

Enzymatic sensing with laccase-functionalized textile organic Biosensors

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ABSTRACT

Herein we present a textile wearable electrochemical transistor by functionalizing a single cotton yarn with semiconducting polymer. The organic electrochemical transistor (OECT), which is low cost and completely integrated e-textile, is decorated by adsorption of the fungal laccase POXA1b, and is used as biosensor for the direct detection of Tyrosine (L-Tyr) without the use of electron mediators. The detection of Tyr in real-case scenario such as human physiological fluids would own a paramount importance in noninvasive analysis of the patient's condition, monitoring and preventing several pathologies. To assess the reaction progression, the redox process is studied by UV-visible absorption with test reference molecule of 2,20-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS): the results confirmed that the oxidation reaction is driven by the presence of laccase enzyme and direct electron transfer occurred. The modulation of the signal response and the kinetic of the signal is used to detect Tyr molecule in aqueous solution and the role of the enzyme adsorption on the textile is analyzed. A kinetic analysis of the characteristic modulation times of the sensing curves, confirm the sensing properties of the textile device. The textile-based biosensor is demonstrated to monitor human health biomarkers through wearable applications in a non-invasive way, finding potential application in sport, healthcare and working safety.

1. Introduction

There is an increasing interest in combining non-conventional and still commercially available materials into sensing devices [1]. As for an example, conventional textile materials are being redesigned and re-engineered to bring innovative functions with major applications in wearable electronics, which represents a high challenge in research. The advantages of using textile substrates as the backbone for new devices are manifold: from comfort and low cost, to enabling physiological monitoring and measurements that are not otherwise feasible with traditional substrates [2,3].

Different materials have been tested as smart textile materials to replace or improve conventional sensing functions: conducting polymers, such as Poly(3,4-ethylenedioxythiophene): poly-styrenesulfonic acid (PEDOT:PSS), have been successfully employed as the active layer of OECTs and have shown excellent results because of their good conductivity, high stability and biocompatibility [4]. Thanks to their low cost, non-invasive and easy to read features, organic electrochemical transistors (OECTs) have been widely exploited in the sensing of simple analytes such as hydrogen peroxide [5], ions [6], and have been challenged with complex bio-structures such as micelle [7], liposomes [8], DNA [6] and pigments [9].

In addition, organic biosensors based on conducting polymer have been integrated on cloths, yielding to efficient e-textile organic devices [10,12]. The output characteristics of textile-OECT have been demonstrated for the first time using a gel electrolyte for ionic exchange [13]. Moreover, textile based OECT has been demonstrated in aqueous solutions, for example in monitoring saline concentration in human sweat [14], or in the detection of adrenaline neurotransmitters in biological mixtures [15]. High sensitivity, simple and handy structure of OECTs are key features for the ongoing interest in developing efficient analytical systems that provide critical output for the diagnosis and treatment of diseases. Despite of their strengths, however OECTs are limited by poor selectivity and responsivity to different biomolecules. Recently, different approaches have been tested to overcome these limitations, including polymeric membranes that, on surrounding the OECT active layer, may selectively adsorb and detect potassium ions [16], or biochemical functionalization through lipid membranes which discriminates between monovalent and divalent ions in a sensor [17], or kinetic models for the analysis of diffusion coefficient of different ions [18]. One of the most employed approaches to increase biosensor selectivity involves the use of enzymes which catalyze specific reactions: immobilization of glucose oxidase in the polymer matrix has been recently proposed for glucose detection or lactate oxidase for lactate detection [19,20]. Even though promising, such approaches are still limited by the use of mediators to promote the electron transfer and the type of electrode material that can be utilized, which may degrade the applicability of the electrode design. In this perspective our research is focused on expanding the sensing ability of cotton based OECTs sensor to enzyme-catalyzed reactions by phenol oxidase enzyme laccase. Laccase has a relatively broad substrate spectrum and is a thermostable and environmentally friendly catalyst. Recently, that class of enzymes has been used to develop biosensors able to recognize a large number of phenolic substrates. One of the most promising advantage of such enzymes relies on the possibility to have a mediatorless electron transfer that significantly improve the sensor design, diminishing the potential losses by the mediated component and increasing the sensitivity of the devices [21].

Here, we present a set-up based on the PEDOT:PSS cotton fiber decorated by the recombinant high redox potential laccase POXA1b (p0.650 V) from *Pleurotus ostreatus* (Jacq.: Fr.) Kummer (type: Florida) (rPOXA1b) [22,23]. The described laccase decorated biosensor is completely wearable and textile-based. In what follows, the device is first tested with ABTS molecule to verify the effective role of the enzyme in the sensing process; then we characterize its sensitivity against several saline solutions containing Tyrosine (Tyr) at increasing concentrations. The detection of L-Tyrosine is a natural phenolic amino acid, whose role as precursor in biosynthetic pathways of neurotransmitters, hormones, alkaloids, natural phenols and pigments is widely reported [24,25]. Unbalanced Tyr concentration in humans can promote diseases like hyperthyroidism, hypochondria, dementia, hypothyroidism or Parkinson's [26-28]. In particular the physiological concentration range for Tyr in blood is around tens to hundreds of mg/mL [29], rather in other biological fluids its content can be very variable reaching very low levels in sweat and down to tens of ng/mL [30,31]. Finally, kinetic and time constants of the measurements are analyzed to understand the dynamic of sensing process.

2. Materials and methods

2.1. Fabrication of PEDOT:PSS cotton sensor and measurements

The OECTs are fabricated on cotton fibers (the yarn) by a simple soaking process, as reported in Ref. [14]. Briefly the yarn is immersed into an aqueous solution of PEDOT:PSS (CleviosPH500, Starck GmbH) with addition of ethylene glycol (15%) and dodecyl benzene sulfonic acid (DBSA) surfactant (1%) for 5 min, followed by baking on a hot plate at 130 °C for 1 h. The cotton-OECT is integrated in electrical circuit as schematically reported in Fig. 1e.

To test the sensing performance of the proposed OECT, a 200 μ L aliquot of solution is placed on the yarn between the source and drain contacts, allowing the liquid to reach the gate electrode. The gate electrode

consists of a thin platinum wire, set at 3 mm distance from the functionalized cotton yarn. OECT performance is evaluated by measuring I_{ds} vs time, for a fixed $V_{ds} = -0.05$ V and pulsing V_{gs} in the 0-1 V range using 0.2 V steps. Transfer characteristics are expressed as current modulation $(I_{ds} - I_{ds0})/I_{ds0}$ vs V_{gs} . Current values are determined as the steady state values of the time varying current. The application of V_{ds} induces a drift of the holes along the PEDOT:PSS channel, generating a drain-source current (I_{ds}). Upon application of a positive gate voltage (V_{gs}), cations (Y^+) from the electrolyte enter the PEDOT:PSS channel causing its de-doping according to equation (1):



In the “de-doping process” [32] a decrease of the module of drain current $|I_{ds}|$ (i.e. less holes available for conduction) is induced as a consequence of cation incorporation into the PEDOT:PSS backbone and reduction of the oxidized PEDOT⁺ to PEDOT⁰. This process is reversible and when V_{gs} is switched off ($V_{gs} = 0$ V), ions diffuse from PEDOT:PSS to the electrolyte, increasing the number of conducting holes and, consequently, $|I_{ds}|$. Such a process is referred to as “doping” [33].

2.2. Enzyme immobilization and enzymatic assay

Recombinant POXA1b (rPOXA1b) laccase (EC 1.10.3.2) from *P. ostreatus* is provided by Biopox srl (Italy) and immobilized by surface adsorption onto the PEDOT-modified cotton fiber. Functionalization is performed after fixation of the conductive polymer to the yarns. To do so, a 2 cm dry PEDOT:PSS cotton fiber is immersed for 1 h in a solution of 0.1 M sodium citrate buffer containing 30 Uml⁻¹ enzyme at its isoelectric point (pH 6.7), afterwards the fiber is washed with the same buffer and stored at 4 °C until use. Standard rPOXA1b activity is assayed at 25 °C by monitoring the oxidation of ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) at 420 nm ($\epsilon_{420} = 36 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) as reported by Lettera et al. [22]. The assay mixture contains 2 mM ABTS in 0.1 M sodium citrate buffer (pH 3.0). Enzyme activity is expressed in international units (IU). To determine the efficiency of immobilization the apparent Michaelis-Menten constants K_M values have been estimated for laccases immobilized on the PEDOT:PSS cotton fibre using the software GraphPad Prism (GraphPad Software, USA; <http://www.graphpad.com/>) operated over a wide range of substrate concentrations (0.05-3 mM) through the following equation (2):

$$V = \frac{V_{max}[S]}{K_M + [S]} \quad (2)$$

where V is the velocity of the reaction, V_{max} is the maximum velocity of the reaction and $[S]$ is the concentration of the substrate.

2.3. L-Aminoacid sensing

The response of differently functionalized OECTs was tested against L-Tyr (Sigma Aldrich, Milan IT), in the same standardized conditions of ABTS assay. Indeed the measurement chamber was filled with 0.1 M sodium citrate buffer (pH 3.0) and the concentration of Tyr was varied in the 10^{-8} - 10^{-2} M range. Tests were performed on samples (i) with and (ii) without the rPOXA1b laccase enzyme. Measurements with the enzyme, in turn, were performed with either (i) the enzyme uniformly dissolved in solution, or (ii) the enzyme immobilized to the yarn surface.

2.4. Statistical analysis

All the data are reported as statistical mean plus and minus the standard deviation (SD). Details on error estimations and pair wise comparisons are reported in [electronic supplementary information](#).

3. Result and discussion

The results are organized as follows. First, (i) we demonstrate the PEDOT:PSS fiber device in presence of Laccase and verify its performance on ABTS as test system. The device is tested as a function of the position of the enzyme in the system (that is, dissolved in the electrolyte or immobilized to the fiber surface), results are grouped in [Figs. 1 and 2](#). This permitted to identify the optimal configuration of the system, that is, when the fiber is decorated with the enzyme. Then, (ii) we compare the performances of the device, with and without the laccase enzyme, to detect tyrosine in solutions, varying the concentrations over a significant range. In doing so, we demonstrate that the enzyme integrated system is capable of detecting selectively tyrosine in very low abundance ranges, down to 10^{-8} M, with good sensitivity, high resolution and repeatability. Differently, the PEDOT:PSS device without the laccase enzyme shows limiting sensing capability. Results are reported in [Figs. 3 and 4](#). Finally, (iii) we analyse the time evolution of the system to find the time constants at different concentrations and operation regimes. The time constants indicate the time that the system necessitates to reach the 90% of the steady state value of the I_{ds} current (the output). Results in [Fig. 5](#) show that the time constants are proportional to the concentration and, perhaps more interestingly, that there is an optimal value of V_{gs} voltage for which the system responds more promptly to the input.

3.1. PEDOT:PSS cotton sensor fabrication and enzyme immobilization

The functionalization of cotton yarn through PEDOT:PSS is chosen as eligible method for the fabrication of wearable sensors because the process does not affect the mechanical properties of the fiber (e.g. flexibility, breaking point). A natural cotton thread is shown in [Fig.1a](#) without functionalization. The brighter areas in the image are due to charging effects caused by the high resistivity of the cotton fibers. [Fig. 1b](#) shows a FE-SEM image of a cotton thread after functionalization, covered with a thin layer of PEDOT:PSS. The darker colour of the fibers is related to the increased conductivity, that reduces the charging effects. The polymer layer is uniformly distributed over the single yarn at a nanometer scale ([Fig. 1c](#)), with an estimated thickness of about 100 nm ([Fig. 1b](#)). Noteworthy no charge effect are observed due to the PEDOT:PSS conductivity and the resistivity of functionalized yarn is measured as 380 Ohm cm^{-1} . [Fig.1d](#) displays the actual device integrated on a textile support: the lower fibre is a functionalized cotton yarn, while the top is the platinum gate electrode. A schematic configuration of the electrode position on the device is reported in [Fig. 1e](#). The OECT response depends on the potential drops occurring at the gate/electrolyte and polymer/electrolyte interfaces. Under the OECT faradaic regime of operation [[34](#)], the drop of potential at the gate/electrolyte interface decreases, and the effective gate voltage ($V_{gs,eff} = V_g + V_{gs,drop}$) increases, which forces cations to move towards the polymer surface and de-dope the PEDOT:PSS polymer. The described mechanism of sensing is similar to the scheme used to detect hydrogen peroxide [[35](#)], dopamine [[36](#)] or melanin pigment [[9](#)] in OECT-based sensors recently reported in literature.

The PEDOT:PSS cotton fiber was decorated with rPOXA1b to impart to the device increased sensitivity to Tyr. rPOXA1b is a fungal laccase enzyme able to catalyze single-electron oxidation of phenolic compounds with associated four-electron reduction of oxygen to water. In particular, rPOXA1b has been extensively studied and largely produced in recombinant systems, due to its industrially relevant capability to oxidize phenolic substrates. This enzyme has been employed less than other enzymes for catalyzing reactions with Tyr as substrate [[37](#)], however unlike tyrosinases, laccases are potentially more suitable for Tyr bio-sensing in humans, because of its higher oxidizing capability. Indeed, laccases have a redox potential (E_0) ranging from 450 to over 750 mV which exceeds to a large extent the redox potential of tyrosinase [[38](#)]. In

biosensor design, laccase electron transfer (ET) devices are typically realized in two manners [21]. The first is realized using a mediator (introducing a redox compound) to shuttle the electron between the electrode surface and the active centers. In this way mediated electron transfer (MET) is used to realize the ET and among synthetic mediators 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) is one of the most common [39,40]. Instead, direct electron transfer (DET) can also be accomplished when an appropriate positioning and interaction might occur between laccase molecules and the conductive polymer chains [21]. Here we first assessed the role of the enzyme in the sensing process, thus the laccase decorated biosensor was assayed against ABTS, the substrate most used as reference for catalytic characterization of laccases. Apparent K_M constants were determined against ABTS (supporting information Fig. S1) in the case of enzyme immobilized on the fiber and compared to the constants associated to the enzyme dissolved in the solution [22]. A significant decrease of substrate affinity is observed for the immobilized enzyme: K_M values for the immobilized enzyme vs ABTS are approximately two times larger than the values of K_M for the soluble rPOXA1b ($0.132 \pm 0.020 \text{ mmol}^{-1}$ against $0.063 \pm 0.004 \text{ mmol}^{-1}$). This difference could be ascribed to the steric hindrance and the limited diffusion of solute into the polymer matrix upon the enzyme adsorption [22,23]. Fig. 2 shows the response of ABTS measured with the textile-based sensor. The I_{ds} amplitude modulation signals are compared for two cases: the enzyme dissolved in solution (Fig. 2a) and the enzyme adsorbed on the textile fiber with a previous functionalization (Fig. 2b). The response of the device is verified against 100 and 200 mM ABTS solutions and compared to the one measured without ABTS. V_{ds} is hold fixed as $V_{ds} = -0.05 \text{ V}$ while the gate voltage is initially set to 0 V and then raised from 0.2 V to 1.2 V by an increasing ramp, alternating 0 V after every increase, with a period of 120 s. The modulation of I_{ds} current (i.e. $(I_{ds} - I_{ds0})/I_{ds0}$) as a function of the gate voltage is reported in Fig. 2a: the modulation increases with the ABTS concentration, and the growth is monotonic with the concentration. The presence of a free form of rPOXA1b in the solution allows the sensing process, nevertheless the signal measured by the device is very low and the ability of the device to distinguish the signal variations across different ABTS concentrations is limited, at lower gate potentials the signal is undetectable. Differently, when the measurements are carried out using a fiber biosensor functionalized by rPOXA1b adsorption (Fig. 2b), the I_{ds} current modulation is higher, with larger differences among the same ABTS concentrations and a signal clearly detectable over all the set voltages. Fig. 2c shows the modulation of the sensor as a function of ABTS concentration for a fixed $V_g = 1.2$. From the figure, one can notice at a glance the difference in sensitivity (derivative of the signal with respect to the ABTS concentration, that is, the slope of the curve) in the case of laccase adsorbed to the fiber (red trace) and laccase dissolved in solution (black trace). Adsorption of the enzyme onto the textile surface improves the sensing, both in modulation and response time, probably due to the widespread contact of rPOXA1b with the PEDOT:PSS conductive layer. In our opinion, the close enzyme-polymer interaction may produce a synergic effect among catalytic activity, conductivity, and sensitivity to result in a significant increase of redox potential and signal amplification of the sensor. Furthermore, is noteworthy to report that the amplification of signal produced by OECT allowed the direct sensing of the catalytic enzyme activity without the use of any mediator. As already reported by Bergelyn et al. [21] an effective direct electron transfer (DET) can be obtained when laccase molecules are oriented in a favourable manner to allow the electron tunnelling from the electrode surface to the T1 Cu site that is positioned 6 Å away from the protein surface. In our case the enzyme molecules can be strongly entrapped into the cotton fibres, coated by conductive polymer PEDOT:PSS, thanks to the action of capillary forces, generated at interface by the textile natural nano and micro structure itself. In this condition, when the solution comes in contact during the adsorption with the textile, the enzyme is naturally forced to adhere on the surface. Furthermore, the highly charged polysulphone part of the conductive layer would play a role in leading the electrostatic interactions increasing the enzyme entrapment ability of our sensor [41]. ABTS is a water-soluble compound that undergoes one electron oxidation to the relatively stable blue coloured radical cation $\text{ABTS}^{\bullet+}$, at a redox potential (0.69 V versus NHE) that almost matches that of POXA1b [38]. Indeed, the standard assay of laccase activity involves spectrophotometric monitoring of the generation of $\text{ABTS}^{\bullet+}$ with time. One more one electron oxidation of $\text{ABTS}^{\bullet+}$ to di-cation ABTS^{++} occurs electrochemically at

higher potential (1.1 V versus NHE), but this red coloured species is less stable. Finally, oxidation of ABTS^{•+} to ABTS⁺⁺ is endoergonic for laccases (Fig. S2). Therefore, the enzyme adsorbed to the electrode is able to promote a specific electrode reaction and acts as a 'molecular transducer' in mediatorless manner. The substrate can be detected by the interaction of positive charged ABTS molecule with the conducting polymer, producing a de-doping effect and modifying I_{ds} current in the PEDOT:PSS conductive layer, while the generated electrons are collected by the Pt gate electrode.

3.2. Verifying Tyr concentrations in diluted solutions

The PEDOT:PSS cotton fiber was verified for Tyr sensing as reported in Fig. 3. The response of the device was tested on Tyr without (Fig. 3a) and with (Fig. 3a) the POXA1b enzyme adsorbed onto the textile thread. In Fig. 3a the I_{ds} modulation amplitude shows the same increment for different gate voltages. Without any enzymatic action, the differences across different Tyr concentrations are vanishingly small. Instead in the I_{ds} modulation analysis performed on the laccase decorated biosensor (Fig. 3b), the modulation values are higher, reflect more clearly changes in the Tyr concentration, and are proportional to the concentration. Moreover, higher gate voltages exaggerate the differences among the Tyr concentrations, showing an effective sensing of the Tyr molecule. The time trace of the I_{ds} current is reported in Fig. 3c. In the course of the measurements, Tyr has been injected in the system in the 10⁻⁸ and 10⁻⁷ M concentration at specific time frames. The injection events induce a significant shift of the signal observable at the considered low concentrations. The sensitivity of the sensor for Tyr is clearly dependent on laccase presence: the biosensor has a limit of detection in the order of 10⁻⁸ M, a sensitivity that allows to detect Tyr in real human physiological fluids, whose reported concentration is in the 10⁻⁶ M range [42]. Finally, the sensing response with rPOXA1b adsorbed in the textile fiber (black) is compared with the sensor without laccase (red) as a function of Tyr concentration (Fig. 4). In the case of adsorbed laccase biosensor, an evident increase of the response occurs starting from a solution containing 10⁻⁶ M Tyr, with a constant growing rate for higher concentrations. Differently, when the assay is performed without the enzyme, even for high concentrations of Tyr, the device results unable to sense Tyr.

3.3. Time evolution of the system

The characteristic time constant for each concentration is reported in Fig. 5a. These values are calculated by fitting the I_{ds} current, for different V_g potentials, with an exponential curve (Fig. 5b). The time constants are related with the kinetic of the de-doping process of the PEDOT:PSS polymer, as described in Ref. [43]. The process depends on the concentration of positive charged species in the fluid and on their diffusive properties. Hence, the overall sensor response is faster when the de-doping species have higher concentration and higher diffusion coefficient. Fig. 5 shows the dependence of tau constant to different Tyr concentration by varying V_g. It is worth noting the optimal gate voltage is in the 0.4e0.6 V range, in such conditions PEDOT:PSS de-doping result faster and such trend is observed for all Tyr concentrations. The reaction responsible of the Tyr sensing is derived on the basis of the experimental data reported by Mattinen et al. [37]. Laccase-catalyzed oxidation of Tyr and Tyr- containing peptides is proposed to proceed through tyrosyl radicals in the hydroxyl group of the phenolic ring and then rapidly delocalize into the different positions of the aromatic ring. *Iso-di-* tyrosine bonds are formed when hydroxyl and tyrosyl radicals located indifferent molecules react with each other (Fig. S3). The production of H⁺ cation in the reaction is responsible of the increment of the de-doping in the fiber sensor channel. The presence of the enzyme at the fiber surface results more efficient in the generation of the de-doping effects due to the reactions showed in Fig. S4. Thus, the already known different reactivity of the POXA1b against phenolic and other aromatic compounds also affects the selectivity of the textile sensor and allows to discriminate Tyr from other molecules and structurally similar amino-acids present in human physiological fluids.

4. Conclusion

The specific detection of biomolecules in real human physiological fluids would own a crucial importance in the non-invasive analysis of the patients' conditions, preventing several pathological states. Herein, we showed the setting of a mediatorless catalyzed reaction for L-Tyr based on the non-covalent immobilization of rPOXA1b laccase onto yarn cotton coated by PEDOT:PSS. The sensing performances have been first characterized using ABTS assay and then the modulation of the signal response and the kinetic analysis of the signal is used to detect Tyr in aqueous solutions, displaying a limit of detection of 10^{-8} M for this amino acid. The textile-based biosensor, properly decorated by enzyme adsorption, has been demonstrated to provide direct electron transfer to monitor biomolecules, with wearable, non-invasive, low-cost performances, finding large application in sports, health care and working safety.

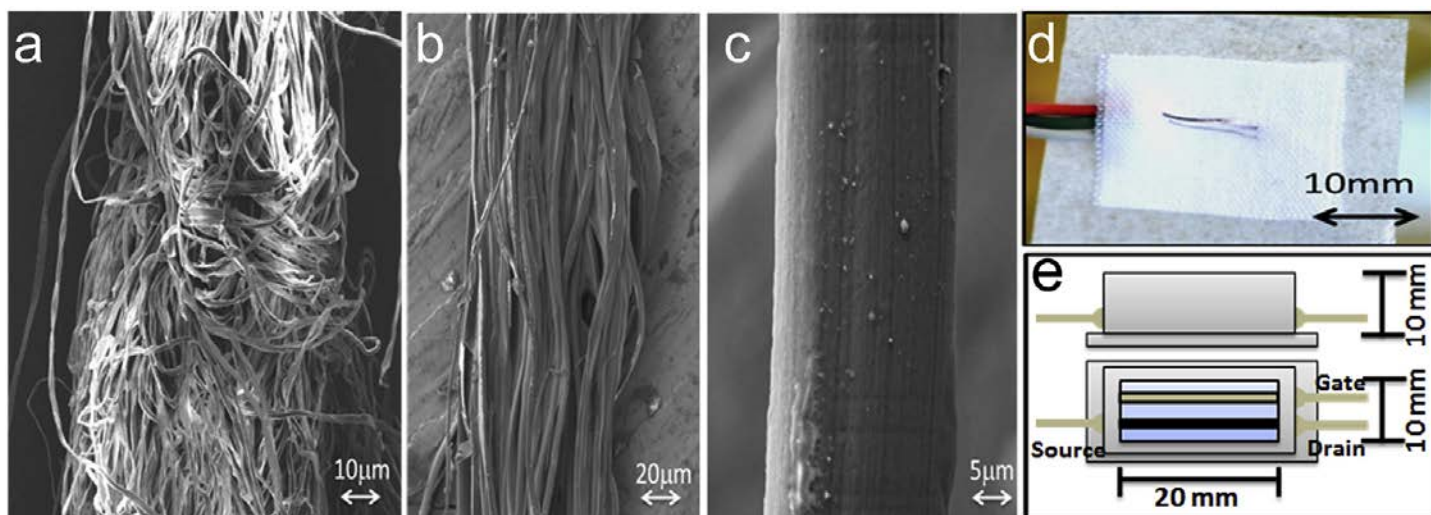


Fig. 1. (a) SEM image of natural cotton thread without functionalization (reference); (b) SEM image of cotton thread functionalized with PEDOT:PSS thin film; (c) SEM image of single cotton yarn functionalized with conductive polymer; (d) Integration of the functionalized e-textile (e) Sketch of the proposed transistor device on textile.

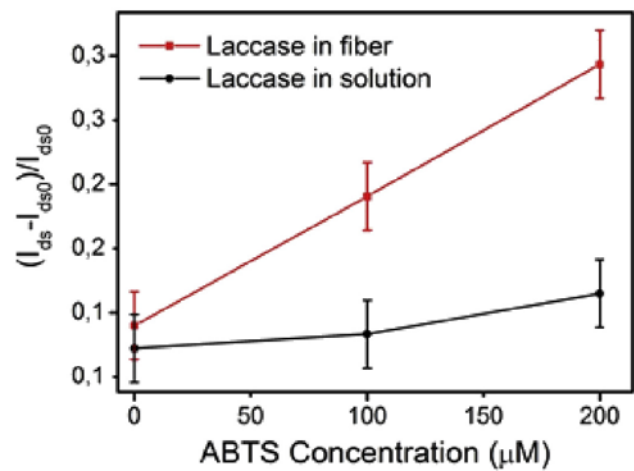
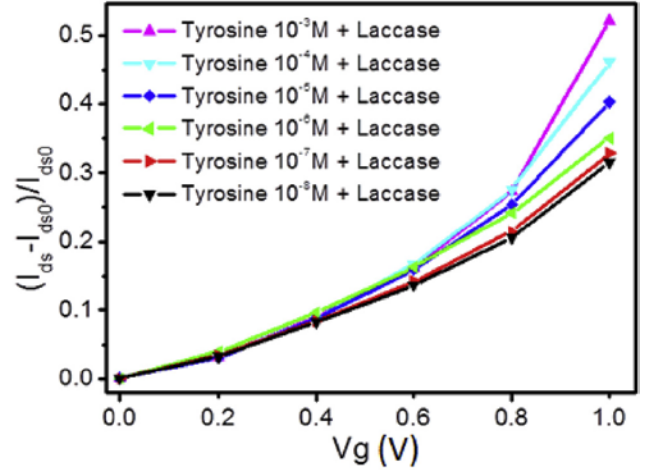
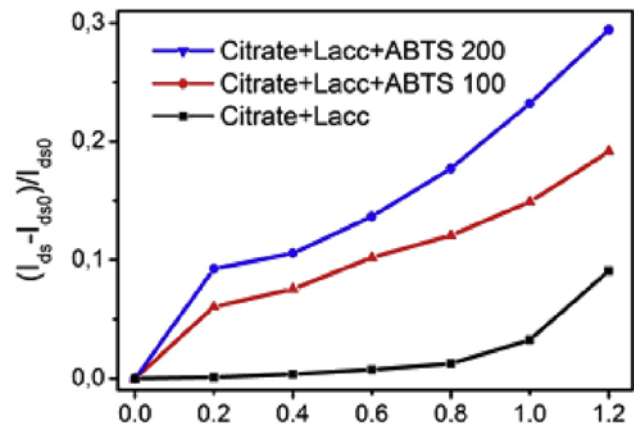
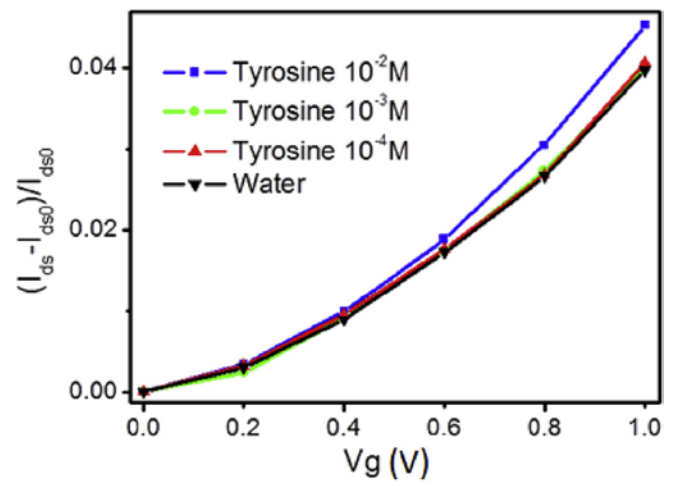
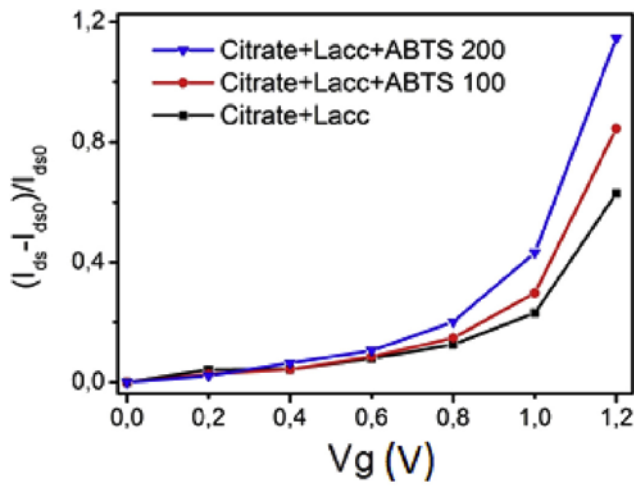


Fig. 2. (a) Sensor response towards ABTS (100 mM and 200 mM) with rPOXA1b in citrate buffer solution (b) ABTS response for different concentrations in citrate solution with laccase enzyme adsorbed in the textile fiber; (c) Comparison of ABTS response as a function of the concentration: enzyme in solution (black) is compared with enzyme adsorbed into the cotton thread (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. Textile biosensor modulation at different Tyr concentrations, as a function of gate voltage, without POXA1b enzyme (a); and with rPOXA1b adsorbed on textile fiber (b). (c) The real time response of the biosensor functionalized with rPOXA1b for two different Tyr concentration: 10^{-6} M (top curve) and 10^{-8} M (bottom curve), using $V_g = 1$ V.

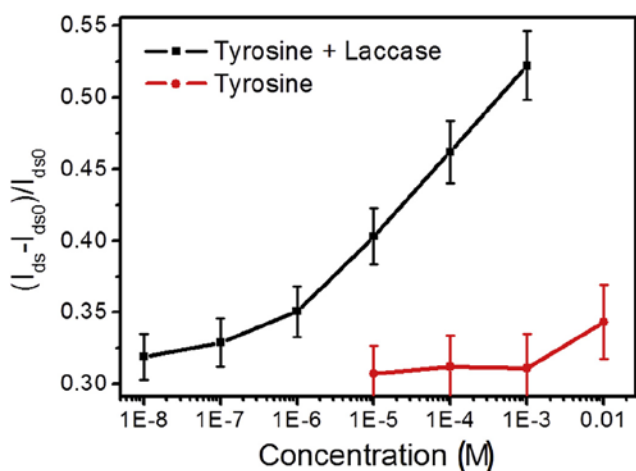


Fig. 4. The intensity of the current modulation is reported as a function of Tyr concentrations. The sensitivity of the sensor functionalized with rPOXA1b enzyme (black) is compared to that of sensor without enzyme (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

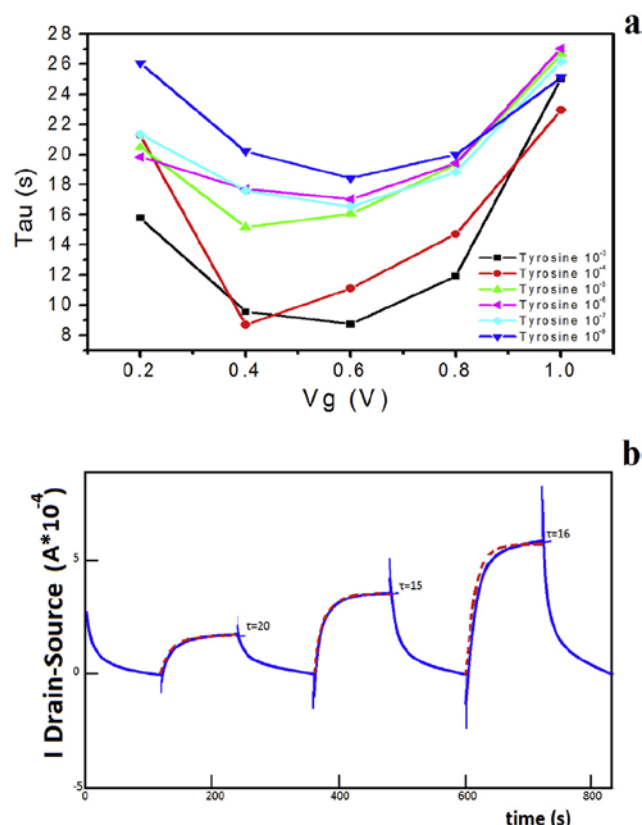


Fig. 5. (a) Time constants (τ) reported as a function of gate voltage for different Tyr concentration with the rPOXA1b enzyme adsorbed in the textile sensor. (b) Example of exponential fitting of I_{ds} current curve and calculation of time constants for each gate voltage applied (gate voltage from 0.2 to 1 V at step of 0.2).

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