adhesion molecules (CAMs) that might offer selectivity or synergism for promoting axon and dendrite extension on the conducting polymer. *In vitro*, L1 bound to plastic or glass substrates potently stimulates axonal elongation [23,24] and selectively promotes neuronal attachment and outgrowth in presence of astrocytes and meningeal cells [25]. Moreover, L1 immobilised on silicon neural probes caused less neuronal death and increased axonal density in the 100 μm perielectrode area when implanted in the rat cerebral cortex [26]. However, substrates coated with L1 are not permissive for dendrite differentiation and growth [27,28]. NCad induces profuse axonal extension on cell culture substrates and, contrary to L1, it also stimulates dendrite elongation and branching [27–29]. Besides its major role in axonal guidance and fasciculation *in vivo* [30,31], NCad is required for neuronal migration [32] and synaptic plasticity [33,34].

The present work investigated the use of PEDOT:PSS-*co*-MA for increasing electric charge transfer of metallic electrodes and carbon microfibres while enabling covalent, multiple functionalisation of the electroactive surface. It likewise addressed the growth and differentiation of dissociated neural cells from the cerebral cortex on PEDOT:PSS-*co*-MA functionalised with recombinant L1 and N-Cadherin. Finally, polymer-coated carbon microfibres were manufactured and tested as substrate for cerebral cortex explants, a culture system that more appropriately models the three-dimensional complexity of the neural tissue.

2. Materials and methods

2.1. Polymerisation of PEDOT:PSS-co-MA

An Autolab PGstat30 galvanostat/potentiostat was used to electrosynthesise PEDOT applying constant anodic current (1 $\mu A/mm^2$) and polymerisation charges of 48, 96, 192, 288 or 384 mC/cm^2 to control film thickness. A Pt foil served as counter-electrode (CE) and a saturated calomel electrode (SCE) as reference electrode (RE). The working electrode (WE) consisted in a gold-coated glass slide (Phasis, Switzerland; 10 nm gold layer deposited on a Ti nanolayer to bind the gold to the glass) or aligned carbon microfibres of 7-μm diameter (Goodfellow, UK). The solution for electrodeposition contained 15 mM EDOT (Sigma–Aldrich) and the respective dopant in aqueous 5 mM potassium phosphate-buffered saline (PBS; Milli-Q water purified to 18.2 $M\Omega\ cm^{-2}$ with 9 g NaCl, 0.8 g $Na_2HPO_42H_2O$ and 0.14 g KH_2PO_4 added per litre). PSS-*co*-MA (20,000 MW) was used as dopant at concentrations equivalent to 20 mM of the negatively charged 4-styrenesulfonate and *co*-maleic acid molecules. Initially we synthesised PEDOT doped with PSS-*co*-MA that had 4-styrenesulfonic acid:maleic acid mole ratios of 1:1 or 3:1 (Sigma–Aldrich references 434,558 and 434,566, respectively) to compare the resulting chemical properties, and advanced experiments were performed only with PEDOT doped with PSS-*co*-MA 1:1. Carbon microfibres were coated with PEDOT:PSS-*co*-MA 1:1 or with PEDOT doped with 20 mM poly(sodium-4-styrene-sulfonate) (PSS; Sigma–Aldrich, 70,000 MW).

2.2. Polymer characterisation

The morphology of PEDOT:PSS-*co*-MA films was studied by scanning electron microscopy (SEM) with a Nova NanoSEM200 microscope (FEI company) at 2–3 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. Film thickness was measured in SEM images perpendicular to the surface. The atomic composition was determined utilising 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 carried out with the PGstat30 using a three-electrode cell configuration in PBS. The WE consisted in 1-cm^2 glass/gold/conducting polymer electrodes or 0.92-mm long polymer-coated carbon microfibres, while a Pt foil and a SCE were used as CE and RE, respectively. For electrochemical impedance spectroscopy (EIS) the potentiostat was configured to sequentially inject 5-mV sine waves at 26 frequencies logarithmically spaced from 10 kHz to 0.1 Hz. The EIS data were fitted to an impedance model with parameters obtained using a complex nonlinear least-squares method [3] programmed in MatLab (MathWorks, USA). 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 −1.05 to +0.55 V (the potential limits of water electrolysis) vs. SCE. CV allowed to evaluate the electrochemical reactions and to calculate the cathodic and anodic charge density (Q_{CV}) of the electrodes by integrating the current enclosed in each phase of the CV and dividing by the sweep rate. For simplification, the data presented correspond to the average of the cathodic and anodic Q_{CV}. For assessing the stability of the CPs, CV at 0.5 V/s was performed for a total of 500 cycles and the Q_{CV} was calculated each 100 cycles. The data were expressed in relative values for comparison. Chronoamperometry was used to study the electric response of carbon microfibres coated with PEDOT:PSS-*co*-MA or PEDOT:PSS. For this, trains of squared, biphasic voltage pulses (0.2, 0.3, 0.4 or 0.5 V; 1 or 5 ms) were applied, setting first the microfibre voltage to 0 V for 10 s and starting always by the cathodic phase. The average charge per phase was calculated to compare the electric response of the polymers.

2.3. Biofunctionalisation of PEDOT:PSS-co-MA

With the aim of confirming its redox activity and suitability for cell adhesion, PEDOT:PSS-*co*-MA was initially functionalised by electroadsorption (−12.5 $\mu A/cm^2$, 3 mC/cm^2) of PLL (45 μg/ml in MQ water) using the same methodology that we developed for PEDOT:PSS [7]. Covalent functionalisation was performed in more advanced experiments. For this, the polymer was reacted with *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) and *N*-hydroxysuccinimide (NHS), both at 45 μg/ml in MQ water, for 1 h at 24 °C. Then, it was washed three times with MQ water and subsequently was incubated for 1 h in 50 mM, pH 8.0 triethanolamine buffer solution containing the molecule to be conjugated (see below). After conjugation the surface was washed four times with MQ water and PBS.

We compared protein binding to PEDOT polymers doped with PSS-*co*-MA 1:1 or 3:1. For this, the EDC/NHS-reacted polymers were incubated with fluorescent antibodies (Alexa-488 goat anti-rabbit IgG, Molecular Probes) at 5, 25 and 45 μg/ml. Control polymer surfaces were processed in the same way but EDC and NHS were omitted from the reaction. 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 from each material 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. Because of its superior protein binding, all subsequent experiments were performed using the polymer doped with PSS-*co*-MA 1:1. After verifying the suitability of PEDOT:PSS-*co*-MA for covalent attachment of antibodies, we proceeded to functionalise the polymer with NCad and/or L1. The EDC/NHS-reacted surface was incubated with an IgG antibody specific against the Fc fragment of human IgG (A-HIgG; 100 μg/ml; Sigma–Aldrich I2136). The antibody was dialysed through cellulose ester membranes (8–10 kD MW cut-off) prior its use. The A-HIgG-functionalised surface was incubated for 1 h with recombinant human NCad and/or L1-Fc chimeric proteins (R&D Systems, ref. 1388-NC and 777-NC) at 5, 10 or 20 μg/ml in pH 7.4 PBS. Control surfaces consisted in PEDOT:PSS-*co*-MA without functionalisation or covalently functionalised with only A-HIgG (100 μg/ml) or PLL (45 μg/ml). In order to evaluate the binding of the recombinant proteins, the functionalised surfaces were immunostained with antibodies anti-N-Cadherin (Sigma–Aldrich C3865, 1:100) and anti-L1 (Chemicon MAB5272, 1:50). For this, the surfaces were incubated for 1 h in PBS containing 5% normal goat serum (NGS) and 0.2% triton, followed by 2 h-incubation in PBS with 0.2 triton and both primary antibodies, and finally 1 h in PBS with Alexa Fluor® 594 goat anti-mouse IgG and Alexa Fluor® 488 goat anti-rat IgG (Molecular Probes, 1:500) antibodies. The surface was imaged by confocal microscopy as described above. The Alexa Fluor® 594 was excited at 594 nm and the fluorescence was collected at 605–750 nm.

2.4. Cell culture and immunohistochemistry

PEDOT:PSS-*co*-MA synthesised on glass/Ti/Au slides was used as the bottom of cell culture polystyrene chambers and the borders were sealed with silicone (MED-