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Label-free C-reactive protein electronic detection with an electrolyte-gated organic field-effect transistor-based immunosensor

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Abstract In this contribution, we propose a label-free immunosensor, based on a novel type of electrolyte-gated field-effect transistor (EGOFET), for ultrasensitive detection of the C-reactive protein (CRP). The recognition layer of the biosensor is fabricated by physical adsorption of the anti-CRP monoclonal antibody onto a poly-3-hexyl thiophene (P3HT) organic semiconductor surface. A supplementary nonionic hydrophilic polymer is used as a blocking agent preventing non-specific interactions and allowing a better orientation of the antibodies immobilized onto the P3HT surface. The whole biomolecule immobilization procedure does not require any pretreatment of the organic semiconductor surface, and the whole functionalization process is completed in less than 30 min. Surface plasmon resonance (SPR) measurements were performed to assess the amount of biomolecules physisorbed onto the P3HT and to evaluate the CRP binding proprieties of the deposited anti-CRP layer. A partial surface coverage of about 23 % of adsorbed antibody molecules was found to most efficiently sense the CRP. The electrical performance of the EGOFET immunosensor was comparable to that of a bare P3HT EGOFET device, and the obtained CRP calibration curve was linear over six orders of magnitude (from 4 pM to 2 μM). The relative standard deviation of the individual calibration points, measured on immunosensors fabricated on different chips, ranged between 1 and 14 %, and a detection limit of 2 pM (220 ng/L) was established. The novel electronic immunosensor is compatible with low-cost fabrication procedures and was successfully employed for the detection of the CRP biomarker in the clinically relevant matrix serum.

Keywords C-reactive protein · Immunosensor · Label-free electronic detection · Electrolyte-gated organic field-effect transistor

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Introduction

In the past few years, electrolyte-gated field-effect transistors (EGOFETs) have been extensively studied for the development of label-free electronic biosensors [1]. So far, this relatively new class of transistors has demonstrated various promising features, such as very low operation voltages [2] and ability to perform sensing in high ionic strength electrolyte solutions, even directly in human serum matrices [3]. The high sensitivity is due to their inherent current gain and selectivity that competes strongly with conventional bioanalytical techniques [4, 5]. In addition to these benefits, EGOFET sensors have a simple layout and can be fabricated on flexible substrates with high throughput and low-cost manufacturing methods.

EGOFETs typically consist of an organic semiconductor channel confined between a source (S) and drain (D) electrode. The conductivity of the channel is controlled by a third top electrode, the gate (G), through an aqueous electrolyte solution. Upon gate biasing, electric double layers (EDLs) are formed at the gate-electrolyte and electrolyte-semiconductor interfaces [6]. The gating system of the EGOFET can therefore be modelled by an equivalent circuit consisting of, at least, two capacitors in series. In an EGOFET biosensor, biological recognition elements (i.e., antibodies, enzymes, etc.) can be introduced into one of these two active interfaces. For a properly tailored sensor, such a bio-layer represents a third and lower capacitance added in series. Being the smallest out of the three, the capacitance of the bio-layer is dominating the gating system capacitance [7]. In such instance, EGOFETs have proven to be extremely sensitive to detecting changes occurring in the capacitance of the bio-layer upon binding of a ligand [8]. Therefore, both charged and neutral ligands can be detected.

As anticipated, both the gate and the organic semiconductor can serve as surfaces for immobilization of biomolecules [4, 8, 9]. The gate functionalization is usually realized through self-assembled monolayers (SAM) of thiolates on gold exploiting the well-known thiol-gold chemistry. The second approach involves placement of the capturing biorecognition layer on the organic semiconductor surface. In this case, different approaches have been proposed, including covalent attachment upon modification of the organic semiconductor surface with proper functional groups [4]. However, biomolecules need to be immobilized onto surfaces with controlled surface coverage and with optimal orientation to retain optimal biological activity and exhibit lowest nonspecific adsorption. As for the organic semiconductor, the deposition of biomolecules on its surface should be carried out without hampering the electronic properties. From a practical application point of view, the process has also to be simple, fast, and low cost.

Among all immobilization methods, physical adsorption is in a practical sense the most straightforward, fast, and inexpensive technique. Biomolecules are adsorbed physically onto solid surfaces via hydrophobic, ionic, and/or Van der Waals interactions. Unlike covalent attachment, adsorption of biomolecules is considered to impact the properties of the semiconductor least. Indeed, covalent attachment of biological recognition elements on the surface of organic semiconductor can be performed only by introducing chemical functional groups on the structure/surface of the organic semiconductor. For instance, polythiophene-based semiconductors have been synthesized with functional groups in the side chains that are then used for anchoring of the biomolecules [10]. However, such an approach can impact dramatically on the electric properties of the semiconductor. Mostly because the functional groups are produced in the bulk of the polymer semiconductor and they are known to detrimentally affect the charge delocalization of conjugated systems with negative effect on their electronic performances. Particularly, the direct adsorption of bovine serum albumin (BSA) protein to the surface of regioregular and regiorandom poly-3-hexyl thiophene (P3HT) was recently studied by Awsiuk et al. [11]. Morphological characterization showed that the deposition of the protein layer could vary depending on the arrangement and packing of the thiophene groups. The underlying semiconductor layer induced changes in protein conformation and degree of denaturation. The highest amount of adsorbed protein was observed in the case of regiorandom P3HT. Protein aggregates and patch-like morphology were found in the case regioregular P3HT, indicating a non-homogeneous coverage. Albers et al. [12] investigated the bioactivity of various types of avidins towards biotinylated antibodies (both anti-h-IgG and anti-C-reactive protein (CRP)) on the surface of spin-coated regioregular P3HT as well as on P3HT bearing carboxylic groups. It was found that certain types of engineered (chimeric) avidins showed enhanced binding levels of the biotinylated antibodies and improved the immobilization efficiency (i.e., yield of binding sites). However, the highest sensitivity (related to the binding constants) for antigen detection was found for direct adsorption of the antibody and especially the $F(ab')_2$ fragment directly onto P3HT. To fill the uncovered areas of the substrate after the deposition of the bio-layer, a nonionic hydrophilic polymer was added. Moreover, the introduction of the polymer served to remove loosely bound protein residues, minimize nonspecific binding, improve the orientation of the bioprobes, and provide a suitable hydrophilic environment for the proteins [13]. The amount of adsorbed protein increased as the hydrophilicity of the semiconductor surface increased. In all cases, the protein exhibited similar specific binding ability regardless of the characteristics of the P3HT surface.

CRP is a plasma protein and a very useful general inflammation and infection biomarker. For instance, the blood plasma concentration of CRP can be indicative of cardiovascular or inflammatory diseases as well as of cancer depending on the concentration in blood serum [3]. According to the American Heart Association and the Centre for Disease Control and Prevention, the risks of cardiovascular diseases are low for CRP concentration below 9 nM, medium from 9 to 27 nM, and high for concentrations greater than 27 nM [14]. For example, high concentrations of CRP (between 0.4 and 1.8 μM) are indicative of bacterial infections and inflammation [15]. Therefore, it is necessary to develop biosensing platforms and analytical techniques capable of detecting CRP in a wide dynamic concentration range (from pM to μM). Conventional bioanalytical techniques that are employed for the quantification of CRP at low concentrations (detection range 45–90 nM) are based on immunoassays using laser nephelometry (light-scattering immunoassays),

fluorescence, and chemiluminescence measurements. These techniques require not only expensive and sophisticated instrumentation but also require sample pretreatment and trained personnel. Last but not least, the tests are also time consuming. Hence, a label-free CRP immunosensor is a welcome alternative to existing clinical tests, eventually yielding a low-cost, fast, and easy-to-handle portable device. To this aim, several label-free biosensing platforms for CRP, including electrochemical sensors [16], capacitors [14, 17], transistors [15, 18], and surface plasmon resonance (SPR) [13], have been reported. However, in most cases, CRP is detected within a narrow concentration range, and multi-step, demanding processes are employed for the immobilization of the bioreceptors.

In this study, we report a low-voltage EGOFET immunosensor for CRP detection using an easy and speedy antibody immobilization strategy, based on physical adsorption of monoclonal anti-CRP antibodies directly onto the organic semiconductor surface. The antibody is directly deposited on the surface of the regioregular P3HT without any pretreatment and post-treated with the hydrophilic polymer. The antibody/polymer layer is used to selectively capture the CRP target analyte, and the biorecognition event is then detected by the EGOFET device through direct electronic transduction without aid of labelled reagents. The EGOFET immunosensor proposed here is also able to detect CRP in a wide dynamic range with high sensitivity. Finally, the fabrication of the immunosensor platform is compatible with low-cost printing technologies and the readout electronics is suitable for miniaturization. The overall technology appears appropriate for manufacturing of a disposable sensor device suitable for point-of-care applications.

Materials and methods

Materials

Si/SiO_2 substrates were acquired from Silicon Materials Inc. (Pittsburgh, PA, USA). The regioregular (RR > 98 %) P3HT organic semiconductor was purchased from Rieke Metals and used after purification by successive Soxhlet extractions with methanol and hexane as reported previously [19]. The anti-CRP monoclonal antibody and the CRP protein from human plasma were obtained from HyTest OY (Turku, Finland) and Scripps Laboratories (San Diego, CA, USA), respectively. The nonionic hydrophilic *N*-[tris(hydroxymethyl)methyl]acrylamide-lipoic acid conjugate (pTHMMAA), used as blocking polymer, was produced according to Albers et al. [20]. The phosphate-buffered saline (PBS) tablets and all the other chemicals and solvents were purchased from Sigma-Aldrich.

EGOFET device fabrication

A schematic picture of the EGOFET device used in this study is given in Fig. 1. For the fabrication of the device, interdigitated gold source (S) and drain (D) electrodes (10-μm channel length and 10-mm channel width) were photo-lithographically patterned on Si/SiO_2 substrates using titanium as gold adhesion promoting layer. The S- and D-patterned substrate was first cleaned by ultra-sonication using solvents of increasing polarity [21], then a purified P3HT chloroform solution (2.5 mg mL^{-1}) was spin-coated on the substrate at 2000 rpm for 30 s. The coated device was subsequently annealed at 75 °C for 1 h on a hot plate. The thickness and refractive indices of the P3HT film were determined with the ʙdual medium SPR methodˆ using a SPR-Navi MP-SPR

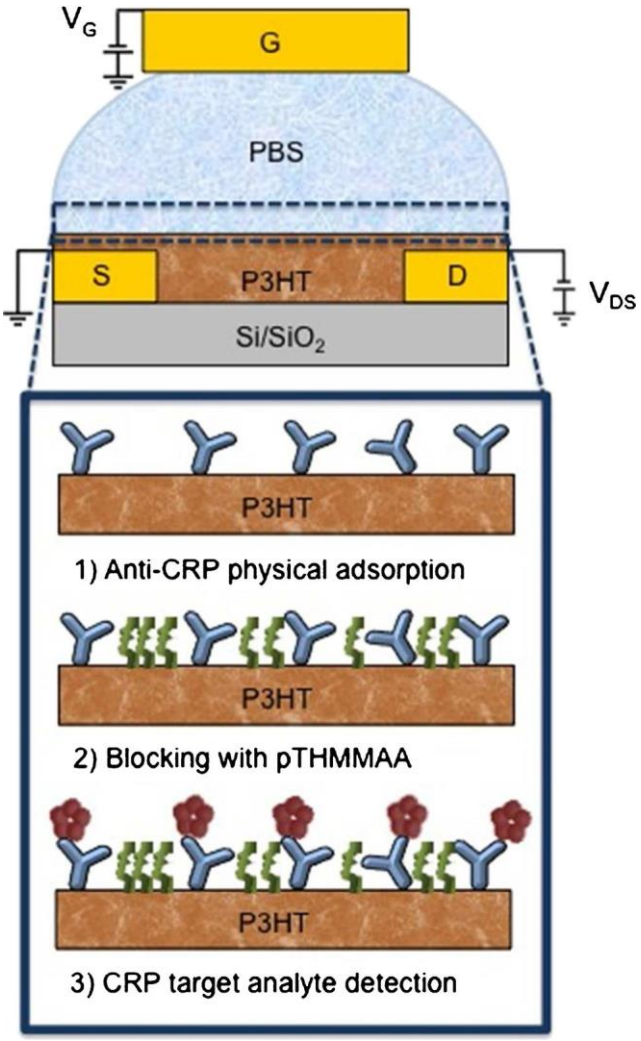

Fig. 1 Schematic structure of the developed EGOFET immunosensor for CRP detection. The anti-CRP antibody is directly adsorbed on the P3HT surface (*panel 1*) and then a pTHMMAA blocking polymer is added to reduce nonspecific interaction (*panel 2*). Finally, the immunosensor is exposed to the CRP target analyte (*panel 3*) and the electrical response is measured

instrument, as reported in a previous publication [12]. The thickness and rms surface roughness (R_q) of the P3HT film were determined by non-contact AFM imaging using a Park Systems XE100 instrument (Suwon, South Korea), as reported in a recent paper [12]. Hydrophobicity of the P3HT surface was evaluated by water contact angle measurements (CA) using a CAM-100 optical contact angle meter (KSV Ltd., Helsinki).

SPR measurements

The anti-CRP monoclonal antibody was deposited on the P3HT surface by physical adsorption. SPR measurements were first performed to assess the concentration of anti-CRP that allows obtaining the highest surface coverage of the P3HT film. A Biacore 3000 instrument (GE Healthcare/Biacore AB, Uppsala, Sweden) was used. For the measurements, cleaned gold SPR chips were spin-coated with P3HT using the same condition used for the Si/SiO_2 substrates. Then, the chips were glued with double-sticking tape in a plastic frame of the Biacore 3000 chip cassette. After recording the baseline in PBS, anti-CRP concentrations ranging from 10 to 125 μg/mL were injected into the flow cell for 15 min. The surface was rinsed with PBS followed by injection of the pTHMMAA blocking polymer solution for 5 min. BSA at a concentration of 0.5 g/L was injected three times to check nonspecific binding of proteins.

SPR measurements allowed an assessment of the binding proprieties of the anti-CRP-functionalized P3HT surface towards the target antigen, CRP. CRP solution of 50 μg/mL was injected for 15 min immediately after BSA. The customary correspondence of 10 resonance units (RU) to 1 ng/cm^2 of adsorbed protein was used for the SPR measurements [22].

Deposition of the anti-CRP on P3HT

For the anti-CRP monoclonal antibody deposition on the P3HT surface of the EGOFET device, 2 μL of the anti-CRP solution in PBS (phosphate 10 mM, KCl 2.7 mM, 137 mM NaCl, pH = 7.4) was placed on the P3HT-covered substrate and different incubation times (from 15 to 30 min) were explored (Fig. 1, panel 1). An antibody concentration of 70 μg/mL was used as this amount allows a good surface coverage as evidenced by SPR measurements performed before. Subsequently, the device was rinsed three times with PBS and incubated with the pTHMMAA blocking polymer solution (0.5 g/L in PBS) for 10 min (Fig. 1, panel 2). After washing three times with PBS, the EGOFET immunosensor was immediately used for the electrical characterization and detection of CRP concentrations (Fig. 1, panel 3).

EGOFET immunosensor electrical characterization and CRP detection

The current–voltage (*I*-*V*) characteristics of the EGOFET immunosensor were measured using a semiconductor parameter analyzer (Agilent 4155C). The electrical measurements were performed in a water vapor-saturated environment employing a gold plate (2×2 mm^2) as gate electrode and a droplet (2 μL) of PBS as electrolyte.

The output characteristics (I_{DS} vs. V_{DS}) were acquired by sweeping the source-drain voltage (V_{DS}) back and forth from 0 to −0.5 V, and the gate bias (V_G) was swept in the same range of voltages with step of −0.1 V. The transfer characteristics (I_{DS} vs. V_G) were measured keeping V_{DS} constant at −0.5 V and sweeping the V_G back and forth from 0 to −0.5 V. The EGOFET immunosensor electrical figures of merit, comprising the field-effect mobility (μ_{FET}), the threshold voltage (V_T), and $I_{on/off}$ ratio, were extracted from the characteristics in the saturation regime [23]. For the μ_{FET} estimation, a gate-channel capacitance per unit area of 3 μF cm^{-2} was used [2].

The CRP detection was performed by measuring the I_{DS}–V_G transfer characteristics in PBS after incubation of the protein solution on the EGOFET immunosensor. Particularly, the CRP solution in PBS was incubated on the EGOFET device for 10 min, followed by rinsing three times with PBS to remove the loosely bound protein fraction. The immunosensor response was evaluated as $\Delta I/I_O = [(I_D \text{ (analyte)} - Io) / Io]$, where I_D (analyte) and Io are the I_D current at $V_G = -0.5$ V upon exposure to the CRP solution and to PBS (baseline), respectively. This procedure allows normalization of the inter-device variation and obtaining a satisfactory reproducibility [24]. The CRP calibration curve was constructed by measuring CRP solutions of increasing protein concentration (from 4 to 2×10^6 pM) on the same EGOFET immunosensor. The detection limit (LOD) was estimated from the calibration curve as the concentration corresponding to the average value (n = 10) of response to the blank sample (PBS buffer with no CRP) plus three times the standard deviation [25]. Further on, the EGOFET immunosensor response at the CRP LOD concentration was compared with that of blank samples. For this purpose, PBS samples (n = 10) with 2 pM CRP concentration were tested. The immunoassay inter-device reproducibility was evaluated by comparing the calibration curves obtained from three EGOFET immunosensors fabricated on different chips.

Spiked human serum samples

Human serum was voluntarily donated by one of the authors. The CRP concentration detected in the sample by a nephelometric assay was below 25 nM (2.5×10^4 pM). The bare serum and two spiked samples with CRP concentrations of 5×10^4 and 2.5×10^6 pM were tested with the EGOFET

immunosensor. For the assay, the samples were diluted 1:100 *v*/*v* in PBS, 10 mM, pH = 7.4 and six replicates were measured.

Results

Characterization of the P3HT film

The spin-coated P3HT film was characterized by MP-SPR, AFM, and CA measurements. On gold surfaces, MP-SPR yielded a film thickness of 18 ± 1 nm and a refractive index of 1.66 ± 0.02. The literature refractive index of the P3HT was 1.58 ± 0.02 [12]. AFM analysis of scratches in the film revealed a P3HT film thickness of 25 ± 2 nm and an *rms* surface roughness (R_q) of 0.83 ± 0.08 nm. An example of a P3HT coating is shown in Fig. 2b, with a total area surface roughness of 1.3 nm. The film was composed of granular domains, in agreement with earlier data published on P3HT morphology [26]. Furthermore, good film uniformity was obtained and only few voids and nano-particulates were visible on the surface.

With respect to the chemical structure of P3HT (Fig. 2a), the hydrophobicity of the spin-coated layer was expected to be fairly high, and this was confirmed by water contact angle measurements (103 ± 3°, *n* = 15). The long alkyl chains afford strong direct adsorption of biomolecules onto the organic semiconductor surface. Physical adsorption of proteins on the P3HT surface was earlier demonstrated for chimeric avidins [12] and bovine serum albumin [11] with a good level of surface coverage and uniformity. However, a fairly high level of denaturation and unfavorable orientation can occur according to the biomolecule structure as demonstrated by Awsiuk et al. [11]. As demonstrated in an earlier study, the affinity constant of adsorbed anti-hIgG remains yet relatively high [12].

SPR measurements

The amount of anti-CRP physically adsorbed on the P3HT surface was determined by SPR measurements using a range of antibody concentrations. As antibodies were allowed to physisorb on the surface, there was an abrupt increase in SPR intensity. This increase was related to the amount of antibodies in solution. As shown in panel 1 of Fig. 3a, a surface saturation was reached at anti-CRP concentrations above 50 µg/mL with a surface coverage of 200 ± 30 ng/cm^2. The P3HT-functionalized surface was post-treated with a pTHMMAA blocking polymer in order to reduce the non-specific binding and allow a better orientation of the

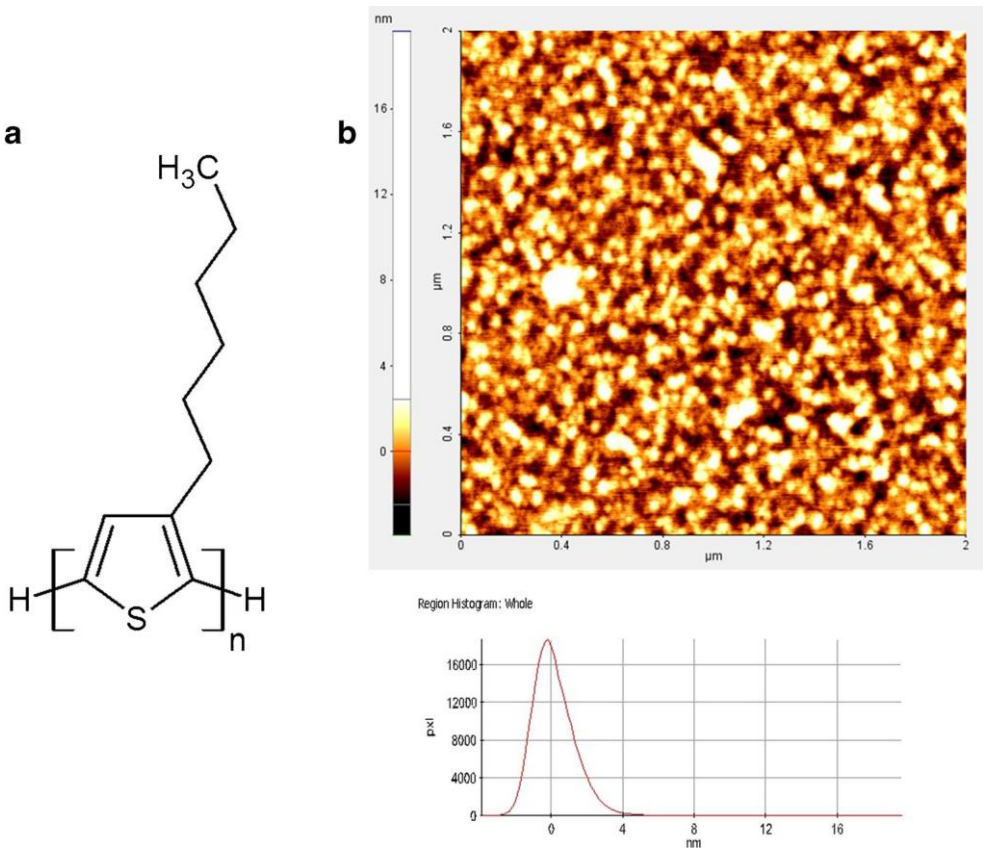

Fig. 2 a Chemical structure of the P3HT organic semiconductor. b AFM non-contact image of the P3HT layer deposited on a gold substrate by spin-coating

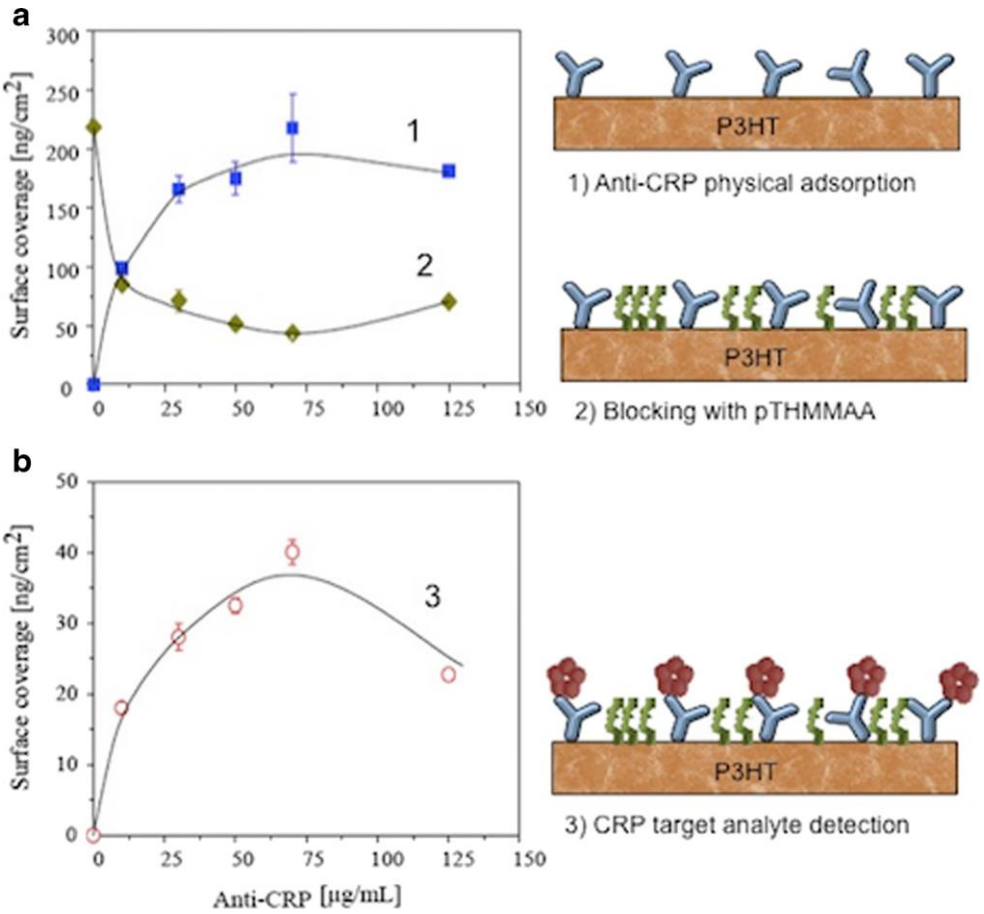

Fig. 3 SPR measurements performed on P3HT spin-coated substrates using different anti-CRP concentrations (*panel 1*) and blocking the surface with pTHMMAA polymer (*panel 2*). a The amount of the antibodies physisorbed on P3HT layer for different anti-CRP concentrations is reported (*blue squares*) along with the measured quantity of the pTHMMAA polymer adsorbed on P3HT upon binding of the anti-CRP (*green diamonds*). b Amount of the CRP protein captured by the anti-CRP layer deposited on the P3HT surface at different concentrations (from 10 to 125 μg/mL) upon injection of a CRP solution of 50 μg/mL. *Error bars* are from four repeated measurements. The *lines* are mere guide for eye and the data were not modelled

antibodies on the organic semiconductor surface (panel 2 of Fig. 3a). The pTHMMAA polymer holds a hydrophobic lipoate group and can be physisorbed on the P3HT surface and intercalated between the antibody molecules, thanks to hydrophobic interactions with the organic semiconductor [12]. Specifically, the amount of pTHMMAA adsorbed on P3HT is strictly related to the anti-CRP concentration and the higher the anti-CRP coverage is, the lower the amount of pTHMMAA adsorbed is (panel 2 of Fig. 3a). However, at very high anti-CRP concentration, pTHMMAA might remove loose antibodies from the surface and more pTHMMAA could be adsorbed. The amount of nonspecific binding of BSA was considerably reduced by the polymer as also previously shown [12, 13, 20]. The pTHMMAA polymer can also provide a better orientation of the anti-CRP molecules on the P3HT surface as discussed later in the text.

The CRP binding to the physisorbed anti-CRP layer post-treated with pTHMMAA was also evaluated. As reported in Fig. 3b, the highest CRP surface coverage (40 ± 2 ng/cm^2) was obtained using an anti-CRP concentration of 50–70 μg/mL. This was in the same concentration range as observed for antibody fragments and pTHMMAA covalently linked to gold [13]. At this concentration, about 23 % of the adsorbed antibody molecules were bonded to the antigen and there was no improvement in the CRP binding by increasing the antibody concentration. On the other hand, lower amount of antigen was bound if the antibody concentration was too high. Such an effect is most probably due to steric hindrance caused by the overlapping of the biorecognition elements. Besides, no CRP binding was observed by covering the P3HT surface with only the pTHMMAA polymer layer.

Electrical and analytical features of the EGOFET immunosensor

In Fig. 4, the I_{DS}–V_{DS} output curves representative of the EGOFET immunosensor fabricated by exposure to 2 μL of 70 μg/mL of anti-CRP for 20 min and subsequent post-treatment with the pTHMMAA blocking polymer are reported. A good field-effect current modulation was achieved with a negligible level of hysteresis, as demonstrated by the back sweep current. The estimated μ_{FET} was in the 10^{-2}-cm^2 V^{-1} s^{-1} range, while the $I_{on/off}$ ratio and V_T were 10^2 and −0.07 V, respectively. These values were comparable with those obtained with a bare P3HT EGOFET device, thus indicating that the properties of the organic semiconductor were not affected by the physical adsorption of biomolecules and pTHMMAA on the surface. A comparison with the

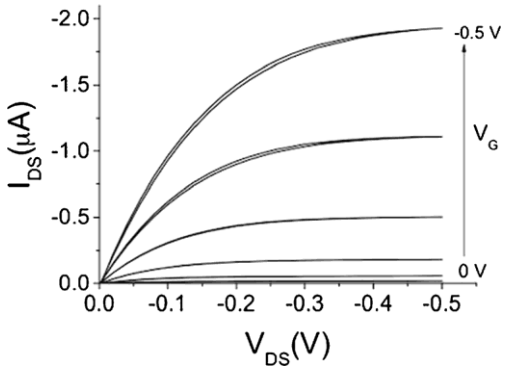

Fig. 4 Current–voltage (I–V) output characteristics (I_{DS} vs. V_{DS}) acquired for the EGOFET immunosensor fabricated by depositing 70 μg/mL of anti-CRP and blocking the surface with the pTHMMAA polymer. The source-drain voltage (V_{DS}) was swept from 0 to −0.5 V back and forth, while the gate voltage (V_G) was swept from 0 to −0.5 V with steps of −0.1 V

performances of EGOFET sensors for CRP whose P3HT semiconductor surface was modified with a hydrophilic coating, and the biorecognition elements anchored by covalent attachment [4] indicate that the electrical features of our EGOFET immunosensor fabricated by physical absorption of anti-CRP are much better especially in terms of μ_{FET} and hysteresis. This is likely because physical adsorption is a very mild deposition process that does not require any pre-modification of the P3HT layer, and during the biomolecule deposition, the organic semiconductor is exposed to an aqueous environment for a limited time frame (20 min vs. more than 2 h for the covalent attachment).

The EGOFET immunosensor response towards the CRP was reliably evaluated by measuring the device transfer characteristics (I_{DS} vs. V_G at V_{DS} constant) before and after exposure to the target analyte. As reported in Fig. 5a, reduction in the I_{DS} current was measured by exposing the EGOFET immunosensor to CRP solutions of increasing concentration, while a negligible shift in the threshold voltage was observed. The field-effect current reduction was most probably due to a

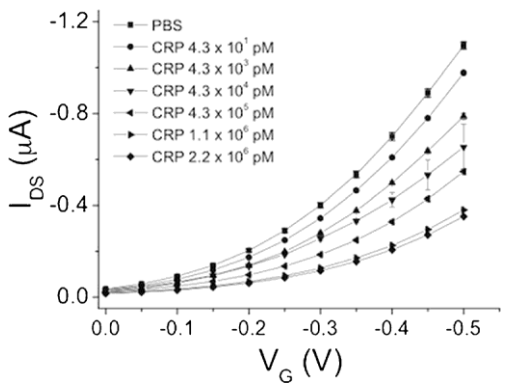

Fig. 5 Transfer characteristics (I_{DS} vs. V_G) measured for an EGOFET immunosensor exposed to increasing concentration of CRP (from 4 to 2×10^6 pM)

change in the capacitance of the gating system upon binding of the CRP to the antibodies [8].

The incubation time of the anti-CRP solution on the P3HT surface of EGOFET devices was optimized by comparing the CRP calibration curves obtained using different incubation times (from 15 to 30 min). The best analytical performance, in terms of sensitivity and dynamic range, were obtained by incubating the anti-CRP solution for 20 min and no improvement was observed at longer incubation time (see Electronic Supplementary Material (ESM) Fig. S1).

The calibration curve obtained for the EGOFET immunosensor, fabricated incubating the anti-CRP solution for 20 min, upon exposure to CRP solutions is reported in Fig. 6. As shown, a wide dynamic range (from 4 pM to 2 μM) was spanned and a detection limit of 2 pM (220 ng/L) was extrapolated. This was experimentally confirmed measuring (10 replicates) the response at 2 pM that was found to be 10 times higher than that of blank samples. The relative standard deviation of the individual calibration points, measured on three immunosensors fabricated on different chips, ranged between 1 and 14 %, indicating a good inter-device reproducibility. Furthermore, the response of EGOFET devices fabricated depositing on the P3HT surface biorecognition elements (anti-IgG) non-selective for CRP or only the pTHMMAA blocking polymer was negligible, even at high CRP concentrations (Fig. 6). In addition, the CRP amount detected with the EGOFET immunosensor for the bare serum and the two spiked samples (with CRP concentrations of 5×10^4 and 2.5×10^6 pM) was $2.1\times 10^4 \pm 1.2\times 10^3$, $4.7\times 10^4 \pm 3.2\times 10^3$, and $2.9\times 10^6 \pm 1.8\times 10^5$ pM,

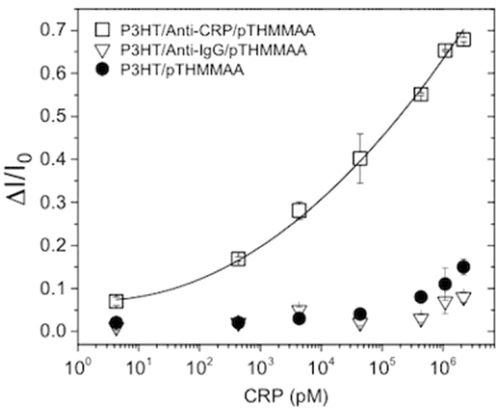

Fig. 6 The *square symbols* comprise the CRP calibration curve obtained for the EGOFET immunosensor, fabricated with the anti-CRP physisorbed on P3HT, and treated with pTHMMAA. Each data point is the average value obtained by measuring the CRP concentration on three immunosensors fabricated on different chips. The *solid line* is the second-order polynomial fitting in log [CRP]. The *triangle symbols* show the CRP calibration curve obtained with an EGOFET immunosensor fabricated with a CRP non-selective antibody (anti-IgG) physisorbed on P3HT and treated with pTHMMAA. The *circle symbols* are the CRP calibration curve obtained for an EGOFET device with no anti-CRP physisorbed on P3HT and treated only with the pTHMMAA polymer

respectively. While the determined relative standard deviation (RSD %) was 5.7, 6.8, and 6.2 %, respectively (see ESM Table S1).

Discussion

EGOFET devices are a very attractive class of transducers for the development of label-free biosensors as they operate at very low voltages (below 1 V) using gating media such as water or buffer solution, which are highly compatible with biorecognition processes and biomolecules in general. Furthermore, beside the possibility to realize these devices with printing technologies at low cost using flexible and disposable formats, they do not require signal amplification and an extremely high sensitivity can be achieved. Two different EGOFET biosensor geometries have been proposed so far for biosensor fabrication. In the first, biorecognition elements are anchored on the gate electrode [27], while in the second, they are placed directly on the organic semiconductor surface. In the latter case, the most exploited approach is the covalent attachment of biomolecules to proper functional groups present on the organic semiconductor surface [4, 28]. Although such a method allows a stable integration of the biomolecules in the EGOFET device, the major drawbacks are the need to modify the organic semiconductor surface with proper functional groups with the risk of denaturating the biomolecules during the anchoring step.

In the present study, the direct physical adsorption of anti-CRP recognition elements on P3HT was explored in order to develop a highly sensitive label-free EGOFET immunosensor for CRP detection (Fig. 1). The procedure to deposit the biomolecules on the organic semiconductor surface is very simple. The whole process occurs in a single step and the biomolecule immobilization is completed in only 30 min, while for the covalent attachment two or three steps and more than 2 h are needed [4, 28].

Particularly, the SPR measurements depicted in Fig. 3 demonstrate that the amount of anti-CRP physisorbed on the P3HT surface is high and in good agreement to what is reported by Albers et al. for direct adsorption of anti-h-IgG antibodies on a bare P3HT surface [12]. Furthermore, thanks to the aid of the pTHMMAA blocking polymer, it was possible to limit the nonspecific interactions and to assist the orientation of the antibodies on the organic semiconductor surface. The antibody molecules can be roughly modelled as ellipsoids with a diameter of about 15 nm and a thickness of about 3 nm. Therefore, if a dense packing of antibodies with a molecular weight of 160 kDa is considered, an end-on (upright) orientation of the biomolecules would give a surface coverage of 750 ng/cm^2, while a surface coverage of 150 ng/cm^2 is expected in the case they are oriented in a side-on (lying flat) manner. Considering that in the case of the present study the highest amount of anti-CRP on the P3HT surface was estimated to be around 200 ± 30 ng/cm^2, a side-on orientation of the biomolecules is inferred. Using such an immobilization procedure, more than the 23 % of the deposited antibodies were capable to bind the target analyte. Usually, only 10 % of physisorbed antibodies remain active [12, 29]. The higher activity of the antibodies is ascribed to the pTHMMAA polymer being intercalated between the antibodies.

Interestingly, the electrical features of the EGOFET immunosensor were comparable with those of a bare P3HT EGOFET device (Fig. 4) and a clear response was measured by exposing the biosensor to increasing concentrations of the target CRP analyte (Fig. 5). In addition, the results reported in Fig. 6 show that the EGOFET immunosensor was able to detect CRP concentrations in a wide dynamic range (six orders of magnitude), with good selectivity and inter-assay reproducibility. Although several methods have been reported for CRP detection, the analytical features of the proposed label-free electronic immunosensor were better than most of the strategies found in the literature [30], especially in terms of dynamic range and detection limit. The estimated detection limit of the EGOFET immunosensor was, in fact, 220 ng/L (2 pM) while most of the optical or electronic immunosensors show a value in the range of 0.1–20 mg/L. Nevertheless, there are a few studies where a better detection limit is reached. For instance, a detection limit of 25 ng/L was achieved with a capacitive immunosensor based on gold-interdigitated electrodes [17], while 800 pg/L (6 fM) was the detection limit reported for a chemiluminescent immunoassay for CRP based on electrospun TiO_2 nanofiber [31]. However, the majority of these analytical approaches are not able to detect CRP in a wide dynamic range and over the whole concentration range of clinical interest (from ng/L to mg/L). In addition, they involve time-consuming immobilization procedure of the biorecognition elements, often require labelled reagents to measure a sensitive signal, and sophisticated tools are needed to record the signal. Furthermore, the results obtained for the detection of CRP in spiked serum samples demonstrate that developed EGOFET immunosensor was suitable for the analysis of real samples.

The developed EGOFET immunosensor is label-free, compatible with low-cost fabrication procedures, and allows the reliable and highly sensitive detection of CRP in a wide dynamic range and in real samples such as human serum. Besides, the immunosensor fabrication procedure is fully compatible with printing technologies employed for electronic device fabrication [6] and the biomolecules can be eventually physically adsorbed on P3HT using printing process as well [32].

Conclusions

A label-free electronic immunosensor for CRP detection was successfully demonstrated. The advantages of this biosensor over other analytical approach include the label-free nature, the direct signal readout in the form of electrical signal, and the possibility to fabricate the whole device with low-cost printing technologies even on a disposable substrate such as plastic or paper. Furthermore, the EGOFET immunosensor format is compatible with the development of label-free electronic arrays where multiple target analytes can be measured simultaneously in clinical relevant fluids such as serum, saliva, or tear fluid.

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Compliance with ethical standards The study has been approved by the ethics committee of the University of Bari ʙAldo Moroˆ and has been performed in accordance with the ethical standards.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Cramer T, Campana A, Leonardi F, Casalini S, Kyndiah A, Murgia M, et al. Water-gated organic field effect transistors—opportunities for biochemical sensing and extracellular signal transduction. J Mater Chem B. 2013;1(31):3728–41.
2. Kergoat L, Herlogsson L, Braga D, Piro B, Pham M-C, Crispin X, et al. A water-gate organic field-effect transistor. Adv Mater. 2010;22(23):2565–9.
3. Palazzo G, De Tullio D, Magliulo M, Mallardi A, Intranuovo F, Mulla MY, et al. Detection beyond Debye's length with an electrolyte-gated organic field-effect transistor. Adv Mater. 2015;27:911–6.
4. Magliulo M, Mallardi A, Mulla MY, Cotrone S, Pistillo BR, Favia P, et al. Electrolyte-gated organic field-effect transistor sensors based on supported biotinylated phospholipid bilayer. Adv Mater. 2013;25(14):2090–4.
5. Magliulo M, Mallardi A, Gristina R, Ridi F, Sabbatini L, Cioffi N, et al. Part per trillion label-free electronic bioanalytical detection. Anal Chem. 2013;85(8):3849–57.
6. Kim SH, Hong K, Xie W, Lee KH, Zhang S, Lodge TP, et al. Electrolyte-gated transistors for organic and printed electronics. Adv Mater. 2013;25(13):1822–46.
7. Manoli K, Magliulo M, Mulla MY, Sing M, Sabbatini L, Palazzo G, et al. Printable bioelectronics to investigate functional biological interfaces. Angew Chem Int Ed. 2015;54:12562.
8. Mulla MY, Tuccori E, Magliulo M, Lattanzi G, Palazzo G, Persaud K, et al. Capacitance-modulated transistor detects odorant binding protein chiral interactions. Nat Commun. 2015;6:6010.
9. Casalini S, Leonardi F, Cramer T, Biscarini F. Organic field-effect transistor for label-free dopamine sensing. Org Electron. 2013;14(1):156–63.
10. Suspène C, Piro B, Reisberg S, Pham M-C, Toss H, Berggren M, et al. Copolythiophene-based water-gated organic field-effect transistors for biosensing. J Mater Chem B. 2013;1(15):2090.
11. Awsiuk K, Budkowski A, Marzec MM, Petrou P, Rysz J, Bernasik A. Effects of polythiophene surface structure on adsorption and conformation of bovine serum albumin: a multivariate and multitechnique study. Langmuir. 2014;30(46):13925–33.
12. Albers WM, Pelto JM, Suspène C, Määttä JA, Yassar A, Hytönen VP, et al. Structural and functional characteristics of chimeric avidins physically adsorbed onto functionalized polythiophene thin films. ACS Appl Mater Interfaces. 2012;4(8):4067–77.
13. Vikholm-Lundin I, Albers WM. Site-directed immobilisation of antibody fragments for detection of C-reactive protein. Biosens Bioelectron. 2006;21(7):1141–8.
14. Quershi A, Gurbuz Y, Kang WP, Davidson JL. A novel interdigitated capacitor based biosensor for detection of cardiovascular risk marker. Biosens Bioelectron. 2009;25(4):877–82.
15. Justino CIL, Freitas AC, Amaral JP, Rocha-Santos TAP, Cardoso S, Duarte AC. Disposable immunosensors for C-reactive protein based on carbon nanotubes field effect transistors. Talanta. 2013;108:165–70.
16. Gupta RK, Periyakaruppan A, Meyyappan M, Koehne JE. Label-free detection of C-reactive protein using a carbon nanofiber based biosensor. Biosens Bioelectron. 2014;59:112–9.
17. Qureshi A, Niazi JH, Kallempudi S, Gurbuz Y. Label-free capacitive biosensor for sensitive detection of multiple biomarkers using gold interdigitated capacitor arrays. Biosens Bioelectron. 2010;25(10):2318–23.
18. Kwon SM, Kang GB, Kim YT, Kim YH, Ju BK. In-situ detection of C-reactive protein using silicon nanowire field effect transistor. J Nanosci Nanotechnol. 2011;11(2):1511–4.
19. Urien M, Wantz G, Cloutet E, Hirsch L, Tardy P, Vignau L, et al. Field-effect transistors based on poly(3-hexylthiophene): effect of impurities. Org Electron. 2007;8(6):727–34.
20. Albers WM, Munter T, Laaksonen P, Vikholm-Lundin I. Improved functionality of antibody-colloidal gold conjugates with the aid of lipoamide-grafted N-[tris(hydroxymethyl)methyl]acrylamide polymers. J Colloid Interface Sci. 2010;348:1–8.
21. Dinelli F, Moulin JF, Loi MA, Da Como E, Massi M, Murgia M, et al. Effects of surface chemical composition on the early growth stages of alpha-sexithienyl films on silicon oxide substrates. J Phys Chem B. 2006;110(1):258–63.
22. Biacore 3000 instrument manual. Biacore AB, Uppsala, Sweden, 1999.
23. Braga D, Horowitz G. High-performance organic field-effect transistors. Adv Mater. 2009;21(14):1473–86.
24. Ishikawa FN, Curreli M, Chang H-K, Chen P-C, Zhang R, Cote RJ, et al. A calibration method for nanowire biosensors to suppress device-to-device variation. ACS Nano. 2009;3(12):3969–76.
25. Torsi L, Magliulo M, Manoli K, Palazzo G. Organic field-effect transistor sensors: a tutorial review. Chem Soc Rev. 2013;42:8612.
26. Cho S, Lee K, Yuen J, Wang G, Moses D, Heeger AJ, et al. Thermal annealing-induced enhancement of the field-effect mobility of regioregular poly(3-hexylthiophene) films. J Appl Phys. 2006;100(11):114503.
27. Casalini S, Dumitru AC, Leonardi F, Bortolotti CA, Herruzo ET, Campana A, et al. Multiscale sensing of antibody-antigen

interactions by organic transistors and single-molecule force spectroscopy. ACS Nano. 2015;9(5):5051–62.
28. Mulla MY, Seshadri P, Torsi L, Manoli K, Mallardi A, Ditaranto N, et al. UV crosslinked poly(acrylic acid): a simple method to biofunctionalize electrolyte-gated OFET biosensors. J Mater Chem B. 2015;3:5049–57.
29. Harris LJ, Larson SB, Hasel KW, McPherson A. Refined structure of an intact IgG2a monoclonal antibody. Biochemistry. 1997;36: 1581–97.
30. Algarra M, Gomes D, Esteves da Silva JCG. Current analytical strategies for C-reactive protein quantification in blood. Clin Chim Acta. 2013;415:1–9.
31. Lee WS, Sunkara V, Han JR, Park YS, Cho YK. Electrospun TiO2 nanofiber integrated lab-on-a-disc for ultrasensitive protein detection from whole blood. Lab Chip. 2015;15(2):478–85.
32. Aleeva Y, Pignataro B. Recent advances in upscalable wet methods and ink formulations for printed electronics. J Mater Chem C. 2014;2(32):6436.