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

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The regulation of stem cell fate decisions builds on a dynamic interplay between extrinsic signals and cell-intrinsic genetic and epigenetic programs. Growth factors (GFs) are a class of extrinsic signals critical for stem cell maintenance and differentiation. During embryonic development, the patterns of expression and activity of GFs are under precise temporal regulation. Importantly, in vivo many GFs are presented to cells anchored to components of the extracellular matrix (ECM).[1] Several GFs, including fibroblast growth factors (FGFs), strongly interact with heparin and heparan sulfate, common components of ECM proteoglycans.[2a,b] Heparin binding stabilizes these GFs and is required for GF clustering and activation of membrane-bound receptors, thereby modulating the signaling strength and duration.[3] Although GF anchoring reportedly affects fundamental aspects of GF signaling, in vitro stem cell differentiation protocols typically rely on sequential treatment with soluble GFs. Previous attempts to anchor GFs have commonly involved covalent immobilization or physical entrapment,[4a–d] which are likely to affect the activity of the GFs and cell accessibility. Importantly, temporal control in these approaches, if available, relies on enzymatic or hydrolytic degradation of a bulk material,[5] rendering these methods generally incompatible with adherent stem-cell culture and affording poor temporal resolution of GF presentation.

To achieve an in-vivo-like anchoring method of GFs with exact temporal control of their activity, we developed and evaluated an electroactive material that enables GF presentation and on-demand switch in GF bioavailability. The electroactive material is the conjugated polymer poly(3,4-ethylenedioxythiophene) (PEDOT),[6a–c] which incorporates anionic counter ions to counteract positive charges arising on the polymer backbone during oxidative electrosynthesis. Incorporation of heparin as a counter ion generated a material with good biocompatibility and stability, previously discussed in the context of neural prosthetics.[7a,b] Electrochemical reduction of the pristine, as synthesized, material caused a decrease in ionic binding of heparin to PEDOT, hence facilitating surface exposure of heparin and GF binding to heparin. Electrochemical oxidation of PEDOT led to a more intimate association between heparin and the polymer, thus decreasing the bioavailability of the bound GF (Figure 1 a,b).

Embryonic neural stem cells (NSCs) depend on fibroblast growth factor-2 (FGF2) to remain in a proliferative and undifferentiated state. Herein we show that FGF2 anchored to PEDOT through heparin supports proliferation and

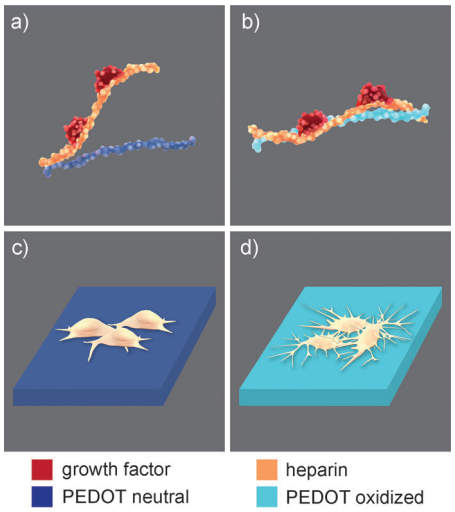

Figure 1. Electrochemical control of GF bioavailability to steer neural stem cell differentiation. a, b) Stronger electrostatic interactions between PEDOT and heparin as a result of electrochemical oxidation of PEDOT:heparin cause a decrease in the bioavailability of heparin-binding GFs; c, d) NSC differentiation induced by the decrease in GF availability caused by electrochemical oxidation of PEDOT:heparin; a, c) neutral PEDOT:heparin/GF; b, d) oxidized PEDOT:heparin/GF; Note that the structures are not drawn to scale.

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suppresses differentiation of NSCs cultured on PEDOT:heparin/FGF2 substrates. Remarkably, this process significantly stabilizes FGF2, eliminating the requirement for daily treatment of NSCs with soluble FGF2.[8a,b] Further, electrochemical oxidation of PEDOT decreases the bioavailability of FGF2, causing a reduction in cell proliferation and increased differentiation into neural cell types (Figure 1 c,d). Together, these data demonstrate a method for temporal control of signaling cues to stem cells, a cornerstone for the development of devices for stem cell culture and cell therapy.

Electrosynthesis of 3,4-ethylenedioxythiophene (EDOT) in a Clexane-solution (pharmaceutical grade, low-molecular-weight heparin) onto a conducting substrate gave dark blue, semi-oxidized films,[9] carrying a net positive charge on the PEDOT chains balanced by negatively charged heparin molecules. Electrochemical reduction of the films rendered the polymer nearly neutral, in the following referred to as "neutral PEDOT". Electrochemical oxidation of neutral PEDOT led to clear blue, fully oxidized films, denoted here as "oxidized PEDOT". The electrochemical characteristics of the material were evaluated by cyclic voltammetry. The redox peaks were at -0.4 V and $+0.3$ V, comparable to other PEDOT-based materials (Figure S1). XPS S(2p) core level spectra at various oxidation states were used to evaluate the presence of heparin on the surface of the films (Figure S2a–c).[10] We observed increased PEDOT-to-heparin sulfur ratios in neutral PEDOT, consistent with a model where there is an increased freedom of movement of the heparin molecules on the surface of the neutral films compared to the ionically attached molecules of the oxidized and pristine films. Heparin molecules on the surface of the films can then be removed by blow-drying the samples with a stream of helium, whereas heparin molecules deeper into the bulk are sterically hindered from escaping the film. To evaluate the accessibility of the anionic sulfate of heparin in the films, a toluidine blue (TB) assay was carried out,[11] omitting the blow drying procedure used for the XPS. In these experiments, the redox state of the surfaces was changed prior to the addition of TB. Oxidized PEDOT surfaces could only bind approximately 53 % of TB compared to neutral surfaces (Figure 2 a), in consistency with a previous report on TB binding to heparin in reduced and oxidized polypyrrole, a widely used conducting polymer.[11] The decrease in TB binding to oxidized films was larger when PEDOT was reduced to the neutral state prior to oxidation than when pristine films were oxidized directly (data not shown), hence demonstrating the greater accessibility of the negative sulfate groups for TB binding in the neutral state. Together, the XPS and TB assay data suggest that the sulfate groups of heparin are more available as interaction sites in neutral compared to oxidized PEDOT. In all subsequent experiments, polymer oxidation was preceded by reduction to neutral PEDOT to maximize accessibility of sulfate groups of heparin. This two-step procedure will hereafter be denoted as "oxidation". Electrochemical oxidation of neutral PEDOT is illustrated in the following reaction [Eq. (1)]:

$$\begin{aligned}&\mathrm{PEDOT}^{0} : \mathrm{heparin}^{n-} : n\mathrm{M}^{+} \leftrightarrow \\ &\mathrm{PEDOT}^{d+} : \mathrm{heparin}^{n-} : (n-d)\mathrm{M}^{+} + d\,\mathrm{M}^{+}(\mathrm{aq}) + d\,\mathrm{e}^{-}\end{aligned} \quad (1)$$

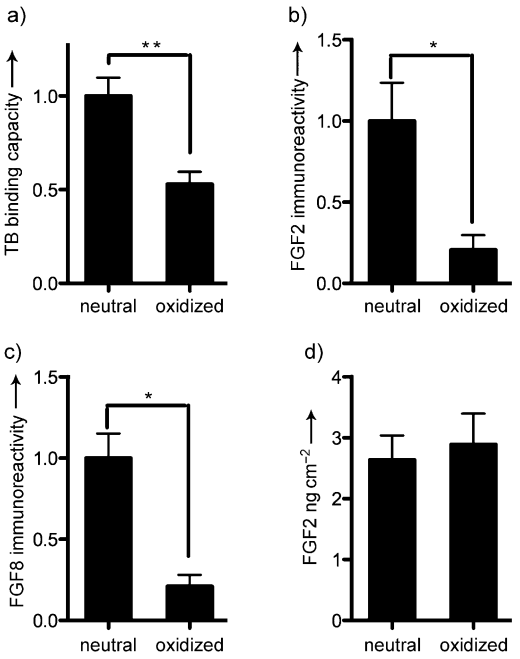

Figure 2. Characterization of PEDOT:heparin surfaces. a) Quantification of exposed heparin on neutral and oxidized PEDOT:heparin surfaces using toluidine blue assay, $n=6$, $p=0.005$; b) antibody-accessible FGF2 associated to neutral PEDOT:heparin incubated with FGF2 (200 ng mL^{-1}) and left in a neutral state (neutral) or oxidized after FGF2 incubation (oxidized), $n=4$, $p<0.05$; c) antibody-accessible FGF8 bound to PEDOT:heparin on neutral surfaces incubated with FGF8 (500 ng mL^{-1}, neutral) or surfaces oxidized post FGF8 incubation (oxidized), $n=3$, $p<0.05$; d) total amount of anchored FGF2 on neutral and oxidized PEDOT:heparin surfaces incubated with I-125-labeled FGF2 (200 ng mL^{-1}), quantified by gamma counting, $n=3$.

M^{+} represents cations, n equals the total number of negative charges on heparin, and d equals the induced positive charges on PEDOT.

AFM and goniometry were used to investigate the surface roughness and energy changes upon oxidation, respectively (see the Supporting Information for a description of methods). The surface roughness of neutral PEDOT, (15.88 ± 3.84) nm, did not significantly differ from that of oxidized PEDOT, (15.46 ± 2.57) nm (Figure S3). Neutral PEDOT:heparin surfaces showed significantly lower water contact angles of $(16 \pm 2.6)°$ compared to oxidized surfaces, $(21 \pm 3.2)°$ ($p = 0.05$), which is consistent with the model of higher accessibility of charged species in the neutral state.

Having characterized the electrochemically driven switch in heparin accessibility of PEDOT:heparin, we investigated the possibility of developing a switch for the presentation of heparin-binding growth factors based on this method. In vitro maintenance of NSCs requires daily addition of soluble FGF2 to the culture. PEDOT:heparin films were reduced to the neutral state, to facilitate heparin interactions, and were then incubated with FGF2 at various concentrations (Figure 2 b and Figure S4a,b). Following surface adsorption of FGF2, samples were coated with poly(ornithine) and fibronectin that are required for NSC culture. Finally, samples were either kept in a neutral state or were electrochemically oxidized in

NSC culture medium. To demonstrate the generality of the procedure, we also used FGF8, another member of the FGF family of GFs with diverse roles in the regulation of stem cells. Immunolabeling showed that oxidation of PEDOT:heparin/FGF2 resulted in a significant drop in the amount of antibody-accessible FGF2. Oxidized PEDOT:heparin/FGF2 showed approximately 20% of antibody-accessible FGF2 compared to the neutral surface (Figure 2b). Reduction of the oxidized surfaces back to the neutral state did not reverse the drop in antibody accessibility of FGF2 observed after oxidation. Importantly, the oxidation conditions selected did not cause significant effects on the amount of accessible fibronectin (Figure S4c), as shown for conjugated polymer films oxidized for longer periods.[12] Oxidized surfaces showed 21% of the amount of antibody-accessible FGF8, compared to neutral surfaces (Figure 2c), thus validating the applicability of the method to other heparin-binding GFs.

The observed decrease in detected FGF2 upon oxidation of PEDOT:heparin/FGF2 could originate from a loss of FGF2 from the film surface or from a conformational change, either in the protein structure or in the polymer film, hiding antibody epitopes. An assay with radiolabeled FGF2–I125 demonstrated that the amount of GF associated to the PEDOT:heparin remained the same on the neutral and oxidized surfaces (Figure 2d). These data demonstrate that FGF2 remains in the polymer film upon oxidation of PEDOT:heparin/FGF2. Importantly, heparin bound to FGF2 carries free sulfate groups available for interactions with other molecules.[13] Together, these data support a model, in which oxidation of the polymer results in stronger electrostatic interactions between heparin and PEDOT, while the association of FGF2 to heparin is maintained.

Next, we elucidated whether PEDOT:heparin/FGF2 can serve as an electrochemical switch for the proliferation and differentiation of NSCs. NSCs at passage 2 were seeded onto neutral or oxidized surfaces, as described above. After 4 days of daily treatment with soluble FGF2, NSCs cultured on neutral and oxidized PEDOT:heparin surfaces showed similar numbers of live cells. These data show that the redox state of the polymer does not affect NSC proliferation when soluble FGF2 is present in the medium (Figure 3a,b). As expected, cultures to which no FGF2 was added show low cell numbers, irrespective of the redox state of the surfaces. In contrast, neutral PEDOT:heparin/FGF2 surfaces supported significantly higher levels of NSC survival than oxidized surfaces. In fact, cell numbers on neutral PEDOT:heparin/FGF2 were at the same levels as on PEDOT:heparin surfaces to which soluble FGF2 was added daily. It is noteworthy that the requirement for daily addition of soluble FGF2 for NSC maintenance stems from the short half-life of FGF2 in solution of only a few hours.[14] Therefore, presentation of FGF2 bound to PEDOT:heparin led to a stabilization of the biological activity of FGF2, effectively replacing the proliferative effects of soluble FGF2. Importantly, the oxidized PEDOT:heparin/FGF2 surfaces showed cell numbers similar to the neutral or oxidized PEDOT:heparin surfaces with no FGF2 added, thus suggesting that the function of surface-bound FGF2 was abrogated by the oxidation process (Figure 3a,b). On conventional tissue culture plastics (TCPS), cell

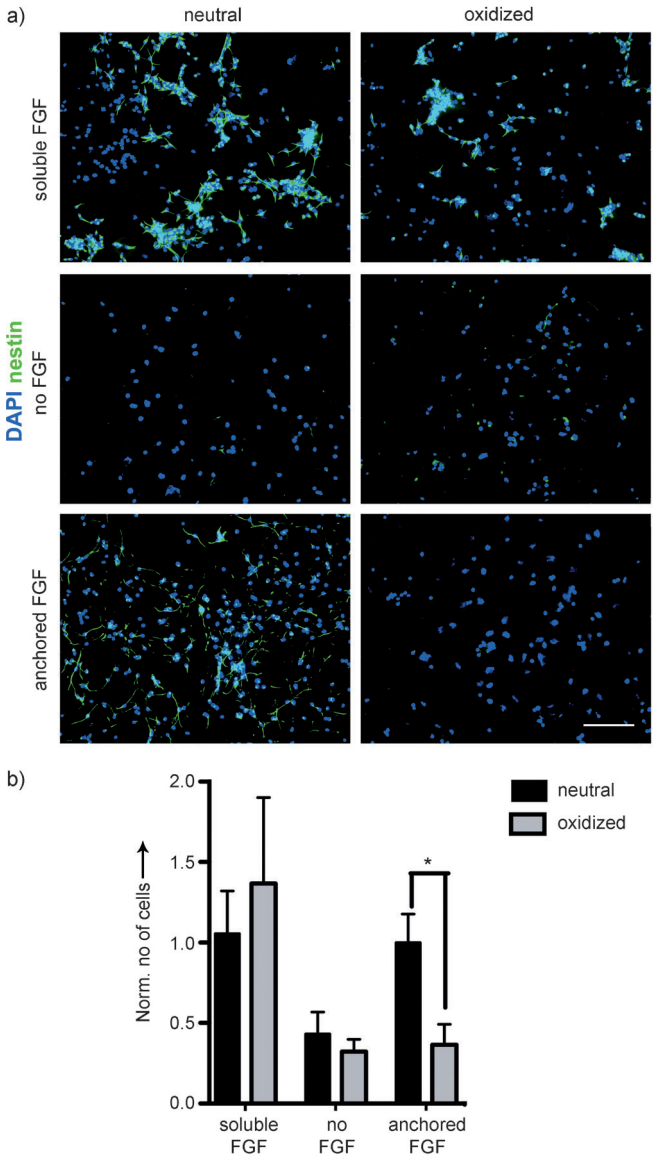

Figure 3. NSC culture on neutral and oxidized PEDOT:heparin/FGF2 surfaces demonstrating the biological activity of anchored FGF2. a) NSCs cultured for 4 days on PEDOT:heparin either kept in the neutral state or oxidized prior to cell seeding. FGF2 concentration was 10 ng mL^{-1} where FGF2 was added daily to the medium (soluble FGF) and 200 ng mL^{-1} in samples preincubated with FGF2 (anchored FGF). Control samples were neither treated with soluble nor preadsorbed FGF2 (no FGF). Scale bar: 150 μm. Green: Nestin staining (neural stem cell marker), blue: 4′,6-diamidino-2-phenylindole (DAPI, nuclear counterstaining). b) Number of live cells on the surfaces ($n = 3$, $p < 0.05$, Student's t-test).

numbers were six times higher where soluble FGF2 was added daily compared to surfaces incubated with FGF2 prior to cell seeding (Figure S5a,b), hence demonstrating minimal activity of adsorbed FGF2 on TCPS surfaces.

In NSC culture, removal of FGF2 initiates differentiation along the neuronal or glial lineages. Immunocytochemical staining for the astrocytic marker GFAP showed suppressed astrocytic differentiation on neutral and oxidized surfaces where FGF2 was added daily, as expected (Figure S6a).

Similarly, no astrocytic differentiation was detected on neutral PEDOT:heparin/FGF2 surfaces, confirming the presence of active FGF2. In contrast, on the oxidized PEDOT:heparin/FGF2 surfaces, the presence of differentiated astrocytes was evident (Figure S6a). Real-time reverse-transcription polymerase chain reaction (qRT-PCR) further demonstrated significantly higher mRNA expression of GFAP on the oxidized surfaces compared to neutral PEDOT:heparin/FGF2 or surfaces where FGF2 was added daily (Figure S6b). The trend of increased differentiation on oxidized compared to neutral PEDOT:heparin/FGF2 was similar for neuronal differentiation (Figure S7a).

Next, we examined the possibility to perform the oxidation process in situ during live cell culturing. NSCs were cultured overnight on neutral PEDOT:heparin/FGF2. Electrochemical oxidation was then performed or alternatively the system was kept as an open circuit. Comparison of the oxidized and open-circuit samples to which FGF2 was added in solution revealed that the oxidation process as such had no impact on the expression of GFAP, although the NSC viability was slightly decreased (Figure 4a). Importantly, the expression of GFAP clearly increased upon oxidation of PEDOT:heparin/FGF2 in situ (Figure 4a,b) and cells acquired an astrocytic morphology. A similar trend of increased neuronal differentiation upon oxidation of PEDOT:heparin/FGF2 was observed (Figure S7b). Together, these results demonstrate that electrochemical switching of PEDOT:heparin/FGF2 decreases the bioavailability of FGF2, thus creating a defined onset of NSC differentiation.

Herein we describe a method that allows for presentation of heparin-binding GFs to adherent stem cells. Association of GFs to heparin has been widely explored as a biomimetic strategy to immobilize GFs.[15] Importantly, in this study we demonstrate that the bioavailability of anchored GFs can be changed electrochemically through a straightforward oxidation step, offering precise temporal control of the stem cell state. This on-demand switch for GF bioavailability represents a significant improvement over methods that rely on enzymatic or hydrolytic bulk degradation, processes which are generally slow and afford little control over the onset of GF release.

We have validated a versatile tool, which offers a new dimension of control over cytokine presentation to cells. We suggest that it can be applied to a wide range of heparin-binding GFs that undergo distinct temporal changes in activity, fundamental for cell regulation. Further, the approach has great potential to be tailored for 3D applications. Conducting polymers have previously been polymerized as coatings onto 3D scaffolds composed of, for example, poly(ethylene terephthalate) (PET)[16] or biodegradable polymers.[17] 3D scaffolds with active control of GF presentation are of true interest for stem cell therapy, which presently struggles with poor cell survival and limited control over differentiation of grafted cells.[18] It is noteworthy that we show stabilization of the activity of GFs anchored to PEDOT:heparin, a prerequisite for using this approach to control the stem cell microenvironment in vivo. We propose that the strategy presented in this study, in which cytokine bioavailability is regulated through the electrochemical properties of a conjugated polymer, has the potential to be a fundamental technique for basic studies of stem cell function as well as stem cell therapy.

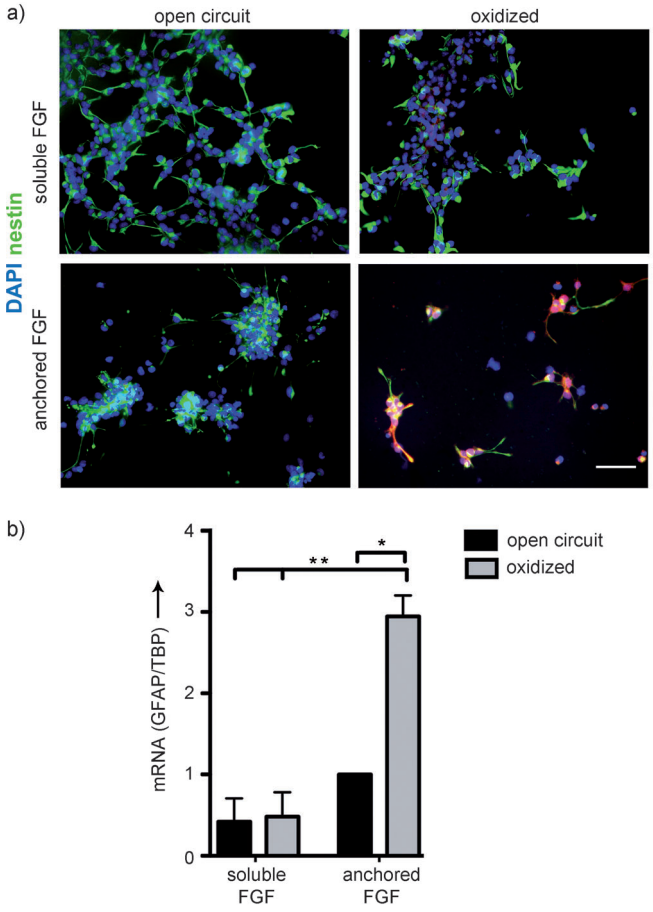

Figure 4. Oxidation of PEDOT:heparin/FGF2 surfaces decreases FGF2 activity during live cell culture. a) NSCs cultured for 4 days on PEDOT:heparin surfaces were oxidized with live cells or kept at open circuit. FGF2 concentration was 10 ng mL^{-1} for the daily treatment (soluble FGF) and 200 ng mL^{-1} for the preincubated samples (anchored FGF). Scale bar: 75 μm. Green: Nestin staining (neural stem cell marker), blue: 4′,6-diamidino-2-phenylindole (DAPI, nuclear counterstaining). b) qRT-PCR quantification of GFAP mRNA expression ($n=2$, one way ANOVA).

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[1] R. O. Hynes, *Science* **2009**, *326*, 1216 – 1219.
[2] a) I. Capila, R. J. Linhardt, *Angew. Chem.* **2002**, *114*, 426 – 450; *Angew. Chem. Int. Ed.* **2002**, *41*, 390 – 412; b) S. Tumova, A. Woods, J. R. Couchman, *Int. J. Biochem. Cell Biol.* **2000**, *32*, 269 – 288.
[3] L. Pellegrini, D. F. Burke, F. von Delft, B. Mulloy, T. L. Blundell, *Nature* **2000**, *407*, 1029 – 1034.

[4] a) K. Alberti, R. E. Davey, K. Onishi, S. George, K. Salchert, F. P. Seib, M. Bornhauser, T. Pompe, A. Nagy, C. Werner, P. W. Zandstra, *Nat. Methods* **2008**, *5*, 645 – 650; b) N. Huebsch, D. J. Mooney, *Nature* **2009**, *462*, 426 – 432; c) M. P. Lutolf, P. M. Gilbert, H. M. Blau, *Nature* **2009**, *462*, 433 – 441; d) H. Yamazoe, Y. Murakami, K. Mizuseki, Y. Sasai, H. Iwata, *Biomaterials* **2005**, *26*, 5746 – 5754.

[5] P. Tayalia, D. J. Mooney, *Adv. Mater.* **2009**, *21*, 3269 – 3285.

[6] a) B. L. Groenendaal, F. Jonas, D. Freitag, H. Pielartzik, J. R. Reynolds, *Adv. Mater.* **2000**, *12*, 481 – 494; b) A. J. Heeger, *Angew. Chem.* **2001**, *113*, 2660 – 2682; *Angew. Chem. Int. Ed.* **2001**, *40*, 2591 – 2611; c) R. H. Karlsson, A. Herland, M. Hamedi, J. A. Wigenius, A. Aslund, X. J. Liu, M. Fahlman, O. Inganäs, P. Konradsson, *Chem. Mater.* **2009**, *21*, 1815 – 1821.

[7] a) M. Asplund, E. Thaning, J. Lundberg, A. C. Sandberg-Nordqvist, B. Kostyszyn, O. Inganäs, H. von Holst, *Biomed. Mater.* **2009**, *4*, 045009; b) E. M. Thaning, M. L. M. Asplund, T. A. Nyberg, O. W. Inganäs, H. von Holst, *J. Biomed. Mater. Res. Part B* 2**010**, 93B, 407 – 415.

[8] a) K. K. Johe, T. G. Hazel, T. Muller, M. M. Dugich Djordjevic, R. D. G. McKay, *Genes Dev.* **1996**, *10*, 3129 – 3140; b) A. I. Teixeira, J. K. Duckworth, O. Hermanson, *Cell Res.* **2007**, *17*, 56 – 61.

[9] S. K. M. Jonsson, J. Birgerson, X. Crispin, G. Greczynski, W. Osikowicz, A. W. D. van der Gon, W. R. Salaneck, M. Fahlman, *Synth. Met.* **2003**, *139*, 1 – 10.

[10] G. Zotti, S. Zecchin, G. Schiavon, F. Louwet, L. Groenendaal, X. Crispin, W. Osikowicz, W. Salaneck, M. Fahlman, *Macromolecules* **2003**, *36*, 3337 – 3344.

[11] B. Garner, A. Georgevich, A. J. Hodgson, L. Liu, G. G. Wallace, *J. Biomed. Mater. Res.* **1999**, *44*, 121 – 129.

[12] K. Svennersten, M. H. Bolin, E. W. H. Jager, M. Berggren, A. Richter-Dahlfors, *Biomaterials* **2009**, *30*, 6257 – 6264.

[13] S. Faham, R. E. Hileman, J. R. Fromm, R. J. Linhardt, D. C. Rees, *Science* **1996**, *271*, 1116 – 1120.

[14] A. Nur-E-Kamal, I. Ahmed, J. Kamal, A. N. Babu, M. Schindler, S. Meiners, *Mol. Cell. Biochem.* **2008**, *309*, 157 – 166.

[15] E. S. Place, N. D. Evans, M. M. Stevens, *Nat. Mater.* **2009**, *8*, 457 – 470.

[16] M. H. Bolin, K. Svennersten, X. J. Wang, I. S. Chronakis, A. Richter-Dahlfors, E. W. H. Jager, M. Berggren, *Sens. Actuators B* **2009**, *142*, 451 – 456.

[17] J. Y. Lee, C. A. Bashur, A. S. Goldstein, C. E. Schmidt, *Biomaterials* **2009**, *30*, 4325 – 4335.

[18] D. E. Discher, D. J. Mooney, P. W. Zandstra, *Science* **2009**, *324*, 1673 – 1677.

