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Sub-picomolar, label-free procalcitonin analytical detection with an electrolyte-gated organic field-effect transistor based electronic immunosensor.

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ABSTRACT

Herein a label-free immunosensor based on electrolyte-gated organic field-effect transistor (EGOFET) was developed for the detection of procalcitonin (PCT), a sepsis marker. Antibodies specific to PCT were immobilized on the organic semiconductor surface through direct physical adsorption followed by a post-treatment with bovine serum albumin (BSA) which served as the blocking agent to prevent non-specific adsorption. Antibodies together with BSA (forming the whole biorecognition layer) served to selectively capture the procalcitonin target analyte. The entire immunosensor fabrication process was fast, requiring overall 45 min to be completed before analyte sensing. The EGOFET immunosensor showed excellent electrical properties, comparable to those of bare poly-3-hexylthiophene based EGOFET confirming reliable biosensing with bio-functional EGOFET immunosensor. The detection limit of the immunosensor was as low as 2.2 pM and within a range of clinical relevance. The relative standard deviation of the

individual calibration data points, measured on immunosensors fabricated on different chips (reproducibility error) was below 7 %. The developed immunosensor showed high selectivity to the PCT analyte which was evident through control experiments. This report of PCT detection is first of its kind among the electronic sensors based on EGOFETs. The developed sensor is versatile and compatible with low-cost fabrication techniques.

KEYWORDS: Electrolyte-gated organic field-effect transistors, immunosensor, procalcitonin, label-free detection.

1. Introduction

A biosensor is an analytical device, incorporated with biological recognition elements that converts a biorecognition event into a quantifiable and processable signal with the help of a transducer (Turner, 2015). Such biosensors are inevitable components in many domains, notably, clinical laboratories, personalized health and fitness, food and environment. Nowadays, a lot of research is focused on developing self-contained, patient bedside sensors, the so-called Point-of-Care sensors (Gubala et al., 2012; Song et al., 2014; Zarei, 2017). These portable devices are supposed to provide to a clinician, fast and reliable analytical quantification of biomarkers right at the place where they are more needed. While organic field effect transistors based on solid dielectrics have been gathering attention as sensors since decades, electrolyte-gated organic field-effect transistors are a relatively new class of bioelectronic sensors (Magliulo et al., 2016; Marinelli et al., 2009). EGOFETs offer advantages such as label-free detection, low-voltage operation (below 1 V), biocompatibility and ability to detect analyte in complex matrices such as serum (Palazzo et al., 2015). They possess inherent gain amplification with a direct read-out making them highly desirable for biosensing (Torsi et al., 2013). They also offer multiparametric analysis of the obtained output signal (Torsi et al., 2000). Besides, they are highly versatile in terms of fabrication, they could be miniaturized and are also compatible with high throughput, inexpensive methods like printing (Manoli et al., 2015).

EGOFETs are three terminal devices which consist of source, drain and gate electrodes. Source and drain are connected via an organic semiconductor, forming the so called transistor electronic channel. The latter is separated from the gate by means of a dielectric medium. The channel conductivity is modulated by the capacitive coupling of the gating system (gate electrode and dielectric medium), but unlike conventional transistors, the gating medium that connects the gate with the channel is an electrolyte solution, more often an aqueous based one. Upon biasing, an electric double layer is formed both at the gate/electrolyte interface and the electrolyte/organic semiconductor interface (Kergoat et al., 2012). These two, constitute the active electronic layers wherein the biomolecules can be conveniently immobilized on either one of these interfaces (Mulla et al., 2015b; Palazzo et al., 2015). EGOFETs could be functionalized by

innumerable ways which could be broadly classified into physical adsorption and covalent modification. Owing to the lack of suitable functional groups on the organic semiconductor (OSC) to anchor the incoming biomolecules, covalent modification is performed either through modification of OSC backbone by introducing functional groups (Lohwasser and Thelakkat, 2010) or through surface treatments such as plasma enhanced chemical vapor deposition (PE-CVD) (Magliulo et al., 2013). Physical adsorption is a relatively simple, fast and inexpensive immobilization process as compared to covalent modification. Physical adsorption is the immobilization of biomolecules through intermolecular forces such as Van der Waals, hydrophobic, and ionic interactions without involvement of a chemical bond between the receptors and the sensor's interface (Jung et al., 2008). Intermolecular forces that exactly take part in the interaction will depend on the protein and the surface involved (Rusmini et al., 2007). The resulting layer is likely to be heterogeneous and randomly oriented. Although covalent modification ensures robust and stable integration of biomolecules, the risk of OSC degradation and biomolecule denaturation is more when compared to physical adsorption due to complex functionalization process (Magliulo et al., 2016).

There are quite a few reports based on physical adsorption of proteins onto the OSC aiding in the deeper understanding of the immobilization process (Albers et al., 2012; Awsiuk et al., 2014). More recently, Magluilo *et al.* (Magliulo et al., 2016) developed a highly sensitive, label-free EGOFET based immunosensor for the detection of C-reactive protein (C-RP) based on the physically adsorbed anti-CRP on regio-regular P3HT. Receptor immobilization through physical adsorption did not affect the electronic performance of the OSC. A good level of surface coverage and uniformity of the adsorbed proteins on the surface of the P3HT was also observed. The whole functionalization step was finished in about 30 min with a detection limit of 2.2 pM.

Sepsis is defined as a systemic inflammatory response to an infection. It is reported to be one of major causes leading to mortality worldwide in hospitals and intensive care units (Knoop et al., 2017). Procalcitonin is being thoroughly explored as a sepsis marker and as an aid for antibiotic stewardship (Sager et al., 2017; Schuetz et al., 2017). The level of PCT in the blood stream rises considerably during sepsis. This physiological increase serves as a marker for sepsis and as a tool for differential diagnosis to determine the severity of the condition. PCT becomes detectable within 3-4 h of stimulus aiding in fast diagnosis (Markanday, 2015).

Life threatening conditions such as sepsis demand high quality diagnosis with fast therapeutic turnaround time (TAT). PCT sensing has been explored through various sensing platforms which includes electrochemical (Lim et al., 2017; Mahe et al., 2014; Shen et al., 2015), optical sensing methods (Baldini et al., 2009a; Baldini et al., 2009b; Vashist et al., 2016). Even commercial sensors for PCT detection exist in the market aiding in clinical decisions (Fortunato, 2016; Kutz et al., 2015). However, more PCT sensors

that are reliable, sensitive, fast, less costing, self-contained, and user friendly, if developed could benefit patient outcomes by helping the clinicians to make quick decisions. EGOFETs are viable alternatives with exceptional credentials that could meet the aforementioned demands.

Herein, an EGOFET immunosensor to detect procalcitonin based on physically adsorbed capturing antibodies has been developed and explored. Anti-PCT antibodies (monoclonal) were immobilized on the surface of the OSC (P3HT) through direct physical adsorption without any pre-treatment, followed by a blocking of the surface with bovine serum albumin (BSA). The antibody/BSA layer served as the biorecognition layer to selectively detect PCT in clinical relevant range. Notably, to the best of our knowledge, this study is the first report of procalcitonin detection through an electronic sensor based on organic electrolyte gated field-effect transistors.

2. Materials and methods

2.1 Materials and Reagents

Si/SiO_2 substrates were purchased from Silicon Materials Inc (Pittsburg, PA, USA). Poly-3-hexylthiophene (P3HT), purchased from Rieke Metals (regio-regularity > 98%, Sepiolid^{TM} P200), was purified following the protocol reported by Urien *et al* (Urien et al., 2007). Indeed, thiophene based organic semiconductors are known for being characterized by a polycrystalline morphology, suitable for sensing applications (Lovinger et al., 1996; Torsi et al., 1995).The anti–PCT (CALCA 4A6) monoclonal antibodies were sourced from Naturwissenschaftliches und Medizinisches Institut (NMI), Reutlingen, Germany. The protein, Human Procalcitonin (HOR-304) was purchased from Prospec. Bovine serum albumin (BSA) (blocking agent) was purchased from Sigma Aldrich. Phosphate buffered saline tablets and all other chemicals, unless mentioned, were purchased from Sigma Aldrich.

2.2 EGOFET device fabrication

The schematic representation of the immunosensor development with EGOFET is shown in the fig. 1. Si/SiO_2 substrates were photolithographically patterned with interdigitated gold source (S) and drain (D) electrodes (10 μm channel length and 10 mm channel width). Afterwards, gold was e-beam evaporated (50 nm) using titanium as adhesion promoting layer of 5 nm thickness. The substrates were then cleaned by ultra-sonication using solvents of increasing polarity (Dinelli et al., 2005). Subsequently, filtered P3HT solution (2.5 mg/mL in chlorobenzene) was spin coated (2000 rpm for 20 s) on the gold patterned substrates. The samples were then annealed at 75 °C for 1 h.

2.3 Deposition of anti-PCT on P3HT

Monoclonal antibodies specific to PCT (CALCA 4A6) were immobilized on the device coated with P3HT by means of physical adsorption, as shown in the Fig.1 (panel 1). To this end, 2 µL of anti-PCT solution in PBS (phosphate 10 mM, KCl 2.7 mM, 137 mM NaCl, pH = 7.4) was drop casted on the P3HT surface in correspondence with the EGOFET electronic channel and incubated for 30 min to allow the receptors to get adsorbed onto the surface. An antibody concentration of 36 µg/mL yielded a good coverage which was in reference with previous studies with the same antibodies to detect PCT using surface plasmon resonance (SPR) technique (Rascher et al., 2014). The surface was then thoroughly rinsed three times with wash buffer (PBS, 10 mM, pH 7.4 with 0.05 % tween) to remove the unabsorbed antibodies. This was followed by exposing the receptor functionalized surface to 2 µL BSA (300 µg/mL in PBS) solution for 15 min in order to block and avoid any non-specific binding (Fig. 1, panel 2). A washing step involving a three times rinsing with wash buffer was finally carried out. The EGOFET immunosensor is now ready for electrical measurements and procalcitonin detection (Fig. 1, panel 3). PCT solutions (10 to 6000 pg/mL) were prepared in PBS (10 mM, pH 7.4). Worth the mention is that the PBS ionic strength and pH are those typical of real samples such as human blood serum. Same range of concentrations was also prepared for the negative control experiments, performed by using a milk powder as interference analyte.

2.4 Electrical characterization

Current voltage characteristics (I-V) were measured with an Agilent 4155 C semiconductor parameter analyzer. A gold L - shaped plate (2 x 2 mm) served as the gate electrode, while 2 µL droplet of PBS (10 mM, pH = 7.4) was used as electrolyte gating solution. The electrical measurements were carried out in a water vapor saturated environment to prevent evaporation of the electrolyte droplet. The output characteristics (I_{DS}-V_{DS}) were recorded by varying the gate voltage (V_{GS}) from 0 to -0.7 V in steps of 0.1 V and the source drain bias V_{DS} was swept from 0 to -0.5 V in steps of 0.05 V. The transfer (I_{DS}-V_{GS}) characteristics were measured by keeping the drain voltage (V_{DS}) constant at -0.5 V and sweeping the gate voltage from 0 to -0.7 V in step of 0.02 V. The hysteresis was also evaluated by sweeping the curves back and forth. Field-effect mobility (μ_{FET}) and threshold voltage (V_T) were extracted from the characteristics in the saturation regime as described elsewhere (Torsi et al., 2013). A gate channel capacitance value of 3 µF cm^{-2} per unit area was used for the field-effect mobility (μ_{FET}) estimation (Kergoat et al., 2010). The sensing measurements to detect the analyte (PCT) were performed by measuring the transfer characteristics in PBS each time after the exposure of functionalized P3HT surface to increasing PCT concentrations (0.8 pM to 4.7 nM) successively on the same EGOFET immunosensor. Typically, the bio-functionalized devices were incubated in each PCT concentration for 20 min. Afterwards, the devices were rinsed three times with wash buffer to remove unbound proteins followed by measurements. Prior to the analyte exposure, blank measurements were taken in PBS. The immunosensor response was estimated as the relative change in drain current at maximum gate voltage

(supplementary information). The inter-device reproducibility was evaluated by comparing the calibration curves obtained from at least three EGOFET immunosensors fabricated on different chips. The limit of detection (LOD) was estimated using the equation $Y_{LOD} = Y_{blank} + 3S_{blank}$, where Y_{blank} and S_{blank} are the average value of the blank response and its corresponding standard deviation respectively that are obtained by measuring at least a minimum of 10 independent sample blanks (González and Herrador, 2007). The responses of the negative controls were taken into consideration as blank responses for the estimation of LOD.

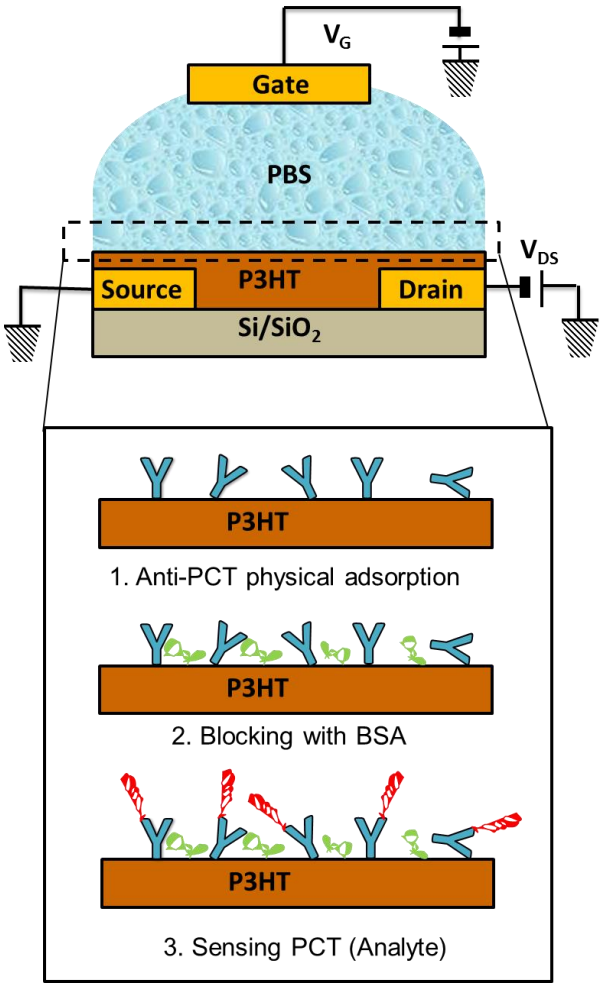

Fig. 1. Schematic representation of the developed EGOFET immunosensor for PCT detection. The anti-PCT antibody was directly adsorbed on the P3HT surface (panel 1) followed by surface blocking with BSA to reduce nonspecific interaction (panel 2). The constructed immunosensor is exposed to the analyte (panel 3) and the electrical response is measured. (single column)

3. Results and Discussion

3.1 EGOFET immunosensor electrical characterization

A comparison of the current-voltage curves; output curve (I_{DS} vs. V_{DS}) and transfer curve (I_{DS} vs. V_{GS}/V_G) of the EGOFET before bio-functionalization (bare P3HT) and after the deposition of the bio-recognition layer by direct adsorption of anti-PCT receptors and BSA (blocking agent), is shown in Fig. 2. A good field-effect current modulation was observed after bio-functionalization with a negligible level of hysteresis, evident from the forward and back sweep current matching. A drop in the source-drain current along with a decrease in the field-effect mobility of about an order of magnitude from 10^{-2} to 10^{-3} cm^2/V s was observed after bio-functionalization. On the other hand, the threshold voltage did not change significantly.

Physical adsorption, being a mild deposition process, does not involve large modification of the OSC layer. Indeed, this is the case for this study, as the physical adsorption of the biological recognition elements, resulted only in slight changes in the EGOFET figures of merit (Fig. 2). This is not surprising, as the reduction in the drain current and the change in the mobility could possibly be attributed to factors such as the penetration of ions into the OSC, degradation of the OSC due to anti-PCT adsorption and exposure to aqueous environment (Mulla et al., 2015a). Those factors did not alter the OSC enough to bring a significant change in the threshold voltage after biofunctionalization. Often, depending upon the biomolecule and the OSC involved, ions penetration might have resulted in a threshold voltage shift either through charge trapping or provision of additional charges which was not the case observed as a consequence of biofunctionalization (Cramer et al., 2013). However, the most important fact to be considered is that the process of bio-functionalization involving the physical adsorption did not render the OSC unfit or hampered for the sensing process. The whole process of sensor fabrication could be completed in 45 min and this is a very short time-frame as compared to covalent immobilization which could at least consume 2-4 h, often encompassing two or more steps.

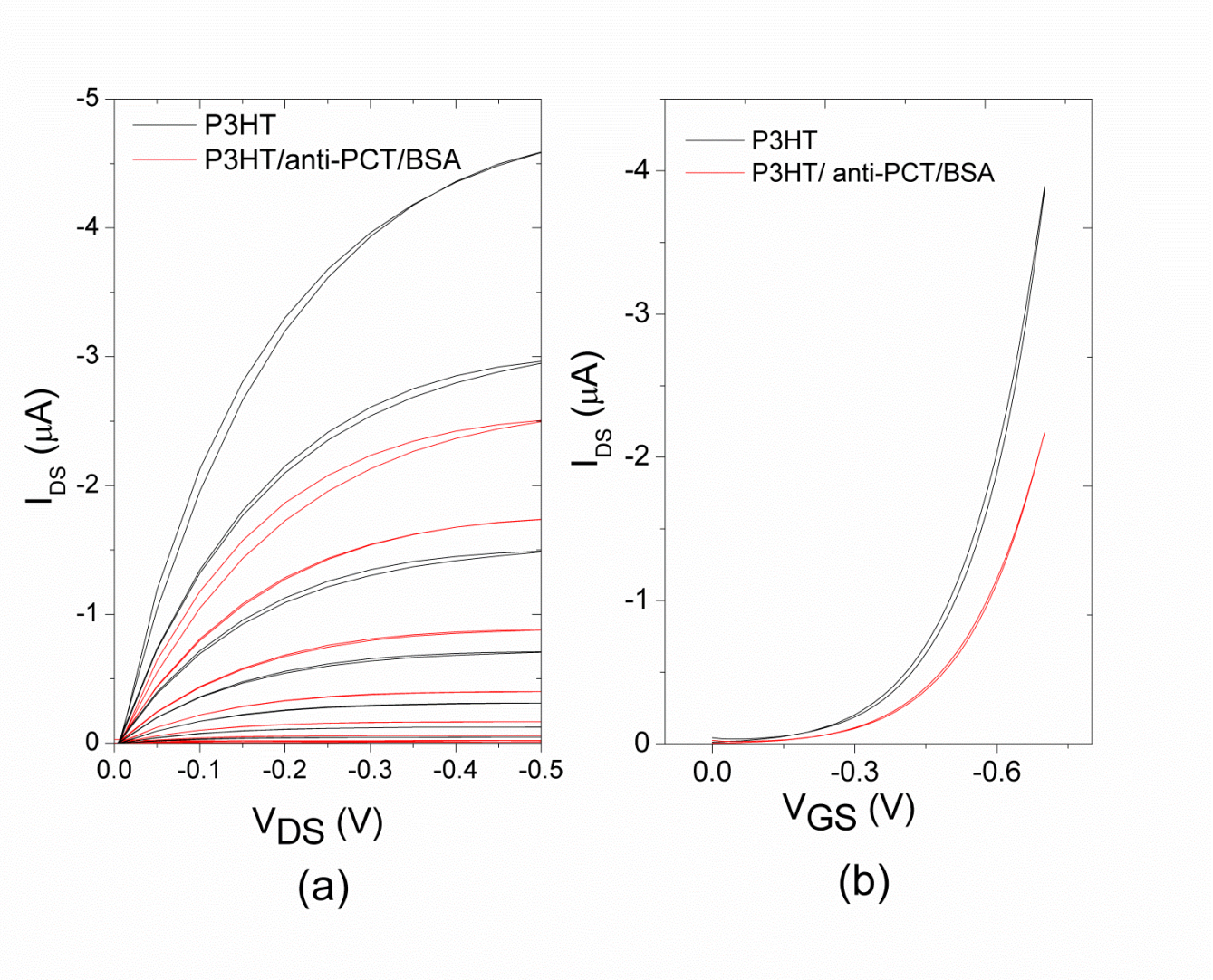

Fig. 2. (a) Characteristic output curves of drain current versus the drain voltage (I_{DS} vs V_{DS}) at different gate voltages; (b) Characteristic transfer curves of the drain current versus the gate voltage (I_{DS} vs V_{GS}) at constant drain voltage V_{DS} = -0.5 V for devices with bare P3HT (black) and P3HT/anti-PCT/BSA (red). (double column)

3.2 Procalcitonin sensing with EGOFET immunosensor

The transfer characteristics (I_{DS} vs. V_{GS} for a constant V_{DS}) of an EGOFET immunosensor obtained upon exposure to increasing concentrations of the analyte procalcitonin along with blank is depicted in Fig. 3.

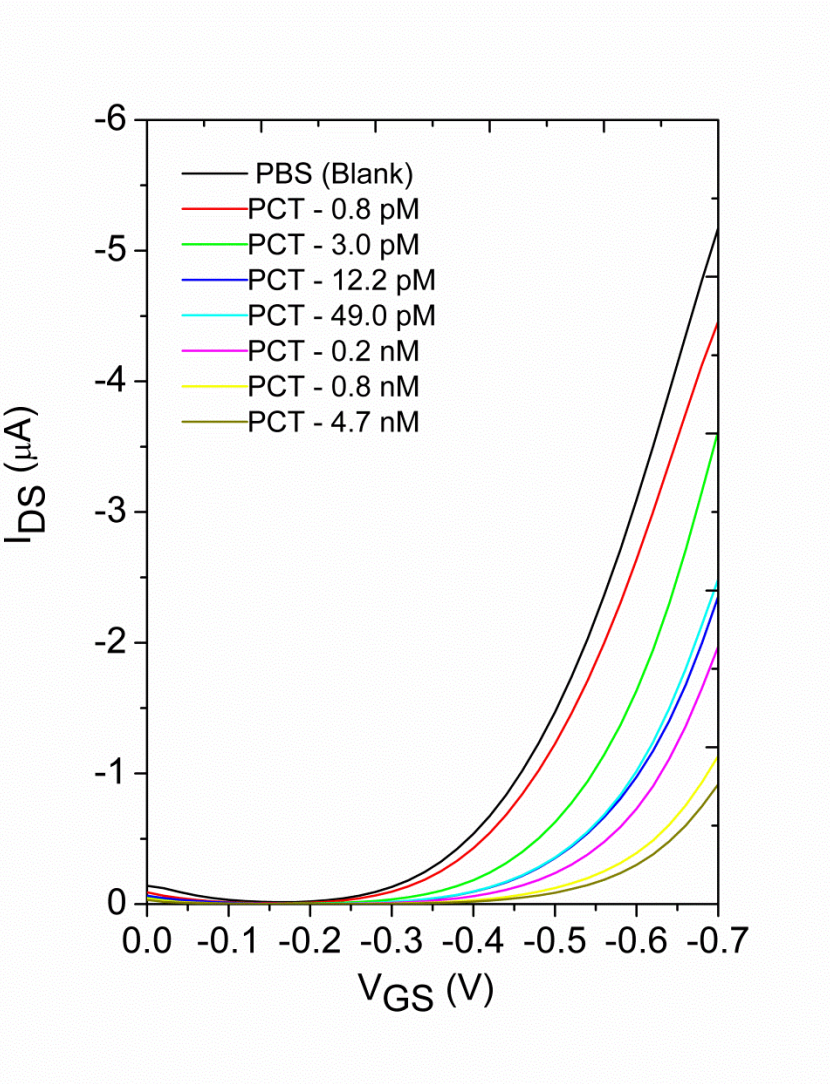

Fig. 3. Transfer characteristics measured for an EGOFET immunosensor exposed to increasing concentrations of PCT along with blank. (single column)

As shown in Fig. 3, a reduction in the drain current was observed as the immunosensor was exposed to increasing concentrations of PCT. Interestingly, the threshold voltage (V_T) was also changed upon exposure to PCT. As more PCT molecules bonded to the surface immobilized with anti-PCT, the value of the threshold voltage increased shifting to more negative values, as reported in Fig. 4a. This trend is more clearly affirmed by plotting the relevant V_T values against PCT concentration (Fig. 4b, *upper panel*). In Fig. 4b (*lower panel*), the mobility is plotted versus the analyte concentration. As we can observe no significant changes are seen in the value of mobility upon exposure to PCT.

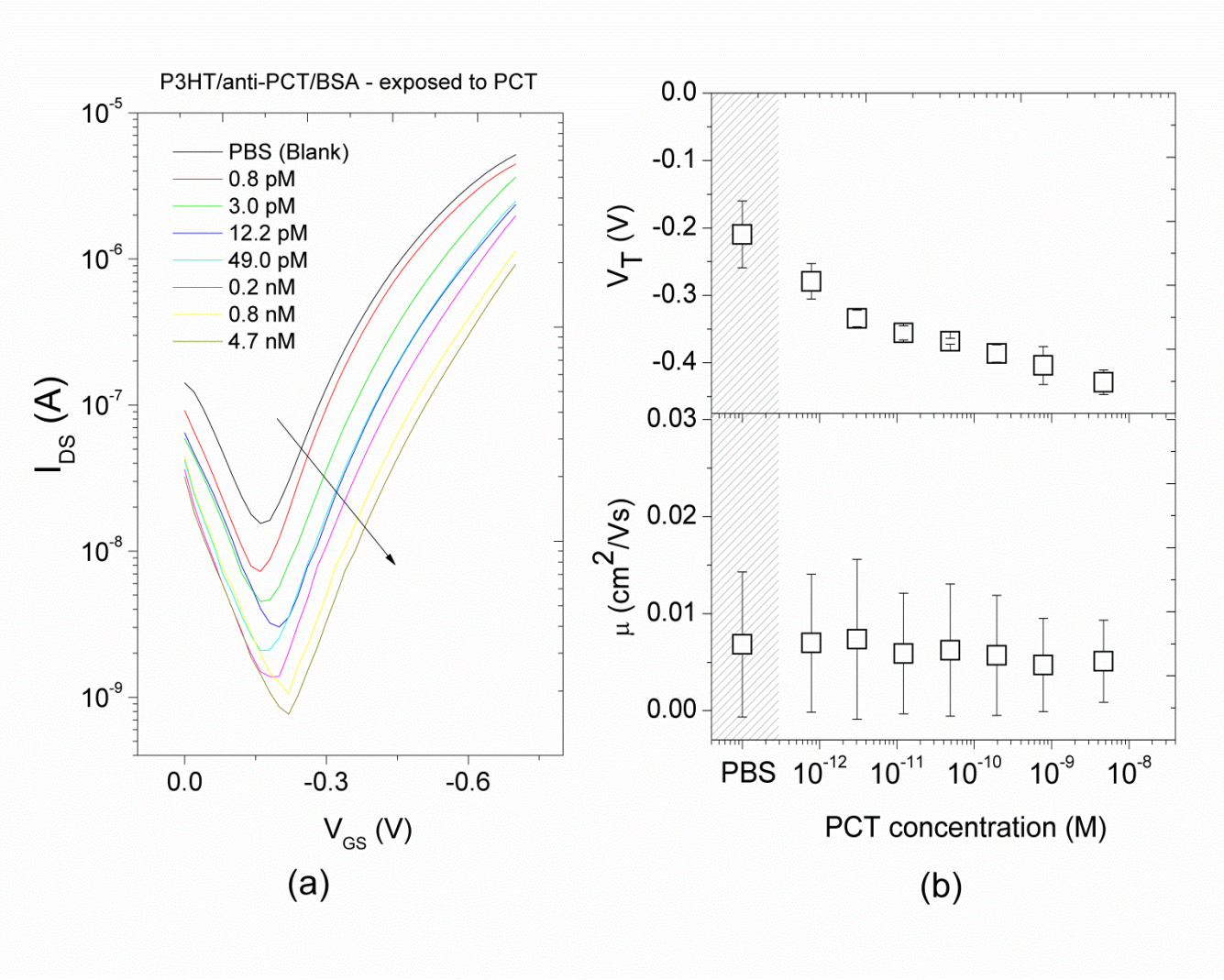

Fig. 4. (a) Transfer characteristics (I_{DS} vs. V_{GS}) of the P3HT/anti-PCT/BSA - exposed to different concentrations of PCT; (b) V_T (upper panel) and μ_{FET} (lower panel) versus the concentration of PCT. (Double column)

In the case of EGOFET biosensors, successful analyte – receptor binding, evokes a response which is seen as a distinct change in the transistor figure of merits. The ligand binding to the receptor changes the receptor conformation and/or the charge distribution at the bio-modified interface, eventually causing a shift in the threshold voltage of the transistor, and/or impacting the field-effect mobility, and/or the capacitance. The first possibility is that the analyte molecules could create trap states or provide additional charges to the semiconductor. As a consequence, the density of the mobile carriers changes and the threshold voltage shifts. Secondly, the interaction between the analyte molecule and the OSC could elicit a change in the electronic coupling along the charge carrier transfer path in the semiconductor which is often attributed to irreversible morphological changes or interactions at grain boundaries. As a result, the charge carrier mobility is altered and experimentally this effect could be seen as a change in transconductance (Cramer et al., 2013). Thirdly, by depositing the capturing biolayer on one of the

electrolyte interfaces (*i.e.* semiconductor and/or gate), we add an extra capacitance to the total capacitance of the gating system. Typically, the biomodified interface exhibits smaller capacitance therefore becomes dominant (Manoli et al., 2015).

In the current study, the receptors specific to PCT were immobilized at the interface between the electrolyte and the organic semiconductor. The anti-PCT layer indeed induced changes to the charge carrier path in the semiconductor seen as change in the mobility. On the other hand, upon binding of PCT to the anti-PCT/P3HT surface, a decrease in the drain current was observed along with a negative shift in the threshold voltage while mobility remained constant (Fig. 4). As mentioned above, the assayed analyte-receptor properties play a major role in the transistor response to sensing. At pH 7.4, in which the study was conducted, PCT holds a net negative charge (pI = 5.1) (Kozlowski, 2016). A possible explanation for this behavior is that the surface negative charge of the tested analyte PCT, at pH 7.4, might have acted as traps for the positive charge carriers (holes) of the OSC (P3HT), assuming that the bioreceptor layer on top of the semiconductors is permeable to ions. Consequently, the impact is seen as decrease in the drain current and as a shift of the threshold voltage towards more negative values with respect to the increasing analyte concentrations (Cramer et al., 2013; Tiwari et al., 2012; Tremblay et al., 2011). Moreover, threshold shifts can be also associated to capacitance changes. The presence of the capturing biolayer on top of the semiconductors can contribute to the total capacitance and in fact dominate as being the smallest. The capacitance at the semiconductor-anti-PCT/electrolyte interface is reduced due to low permittivity of adsorbed biomolecules relative to the electrolyte (Mulla et al., 2015b). The electrostatic capacitance reduction due to adsorbed biomolecules can only become apparent in case of near-full coverage under the assumption that ions cannot permeate through this protein layer.

3.3 Control experiments

The selectivity of the developed immunosensor was challenged by performing two control experiments. In the first case, EGOFET devices without the anti-PCT receptors (P3HT/BSA) were exposed to the different PCT concentrations. Secondly, a fully bio-functionalized EGOFET immunosensor (P3HT/anti-PCT/BSA) was exposed to an interference analyte (*i.e.* milk powder). Fig. 5(a-c) shows the comparison between the I-V transfer curves of the PCT sensing and the control experiments. Clearly, the graphs suggest a distinctive change in the drain current with respect to PCT concentration, only in the case of fully functionalized immunosensors (P3HT/anti-PCT/BSA). This distinctive trend is not seen in the other two experiments. This proves the selectivity and the robustness of the constructed EGOFET immunosensor towards PCT detection.

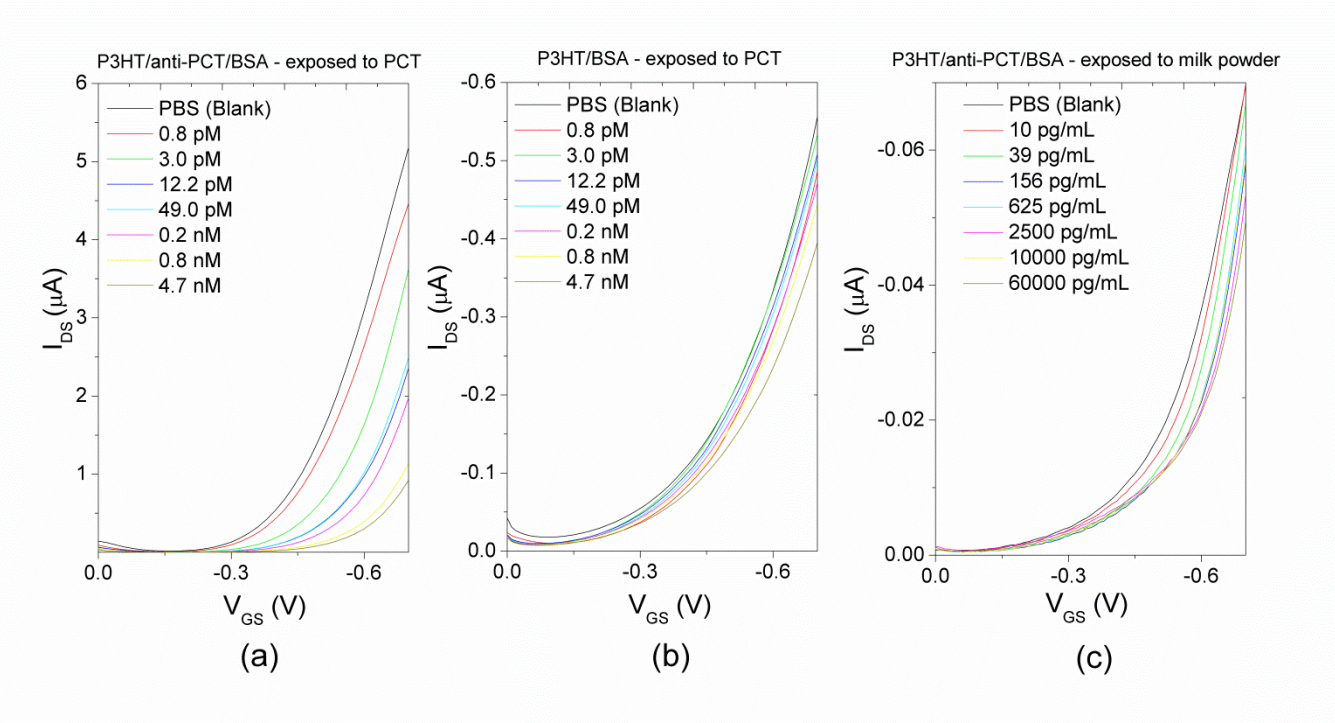

Fig. 5. Transfer characteristics (I_{DS} vs. V_{GS} at constant V_{DS}) for: (a) P3HT/anti-PCT/BSA - exposed to different concentrations of PCT; (b) P3HT/BSA - exposed to different concentrations of PCT (control); (c) P3HT/anti-PCT/BSA - exposed to different concentrations of milk powder (negative control). (double column)

3.4 Analytical features of the EGOFET immunosensor

The calibration curve obtained for the developed EGOFET immunosensor is shown in Fig. 6a. A range of PCT concentration spanning from 0.8 pM to 4.7 nM could be detected. Under normal conditions, the concentration of PCT is below < 4 pM in the blood stream. However during sepsis, the level of PCT rises considerably to several thousand folds (> 4 nM) (Maruna et al., 2000; Pfafflin and Schleicher, 2009). Accordingly, the dynamic range of PCT detection reported in the present study is clinically relevant. The EGOFET immunosensor response to the different analyte concentrations ranged between 20 – 80 %. A detection limit of 2.2 pM was extrapolated from the PCT calibration curve. Also, the corresponding relative responses of V_T vs. the analyte concentration in the Fig. 6b (*upper panel*) asserts the pronounced threshold voltage shift upon analyte exposure. The relative responses of the mobility versus the concentration of PCT is depicted in Fig. 6b (*lower panel*) and shows no considerable trend. The relative response of the anti-PCT functionalized sensor to different concentrations of milk powder (non-specific protein) can reach up to 20%. Similar response is obtained from control experiments carried out for devices prepared only with BSA on the surface of the semiconductor. The relative standard deviation of the individual data points of the sensor response measured on three immunosensors from different chips was ranging from 0.01 to 7 %, which is a sound proof for the good inter-device reproducibility.

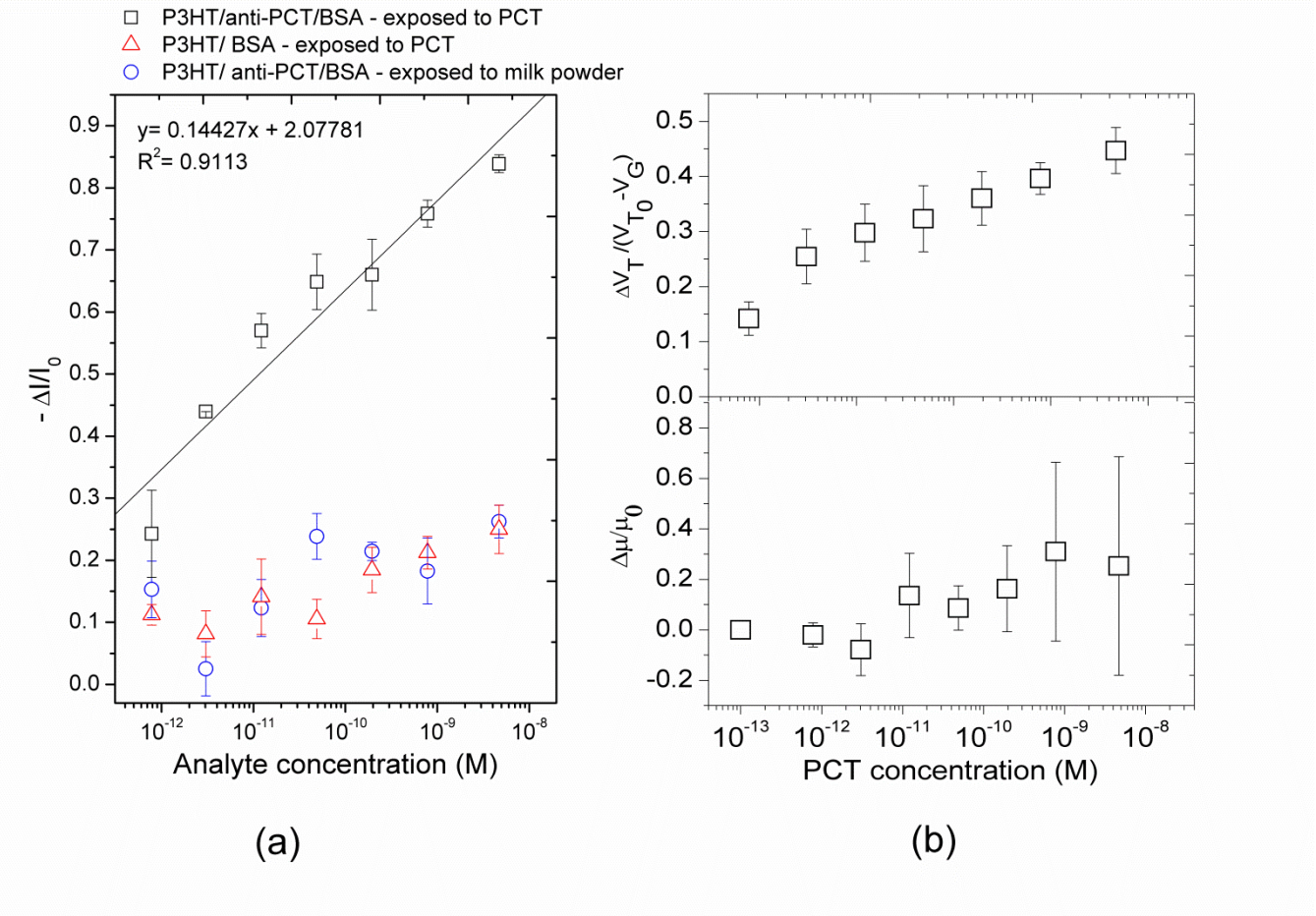

Fig.6. (a) Calibration curve obtained for the EGOFET immunosensor ($\Delta I/I_0$ vs. log [analyte]); the line is a linear fitting of the data in semi-logarithmic scale. (b) Relative response of the threshold voltage (V_T) (upper panel), field-effect mobility (μ) (lower panel) versus the concentration of PCT. Each data point in the calibration curve is an average value of at least three measurements from three immunosensors fabricated on different chip (double column).

4. Conclusions

A label-free immunosensor electronic biosensor for the detection of procalcitonin was developed by immobilizing anti-PCT on the surface of the semiconductor, through direct physical adsorption. The proposed fabrication process is quite simple and has a higher commercial potential due to its simplicity. The developed sensor could selectively detect procalcitonin at sub-picomolar concentrations and within a physiologically relevant range. Regarding the sensing mechanism, noticeable changes in the threshold voltage of the device are observed upon exposure to the analyte, while the semiconductor's mobility did not show any particular trend with the analyte concentration. Such a behavior suggests that the electronic detection is based on charge variations induced in the semiconductor/electrolyte interface upon ligand binding and/or capacitance changes. Further work involves studies for identifying the sensing mechanism and measurements carried out in serum samples.

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