Electrochemical Control of Growth Factor Presentation To Steer Neural Stem Cell Differentiation**

Anna Herland,* Kristin M. Persson, Vanessa Lundin, Mats Fahlman, Magnus Berggren, Edwin W. H. Jager, and Ana I. Teixeira*

anie_201103728_sm_miscellaneous_information.pdf
Materials and Methods

PEDOT:heparin synthesis

PEDOT:heparin was electrosynthesized on conducting films of PEDOT:PSS on polyethyleneterephthalate substrates (Agfa-Gevaert Orgacon™ F-350) using a three electrode setup, in a polymerization solution of 3,4-ethylenedioxythiophene (EDOT) (Sigma) and Klexane (Sanofi Aventis). A platinum grid was used as counter electrode and Ag/AgCl as reference electrode, controlled by an Ecochemie µAutolabIII (Metrohm Autolab, Utrecht, NL) potentiostat. A solution of 3 ml of 10 mg mL⁻¹ Klexane (Sanofi Aventis), 27 ml H₂O, and 300 µl 3,4-ethylenedioxythiophene (EDOT) (Sigma) was stirred overnight. Electrosynthesis was carried out for 1200 s at 400 µA. The size of the PEDOT:PSS substrate was about 6 cm². Polymerization at approximately 65 µA/cm² for 1200 s resulted in a film thickness of about 400 nm.

PEDOT:heparin characterization

The thickness of the synthesized PEDOT:heparin films was measured with a Dektak 3ST surface profiler (Veeco Instruments, Plainview, NY, USA). The surface energy of PEDOT:heparin was evaluated by measuring and analyzing the water contact angles with a goniometer (Cam 200, KSV Instruments, Helsinki, Finland) and accompanying software. Electrochemical characterization of PEDOT:heparin surfaces was done using cyclic voltammetry at 20 mV/s in a setup as described above with 0.1M NaCl as electrolyte. Atomic force microscopy of PEDOT:heparin was done with a Dimension 3100 instrument from Veeco in tapping mode using a 10 µm scan size. The X-ray photoelectron spectroscopy (XPS)
experiments were carried out using a Scienta ESCA 200 spectrometer. The vacuum system consisted of an analysis chamber and a preparation chamber. XPS was performed in the analysis chamber at base pressure of $10^{-10}$ mbar, using monochromatized Al K α X-rays, at $h\nu = 1486.6$ eV. The experimental conditions were such that the full width at half maximum (fwhm) of the gold Au(4f7/2) line was 0.65 eV. The binding energies were obtained with an error of ± 0.05 eV. XPS measurements were carried out on pristine, oxidized (0.8 V 5 min) and reduced (-0.8 V 5 min) PEDOT:heparin films. The oxidized and reduced films were blow-dried using He-gas prior to insertion into the vacuum system.

**Toluidine blue assay**

Briefly, PEDOT:heparin surfaces were reduced to the neutral state or oxidized (first reduced and then switched to oxidized by ramping 0.1 V/s) in an electrochemical setup as above followed by incubation in 2 ml TB solution for 2 hours. The TB solution was composed of 0.05 mg ml$^{-1}$ TB (Sigma) in 0.01 M HCl and 0.2% w/v NaCl. After incubation, the solution was diluted 1:10 in ethanol (99.5%) and absorption was measured between 500 and 800 nm, with an absorption maximum at 631 nm. Absorption was measured with an UV-VIS-NIR spectrometer Lambda 900 from Perkin Elmer. For incubated polymer samples, shaking against n-hexane was omitted as the TB:Klexane complex formed during the assay would be present on the polymer surface, not in solution. Therefore, there was no need to remove the complex.

**Cell culture and surface preparations**

For growth factor (GF) association and cell culture studies PEDOT:heparin surfaces were sterilized by immersion in ethanol (70%) for 15 min followed by air drying. Surfaces were then immersed for 48 h in PBS. For association of GFs to PEDOT:heparin surfaces, the surfaces were reduced (-0.8 V 5 min) to the neutral state in PBS using a three electrode setup as above. Thereafter, the surfaces were immersed in 200 ng ml$^{-1}$ basic fibroblast growth factor, bFGF (denoted as FGF2 in this work), (RnD Systems) in 2 mg ml$^{-1}$ BSA in PBS for 1h at 37 degrees, followed by 2 times of rinsing in PBS. Prior to cell seeding the surfaces were coated with 15 µg ml$^{-1}$ poly-ornithine (SigmaAldrich) followed by 1 µg ml$^{-1}$ fibronectin. To evaluate the decrease of surface exposed FGF2, surfaces were cycled 3 times at -0.8 to 0.8 V, 0.1 V/s followed by a constant oxidizing potential 0.8 V for 5 min in cell culture media described below.

Primary neural stem cells (NSCs) were obtained from cerebral cortices of Sprague-Dawley rat embryos at embryonic day 15.5. Animals were treated according to institutional and national guidelines (Ethical permit no. N79/08). NSCs were expanded on poly-ornithine and fibronectin coated tissue culture polystyrene in serum-free DMEM:F12 medium with N2 supplement $^{[12a]}$. 10 ng ml$^{-1}$ FGF2 was added daily to maintain the proliferative NSC state. Passaging of NSCs was done by dissociation in HBSS, HEPES and NaHCO$_3$. In all experiments, cells at passage two (P2) were used.

Cells were seeded at ~16000 cells/cm$^2$ and cultured for 4 days. For samples with FGF2 in solution, the GF was added daily. Experiments in which oxidation was done during cell culture were performed after overnight culturing, with cycling of the potential during oxidation being omitted. Instead, a potential was swept from -0.8 to 0.8 V at 0.05 V/s. Reference surfaces were kept at open circuit.

**Immunoreactivity of PEDOT:heparin/GF, and cell assays**

To quantify the amount of antibody accessible growth factors, FGF2 and FGF8 (RnD systems), surfaces were treated as above with the exception that FGF8 was incubated at 500 ng ml$^{-1}$. The surfaces were then fixated in 10 % formalin for 15 min at RT. Blocking was
done in 100 mg ml$^{-1}$ BSA in PBS/0.1% Triton-X (PBST) for 20 min in RT, followed by two times rinsing in PBST. Primary antibodies 5 µg ml$^{-1}$ goat anti-FGF basic (RnD Systems) or 5 µg ml$^{-1}$ goat anti-FGF 8b (RnD Systems) and 1.2 µg ml$^{-1}$ rabbit anti-fibronectin (Sigma) were incubated overnight in 10 mg ml$^{-1}$ BSA PBST. Next, rinsing in PBST was done six times for 5 min. Species specific secondary antibodies conjugated with Alexa-488 or Alexa-594 at 4 µg ml$^{-1}$ in 10 mg ml$^{-1}$ BSA PBST were incubated in darkness at RT for 1h. Rinsing in PBST, followed by PBS was done prior to evaluation in fluorescence Zeiss Axioskop2 microscope and a Zeiss AxioCam MRm camera with Axiovision Rel 4.6 software.

Fixation of NSCs was done in 10 % Formalin for 15 min. Permeabilization was done through three times 5 min incubation in PBST. Primary and secondary antibody incubations were done as described above, without a blocking step. The following primary antibodies were used: mouse anti-Nestin from BD Biosciences Pharmingen (1:500) and rabbit anti-glial fibrillary acidic protein (GFAP) from Dako (1:500). Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) from Invitrogen.

Quantitative real-time PCR (RT-QPCR) was performed after 4 days of culturing. RNA was extracted using RNeasy microkit including RNase free DNase kit (Qiagen). cDNA was synthesized from 200 ng of RNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time PCR was carried out using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) and the 7300 Real Time PCR System using the following thermo cycling program: 50°C for 2 min, 95°C for 2 min and 40 cycles of 95°C for 15 s and 60°C for 30 s, and finally 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. Each sample was run in triplicates. The $\Delta\Delta$C$_T$ method was used to evaluate mRNA levels relative to TATA binding protein (TBP), which was the endogenous control. Primers are available upon request.

**Quantification of surface bound FGF2**

For quantification of surface bound FGF2, $[^{125}$-I]FGF2 (PerkinElmer and a gamma counter (LKB Wallac Compugamma) was used. Exact area of each surface was determined with a phosphoimager.

**Statistical analysis**

All statistical analysis was done in Graphpad Prim 5 using one-way ANOVA with post analysis Tukey test, alternatively a two-tailed t-test was used. Significance level was set to 0.05, ** = p<0.01, *** = p< 0.001.
Supplementary Figure 1. Cyclic voltammogram of PEDOT:heparin at 20 mVs$^{-1}$ in 0.1 M NaCl (aq). Redox peaks observed at -0.4 V and +0.3 V.
**Supplementary Figure 2.** S(2p) XPS core levels spectra of a) pristine; b) neutral (-0.8 V, 5 min); and c) oxidized (0.8 V, 5 min) PEDOT:heparin films. The S(2p) core level was fitted with two spin split doublets corresponding to sulfur atoms in PEDOT (low binding energy) and in heparin (high binding energy), respectively. Dots mark the raw data that have been fitted with spin-split doublets and a Shirley background. a) For the pristine film, the PEDOT-to-heparin sulfur ratio was ~3.5. b) For the neutral sample, the PEDOT-to-heparin sulfur ratio was significantly increased to ~7.1. This loss of heparin on the film surface is due to a decrease in the amount of positive charge of PEDOT upon reduction, resulting in weaker electrostatic interactions between the heparin molecules and PEDOT. The heparin molecules on the surface of the films can then be removed by blow-drying the samples with a stream of helium. c) Upon electrochemical oxidation of neutral PEDOT and blow-drying with helium gas, the PEDOT-to-heparin sulfur ratio was ~4.4. The relative increase in surface heparin in oxidized PEDOT compared to the neutral films is consistent with the presence of tighter interactions between heparin and PEDOT in oxidized films.
Supplementary Figure 3. AFM images of a) pristine, b) neutral and c) oxidized electropolymerized PEDOT:heparin films on Orgacon-foil, the scan size is 10 µm, the scan rate 0.5 Hz and the height scale 150 nm.
**Supplementary Figure 4.** Quantification of bound FGF to PEDOT:heparin. Antibody accessible FGF2 anchored to neutral or oxidized PEDOT:heparin, pre-incubated with a) 100 ng ml⁻¹ or b) 500 ng ml⁻¹ of FGF2. Of note, for neutral samples, increasing FGF2 incubation concentrations correlated with increasing FGF2 immunoreactivity. FGF immunoreactivity for incubation with 200 ng ml⁻¹ and 500 ng ml⁻¹ of FGF2 was 2.2 and 5.1 times larger than incubation with 100 ng ml⁻¹ FGF2, respectively; c) Antibody accessible fibronectin (FN) associated to PEDOT:heparin on neutral or oxidized surfaces post FN incubation.
Supplementary Figure 5. NSCs cultured on TCPS for 4 days. a) NSCs cultured on TCPS substrates, treated daily with 10 ng ml\(^{-1}\) FGF2 (soluble FGF) or without FGF2 treatment (no FGF). Alternatively, TCPS was pre-incubated with 200 ng ml\(^{-1}\) FGF2 (anchored FGF). Green shows Nestin staining for neural stem cells and blue is DAPI for nuclear counterstaining; b) Number of live cells on the corresponding surfaces in (a) (n=3, ** = p<0.01). Notably, coating TCPS with heparin prior to FGF2 incubation led to a severe decrease in NSC adhesion.
Supplementary Figure 6. Astrocytic differentiation of NSCs on neutral and oxidized PEDOT:heparin/FGF2. a) NSCs cultured for 4 days on neutral PEDOT:heparin surfaces or on surfaces oxidized prior to cell seeding. Soluble FGF2 of 10 ng ml\(^{-1}\) was added daily to the medium (soluble FGF) or samples were pre-incubated with 200 ng ml\(^{-1}\) FGF2 (anchored FGF). Scale bar corresponds to 75 µm. Red shows immunolabeling of the astrocytic marker GFAP and blue corresponds to DAPI nuclear counterstaining; b) RT-QPCR quantification of GFAP mRNA expression (n=3, one way ANOVA).
Supplementary Figure 7. RT-QPCR quantification of Tubb3 mRNA expression.

a) RT-QPCR analysis of Tubb3 mRNA expression in NSCs cultured on PEDOT:heparin surfaces, either kept in a neutral state or oxidized prior to cell seeding. FGF2 concentration was 10 ng ml\(^{-1}\) for daily treatment (soluble FGF) and 200 ng ml\(^{-1}\) for samples pre-incubated with FGF2 (anchored FGF) (n=3); b) NSC cultured 4 days on PEDOT:heparin where oxidation or open circuit was applied after overnight culturing. FGF2 concentration was 200 ng ml\(^{-1}\) in the FGF adsorbed samples, whereas in FGF solution 10 ng ml\(^{-1}\) FGF2 was added daily (n=2).