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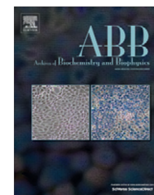

Directed differentiation of embryonic P19 cells and neural stem cells into neural lineage on conducting PEDOT–PEG and ITO glass substrates

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ABSTRACT

Differentiation of pluripotent and lineage restricted stem cells such as neural stem cells (NSCs) was studied on conducting substrates of various nature without perturbation of the genome with exogenous genetic material or chemical stimuli. Primary mouse adult neural stem cells (NSCs) and P19 pluripotent embryonal (P19 EC) carcinoma cells were used. Expression levels of neuronal markers β-III-tubulin and neurofilament were evaluated by immunochemistry and flow cytometry. It was shown that the ability of the substrate to induce differentiation directly correlated with its conductivity. Conducting substrates (conducting oxides or doped π-conjugated organic polymers) with different morphology, structure, and conductivity mechanisms all promoted differentiation of NSC and P19 cells into neuronal lineage to a similar degree without use of additional factors such as poly-L-ornithine coating or retinoic acid, as verified by their morphology and upregulation of the neuronal markers but not astrocyte marker GFAP. However, substrates with low conductance below ca. 10^{-4} S cm^{-2} did not show this ability. Morphology of differentiating cells was visualized by atomic force microscopy. NSCs cells increased β-III-tubulin expression by 95% and P19 cells by over 30%. Our results suggest that the substrate conductivity is a key factor governing the cell fate. Differentiation of P19 cells into neuronal lineage on conducting substrates was attributed to downregualtion of Akt signaling pathway and increase in expression of dual oxidase 1 (DUOX 1).

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Introduction

Differentiation of stem cells towards specific functional cell types is crucial in applications such as cell-based therapy and tissue engineering. It would be valuable to accelerate stem cell differentiation and direct them into desirable cell type over the course of their development for potential cell replacement and regeneration. Furthermore, pre-differentiation also allows control over the factors influencing the development of mature phenotypic behavior. The extracellular environment plays a crucial role during neuronal development and tissue formation, and its electrical properties were shown to greatly affect neuron adhesion and neurite outgrowth and orientation [1]. Electrically conductive materials hold promise for developing bionic systems and provide new approach in controlling cell state and fate via regulation of cell volume, conductivity, redox status and mechanical properties [2–4].

In this work we have been interested in finding how various conductive substrates are able to control cell conducive environment for neural stem cell differentiation. Electrically conducting materials were shown to support neural cell growth and promote neural repair and neural cell differentiation. Among most extensively studied conductive materials are so-called electronically conducting polymers (ECPs), such as polypyrrole, polyaniline, and various polythiophenes, which are reasonably stable, conductive under appropriate conditions and biocompatible materials. The pioneering work by Schmidt and coauthors showed that neuron-like pheochromocytoma cells PC12 cells and primary chicken sciatic nerve explants attached and extended neurites on polypyrrole (PPy)[1] films [5]. When modified in certain ways (doped with immobilization factors such as extracellular matrix cell adhesion proteins or peptides), PPy has showed the ability to promote growth of primary neurons [6], neural stem cells, and mesenchymal stem cells [7,8].

Poly(3,4-ethylenedioxythiophene) doped with polystyrene sulfonate (PEDOT:PSS) is another electronically conducting polymer that have gained a growing interest in various applications. Similar to polypyrrole, PEDOT:PSS was investigated for biomedical applications [4,9–11]. Although PEDOT:PSS exhibits superior physicochemical properties, biocompatibility issues have limited its use. PEDOT:PSS is itself acidic and therefore corrosive and toxic because

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[1] *Abbreviations used:* NSC, neural stem cells; PEDOT, poly(3,4-ethylenedioxythiophene); ITO, indium-tin oxide; FTO, fluorine-tin oxide; PBT, poly(2,2′-bithiophene); P3HT, poly(3-hexylthiophene); AFM, atomic force microscopy; DUOX1, dual oxidase 1.

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each phenyl ring of the PSS component has one acidic SO_3H (sulfonate) group [12,13]. To avoid this problem, electrodeposition of polylysine of collagen was employed. Thus, electro-adsorbed polylysine on PEDOT:PSS enabled long-term neuronal survival and growth [14]. To avoid high acidity of PEDOT:PSS, conductive PEDOT copolymerized with poly(ethylene glycol) (PEG) (i.e., PEDOT:-PEG), which is virtually non-acidic, was developed and is now commercially available [15]. PEDOT:PEG has relatively high conductivity (0.4 S cm^{-1}). It showed the work function of 4.33 eV and has a comparable conductivity to the PEDOT:PSS [15].

Both PPy and PEDOT doped with polystyrene sulfonate (PEDOT:PSS) or tosylate showed the ability to promote differentiation of neural stem cells [2,7,14]. However, the presence of acidic PSS limited the materials potentials. The positive effect of electronically conducting polymers on cell differentiation and growth is typically related to the conductivity of the materials. However, in most of those studies, conducting polymer was modified with some other biologically active agents. The electroactive materials were engrafted or coated with various extracellular matrix proteins and cell adhesion promoting peptides which mimic the in vivo environment.

Here, we are interested in comparing the effects of electronically conducting polymers, such as PEDOT:PEG and electrodeposited PEDOT, with those of other conducting substrates, such as electrically conducting indium (ITO) and fluorine (FTO) doped tin oxide glass and polymers of polythiophene series like poly(3-hexylthiophene) (P3HT) and poly(2,2′-bithiophene) (PBT), for their ability to control cell differentiation. We found that PEDOT (either copolymerized with poly(ethylene glycol) (PEG) or electrodeposited) and ITO coated glass substrates both sustained differentiation of lineage restricted mouse adult neural stem cells (NSC) and pluripotent P19 embryonic carcinoma (P19 EC) cells into cells with neuronal phenotype without substrate modification with any additional biological agents or stimulation, in the case of P19 EC cells. We also show that suppression of Akt activity and upregulation of dual oxidase 1 (Duox 1) contribute to neural differentiation of P19 cells on electrically conductive PEDOT. PEDOT:PEG can be an appealing candidate for augmenting the endogenous neural/progenitor cells differentiation for tissue replacement. This finding may open new horizons for understanding the initiation of stem cell differentiation and new avenues in the development of cell-based tissue replacement strategies and neuroprosthetic devices. Most importantly, the ability of studied materials to selectively produce one differentiated cell type from pluripotent stem cells would be of great importance in investigating the effects of drugs and environmental factors on differentiation and cell function in the human nervous system.

Experimental procedures

Dispersions of PEDOT:PEG in acetonitrile, at 0.1 wt.% were used to prepare films on either non-conducting borosilicate glass or ITO-glass. The dispersion were prepared by diluting commercially available (Sigma–Aldrich) PEDOT:PEG dispersion in nitromethane. Drop casting and spin coating were used for deposition. After the deposition, the slides were washed three times in PBS and air dried. The thickness of deposited PEDOT film layer as measured by AFM was 70–80 nm on glass and 160–170 nm on ITO. Poly-ornithine coatings were prepared using the literature procedure [16]. The thickness of poly-ornithine coatings was ca. 1 nm. PEDOT:PSS coatings were made using a 2.8 wt.% dispersion in H_2O (Sigma–Aldrich) with ca. 2.6% PSS content. 2,2′-Bithiophene (PBT) (Aldrich, 97%) was purified by vacuum sublimation prior to use. Electronic grade poly(3-hexylthiophene) (93–95%, MW = 40–60 kDa) was purchased from Solaris Chem Inc. Poly(3-hexylthiophene) (P3HT) was dissolved in anhydrous 1,2,4-trichlorobenzene (Sigma–Aldrich, >99%) at a concentration of 20 mg mL^{-1}. ITO (G-50in-CUV cuvette slides with resistance 8–12 ohm) and FTO glass were obtained from Delta Technologies and Pilkington TEC Glass, respectively. The conducting glass slides were washed as described below prior to use.

Cell cultures

Primary neurosphere cultures

Primary neural stem cells (neurospheres) were isolated from postnatal day 1–3 mouse brains. The minced periventricular tissue of brain was incubated in tissue dissociation medium (NeuroCult Dissociation solution, StemCell Technologies) for 7 min at 37 °C. Cells were mechanically dissociated by trituration and cell suspension was filtered through 45-μm cell strainer. Details of the method of neurosphere culture have been described by Rietze and Reynolds [16]. In three washing steps, the homogenate was centrifuged for 5 min at 110 g, and the pellet was re-suspended in 10 mL of hormone-supplemented NeuroCult Resuspension solution. Cells were plated at a density of 3500 cells/cm^2 and cultured in serum-free hormone-supplemented growth medium (NeuroCult NSC proliferation medium, StemCell Technologies) with 20 ng/mL EGF (R&D system) in a humidified incubator at 37 °C with 5% CO_2 in air. Nonadherent cell clusters were collected and re-cultured. After 5–7 days, distinct floating spherical colonies (also referred as neurospheres) were observed.

To induce neural differentiation, cell suspensions of neurosphere cultures were seeded on polyornithine-coated glass bottom culture plates (Mattek), conductive indium-tin oxide (ITO) or fluorine-tin oxide (FTO) glass slides, or electrically conductive polymer PEDOT and cultured for up to 5 days under differentiation conditions in NeuroCult NSC differentiation medium (StemCell Technologies) supplemented with or without 20 ng/mL rh FGF-b (R&D system) and heparin (StemCell Technologies). Spheres attached to glass bottom differentiated into mature neurons expressing β-III-tubulin.

P19 pluripotent embryonal carcinoma cells (American Type Culture Collection, Manassas, VA) were cultured as previously described [17] in α-minimum essential media (GIBCO) supplemented with 10% fetal bovine serum (Gibco). To induce neuronal differentiation 1.0×10^6 P19 cells, were aggregated in α-minimum essential media supplemented with 5% New born calf serum (GIBCO) with or without 1 μM all-trans retinoic acid (RA) from Sigma. P19 cells were transfected with PI 3-kinase p110γ shRNA (Santa Cruz Biotechnology) or control shRNA lentiviral particles (Santa Cruz Biotechnology) according to the manufacturer's protocol.

PEDOT and PBT electrodeposition

Indium tin oxide (ITO; unpolished float glass, 5–10 ohm) electrodes were sonicated sequentially in soap, deionized water, acetone, ethanol, and deionized water for 15 min each. Residual water was removed from the surface of the ITO electrode using nitrogen gas. The electrodes were placed in an oven at 90 °C to completely dry. 3,4-Ethylenedioxythiophene (Aldrich, 97%) and tetrabutylammonium hexafluorophosphate ($TBAPF_6$) (Aldrich, 98%) were used. A Pyrex glass cell without separation of the anodic and cathodic compartments was used for electrochemical polymerization. The pseudo-reference electrode was silver wire, which was stored in a solution of the supporting electrolyte between experiments. The deposition of poly(3,4-ethylenedioxythiophene) (PEDOT) was carried out in galvanostatic conditions (current density of 0.54 mA/cm^2) in solution of 0.37 M 3,4-ethylenedioxythiophene, 0.1 M TBAPF6 as supporting electrolyte in acetonitrile (<35 ppm H_2O). The film thickness was controlled by varying the

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