Mussel Inspired Electro-cross-linking of Enzyme for the Development of Biosensor

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Abstract

In medical diagnosis and environmental-monitoring, enzymatic biosensors are widely applied due to their high sensitivity, potential selectivity, and their possibility of miniaturization/automation. Enzyme immobilization is a critical process in the development of this type of biosensors with the necessity to avoid the denaturation of the enzymes and ensuring their accessibility towards the analyte. Electrodeposition of macromolecules is increasingly considered the most suitable method for the design of biosensors. Simple and attractive, it finely controls the immobilization of enzymes on electrode surfaces, usually by entrapment or adsorption, using an electrical stimulus. Performed manually, enzyme immobilization by cross-linking prevents enzyme leaching and was never done using an electrochemical stimulus. In this work, we present a mussel-inspired electro-cross-linking process using Glucose Oxidase (GOX) and a homobifunctionalized catechol ethylene oxide spacer as cross-linker in the presence of ferrocene methanol (FC) acting as mediator of the buildup. Performed in one pot, the process takes place in three steps: (i) electro-oxidation of FC, by application of cyclic voltammetry, creating a gradient of ferrocenium (FC+), (ii) oxidation of bis-catechol into bis-quinone molecule by reaction with the electrogenerated FC+ and (iii) chemical reaction of bis-quinone with free amino moieties of GOX through Michael addition and a Schiff's base condensation reaction. Employed for the design of a second generation glucose biosensor using ferrocene methanol as mediator, this new enzyme immobilization process presents several advantages. The cross-linked enzymatic film (i) is obtained in a one pot process with non-modified GOX, (ii) is strongly linked to the metallic electrode surface thanks to catechol moieties and (iii) presents no leakage issues. The developed GOX/bis-catechol film shows a good response to glucose with a quite wide linear range from 1.0 to 12.5 mM as well as a good sensitivity (0.66 µA/mM.cm^2) and a

high selectivity to glucose. These films enable to distinguish between healthy (3.8 and 6.5 mM) and hyperglycemic subjects (> 7 mM). Finally, we show that this electro-cross-linking process allows the development of miniaturized biosensors through functionalization of a single electrode out of a microelectrode array. Elegant and versatile, this electro-cross-linking process can also be used for the development of enzymatic biofuel cells.

Introduction

In the recent years, an increasing interest in the integration of biomolecules onto electronic platforms was observed in order to create functional bioelectronics devices. One of the main challenge is the development of simple and low-cost analytical methods for biomarker detection. [1,2] In biomedical applications, enzymes are commonly used as recognition elements due to their high specificity, selectivity and catalyst efficiency at physiological conditions.[3–5] Thus, main research is focused on the development of enzymatic biosensors based on novel architectures incorporating enzymes able to transduce biorecognition as electric output signals. Enzymatic biosensors are a combination of an electrochemical probe (amperometric, potentiometric, or conductimetric) with a thin layer of an active immobilized enzyme.[6,7] An extensive variety of redox enzymes, such as glucose oxidase (GOX),[8] horseradish peroxidase,[9] lactate oxidase,[10] alcohol dehydrogenase,[11] aldehyde dehydrogenase[12] or urease,[13] has been used for the elaboration of glucose, H_2O_2, lactate, ethanol, aldehyde or urea biosensors, respectively. Micro-enzyme based biosensors have been also developed for short term brain application in animal models to monitor neurotransmitters, such as glutamate or choline, based on immobilized glutamate or choline oxidase, respectively.[14] GOX is the most common enzyme used owing its importance for detection of blood glucose[15] and its effectiveness in the diagnostic analysis of diabetes. A representative case of success is the glucometer with test strips. However, the lack of accuracy of these systems is still a major concern.[16,17] Low accuracy of the fabrication protocols, high sensitivity of enzymes to the immobilization protocol or to the environmental factors can affect the reliability and reproducibility of glucose measurements. Enzyme immobilization is thus a critical process in biosensors development with the necessity to avoid their denaturation.

A variety of techniques has been implemented for the immobilization of enzymes onto solid surfaces, including adsorption, entrapment, covalent binding, cross-linking or affinity.[18–22] Each immobilization method presents advantages and drawbacks. Well-studied, enzyme adsorption and entrapment present enzyme leaking issues. Covalent binding and cross-linking are known for enzyme stability but decrease its activity after immobilization. Electrodeposition of macromolecules is increasingly considered the most suitable method for the creation of biosensors.[23] This simple and attractive bottom up approach finely controls the immobilization of enzymes on electrode surfaces, usually by entrapment or adsorption, using an electrical stimulus. Up to now, three types of electrodeposition were developed to immobilize enzymes: (i) by changing the solubility of polyelectrolytes or enzymes,[24–27] (ii) by polyelectrolytes self-assembly based on electrostatic/ionic interactions,[28–31] and (iii) by electropolymerization of monomers.[32,33] No electro-cross-linking processes applied on enzymes were developed so far. Indeed, enzyme immobilization by cross-linking is usually performed manually by drop- or dip coating of liquid enzyme preparations containing suitable cross-linker, such as glutaraldehyde. Non-manual procedures offer better reproducibility and better control of the immobilization process, especially for the development of miniaturized biosensors through the functionalization of single electrodes out of an assorted microelectrode array.

In the field of electrodeposition of macromolecules, we introduced a new concept based on electro-cross-linking between two polymers in one pot, named the morphogenic electrotriggered self-construction of films. A morphogen is a molecule or an ion generated locally to create a gradient and inducing a chemical reaction or interaction between two species. Cu(I) being the morphogen, our group used Sharpless–Huisgen click reaction between azide and alkyne to obtain different polymeric films.[34] Recently, we developed the electro-cross-linking of

polyamines based on mussel-inspired chemistry using a homobifunctional catechol ethylene oxide molecule, named bis-catechol having the role of morphogen.[35] Indeed, the exceptional ability of mussels to adhere on almost any type of surfaces is based on catechol biochemistry (*i.e.* hydrogen bonds, metal-ligand complexes and covalent bond formation). In particular after the oxidation of catechol into quinone, these new moieties react with nucleophilic groups, such as amines or thiols, through Michael addition and Schiff's base formation.[36,37]

In the present work, we describe mussel-inspired electrotriggered self-construction of enzymatic films for the development of a glucose biosensor by cross-linking of GOX with bis-catechol on the surface of a gold electrode. An electrochemical mediator, ferrocene methanol (FC), was used as morphogen in the buildup solution. Performed in one pot with a bis-catechol/GOX/FC mixture solution, the process takes place in three steps: (i) electro-oxidation of FC, by application of cyclic voltammetry, creating a gradient of ferrocenium (FC^+), (ii) oxidation of bis-catechol into bis-quinone by reaction with the electrogenerated FC^+ and (iii) chemical reaction of bis-quinone with free amino moieties of GOX through Michael addition and a Schiff's base condensation reaction (Figure 1). Efficiently immobilized with no-leaching out, GOX allows the electrochemical detection of glucose using FC as free mediator in solution with a very good sensitivity and selectivity. Finally, the film buildup, occurring exclusively from the surface, can be performed on microelectrodes. This new synthesized biosensor can be classified as a second-generation glucose sensor.[38]

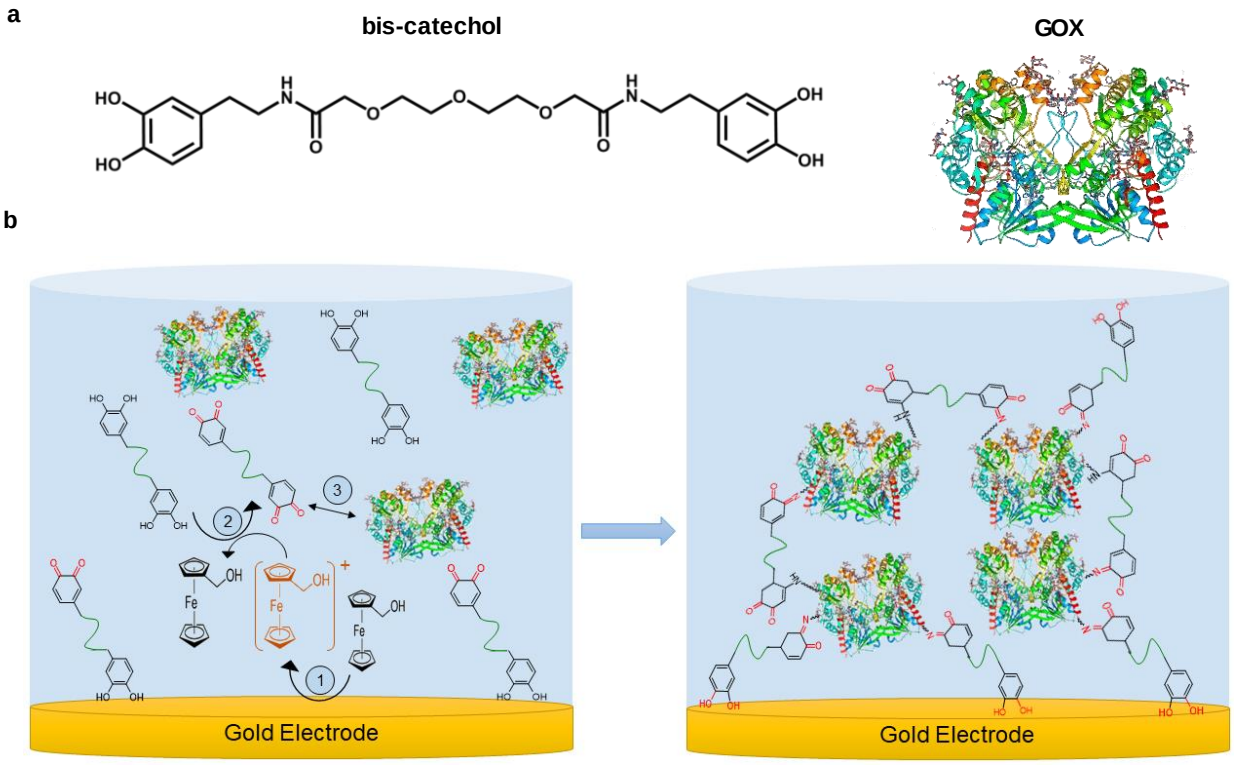


Figure 1: (a) Bis-catechol chemical formulae and GOX quaternary structure (b) Principle of the one pot mussel-inspired electrotriggered self-construction of enzymatic films based on three steps: (1) electro-oxidation of FC, creating a gradient of ferrocenium (FC^+), by application of cyclic voltammetry, (2) oxidation of bis-catechol into bis-quinone molecules by reaction with FC^+ and (3) chemical reaction of bis-quinone with free amino moieties of GOX, through Michael addition and a Schiff's base condensation reaction.

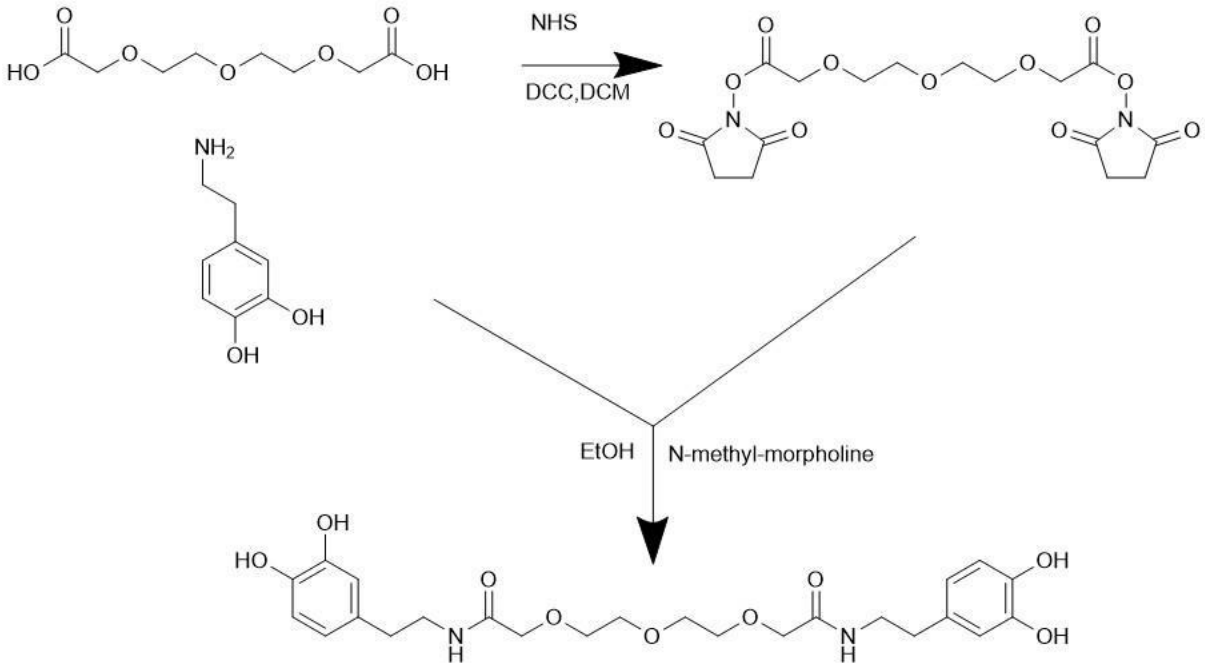
This new enzyme immobilization process presents several advantages: (i) it is a one pot process; (ii) bis-catechol has a strong interaction with gold, allowing a strong stability of the immobilization; (iii) the enzyme is cross-linked avoiding leakage issues; (iv) it is versatile, no chemical modification of the enzymes is needed; (v) electrotriggered, it allows tunable and localized functionalization of electrodes and (vi) composed of phenol moieties, which are known

to be selective towards interferents (notably ascorbate, acetaminophen and uric acid) of clinical significance in amperometric assay.[39,40]

EXPERIMENTAL SECTION

Chemicals. Alkaline Phosphatase (AP from bovine intestinal mucosa, CAS 9001-78-9), Glucose oxidase (GOX from *Aspergillus niger,* CAS 9001-37-0), *para*-nitrophenyl phosphate liquid substrate (pNPP, P7998, CAS 4264-83-9), salicylic acid (SA, M = 138.12 g/mol, CAS 69-72-7), ascorbic acid (AA, M = 176.12 g/mol, CAS 50-81-7) sodium nitrate ($NaNO_3$, M = 84.99 g/mol, CAS 7631-99-4), potassium hexacyanoferrate(II) (M = 422.41 g/mol, CAS 14459-95-1), o–dianisidine peroxidase (M = 244.29 g/mol, CAS 119-90-4), glucose (M = 180.16 g/mol, CAS 50-99-7), phosphate buffered saline tablet (PBS, P4417), tris(hydroxymethyl)aminomethane (Tris, M = 121.14 g/mol, CAS 77-86-1) and ferrocene methanol (FC, M = 216.06 g/mol, CAS 1273-86-5) were purchased from Sigma-Aldrich. Dopamine hydrochloride was purchased from Aldrich. TUDA was purchased from Iris Biotech. All dried solvents were purchased from Acros Organics. Acetaminophen (AP, M = 151.17 g/mol, CAS 103-90-2) and uric acid (UA, M = 168.11 g/mol, CAS 69-93-2) were purchased from Alfa Aesar. All chemicals were used as received and dissolved in aqueous solution using MilliQ water (resistivity of 18.2 MΩ.cm at 25 °C). Phosphate buffer solution was prepared at 150 mM phosphate, 2.06 M NaCl and 0.041 KCl and adjusted at pH 7.4.

Synthesis of bis-catechol. A two steps procedure was used to prepare bis-catechol from dopamine (CAS 62-31-7, Sigma) and 3,6,9-trioxaundecandioic acid (TUDA, CAS 13887-98-4, Iris Biotech) (Scheme 1). ^{1}H NMR spectra were recorded on Bruker Advance DPX400 (400 MHz) spectrometers.



Scheme 1 : Synthetic route for the preparation of bis-catechol in two steps.

N,N-succinimide trioxaundecanediammide. 3,6,9-trioxaundecandioic acid (TUDA, 0.99 g, 4.46 mmol, 1.0 eq.) was mixed with 5.31 g of molecular sieves in CH_2Cl_2 (20 mL). DCC (3.27 g, 16.19 mmol, 3.6 eq.) and NHS (1.83 g, 15.91 mmol, 3.6 eq.) were added and the mixture was stirred overnight. The mixture was filtered over celite. The volume of the solution was reduced under vacuum. Cold Et_2O was then added to induce the precipitation of the product and the mixture was stored in a cold medium overnight. After filtration, the precipitate was purified with flash chromatography (eluent DCM) affording of solid white product (0.28 g, 15 %). ^{1}H NMR (MeOD-d^4, 400 MHz) δ 2.83 (s, 8H) 3.79 (m, 4H) 3.78 (m, 4H) 4.52 (s, 4H).

Bis-catechol. N,N-succinimide trioxaundecanediammide (0.28 g, 0.67 mmol, 1 eq.) was dissolved in 5 ml of chloroform. Then dopamine hydrochloride (0.25 g, 1.34 mmol, 2 eq.), dissolved in 5 ml of EtOH, was added followed by N-methylmorpholine (0.40 ml, 3.35 mmol, 5 eq.) and the solution was stirred for 48 h at RT. The solvent was evaporated, and the residual

solid was purified by flash chromatography eluting with CH_2Cl_2/MeOH (100:0 to 97:3) affording 0.25 g of the desired biscatechol (492.52 g/mol, 70 %). This products was found identical to the biscatechol previously characterized in literature.[35]

Synthesis of Rhodamine labeled GOX. 100 mg of the GOX was dissolved in 80 mL of a solution of Na_2CO_3 (0.1 M, pH 8.5) and stirred at 4°C for 1 h. 340 µL of rhodamine B isocyanate solution (1.5 mg in 0.7 mL of DMSO) was added to the GOX solution and remained stirred at 4°C for 4 h. Rhodamine labeled GOX (GOX^{Rho}) was purified by first dialyzing it (ZelluTrans dialysis membranes cut off 12000-16000 g/mol) overnight in a solution of 0.25 M of NaCl and then in pure water for several days.

Electrochemical Quartz Crystal Microbalance (EC-QCM) with Dissipation Monitoring. Q-Sense E1 apparatus from Q-Sense AB (Gothenburg, Sweden) was used to perform the electrochemical quartz microbalance (EC-QCM) experiments by measuring the resonance frequency f_v and the dissipation factor D_v at ν = first, third, fifth, and seventh overtones, corresponding to 5, 15, 25, and 35 MHz after the excitation of the quartz crystal at its fundamental frequency (5 MHz). Only the third overtone at 15 MHz is presented. CHI660E apparatus (CH instrument, Austin, Texas) was coupled on the QCM-D apparatus with a three electrode system to perform the electrochemical measurements. The gold-coated QCM sensor acted as working electrode, a platinum electrode as a counter electrode on the top wall of the chamber and a no-leak Ag/AgCl as a reference electrode fixed in the outlet flow channel. Before any buildup, the quality of the EC-QCM cell was tested by measuring the capacitive current in the presence of phosphate buffer and the faradic current in the presence of 1 mM of potassium hexacyanoferrate (II) prepared in phosphate buffer by applying 5 cycles between 0 and 0.7 V (vs Ag/AgCl) at 0.05 V/s.

Film buildup procedure. Enzyme/bis-catechol/FC mixture solution was prepared at 1 mg/mL GOX (6.25 mM), 3 mg/mL (6.1 mM) bis-catechol and 0.5 mM FC in phosphate buffer at pH 7.4. To prevent the oxidation of bis-catechol, nitrogen was flushed in the prepared solutions. After the stabilization of the QCM signal with the buffer solution, 600 µL of a mixture of bis-catechol, GOX and FC were injected in the cell at a flow rate of 600 µL/min with a peristaltic pump. To trigger the electro-crosslinking of the enzyme, a cyclic voltammetry was applied between 0.0 and 0.7 V (vs Ag/AgCl, scan rate 0.05 V/s). To stop the film buildup, a rinsing step with phosphate buffer was performed at a flow rate of 600 µL/min until stabilization of the QCM signal. The coated QCM sensor was then kept in the cell for electrochemical characterization of the enzymatic activity or un-mounted and stored into buffer solution for AFM or colorimetric characterizations.

Colorimetric enzymatic activity of GOX/bis-catechol films. A microplate reader (Xenius XC, SAFAS, Monaco) was used to determine the catalytic activity of the enzymes within the films using *o*-dianisidine assay. The films were disposed in a 24 wells plate with 1 mL of a solution containing glucose (1 mg/mL), HRP (1 mg/mL) and *o*-dianisidine (10^{-3} M) prepared in 150 mM $NaNO_3$-10 mM Tris buffer at pH = 8.0. GOX's activity was monitored by using a second enzyme, HRP, which uses the H_2O_2 produced by GOX enzymatic reaction, in the presence of glucose, to react with *o*-dianisidine (colourless) to obtain oxidized *o*-dianisidine, which is brown. The reaction was followed at 440 nm.

Electrochemical enzymatic activity of GOX/bis-catechol films. All measurements were carried out on a CHI 660B electrochemical workstation (CH Instruments, USA). The same electrode set-up used during the electrodeposition, was used for the electrochemical performance study. The gold electrode was chosen as the working electrode. Cyclic voltammetry measurements were

performed by injecting 600 µL of solutions of different concentrations of glucose at 1 mL/min in the absence and in the presence of a mediator (ferrocene methanol, FC). The chronoamperometric measurements of glucose were performed in Argon saturated solutions at constant potential of 0.25 V (vs Ag/AgCl). The current density was calculated using a surface area of 0.8 cm^2 which is the area of QCM sensor exposed to the solution.[41]

Atomic Force Microscopy. The enzymatic films built on QCM crystals were characterized by AFM in contact mode and liquid state using Nanoscope IV from Veeco (Santa Barbara, CA). Deflection and height images were scanned at a fixed scan rate (1 Hz) with a resolution of 512 × 512 pixels using cantilevers (spring constant of 0.03 N/m) with silicon nitride tips (model MSCTAUHW, Veeco). To determine the film thickness by profilometric section analysis, several scratches were achieved using plastic cone tip and imaged perpendicular to the fast scan axis. The film thickness is the minimal z distance between the bare substrate and the surface of the film, which covers the whole substrate. Six areas were imaged to determine the mean and the standard deviation of the film thickness. The film roughness was determined by the RMS value given by the AFM software (NanoScope software version 5.31r1, Digital Instruments, Veeco) on 3 × 3 μm^2 topographic images.

Functionalization of microelectrodes. Immersed in a bis-catechol/GOX^{Rho}/FC mixture (1 mg/mL GOX, 3 mg/mL bis-catechol and 0.5 mM FC in phosphate buffer at pH 7.4 solution), the interdigitated array electrode (IDA ref: A-012125, Biologic) was addressed for 45 min through the application of a CV (between 0.0 and 0.7 V vs Ag/AgCl, scan rate 0.05 V/s) using CHI 660e potentiostat/galvanostat in a 3-electrode cell. The working electrode and the counter electrode were part of the IDA with no-leak Ag/AgCl reference electrode. Coated IDA electrode was

imaged using a station SARFUS IMAGING HR (Nanolane, Le Mans) in bright field and fluorescence mode.

RESULTS AND DISCUSSION

Bis-catechol synthesis and electrochemical characterization. Bis-catechol is a molecule displaying a catechol group at each end of a linear oligoethylene oxide chain. This hydrophilic molecule is stable and water soluble. Unlike our previous study,[35] bis-catechol was prepared in two steps from dopamine and TUDA, both commercially available starting materials (Scheme 1). Briefly, TUDA was first modified by N-hydroxysuccinimide in 15% yield. Then, in the presence of two equivalents of dopamine, the bis-catechol was obtained in 70%. The overall yield of this synthesis was 10%. We first investigated the electrochemical response of bis-catechol and bis-catechol/FC mixture solutions by cyclic voltammetry (CV) in phosphate buffer solution (Figure 2). For bis-catechol solution, the oxidation and reduction peaks were observed at 0.30 V and 0.10 V (*vs* Ag/AgCl), respectively (Figure 2a) which is in good match with the literature.[42,43] In the presence of FC, the redox peaks were slightly shifted at 0.35 and 0.05 V with higher values of intensity measured for the oxidation peak (Figure 2b). In both cases, the intensity of oxidation decreased dramatically with the number of cycles and the intensity of reduction was lower than the one of the oxidation. This is the signature of an irreversible process. Simultaneously, an increase of the normalized frequency shift, measured by QCM and related to a mass deposition, was observed due to the electro-cross-linking of bis-catechol molecules (Figure 2c).

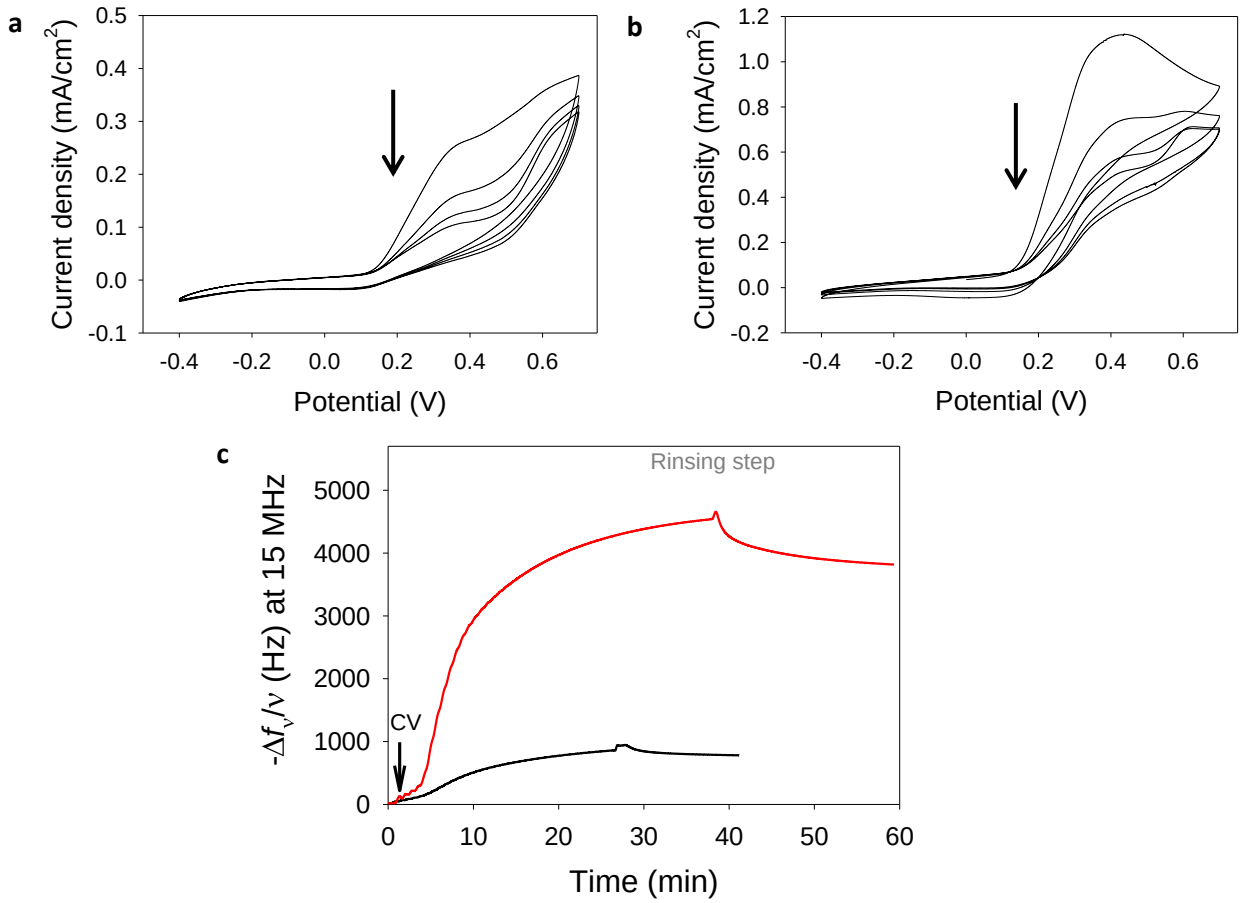


Figure 2: Cyclic voltamogramms, performed at a scan rate of 0.05 V/s, of (a) bis-catechol (6.1 mM) and (b) bis-catechol (6.1 mM)/FC (0.5 mM) mixture solution, the black arrows indicate the evolution of the signal during the CV application and (c) normalized frequency shift as a function of time of bis-catechol (black curve) and bis-catechol/FC mixture (red curve) solutions during CV application (range -0.4 and 0.7 V vs Ag/AgCl, scan rate 0.05 V/s). The supporting electrolyte was phosphate buffer solution at pH 7.4.

A higher increase of the normalized frequency shift was obtained for bis-catechol/FC mixture in comparison to bis-catechol solution, reaching a plateau after the rinsing step at 3800 Hz and 760 Hz, respectively (Figure 2c). This result shows that FC molecules act as a mediator of a chemically irreversible process, favoring the oxidation of bis-catechol into bis-quinone which can further cross-link to deposit a film. This effect was already reported by Kim et al. who found

that FC^+ (oxidized form of FC) can be reduced back to FC by oxidation of grafted catecholic moities.[44] Applying a constant potential of 0.3 V (vs Ag/AgCl) to generate FC^+, a small increase of the normalized frequency shift (420 Hz) was obtained after 50 min (Figure S1 in Supporting Information, SI). In the following, all the film buildup was performed in the presence of FC using CV between -0.4 and 0.7 V (vs Ag/AgCl) with a scan rate of 0.05 V/s.

GOX/bis-catechol film buildup characterizations. Knowing that GOX have 15 on 30 accessible lysine residues for chemical modification,[45] bis-catechol/GOX/FC mixture solutions were prepared in phosphate buffer at pH 7.4 to obtain a catechol/amine ratio of 0.13 to favor the cross-linking of GOX with bis-catechol compared to bis-catechol self-cross-linking. Figure 3a shows the typical buildup of a GOX/bis-catechol film obtained by EC-QCM in the presence of FC.

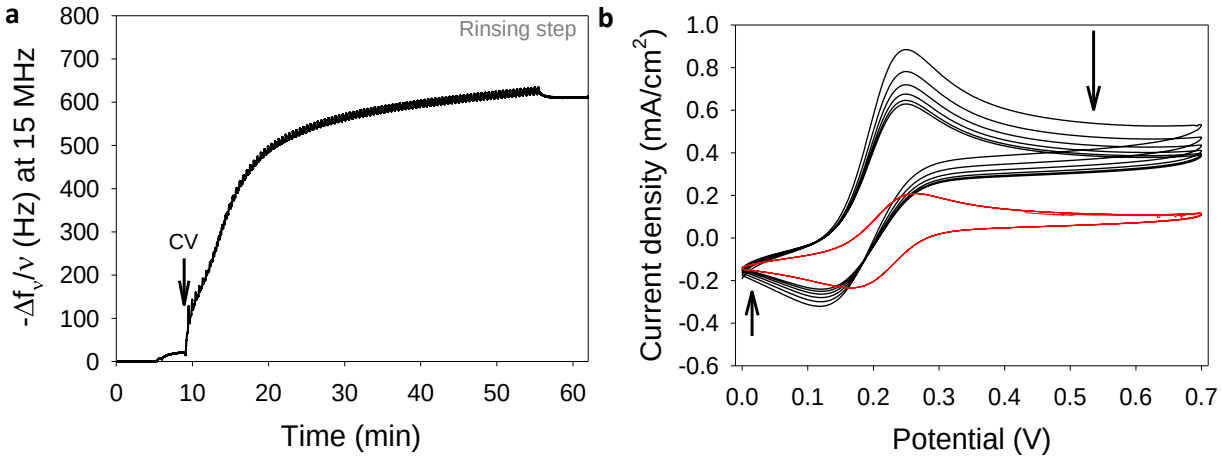


Figure 3. (a) Normalized frequency shift, measured by QCM, as a function of time during the self-construction of GOX/bis-catechol film by application of CV (0.0-0.7 V vs Ag/AgCl, scan rate 0.05 V/s) for 45 min (100 cycles) using bis-catechol (6.25 mM)/GOX (6.1 mM)/FC (0.5 mM) mixture solution in phosphate buffer and (b) measured cyclic voltammograms, (black curve) first five cycles and (red curve) last five cycles, the arrows indicate the evolution of the signal during the self-construction.

A small increase of the normalized frequency was obtained at the injection of the buildup solution due to electrostatic adsorption of GOX and coordination bonding of bis-catechol with gold. A strong increase of the normalized frequency was obtained at the application of CV (range of 0.0 and 0.7, scan rate of 0.05 V/s) followed by a slowdown reaching 610 Hz after 45 min. The oxidation currents decreased dramatically with the number of cycles with a superimposition of the last five cycles (Figure 3b). The oxido-reduction signal was dominated by FC signal. The oxidation peak was observed at 0.25 V and the reduction peak at 0.11 V shifted to 0.17 V at the end of the CV application. In comparison to bis-catechol/FC mixture (Figure 2c), the kinetics of self-construction of bis-catechol/GOX/FC mixture was dramatically slower and the electrochemical signals were clearly different. This indicates that different buildup mechanisms were involved in the two cases.

Topography and thickness of the enzymatic films were determined by AFM in contact mode and liquid state. Figure 4 shows a homogenous morphology of the different films regardless to the deposition time. A scratching of the films was performed to determine their thicknesses. A nice scratched area was difficult to obtain due to the cross-linking of the enzyme and the strong interaction of biscatechol with the gold surface. Thus to ensure a reliable value, the mean thickness of the scratched film was determined by measuring it on at least six different areas. The films covered uniformly the whole substrate with a thickness varying from 55 to 100 nm when the film was built for 15 to 60 min, respectively (Figure 5). The film thickness increased as a function of the current time application, whereas the film roughness (determined on 3×3 μm^2 images) remained constant at about 30 nm until 45 min of self-construction with a little increase at 50 nm for 60 min of buildup.

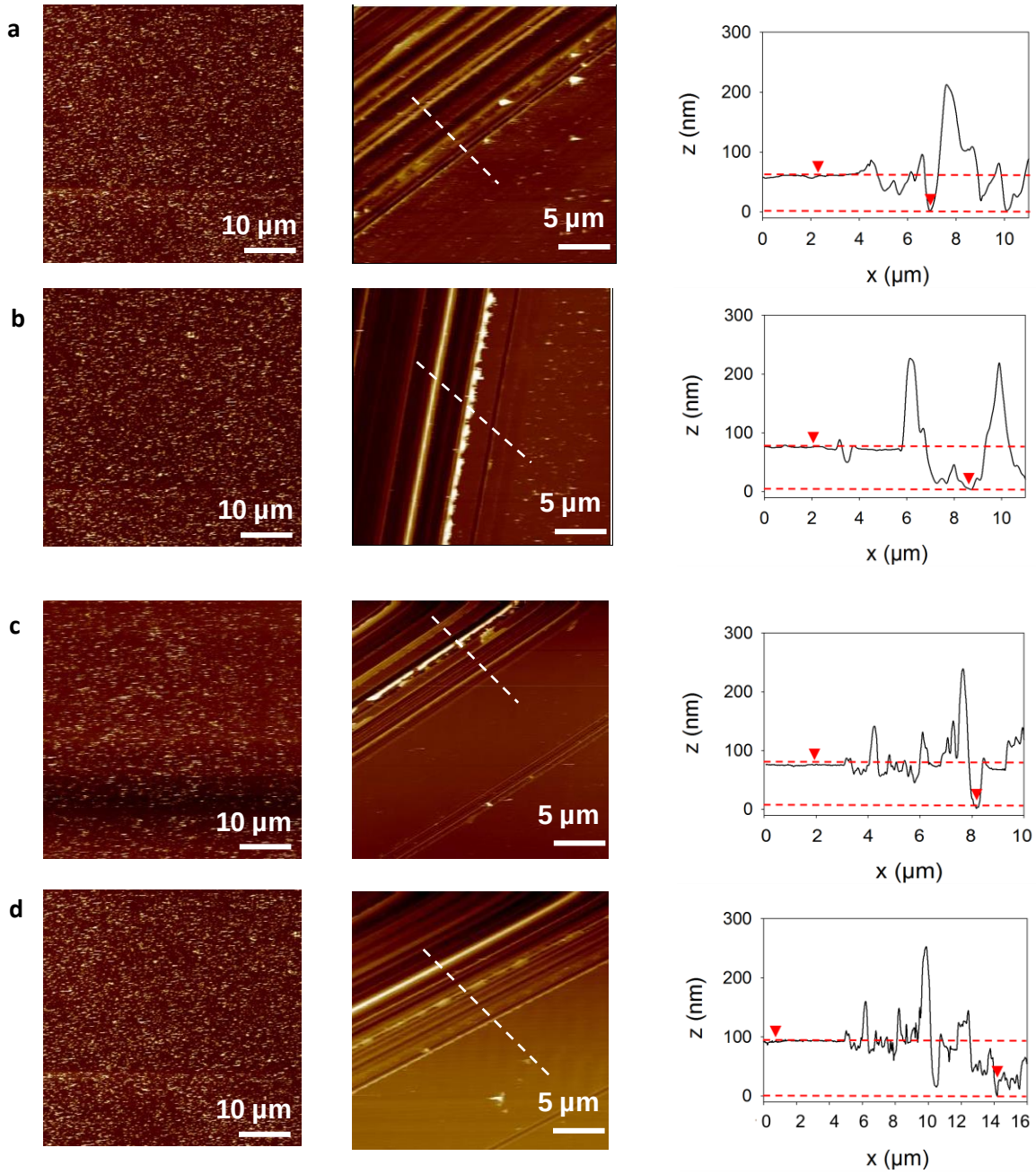


Figure 4. Typical topographic AFM images, obtained in contact mode and liquid state, before (50 × 50 µm^2, *z*-scale = 400 nm), and after scratching (25 × 25 µm^2, z-scale = 300 nm) with cross-section profiles of GOX/bis-catechol film obtained after (a) 15, (b) 30, and (c) 45 min (d) 60 min of buildup using conditions of Figure 3. The white dotted bars represent the cross-section area.

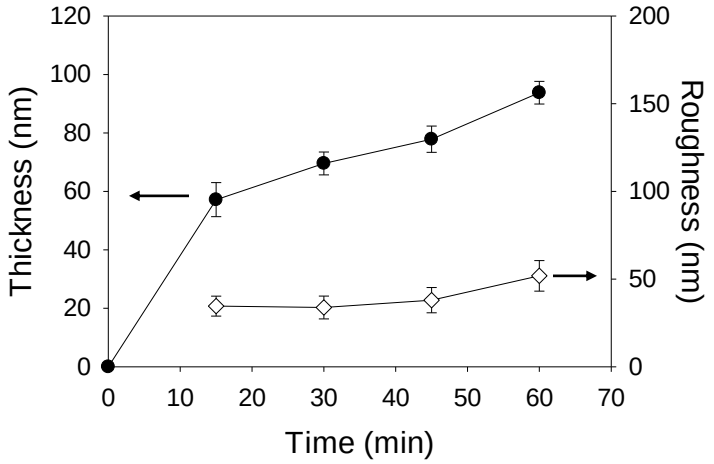


Figure 5. Thickness (●) and roughness (calculated on a 3 × 3 μm^2 AFM images) (◊) of self-constructed GOX/bis-catechol film, measured by AFM in contact mode and liquid state, as a function of self-construction time using conditions of Figure 3. The lines are only drawn to guide the eye.

The enzymatic activity of GOX/bis-catechol film was first investigated using a standard colorimetric test for different electrodeposition times going from 15 to 60 min. The enzymatic analysis of the different films showed the best response for the self-constructed film at 30 min with no leaking of the GOX from the matrix, due to the covalent cross-linking with the bis-catechol in the film (Figure S2 in Supporting Information, SI).

GOX/bis-catechol film biosensing properties. To demonstrate the effective biofunctionalization of the electrode by GOX/bis-catechol film, we examined the electrochemical biosensing capabilities of the deposited enzyme using standard enzyme-catalyzed glucose oxidation in the presence of FC. Indeed, as direct electron transfer of GOX could not been achieved due to inaccessible enzymatic active site from the electrode, FC mediator was used in order to enhance the electron transfer rate between GOX and the electrode. Indeed, diffusional electron mediators have been frequently employed to "shuttle" electrons between enzymes and anodes.[46,47] These mediators must possess an adequate solubility in both

oxidized and reduced states for a rapid diffusion between the redox center of the enzyme and the electrode surface as well as a fast reaction with the reduced form of the enzyme. The sensing mechanism of glucose is based on the following equations:

$$FC \rightarrow FC^{+} + e^{-} \text{ (at electrode)}$$

$$GOX_{ox} + \beta\text{-D-Glucose} \rightarrow GOX_{red} + \text{D-glucono-}\delta\text{-lactone}$$

$$GOX_{red} + 2\ FC^{+} \rightarrow GOX_{ox} + 2\ H^{+} + 2\ FC$$

In a typical electrochemistry test, the reduced form of the mediator, FC, is oxidized into FC^{+} by the application of an appropriate potential. The introduction of glucose triggers an increase of the anodic current caused by the regeneration of FC through the catalytic cycle depicted in Figure 6a. This increase of the anodic current contains information concerning the quantity of glucose. GOX/bis-catechol films were built for 30 min on gold coated QCM crystal and further electrochemically characterized in EC-QCM cell by injection of glucose solutions. Based on the recommendations of Liu et al[48], 10 mM phosphate buffer solution at pH 7.4 was chosen as the optimal electrolyte to obtain maximum sensitivity of the biosensor. Figure 6b shows the different cyclic voltammograms of GOX/bis-catechol film in the absence and the presence of 0.5 mM FC at different concentrations of glucose, performed in Ar-saturated environment. In the presence of FC, the oxidation and reduction peaks were observed at 0.27 and 0.16 V, respectively related to the redox behaviors of FC. No redox peaks were observed in pure phosphate buffer or in the presence of 10 mM glucose without mediator. In the presence of FC, the addition of an increasing concentration of glucose led to a significant increase in the oxidation current and to a decrease in the reduction current of the redox couple of FC mediator demonstrating a good bioelectrochemical catalytic activity of GOX/bis-catechol film toward glucose oxidation. The potential of 0.25 V was selected as the optimal applied potential for further investigations.

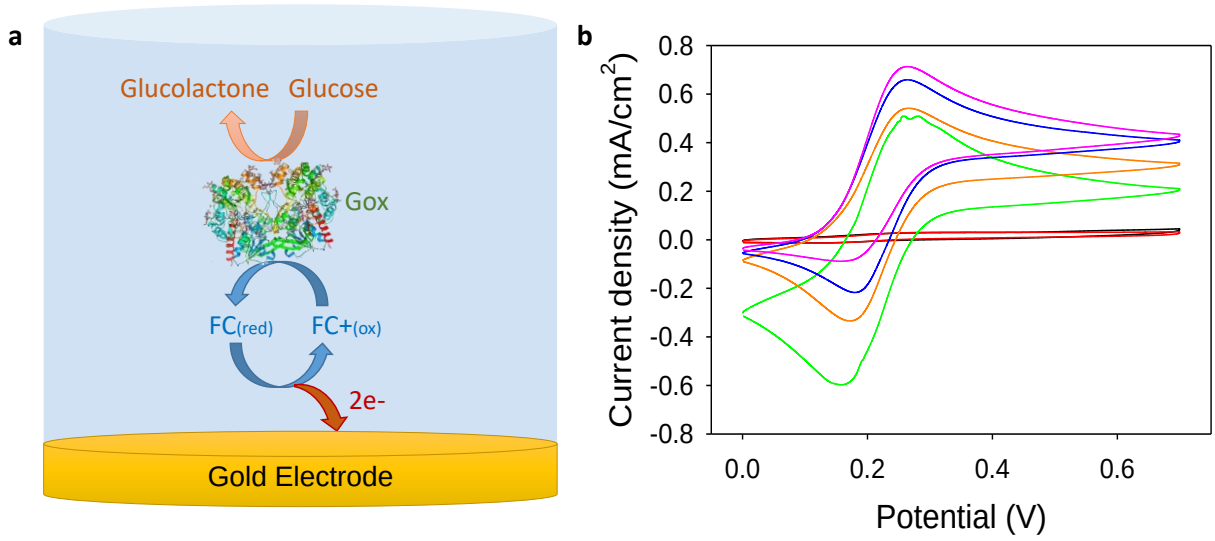


Figure 6 : (a) Schematic representation of GOX catalytic reactions and oxido-reduction of FC in self-constructed GOX/bis-catechol film allowing glucose sensing and (b) Cyclic voltammograms, performed at scan rate of 0.05 V/s, of self-constructed GOX/bis-catechol films in contact with pure 10 mM phosphate buffer saline (black curve), 10 mM glucose (red curve), 0.5 mM FC (green curve), 10 mM glucose and 0.5 mM FC (orange curve), 20 mM glucose and 0.5 mM FC mixture (blue curve), 40 mM glucose and 0.5 mM FC (pink curve) mixture solutions, prepared in 10 mM phosphate buffer saline, measured in Ar-saturated environment.

Figure 7a shows the amperometric response of GOX/bis-catechol self-constructed film. Each fluid replacement led to an electrical current overshoot followed by a period of stabilization at a steady state value. The overshoot happened in the transient period due to artefact noises or a local rise of glucose concentration around the electrode. The steady state value increases with the solution's glucose concentrations. The functionality of GOX and the feasibility of the method for biosensing are confirmed by the increase in current upon addition of successive aliquots of glucose.

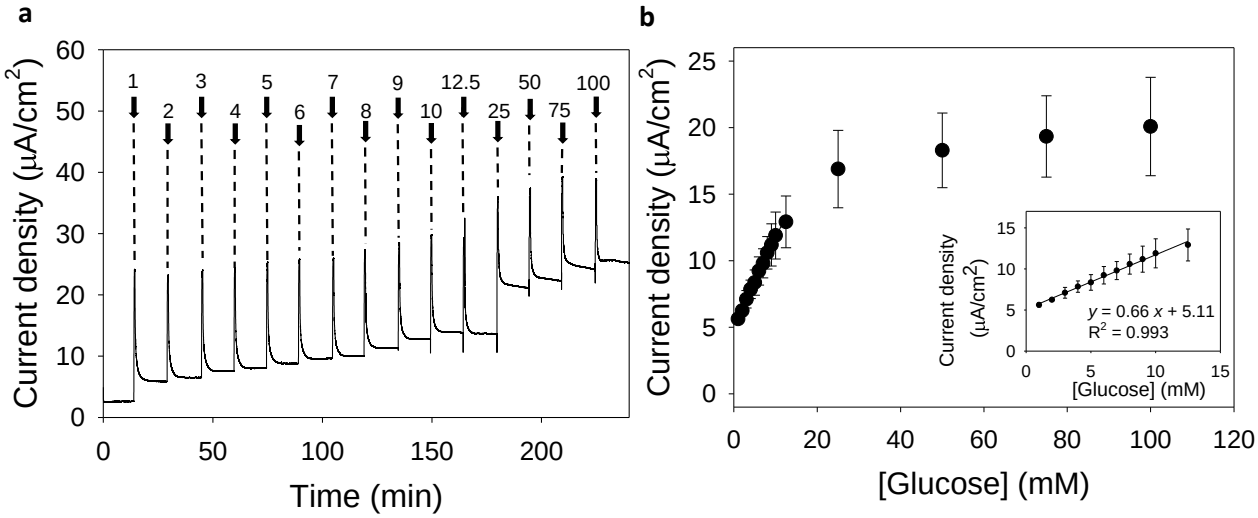


Figure 7. (a) Typical amperometric sensing evaluation and (b) average current density, with in inset the linear fit of average current density data, of self-constructed GOX/bis-catechol film upon addition of 600 µL of different concentrations of glucose (from 0.0 to 100 mM) in the presence of 0.5 mM FC during the application of + 0.25 V vs Ag/AgCl. The average and error bars were evaluated on three independent experiments.

The calibration curve shows a linear range which extends from 1.0 to 12.5 mM (R^2 = 0.993) on glucose concentration which deviates from linearity at higher concentration representing a typical characteristic of Michaelis–Menten kinetics (Figure 7b inset). This system enables to distinguish between healthy (3.8 - 6.5 mM) and hyperglycemic subjects (8.3 – 16.6 mM). The average sensitivity, calculated from the slope of the calibration curve, is of 0.66 µA/mM.cm^2 with a detection capacity of the system at 0.6 mM (Limit of detection, LOD at a signal to noise ratio 3). The observed sensing sensitivity (0.66 µA/mM.cm^{-2}) and linear range (1 - 12.5 mM) of GOX/bis-catechol biosensor are similar to cross-linked GOX biosensors based on human serum albumin/ferrocenyl-polymer (0.33 µA/mM.cm^{-2} with a linear range of 0.1 – 10 mM)[49] or on bovine serum albumin[50] (0.74 µA/mM.cm^{-2} with a linear range of 2.8 – 25 mM). In comparison to cross-linked GOX nanocomposite biosensors, based on ZnO nanotubes[51] (21.7 µA/mM.cm^{-2}), graphite composite electrode[52] (18.3 µA/mM.cm^{-2}) or boron-doped diamond electrode[53] (1.25

µA/mM.cm^{-2}), the sensitivity of the developed biosensor is lower. Indeed, nanocomposite are known to improve the sensitivity of biosensors. In contrast to GOX/biscatechol biosensor, all these developed biosensors were obtained manually by drop casting using a specific cross-linker. This method is not suitable for specific functionalization of a single electrode out of a microelectrode array.

The Michaelis–Menten constant (K_m^{app}) was determined to evaluate the biological activity of the immobilized enzyme and is estimated using the following derived equation from Lineweaver–Burk equation[54]:

$$\frac{1}{i_{ss}} = \left(\frac{K_m^{app}}{i_{max}}\right)\left(\frac{1}{C}\right) + \left(\frac{1}{i_{max}}\right)$$

where i_{ss} is the steady-state current after the addition of substrate, i_{max} is the maximum current measured under saturated substrate condition, and *C* is the bulk concentration of the substrate. A low K_m^{app} indicates a high enzymatic activity of the immobilized GOX[55]. The K_m^{app} of self-constructed GOX/bis-catechol film is about 6.3 mM which is lower than the reported 10.36 mM obtained for GOX/polyaniline,[56] 19 mM for GOX/ZnO nanotubes[51] and 21.4 mM GOX/$CaCO_3$[57] biosensors. The above result further indicates that the electrodeposited films possess a high affinity to glucose with I_{max} = 25 µA/mM.

To prove the covalent immobilization of GOX, three sequential washes (for 5 min) by injection of 0.01% of detergent Tween, prepared in phosphate buffer, were performed during chronoamperometry test. These washes were expected to remove any physically bound GOX. The current density measured before and after washes were similar (Figure S3 in SI). The selectivity of GOX/bis-catechol functionalized biosensor was evaluated using common blood

interfering substances, such as salicylic acid (SA, 0.75 mM), acetaminophen (AP, 0.35 mM), uric acid (UA, 0.5 mM)) and ascorbic acid (AA, 0.15 mM), which could have a contribution on the amperometric signal because of their low redox potentials. Thus, the maximum common concentration of these molecules in blood (Medscape) was added to the buffer solution both in the absence and the presence of 5 mM glucose to measure the current response at 0.25 V. There are not significant differences in the biosensor response due to the presence of these interfering species, suggesting an excellent anti-interference ability of the biosensor (Figure 8).

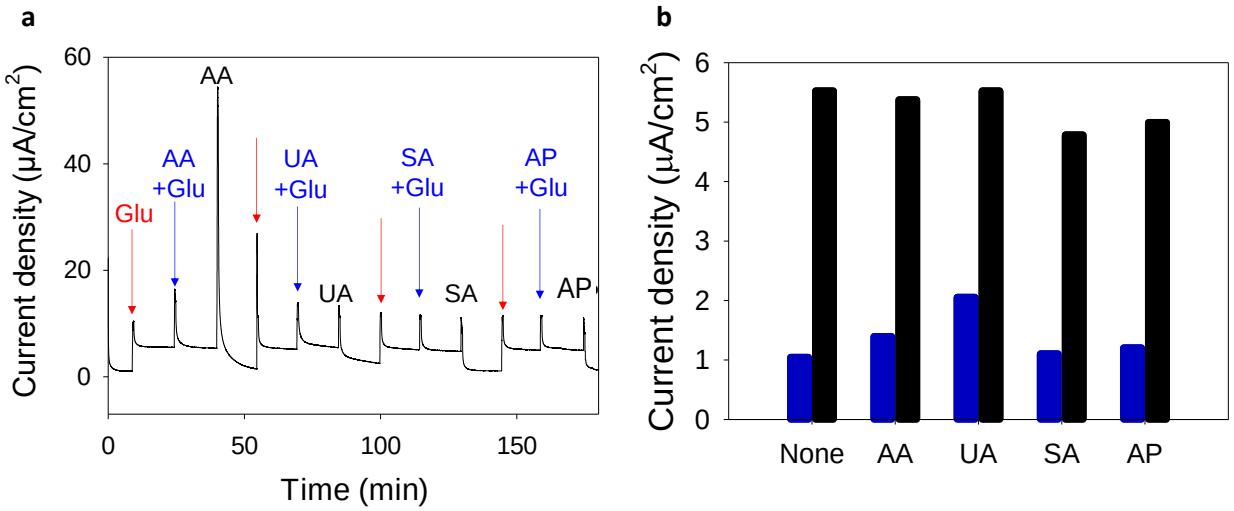


Figure 8 : (a) Typical steady-state current response of self-constructed GOX/bis-catechol film upon addition of 600 µL of different interfering substances, ascorbic acid (AA), uric acid (UA), salicylic acid (SA) and acetaminophen (AP) in the absence and in the presence of 5 mM glucose during the application of + 0.25 V vs Ag/AgCl in the presence of 0.5 mM FC (b) Current density of the film in contact with a solution containing (black) 5 mM glucose and (blue) 0 mM glucose in the absence (None) and in the presence of interfering species (AA, UA, SA, AP).

Since the electro-cross-linking of GOX using bis-catechol is localized near the electrode, we performed the functionalization of interdigitated arrays (IDA) of electrodes using rhodamine labelled GOX (GOX^{Rho}) (Figure S4 in SI) by applying CV on one of the two arrays for 45 min.

The spatial localization of GOX^{rho} on the addressed microelectrodes was checked by optical microscopy in fluorescence (Figure 9). GOX^{Rho}/bis-catechol film exhibited an excellent spatio-selectivity since a high fluorescence was observed only on the addressed microelectrodes.

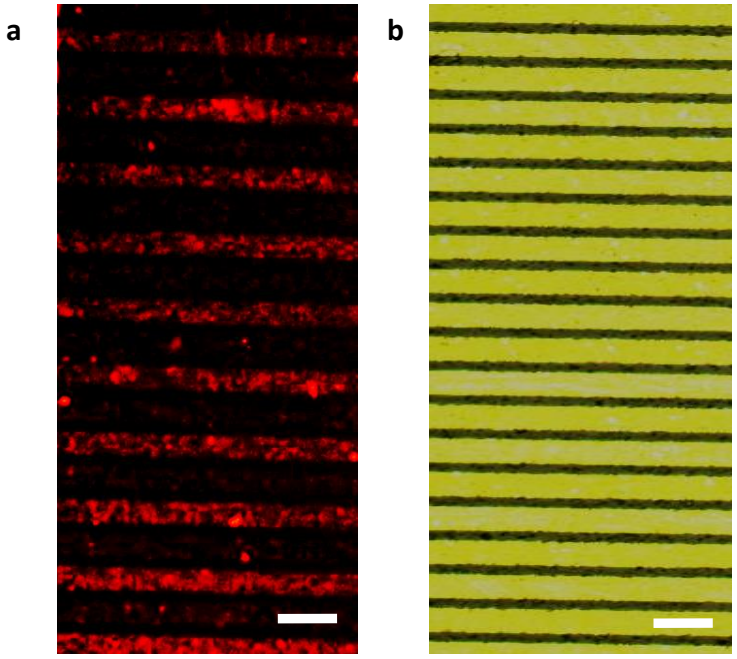


Figure 9. Optical microscope images of IDA electrodes (a) in fluorescence mode and (b) in bright field where one of the two arrays was addressed to self-constructed GOX^{Rho}/bis-catechol films by application of CV (range 0.0-0.7 V vs Ag/AgCl, scan rate 0.05 V/s) for 45 min using bis-catechol/GOX/FC mixture solution in phosphate buffer in contact with IDA. The scale bar represents 25 µm.

CONCLUSION

We demonstrated the application of a mussel-inspired electro-cross-linking process for the development of an electrochemical glucose biosensor of second generation, using bis-catechol as cross-linker in the presence of GOX and FC as mediator. This new enzyme immobilization process presents several advantages. Obtained in a one pot process with non-modified enzyme,

the cross-linked enzymatic film is strongly linked to the metallic electrode surface thanks to catechol moieties, presents no leakage issues and can be deposited selectively on microelectrode array. The developed GOX/bis-catechol film shows a good response to glucose with a quite wide linear range from 1.0 to 12.5 mM as well as a good sensitivity and a high selectivity to glucose. These films enable to distinguish between healthy (3.8 and 6.5 mM) and hyperglycemic subjects (> 7 mM). Compared with other types of biosensor based on cross-linked enzyme, the preparation of this new type of biosensor assembly is simple, fast and reproducible, being promising for the design of micro-biosensors such as biochips. Moreover, this electro-cross-linking process can be also used to develop enzymatic biofuel cells.

ASSOCIATED CONTENT

Supporting Information. The following files are available free of charge. Evolution of the normalized frequency shift as a function of time of bis-catechol/FC mixture during the application of a constant potential, Evolution of the absorbance of *o*-dianisidine as a function of time in contact with GOX/bis-catechol films self-constructed at different time, Typical steady-state current response of self-constructed GOX/bis-catechol film before and after Tween 20 detergent step, Picture and schematic representation of interdigitated array (IDA) electrodes of 10 µm width separated by 5 µm.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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