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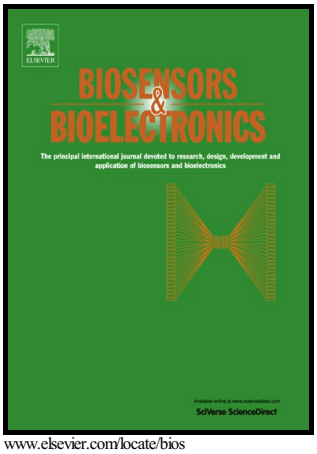

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Aqueous polythiophene electrosynthesis: A new route to an efficient coupling of PQQ-dependent glucose dehydrogenase for sensing and bioenergetic applications

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Abstract

In this study, polythiophene copolymers have been used as modifier for electrode surfaces in order to allow the immobilization of active pyrroloquinoline quinone dependent glucose dehydrogenase (PQQ-GDH) and to simultaneously improve the direct electrical connection of the enzyme with the electrode. Polymer films are electrosynthesized in aqueous solution without the need of surfactants onto carbon nanotubes modified gold electrodes from mixtures of 3-thiopheneacetic acid ($ThCH_2CO_2H$) and 3-methoxythiophene ($ThOCH_3$) using a potentiostatic pulse method. Polythiophene deposition significantly improves the bioelectrocatalysis of PQQ-GDH: the process starts at -200 mV vs. Ag/AgCl and allows well-defined glucose detection at 0 V vs. Ag/AgCl with high current density. Several parameters of the electro-polymerization method have been evaluated to maximize the anodic current output after enzyme coupling. The polymer deposited by this new procedure has been morphologically and chemically characterized by different methods (SEM, EDX, FT-IR, UV-Vis, XPS and Raman spectroscopy). The bioelectrocatalytic response towards increasing glucose concentrations exhibits a dynamic range extending from 1 μM to 2 mM. The low

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applied potential allows to avoid interferences from easily oxidizable substances such as uric acid and ascorbic acid. Short and long-term stability has been evaluated. Finally, the PQQ-GDH electrode has been coupled to a bilirubin oxidase (BOD)- and carbon nanotube-based cathode in order to test its performance as anode of a biofuel cell. The promising results suggest a further investigation of this kind of polymers and, in particular, the study of the interaction with other enzymes in order to employ them in building up biosensors and biofuel cells.

Keywords: polythiophene, biosensor, bioanode, PQQ-GDH, DET, electropolymerization.

1. Introduction

The communication between enzymes and electrode surfaces is of fundamental importance in biosensor development. In this regard, the last twenty years have seen significant progress in the study and efficient improvement of direct electron transfer (DET) between the redox center of proteins and electrodes. In fact, avoiding the use of mediators allows to yield currents from biocatalysis at potentials close to the formal potential of the immobilized enzyme. This can be advantageous in order to prevent interferences and to maximize the performances of the so developed device (e.g. biosensors or biofuel cells). The direct connection between the electrode surface and the enzyme is generally hindered by the redox-active site location, embedded inside the protein structure (Gorton et al., 1999). Several strategies were applied to solve this problem (Nöll and Nöll, 2011; Sarauli et al., 2014a; Schulz et al., 2016; Zayats et al., 2005). Among them, the nanostructuration of the electrode surface has been found a profitable way to have an efficient electrical communication. The roughness of the electrode surface leads to a large increase of the electroactive area and of the number of binding sites for a productive immobilisation of enzymes. Furthermore, the employed nanomaterials may decrease the distance between the redox-active site and the electrode surface, enhancing the rate constant of the electron transfer process (Paolo Bollella et al., 2017; P. Bollella et al., 2017; Katz and Willner, 2004; Lanzellotto et al., 2014). Carbon nanotubes (CNTs) have been widely used for this purpose. They can provide a good microenvironment for the proteins and can be further used in nanocomposite developments, functionalized by oxidation or modified after deposition of other materials in a layer-by-layer fashion or by π-stacking compounds (Göbel et al., 2016; Lalaoui et al., 2016; Lisdat, 2017; Meredith et al., 2011; Tanne et al., 2010; Tasca et al., 2011).

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Conductive polymers, such as polyaniline (Dhand et al., 2011), have been very often employed as electrode modifier in order to allow a good enzyme wiring (Cosnier and Holzinger, 2011). Their (semi)conductive properties, their biocompatibility and their versatility have made these materials very interesting in this field since their discovery in the late 1980s (Ahuja et al., 2007; Bartlett and Birkin, 1993; Gaspar et al., 2001; Gladisch et al., 2016; Sarauli et al., 2015). The polymer properties can easily be modulated by choosing the proper monomers (Sarauli et al., 2013). Furthermore, the electrochemical polymerization allows a direct deposition on top of the electrode and it is much faster than a time and reagents-consuming chemical synthesis (Li et al., 2009).

Despite these features, polythiophene application in biosensing has only partly been seen until now. It has been used mainly in molecular imprinted polymers (Huynh et al., 2015), in supercapacitors (Patil et al., 2014) and for enzyme immobilization (Abasıyanık and Şenel, 2010; Akbulut et al., 2015; Hiller et al., 1996; Krikstolaityte et al., 2014; Kuwahara et al., 2009; Nandini et al., 2014). One of the problems that can impact the enzyme interacting with this kind of polymer is the need to use organic solvents to solubilize the monomers and to reach the high potentials requested for the monomer oxidation in electrosynthesis (Fusco et al., 2017). Further, during the polymerization the upcoming polythiophene structure can be damaged by the high potential applied (polythiophene paradox (Krische and Zagorska, 1989)). Many strategies have been used in order to lower the required oxidation potential, for example starting from functionalized bi- or tri-thiophenes or employing β-functionalized thiophene monomers with electron donating groups like methyl or oxoalkyl groups as in 3,4-ethylenedioxythiophene (EDOT) (Zanardi et al., 2013). The electrosynthesis in micellar aqueous media by using anionic surfactants allows decreasing the oxidation potential of methoxythiophene compared to acetonitrile polymerization (Fall et al., 2001). Ionic liquid/water microemulsions have also been used successfully either as solvents and electrolytes to electrosynthesize poly(3-methoxythiophene) with good electroactivity and conductivity (Dong et al., 2009). Poly(3,4-ethylenedioxythiophene) (PEDOT) has been described as a highly conductive and stable polymer with promising features in the biosensing field: the monomer is slightly soluble in water (especially if surfactants are present) and has a quite low oxidation potential. Nevertheless, until now the advantage of using PEDOT in DET-type of enzyme-electrode contacts has not been exploited and this polymer has generally been used in combination with redox mediators (Kakhki et al., 2013; Rozlosnik, 2009; Xiao et al., 2017).

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Pyrroloquinoline quinone dependent glucose dehydrogenase (PQQ-GDH) is one of the most employed enzymes for glucose conversion. Its oxygen insensitivity simplifies the working conditions. Furthermore, it is possible to enhance its activity and selectivity towards glucose by site–specific mutagenesis (Sode et al., 2002). Researchers have used different strategies to wire PQQ-GDH to electrode surfaces. One of the promising ways has been the use of sulfonated polyaniline as an interlayer between the electrode and the enzyme (Sarauli et al., 2014a, 2014b; Schubart et al., 2012). The enzyme has also been coupled by employing immobilized redox molecules. In this case, the currents from the glucose oxidation were often rather high, but the starting potential of the bioelectrocatalysis was far from the enzyme formal potential (Habermüller et al., 2000; Malinauskas et al., 2004; Wettstein et al., 2012) or the stability of the mediator was insufficient for long-term usage (Nakashima et al., 2016). These are disadvantages in order to employ the enzyme electrodes, for example, as anode of a biofuel cell.

In this study, a polythiophene copolymer has been electrosynthesized from methoxy- and carboxy-modified monomers on multiwalled carbon nanotubes (MWCNT) modified gold electrodes by potentiostatic pulses. The method operates in an aqueous solution without the need of surfactants. The reaction conditions avoid the poisoning of the surface by organic solvents and allow wiring PQQ-GDH in an efficient way reaching high current values at low potential. These features provide the basis for the use of the so developed PQQ-GDH electrodes for sensing but also as an anode in a biofuel cell. Special focus is devoted to the characterisation of the polymer prepared by the new procedure.

2. Material and methods

2.1 Materials

Thiol functionalized multiwalled carbon nanotubes (MWCNT) with a purity of 95%, a diameter of 10 nm and an average length of 1 μm were purchased from Nanocyl (Belgium); 3-thiopheneacetic acid ($ThCH_2CO_2H$), 3-methoxythiophene ($ThOCH_3$), dimethyl sulfoxide (DMSO), N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), potassium chloride, 4-morpholineethanesulfonic acid monohydrate (MES), citric acid monohydrate, sodium phosphate dibasic, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), calcium chloride dehydrated ($CaCl_2$), D-(+)-glucose anhydrous, L-(+)-ascorbic acid and uric acid have been provided by Sigma Aldrich; pyrroloquinolinequinone (PQQ) was

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supplied by Wako Chemicals GmbH (Germany). For all aqueous solutions water was purified with the "ultra-clear direct" system from SG Water (Germany).

2.2 Enzymes

The soluble PQQ-dependent glucose dehydrogenase (PQQ GDH) was obtained as apoenzyme from Roche Diagnostics as a kind gift. It was recombinantly produced in Escherichia coli with the gene of periplasmic glucose dehydrogenase from *Acinetobacter calcoaceticus*. The Michaelis constant (K_M) towards glucose oxidation was determined by the supplier employing dimethyl((6-methyl-7-((4-nitrosophenyl)amino)-1H-imidazo[1,2-b]pyrazol-1-yl)methyl)phosphine oxide as electron acceptor. The holoenzyme was reconstituted by incubating the apoenzyme (to have a final concentration of 30 μM) in 5 mM MES buffer at pH 6.5 with 1 mM $CaCl_2$ and 60 μM PQQ for 3 h at room temperature in the dark. PQQ-GDH solution was stored at 4 ˚C.

Bilirubin oxidase (BOD) from *Myrothecium verrucaria* was purchased from Sigma Aldrich. 2.4 mg of lyophilized powder (around 0.7 mg of pure protein) with a specific activity of 35 U/mg (protein) are dissolved in 0.3 ml of 5 mM citrate-phosphate buffer (CiP), pH 7 with a final concentration of 40 μM and stored in aliquots of 30 μl at -20˚C.

2.3 Electrochemical characterization

Electrochemical measurements were performed in a 1 mL cell. An Ag/AgCl/1M KCl (0.235 V vs. NHE, BASi, USA) electrode and a platinum wire were used as reference and counter electrode, respectively. Every potential in this manuscript has to be referred to Ag/AgCl/1M KCl. A gold wire was used as working electrode (area $2mm^2$ - BASi, MF-2014, USA). The cyclic voltammetry experiments were carried out with the potentiostat PGSTAT 12 (Methrom-Autolab, Netherlands) and the collected data were analysed by GPES software (General Purpose for Electrochemical System, Eco Chemie, Utrecht, Netherlands). Chronoamperometric experiments were performed by a CH Instrument 1230b (Austin, USA) under stirring. Open circuit potential (OCP) and galvanodynamic experiments were carried out with the potentiostat/galvanostat/ZRA Reference 600 from Gamry Instruments. In the biofuel cell assembly, the cathode was connected as counter-reference electrode and the anode as working electrode. Different concentration of air saturated MES buffer, CiP buffer and HEPES buffer with 1 mM $CaCl_2$, pH 6.5, were used to test the bioelectrocatalytic activity of the developed enzyme electrode.

2.4 Preparation of the enzyme electrodes

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The gold electrodes were wet cleaned (H_2O) with abrasive papers (Dieter Schmid, Feine Werkzeuge, Germany) in three steps (grain sizes: 2000, 2500, 3000). After polishing, they have been modified by MWCNT in two steps (in order to homogeneously cover the entire working surface) as in our previous works (Fusco et al., 2017; Schubart et al., 2012). Briefly, 2 mg of thiol functionalized MWCNT were dispersed in 0.8 mL of 5 mM CiP, pH 7, then ultrasonic pulses of 2 s were applied to the mixture for 30 min and finally ethanol was added to reach a concentration of 1 mg/mL. 4 µL of this solution have been dropped and dried twice on top of a clean gold electrode.

For the bioanode preparation, the electropolymerization of polythiophenes (PTh) was performed by cyclic voltammetry (CVs, scan rate 100 mV/s) or by applying a series of potentiostatic pulses in different air saturated aqueous buffer solutions (+100 mM KCl as supporting electrolyte) of the two thiophene derivatives. The monomers have been solubilized after 15 min of sonication at 40 °C. In order to maximize the biocatalytic current, firstly, the start potential (-0.3 V and -0.5 V) and the oxidizing potential (+1.2 V – 1.6 V) in cyclic voltammetric polymer deposition have been systematically varied, then the influence of the polymerization buffer, the pulse oxidation time (from 0.4 s to 1.2 s) and the number of pulses (from 42 to 1000) in potentiostatic pulse electrosynthesis was studied.

After the electrodeposition, the modified gold electrode (PTh/MWCNT/Au) was well washed in water and, after drying, the carboxylic acid groups of the polymer (due to the presence of $ThCH_2CO_2H$ as monomer) were activated for covalent coupling of the enzyme by getting in contact with 20 µL of 25 mM NHS and 100 mM EDC aqueous solution for 15 min. After washing, the electrode was incubating in 30 µM PQQ-GDH solution (20 µL on top of the working electrode surface) for 1 h. After the coupling, loosely bound enzyme was removed by washing with MES buffer.

For the biocathode preparation, the procedure described in our previous work (Göbel et al., 2016) has been followed. Briefly, MWCNT modified gold electrode was brought in contact with a 5 mM CiP buffer solution (pH 7) of 2.5 mg/mL PQQ for 1 h. After stacking of the pyrroloquinolinic compound on top of the modified carbon surface, its carboxylic acid units were activated in 25 mM NHS and 100 mM EDC aqueous solution for 15 minutes in order to covalently couple the enzyme in the following step. Finally, the electrode has been incubated with 10 µL of 20 µM BOD for 1 h. After removing the adsorbed enzyme, the BOD/PQQ/MWCNT/Au electrode was stored at 4 ˚C when not used.

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2.5 Stability measurements

In order to test the PQQ-GDH electrode response over time, chronoamperometric measurements at 0 V by adding 100 μM glucose to 1 mM $CaCl_2$, 100 mM CiP buffer solution, pH 6.5, were performed during the day and day-by-day. The PQQ-GDH/PTh/MWCNT/Au electrodes were stored in 100 μM PQQ, 1 mM $CaCl_2$, 5 mM MES buffer solution, pH 6.5, during the long-term stability test in order to reduce the PQQ release from the enzyme.

2.6 Morphological and spectroscopic characterization

Gold slides from Ssens (Netherlands) were cut in pieces of 2 mm × 10 mm, then areas of circa 2 mm × 2 mm were defined by parafilm, modified with MWCNT (see *Preparation of the enzyme electrodes*) and used as working electrodes for polymer deposition (area of the modified surface around 4 mm^2). Electropolymerization was performed in 5 mM MES buffer (pH 6.5) solution of 6.7 mM $ThCH_2CO_2H$ and 13.3 mM $ThOCH_3$ as monomers and 100 mM KCl as supporting electrolyte at MWCNT/Au electrodes by applying 667 pulses between 1.4 V (0.8 s) and -0.3 V (0.2 s) vs. Ag/AgCl. Unmodified MWCNT/Au surfaces were characterized for comparison.

SEM, EDX: The surfaces have been morphologically characterized by a High-Resolution Field Emission Scanning Electron Microscopy (HR FESEM, Zeiss Auriga Microscopy) equipped with an EDS (energy dispersive x-ray spectroscopy) detector [≤ 123 Mn-Kα eV (Bruker)]. In order to calculate the element percentage in the deposited material (MWCNT or PTh/MWCNT). The gold signal from the bare slide has been excluded from the calculation.

Fourier-transform infrared spectroscopy (FT-IR) characterizations were collected in the range 400 - 4000 cm^{-1} by a 660-IR FT-IR Spectrometer (VARIAN, Agilent Technologies). FT-IR monomer spectra were acquired for comparison by using pure samples from Sigma Aldrich. Raman spectra were recorded using a micro-Raman dispersive spectrometer (SENTERRA, Bruker Optik GmbH) employing a CW diode pumped solid state laser of 0.1 mW operating at 785nm. The spectra were recorded by using a 50 X long distance objective with a resolution of 3 cm^{-1} in the spectral window from 45 to 2600 cm^{-1}.

X-ray photoelectron spectroscopy (XPS) measurements were performed with a modified Omicron NanoTechnology MXPS system equipped with a dual X-ray anode (Omicron DAR 400) and an Omicron EA-127 7-channeltron energy analyser. All binding energies have been

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referenced against the silicon 2s peak (glass slide) taken at 154.4 eV and are considered accurate to ± 0.2 eV.

Ultraviolet–visible spectroscopy (UV-Vis) characterizations were performed by an Evolution 201 UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA) in the range 300 nm - 700 nm. For this purpose, the electrodeposited polymer on the MWCNT/Au electrode was dissolved in DMSO.

3. Results and discussion

MWCNT have been selected as electrode material due to the high electroactive surface and to the compatibility with conductive polymer functionalization. The electrosynthesis of polythiophene derivatives has been performed in buffer solution without employing organic solvents or surfactants. The preparation parameters have been varied in order to maximize the anodic current from the biocatalyzed glucose oxidation and the performance of the developed system, in terms of interference effects, stability and application as anode in a biofuel cell, has been assessed.

3.1 Polymerization by cyclic voltammetry: potential range and monomer mixtures

The polymer electrosynthesis in aqueous solution has been firstly evaluated by cyclic voltammetry. Carboxy-modified thiophene ($ThCH_2CO_2H$) has been used since the enzyme can be bound in a covalent fashion to the prepared polymer film, while methoxythiophene ($ThOCH_3$) is used as spacer between the carboxy-monomers and to lower the oxidizing potential. Whereas $ThCH_2CO_2H$ is well soluble, $ThOCH_3$ has been solubilized by means of ultrasonication. This results in a very fine distribution of nanodroplets in the $ThCH_2CO_2H$-containing aqueous solution (around 170 nm, Fig. S1). Figure 1A-B shows the cyclic voltammetric behaviour of such a mixture of both monomers. A peak at +1.3 V vs. Ag/AgCl indicates the required potential to start the monomer oxidation process. Furthermore, the anodic and cathodic peaks (related to a formal potential of 35 mV vs. Ag/AgCl) are increasing during the scans, demonstrating a conductive polythiophene deposition on top of the electrode. After a covalent coupling of the PQQ-GDH (via EDC/NHS, see *Preparation of the enzyme electrodes*), CVs in buffer in absence and presence of glucose show that the bioelectrocatalysis starts at about -0.2 V vs. Ag/AgCl (Fig. 1C). Compared to a pure MWCNT

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electrode this is a clear improvement with respect to catalytic current magnitude and start potential (Fig. S2).

Monomer composition and oxidizing potential are of fundamental importance for the polymerization reaction and thus, at this point their evaluation is essential. Table S1 lists the catalytic current responses in presence of glucose after the enzyme coupling by systematically varying the end potential in the electrodeposition step in the absence or presence of methoxy-containing monomers. The presence of methoxythiophene in solution results in an increase of the catalytic current between 3 and 8 times (a further increase in $ThOCH_3$ content has not been found beneficial – data not shown). Furthermore, reaching a potential of at least +1.3 V vs. Ag/AgCl is required to have an active polythiophene layer. Going above +1.5 V is not beneficial, but harmful because of the overoxidation of the polymer (Gratzl et al., 1990) reflected by lower catalytic currents. Moving the start potential from -0.3 V to -0.5 V vs. Ag/AgCl does not influence the performances of the polymer electrode after PQQ-GDH coupling (Table S2).

These experiments verify the suitability of a polymer deposition from an aqueous solution and the successful connection of the enzyme with the electrode by the prepared layer.

3.2 Polymerization by potentiostatic pulses: evaluation of the pulse parameters and the operational conditions

A pulsed deposition allows often better control of the polymer features than the cyclic voltammetric electrosynthesis. Figure S3 shows a general scheme of the employed method. Fixing the monomer composition (6.7 mM $ThCH_2COOH$, 13.3 mM $ThOCH_3$) and the start and end potentials as evaluated by the CV experiments (1.4 V and -0.3V, respectively), pulse oxidizing time and the number of pulses have been studied in order to maximize the response of the coupled PQQ-GDH towards glucose. First, different buffers at different pH values have been used to perform the electropolymerization (Fig. 2A). It can be observed that with all conditions a polymer modification of the MWCNT can be prepared, but with higher current values for the bioelectrocatalysis of PQQ-GDH when prepared at neutral pH values. A pH of 6.5 is then chosen for the polymerization solution. Finally, by comparing the results obtained applying MES buffer with different concentrations it is found that a high ionic strength can lower the electrosynthesis rate.

In Figure 2B, the dependency on the number of pulses (n) is shown. Evidently, a pulse number increase from 42 to 667 results in a deposition of a larger amount of conductive

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polymer that can successively bind PQQ-GDH in an efficient DET communication, while by almost doubling n (from 667 to 1000) the outcome has remained the same; this means that a further increase in polythiophene amount is not useful in enhancing bioelectrocatalysis. In Figure 2C, the effect of the pulse oxidation time (t_{ox}) on the response of the enzyme electrode is illustrated. A slight enhancement in the catalytic current can be obtained by increasing t_{ox} from 0.4 s to 0.8 s, but by setting a longer t_{ox} (1.2 s) no significant variation in current response can be found.

In conclusion, the polythiophene electrosynthesis in the following experiments has been performed in 5 mM MES buffer, 1 mM $CaCl_2$, 100 mM KCl, pH 6.5 solution of 6.7 mM $ThCH_2COOH$ and 13.3 mM $ThOCH_3$ by applying 667 potentiostatic pulses between 1.4 V (0.8 s) and -0.3 V (0.2 s). A confirmation that the electrosynthesized polythiophene is really responsible for electro-active PQQ-GDH coupling can be obtained from control experiments: here the MWCNT surface has been treated in the same way, but without the monomers presence. A direct bioelectrocatalysis of the enzyme could be observed, but only around 25% of current value compared to the presence of polythiophene can be obtained. Furthermore, when the MWCNT electrode is immersed in the monomer solution without the application of pulses a negligible current output has been recorded (Fig. S4).

These experiments and the voltammetric curve shown in Figure 1C indicate that a direct electron transfer from the substrate-reduced enzyme via the conductive polymer towards the MWCNT electrode occurs. One could assume that free PQQ acts as a mediator and contributes to the signal, but it has to be emphasized here that the start potential of glucose bioelectrocatalysis is close to the redox potential of the enzyme (Sato et al., 2001) and lower than free PQQ (Willner et al., 1998). Furthermore, there are only minute amounts of free PQQ within the electrode architecture since the holoenzyme is reconstituted with an enzyme – PQQ ratio of only 1:2 and the electrodes are carefully rinsed before use. It shall also be underlined that here the conductive properties of the polymer are exploited and thus, no redox conversion of the polymer is needed in the potential range of bioelectrocatalytic glucose oxidation.

3.3 Characterization of the electrosynthesized polymer

SEM pictures have been taken to morphologically characterize the modified electrodes. Figure 3A-B and Figure 3C-D show the MWCNT/Au and PTh/MWCNT/Au surfaces, respectively, at different magnifications. It is clearly visible that the polymer is homogeneously dispersed as small spheres on top of the carbon nanotubes. In particular, at

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high magnification a nanostructuration of the deposited polythiophene is evident: Smaller particles with a diameter of circa 40 nm seem to aggregate in spheres with a diameter of ~400 nm. From the polymer shape, it can be asserted that the electropolymerization in buffer solution occurs inside the dispersed monomers droplets, that can work as nanoreactors. Since in this study thiophene monomers are electropolymerized for the first time in surfactant-free aqueous solution, care has been taken to verify the successful polymer formation on the MWCNT surface and several structural characterisations have been performed.

The energy-dispersive X-ray (EDX) analysis (Fig. S5) of PTh/MWCNT/Au electrode indicates a significant increase in the sulphur and oxygen content with respect to the MWCNT/Au electrode, that is in line with the polythiophene deposition.

FT-IR spectra of the bare MWCNT surface (a) and the polymer modified MWCNT (b) are given in Figure 4A. FT-IR spectra of $ThCH_2COOH$ (a) and $ThOCH_3$ (b) are shown for comparison in Figure S6A. In the MWCNT spectrum, a broad band at 3200 cm^{-1} relative to the O-H stretching of hydroxyl groups on the oxidized nanotubes ends is clearly visible. In the range between 3000 cm^{-1} and 2850 cm^{-1} some weak peaks refer to the C-H stretching due to some defects in the nanotubes structure. The broad band centered at 1410 cm^{-1} and 1070 cm^{-1} is generated by the aromatic C_α=C_β bending and stretching vibrations and by a convolution of the C-H bending and the C-O stretching signals from the defects, respectively. In the FT-IR spectrum of the PTh/MWCNT surface, in addition to that reported for the MWCNT, many peaks belong to the polythiophene derivative and these signals are strongly broader compared to the monomer ones. In particular, the peaks at 2925 cm^{-1} and 2850 cm^{-1} are attributed to the C-H stretchings of the methyl ($ThOCH_3$) and of the methylene groups ($ThCH_2COOH$) (Alves et al., 2010). The signal at 1650 cm^{-1} is assigned to the C=O stretching, whereas the band at ~1510 cm^{-1} refers to the C_α=C_β stretching of the thiophene rings (as shown in literature for $PolyThOCH_3$ (Dong et al., 2009)). The peak at 1350 cm^{-1} refers to the C-H bending of the thiophene ring and the strong absorption at 1070 cm^{-1} is characteristic of the methoxythiophene copolymers and can be referred to the in plane deformation of the C-O-C stretching of $ThOCH_3$ (Alves et al., 2010). The finger print region is a convolution of all of the monomer absorptions: in particular, the band at 795 cm^{-1} (C_β-H out of plane stretching) is characteristic of 2,5-disubstituted thiophene rings (Zhang et al., 2014) and the absence of relevant peaks between 1250 cm^{-1} and 1150 cm^{-1} (C_α-H in plane stretching, absorptions present in all of the two monomers spectra) suggests that the electrochemical coupling of the thiophene rings occurs preferentially at 2,5 positions (Dong et al., 2009). Finally, the peak at

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615 cm^{-1} is due to the C-S bending mode of the thiophene ring (and of the thiol ends in MWCNT).

Raman spectroscopy is particularly well suited to molecular characterization of carbon materials and generally provides complementary information to the FT-IR. In Figure 4B, localized Raman spectra of MWCNT (a) and PTh/MWCNT (b) are shown. Raman spectra of $ThCH_2COOH$ (B) and $ThOCH_3$ (C) are shown for comparison in Figure S6. In the bare carbon nanotube surface spectrum, the bands at 1586 cm^{-1}, assigned to the in-plane C-C bond (G-band), and at 1300 cm^{-1}, referring to the hybridized vibrational mode associated to the graphene edges (D-band), are present as expected (Bokobza and Zhang, 2012). In the PTh/MWCNT spectrum, the polythiophene contribution is greatly enhanced (due to the possibility to investigate defined micro-areas of the sample and not only the whole surface as for FT-IR). The presence of two different monomers and the possibility to have regio-regular or regio-random polymer chains make the bands assignments rather difficult. The MWCNT G-band seems to shift to lower energy, indicating a favourable interaction between the π-electrons from polythiophene and carbon nanotubes (Karim, 2012). The region between 1600 cm^{-1} and 1300 cm^{-1} includes the C_α=C_β and the C_β-C_β intra-ring stretchings, which are the bands sensitive to π-electron delocalisation (Paternò et al., 2017). In this regard, the signal at 1530 cm^{-1} is generally assigned to the antisymmetric stretching mode of the C_α=C_β bond ring in polythiophene, as well described in the literature (Hernández et al., 1996; Paternò et al., 2017; Yu et al., 2012). The peaks at 1480 cm^{-1}, 1460 cm^{-1} and 1430 cm^{-1} can be referred to the symmetric C_α=C_β stretching in the different configurations and oxidation states of the structures while the bands at 1400 cm^{-1}, 1360 cm^{-1} and 1335 cm^{-1} are related to the C_β-C_β intra-ring stretching (Shi et al., 2002; Yu et al., 2012). Comparing PTh and monomer spectra in this region, it is clear, that a conjugated polymer has been electrosynthesized on the surface. We can also speculate that the regio-random configuration is prominent in the structure, but the presence of a strong and narrow C_β-C_β (1480 cm^{-1}) mode may indicate that more ordered and planar conjugated regions would be present (Tsoi et al., 2011). At lower wavenumbers, other important information can be acquired. The band at 1262 cm^{-1} can be attributed to distorted inter-ring vibrations at the interfaces between quinodic and neutral regions of the polymer (Yu et al., 2012). Signals at 1214 cm^{-1} and 1180 cm^{-1} can be assigned to the combination of inter-ring C_α–$C_{\alpha'}$ symmetric and anti-symmetric stretching, respectively (Hernández et al., 1996; Shi et al., 2002; Yu et al., 2012); the peak at 957 cm^{-1} can be referred to the stretching vibration of $C_ß$–C_δ from $ThCH_2COOH$ monomer (Shi et al., 2002); the band at 871 cm^{-1} (Hernández et al., 1996; Shi et al., 2002) is from the ring C-S anti-symmetric stretching and

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the peaks at 719 cm^{-1}, 700 cm^{-1} and 685 cm^{-1} are related to C-S-C deformation vibrations of the thiophene ring (Paternò et al., 2017; Shi et al., 2002).

These characterizations have not evidenced any signal from polythiophene over-oxidation and consequently sulphur oxidation and thiophene-ring opening. XPS analysis of MWCNT/Au (a) and PTh/MWCNT/Au (b), shown in Figure 4C, confirms the polythiophene preparation and the conclusions above. The peak at 154.4 eV, present in both spectra and related to the silicon 2s from the glass substrate, has been taken as internal reference. In the MWCNT spectrum, two small peaks appear at around 163 eV and 169.6 eV, referring to the thiol functionalization of the carbon nanotubes and to sulphate coming from the glass substrate, since the latter is present in the control spectrum of the pure support (data not shown). In the XPS spectrum of PTh/MWCNT the signal at 164.3 eV is characteristic of the thiophene ring (Fusco et al., 2017; Wang et al., 2016) and a fingerprint of functionalization. No peaks referring to ring-sulphur overoxidation have been found in PTh/MWCNT.

In Figure 4D, a comparison between the UV-Vis spectra of the two monomers and the deposited polythiophene, dissolved in DMSO, is shown. Even though the higher monomer concentrations (20 mM) compared to the polymer one (polythiophene electrodeposited onto one electrode dissolved in 1 mL of solvent), the PTh spectrum presents higher absorbance in the explored wave-length range. In particular, an evident absorption maximum at 325 nm, two convoluted signals at about 480 nm and 520 nm and a fourth weak absorption peak at circa 620 nm appear, referring to π-π^* transitions inside the polymer chains (McCullough, 1998; R. Matthews et al., 2005).

In conclusion, it can be stated that pulsed electropolymerization of $ThCH_2CO_2H$ and $ThOCH_3$ mixtures in an aqueous environment is feasible without the need of surface-active molecules. The resulting polythiophene seems to be of conductive properties without overoxidised sulphur.

3.4 Enzyme electrode behaviour

The bioelectrocatalysis of the immobilised PQQ-GDH has been evaluated first with respect to the influence of the measuring buffer composition at a fixed pH value. Figure S7 shows that the catalytic current output is not dependent on the concentration or the kind of buffer used. This can be considered as a clear advantage since this means variations in the solution composition can be tolerated with this sensing electrode, which will be beneficial for measurements of real samples. For analysis of the concentration dependence, the response of

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the PQQ-GDH/PTh/MWCNT/Au electrode has been studied chronoamperometrically by fixing the potential and adding different glucose concentrations (Fig. 5A). Since PQQ-GDH is in DET with the polythiophene modified electrode, it is possible to detect the glucose- related anodic current at very low applied potential (0 V vs. Ag/AgCl). The response of the developed enzyme electrode after substrate additions is rather fast ($t_{90\%}$= ~ 15 s), with a dynamic range between 1 µM (LOD) and 2 mM glucose and a linear range between 50 µM and 500 µM glucose (sensitivity 246 ± 4 µA/(cm^2 mM), R^2 = 0.999). It has to be emphasized here that the error bars in Figure 5B are not originated from repeated measurements, but from the characterisation of three independently prepared electrodes. Thus, they demonstrate the present status of reproducibility in preparation.

The PQQ-GDH/PTh/MWCNT/Au electrode shows the classical Michaelis Menten-type behaviour and the characteristic parameters have been extracted from fit curves of the experimental data points (K_M^{app} = 0.74 ± 0.08 mM and J^{Max} = 340 ± 80 µA/cm^2). Kinetic and analytical data are summarized in Table S3. When these data are compared with those obtained for a similar PQQ-GDH polythiophene modified MWCNT/Au electrode in which the polymer has been electrosynthesized in acetonitrile solution (Fusco et al., 2017), the clear advantage of the preparation of the bioelectrocatalytic interface described in the present study becomes obvious. The aqueous polymerization allows to significantly increase the maximum current density and to extend the dynamic response range towards higher glucose concentrations. This indicates a much better electron withdrawal from the substrate-reduced enzyme. The K_M values for free diffusing PQQ-GDH are varying strongly with the electron acceptor used from 1 mM (determined by our supplier with a p-nitrosoaniline derivative, see *Enzymes*) to 25 mM (Matsushita et al., 1989; Sode et al., 2000; Tsujimura et al., 2008). The K_M^{app} value obtained here is more in the range as for other PQQ-GDH enzyme electrodes (Flexer et al., 2011; Gladisch et al., 2016; Razumiene et al., 2006).

The low potential required to detect the enzyme substrate is advantageous to avoid interferences from electro-active species during the measurement. In fact, the current responses to 50 µM uric acid (UA) and 50 µM ascorbic acid (AA) are less than 0.1% and 10.5%, respectively, related to the anodic current detected towards 100 µM glucose (Fig. S8). Considering the physiological concentrations of these substances in blood and the ratio between each of them and glucose ([UA]/[Glucose] = 0.1, [AA]/[Glucose] = 0.01, the interferences due to the presence of uric and ascorbic acid in solution would be negligible for practical application.

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Finally, the stability of the PQQ-GDH onto the polythiophene - MWCNT interface has been tested by analysing the glucose response day by day (Fig. 5C). After 15 days 70% of the signal strength from the first day of measurement can be retained. Furthermore, the signal stability during the day has been measured (Fig. S9). Overall it can be stated that for the situation of an enzyme covalently coupled to the surface (with a loosely bound cofactor) a sufficient stability can be achieved.

3.5 Biofuel cell application

Due to the above described performance parameters, in particular the low starting potential and the current density of the bioelectrocatalysis, this enzyme electrode can be used as bioanode in a biofuel cell. For this purpose, PQQ-GDH/PTh/MWCNT/Au electrode has been coupled with a well characterized BOD- and MWCNT-based biocathode (see *Enzyme electrode preparation*). The oxygen insensitivity of PQQ-GDH allows to perform the measurements without the presence of a membrane between the two half cells. In agreement with the redox potential of PQQ within the enzyme (Sato et al., 2001), the open circuit potential (OCP) of the bioanode in presence of 5 mM glucose has been measured as around -220 mV vs. Ag/AgCl. This rather low value reflects the efficient contact between the enzyme and the electrode and seems to be even slightly lower as values reported for other enzyme electrodes based on PQQ-GDH (Gladisch et al., 2016; Göbel et al., 2016; Sarauli et al., 2014b; Schubart et al., 2012; Zayats et al., 2005).

After the assembly of the whole cell, the open circuit voltage (OCV) has been assessed as about 780 mV. It can be clearly explained as the sum of cathode (~ 560 mV vs. Ag/AgCl) and anode potential (Fig. 6A). The biofuel cell power density maximum has been evaluated by measurements with increasing currents flowing through the cell as 53+5 $\mu W/cm^2$ at a potential of around 540 mV (Fig. 6B). They demonstrate the clear potential of this kind of enzyme-electrode contact.

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4. Conclusions

Effective electrosynthesis of a polythiophene copolymer, equipped with methoxy- and carboxy- groups, can be demonstrated in an aqueous solution on top of a carbon nanotube modified electrode. For the polymer preparation no surfactants are needed, but a fine distribution of the monomers has to be ensured by sonication. Advantageously for the

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coupling with biomolecules here no organic solvents have to be applied. Furthermore, the prepared polythiophene is of conducting character and does not possess overoxidized sulphur. It can be used subsequently for the preparation of enzyme electrodes as exemplified with PQQ-GDH allowing efficient electron transfer from the substrate-reduced enzyme towards the electrode.

In order to maximize the bioelectrocatalytic current output after covalent PQQ-GDH coupling a potentiostatic pulse method has been used for the polymer deposition and various parameters have been evaluated. SEM pictures show that the electrode coverage is quite homogeneous and the polymerization seems to occur inside the monomer droplets in a nanometer scale. The polymer chemical features are confirmed by EDX, FT-IR, UV-Vis, XPS and Raman spectroscopy. After the enzyme coupling, the PQQ-GDH bioelectrocatalysis in presence of glucose starts at low potential (-0.2 V vs. Ag/AgCl) reaching already high current values at 0 V vs. Ag/AgCl. The low potential is also beneficial to avoid interferences from easily oxidizable substances as uric acid and ascorbic acid. The dynamic range for glucose detection is between 1 μM (LOD) and 2 mM, here the current follows the Michaelis-Menten kinetics. The PQQ-GDH electrode has been used as a bioanode within a biofuel cell (coupled to a BOD-based cathode), reaching a power density maximum of about 53 $\mu W/cm^2$.

The results obtained within this study demonstrate the great potential in establishing electrode-enzyme contacts by the use of conducting polymers. The performance of the developed PQQ-GDH/polythiophene electrode may be a good starting point for further investigations of this kind of polymer. This can be directed to more fundamental studies enlightening the mechanisms of ET or to polymer interactions with other enzymes in order to employ them in building up biosensors and biofuel cells.

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Conflicts of interest

There are no conflicts to declare

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Figures:

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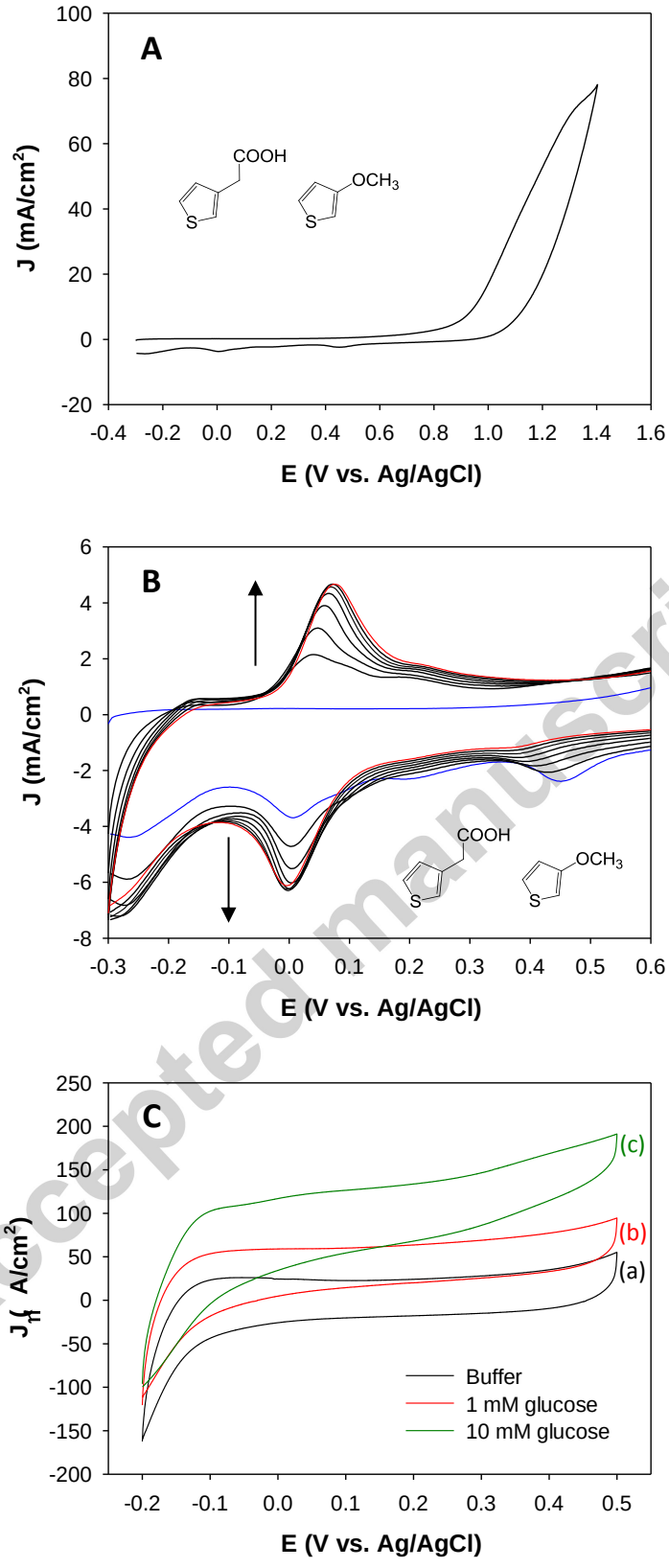

Figure 1. Polythiophene electropolymerization by cyclic voltammetry (scan rate 100mV/s) in 5 mM MES buffer solution (1 mM $CaCl_2$, pH 6.5, 100 mM KCl) of 6.7 mM $ThCH_2CO_2H$, 13.3 mM $ThOCH_3$ at MWCNT-modified gold electrode. First CV scan in the entire potential range (**A**) and a selected potential window of the first eight CV scans (**B**), 1st scan blue, 8th scan red) are shown. (**C**) Cyclic voltammograms of a PQQ-GDH/PTh/MWCNT/Au electrode performed in the absence (a, black curve) and in the presence of 1 mM

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glucose (b, red curve) or 10 mM glucose (c, green curve) in 5mM MES buffer, 1 mM $CaCl_2$, pH 6.5, scan rate 10 mV/s. Polythiophene was electrosynthesized by 20 CV scans (100 mV/s) between - 0.3V and 1.4 V in 5 mM MES solution (1 mM $CaCl_2$, pH 6.5, 100 mM KCl) of of 6.7 mM $ThCH_2CO_2H$, 13.3 mM $ThOCH_3$.

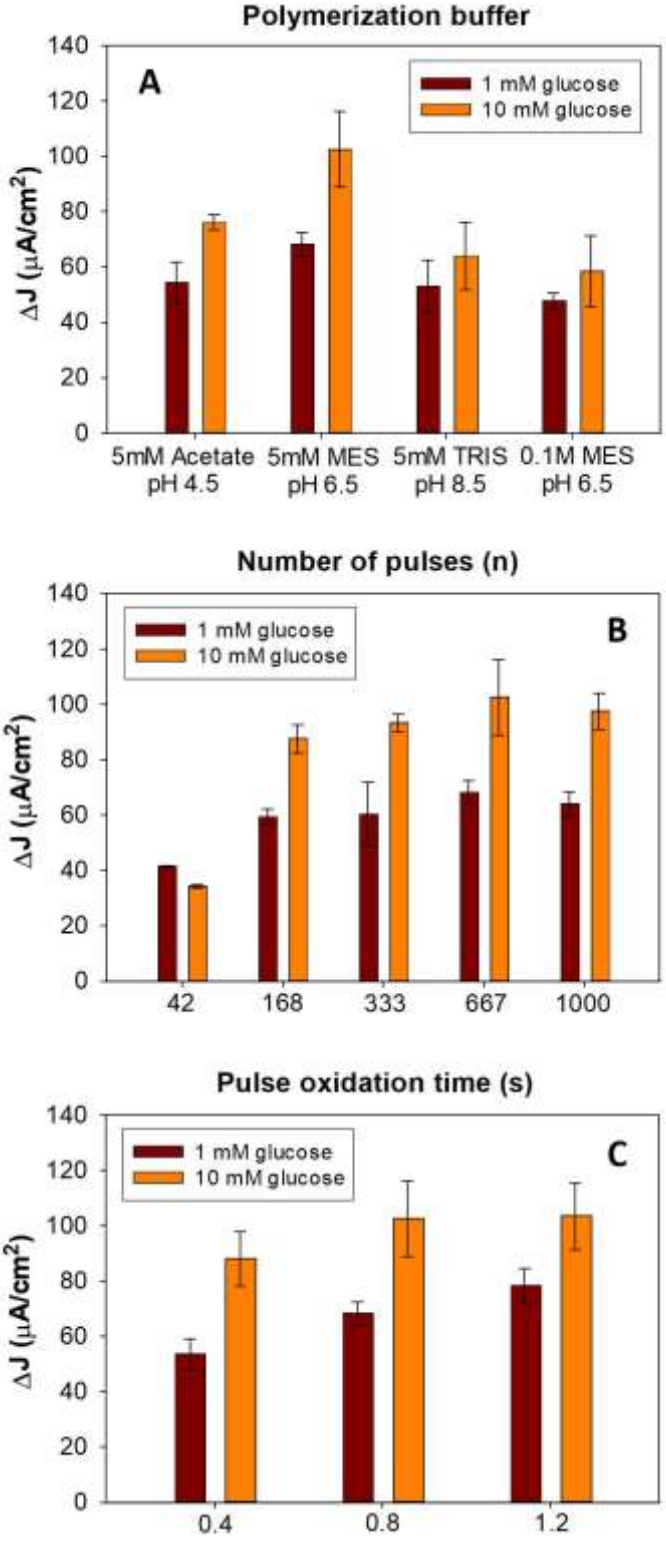

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Figure 2. Catalytic current response of PQQ-GDH/PTh/MWCNT/Au electrodes in dependence of the preparation conditions of the polythiophene layer. Catalytic currents were measured by cyclic voltammetry between -0.2 V and 0.5 V (10 mV/s) in the presence of 1 mM glucose (red) or 10 mM glucose (orange) in 5 mM MES buffer, 1 mM $CaCl_2$, pH 6.5, after coupling of 3 mg/mL PQQ-GDH. The catalytic current was evaluated at 0 V vs. Ag/AgCl. Different parameters are evaluated for electropolymerizing pulse method: (**A**) polymerization buffer, fixing E_{ox} = 1.4V, t_{ox} = 0.8s, E_{red}= - 0.3V, t_{red} = 0.2s, 667 pulses; (**B**) number of pulses, fixing E_{ox} = 1.4V, t_{ox} = 0.8s, E_{red}= - 0.3V, t_{red} = 0.2s; (**C**) pulse oxidation time (t_{ox}), fixing E_{ox} = 1.4 V, E_{red}= - 0.3V, t_{red} = 0.2s, 667 pulses. Electropolymerizations are performed (when not differently specified) in 5 mM MES solution (1 mM $CaCl_2$, pH 6.5, 100 mM KCl) of 6.7 mM $ThCH_2CO_2H$ and 13.3 mM $ThOCH_3$. Error bars refer to the standard deviations calculated from three independent experiments.

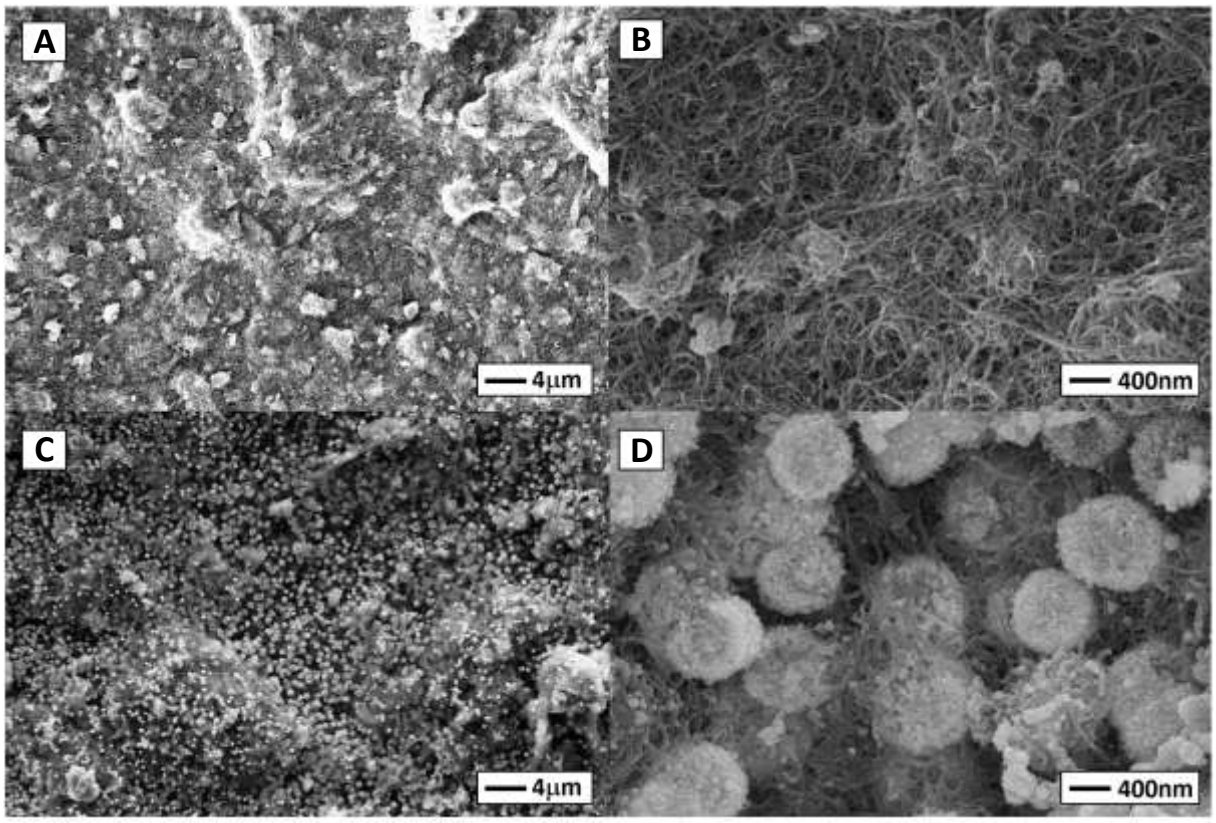

Figure 3. SEM pictures of (**A**-**B**) bare MWCNT/Au electrode and (**C**-**D**) PTh/MWCNT/Au electrode. Polythiophene copolymer was synthesized in 5 mM MES solution (1 mM $CaCl_2$, pH 6.5, 100 mM KCl) of 6.7 mM $ThCH_2CO_2H$ and 13.3 mM $ThOCH_3$ by applying 667 pulses between 1.4 V (0.8 s) and - 0.3 V (0.2 s) at MWCNT modified gold electrodes. Pictures A and C has been acquired with a magnification of 5000× while B and D with a magnification of 50000×.

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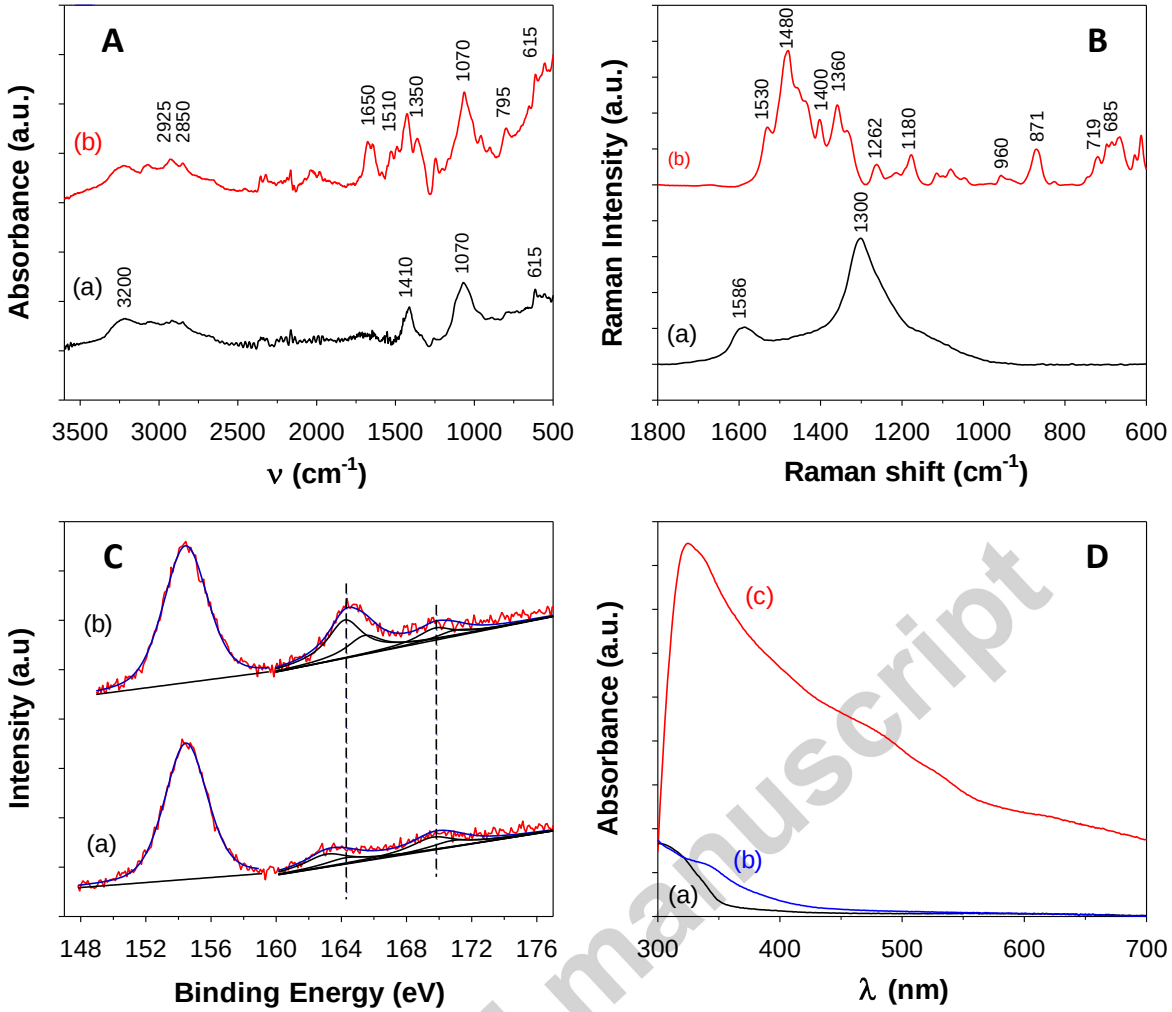

Figure 4. Spectrochemical characterization of the polythiophene-modified and unmodified electrode surfaces: (**A**) FT-IR spectra of bare MWCNT surface (a, black) and polymer-modified MWCNT surface (b, red); (**B**) Raman of bare MWCNT surface (a, black) and polymer-modified MWCNT surface (b, red); (**C**) XPS spectra of bare MWCNT surface (a) and polymer-modified MWCNT surface (b): experimental curves in red, fitting curves in blue and deconvoluted curves in black; (**D**) UV-Vis spectra of 20 mM $ThCH_2COOH$ (a, black), 20 mM $ThOCH_3$ (b, blue) in DMSO and of the polythiophene derivative dissolved in DMSO after the electropolymerizationon process (c, red). Polythiophene copolymer was synthesized in 5 mM MES solution (1 mM $CaCl_2$, pH 6.5, 100 mM KCl) of 6.7 mM $ThCH_2CO_2H$ and 13.3 mM $ThOCH_3$ by applying 667 pulses between 1.4 V (0.8 s) and - 0.3 V (0.2 s) at MWCNT modified gold electrodes.

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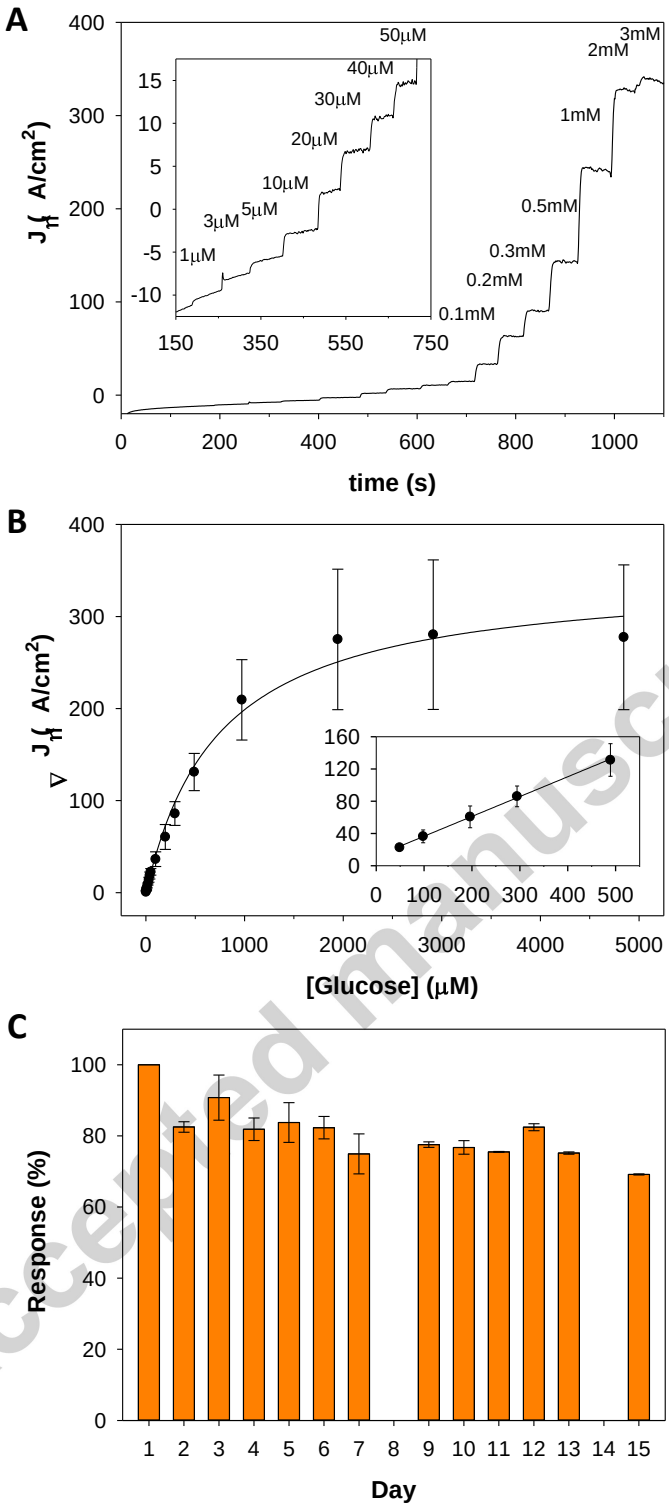

Figure 5. Chronoamperometric experiment with PQQ-GDH/PTh/MWCNT/Au electrodes performed at a fixed potential of 0 V vs. Ag/AgCl (**A**) by measuring the anodic current density by additions of glucose to the stirred 100 mM CiP solution (+1 mM $CaCl_2$, pH 6.5) and (**B**) fitting of the experimental data with the Michaelis Menten equation. (**C**) PQQ-GDH/PTh/MWCNT/Au response after successive chronoamperometric experiments performed at a fixed potential of 0 V vs. Ag/AgCl by measuring the anodic current density with additions of 100 μM glucose to the stirred 100 mM CiP solution (+1 mM $CaCl_2$, pH 6.5) day by day. Polythiophene copolymer has been synthesized in 5 mM MES solution (1 mM $CaCl_2$, pH 6.5, 100 mM KCl) of 6.7 mM $ThCH_2CO_2H$ and 13.3 mM $ThOCH_3$ by applying 667 pulses between 1.4 V (0.8 s) and - 0.3 V (0.2 s) at MWCNT modified gold electrodes. Error bars refer to the standard deviations calculated from three independent experiments.

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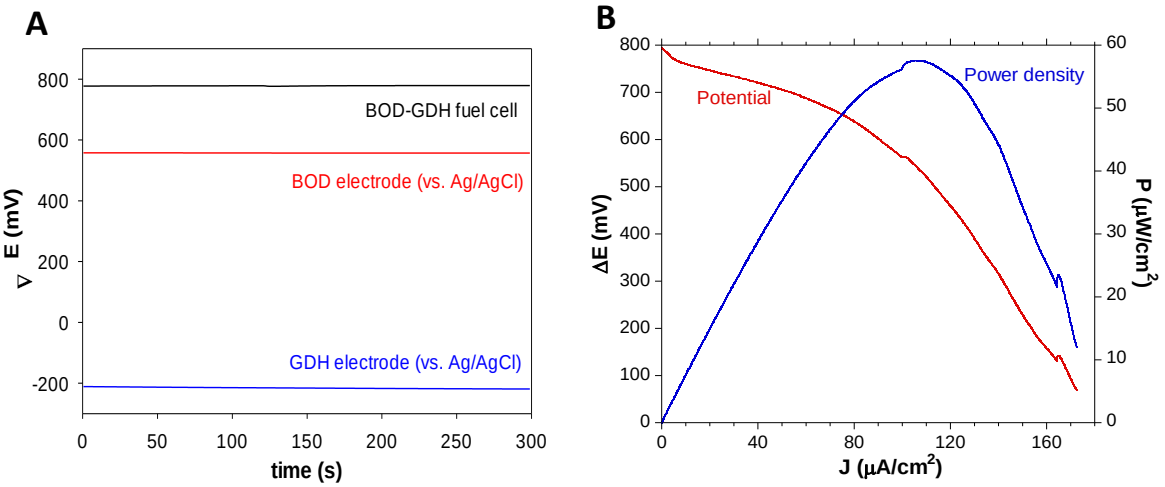

Figure 6. Biofuel cell performance. (**A**) Open circuit potential (vs. Ag/AgCl) measurements of PQQ-GDH/PTh/MWCNT/Au anode (blue, OCP = - 217 ± 4 mV), BOD/PQQ/MWCNT/Au cathode (red, OCP = + 559 ± 4 mV) and the open circuit voltage of the whole biofuel cell (black, OCV = 779 ± 7 mV); (**B**) Biofuel cell power density (blue) and polarization (red) curves (determined by galvanodynamic experiments at 2 nA/s). Power density maximum = 53 ± 4 μW/cm^2; Voltage at power density maximum = 539 ± 14 mV. Measuring conditions: 5 mM glucose, air saturated 100 mM CiP buffer, 1 mM $CaCl_2$, pH 6.5. Errors refer to standard deviations from measurements performed with three different couples of electrodes.

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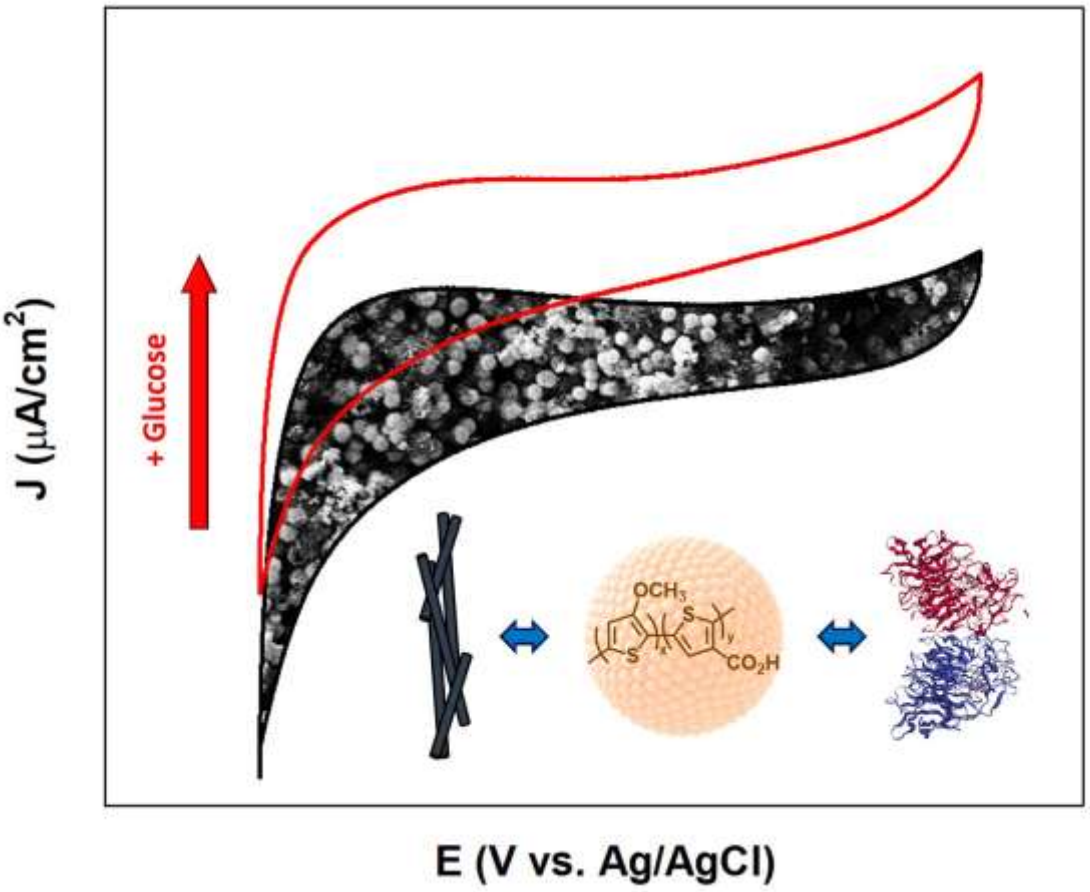
Graphical Abstract:



Highlights

- Polythiophene can be electrosynthesized from an aqueous solution without surfactants
- Electrosynthesis occurs inside the monomer droplets resulting in a nanoscaled polymer
- Avoiding organic solvents improves the activity of the coupled biomolecules
- PQQ-GDH is efficiently wired by the polymer to the MWCNT electrode
- Besides sensing, the PQQ-GDH electrode can be used as bioanode within a biofuel cell

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