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Development and Characterization of Layer-by-Layer Biosensors Based on PEI(+)/GOx(-) Layers Using Label-Free Methods

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Abstract: Biosensors, as analytical devices, demonstrate unique efficiency in translating biochemical events into easily measurable electrical signals by using biological recognition elements, especially enzymes. Of the possible enzyme immobilisation methods, the layer-by-layer (LBL) technique, based on electrostatic interactions between layers, has the advantages of low cost, using small amount of materials, and leads to the formation of highly ordered and reproducible biosensor architectures. In this study, LbL biosensor construction has been evaluated. The substrates used were Au surfaces and mediated carbon-ink screen-printed electrodes. The gold electrodes were first functionalized with amino moieties by covalent linkage of cysteamine (Cys) through Au-S bonds. These allowed the linking of polyethyleneimine (PEI) through hydrogen-bonding to the gold surface and increased the stability of subsequent multilayers. PEI was directly adsorbed on the SPE surface. PEI is a short chain polymer and thence an efficient electron carrier, and being positively charged, it allows the formation of LBL structures with negatively charged enzymes. The multilayer formation of PEI(+)/GOx(-) was monitored by cyclic voltammetry, electrochemical impedance spectroscopy, and gravimetry. The influence of each enzymatic layer on the performance of the developed biosensor was analysed by fixed potential amperometric measurements.

Keywords: Layer-by-Layer biosensors; Enzyme immobilization; Voltammetric determination; Electrochemical impedance spectroscopy; Gravimetry; Amperometric detection.

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Introduction

Biosensors are being developed as selective tools for environmental monitoring, food industry or clinical analysis. Biosensors are analytical devices, which demonstrate unique efficiency in translating biochemical events into easily measurable electrical signals by using biological recognition elements. Some of their attributes which need to be permanently optimized are: initial and long-term activity, analytical range, sensitivity, selectivity, detection limit and reproducibility [1,2]. Electrochemical enzyme-based biosensors use a certain enzyme which catalysis a biochemical reaction, followed by the oxidation or reduction of the by-product. These redox reactions can be detected at the electrode surface either directly [3], or with the help of a redox mediator, such as phthalocyanine compounds [4].

The biosensors stability and analytical performance depends on the immobilization process of the enzyme. There are various immobilization strategies, such as adsorption, covalent linkage, cross-linking, entrapment or affinity [5,6,7], all of them presenting advantages and disadvantages. Self-assembled mono- or multilayers are a good strategy for linking biomolecules because the number of random orientations is reduced, increasing the stability of layers. A disadvantage is that the biomolecules are loosely bound, and desorption may occur towards changes in analytical parameters, such as pH, temperature, etc. [8].

Of the possible enzyme immobilisation methods, the layer-by-layer (LbL) technique, based on electrostatic interactions between layers, has the advantages of low cost, small amount of materials, and highly ordered and reproducible biosensor architectures. This technique is suitable for immobilizing enzymes in a controlled way by keeping its activity preserved for long periods of time [9]. The LbL mechanism reveals the direct electron transfer between enzyme and electrode in different biological systems: biosensors, bioreactors, fuel cells and many more systems and devices [10,11]. A variety of polyelectrolytes alternating oppositely charged enzyme layers are used for LbL deposition. Besides poly (ethyleneimine) PEI [12,13,14] other polycations have been used: poly (dimethyl-diallylammonium chloride) PDDA [15,16], poly (allylamine hydrochloride) PAH [17,18] and chitosan [19,20].

PEI is a short chain polymer and thence an efficient electron carrier used for the entrapment of several molecules in various biosensor configurations. It contains primary, secondary and tertiary amines in its structure. The presence of these amino groups facilitates the adsorption of PEI onto any hydroxylated solid surfaces through hydrogen bonds and Van der Waals forces. Also, the large amount of functional amino groups is capable of forming very stable films [21]. The active -NH_2 groups on the surface can covalently bind proteins, but

mostly, PEI is used for the electrostatic binding of enzymes, since many of them are negatively charged at neutral pH [22]. PEI undergoes protonation (and deprotonation) reactions, in aqueous media, due to its pH-dependency of the nitrogen atoms, thus carrying positive charges that decrease with increasing pH [23,24]. It has been shown that PEI has a good biocompatibility, the electrostatic attraction allowing the enzymes to keep their active conformation and stability. It also significantly reduces the charge transfer resistance and stabilizes the bioelectrodes due to the electrostatic interactions [25,26].

In this study, LbL biosensor construction has been evaluated using label-free electrochemical methods. The substrates used were Au surfaces, and cobalt phthalocyanine (CoPC) mediated carbon-ink screen-printed electrodes (SPE). Both bulk Au electrode and Au crystal microbalance electrode (AuQCM) needed to be first functionalized with amino moieties by covalent linkage of cysteamine (Cys) through Au-S bonds. These allowed the linking of polyethyleneimine (PEI) through hydrogen-bonding to the gold surface and increased the stability of subsequent multilayers. The cobalt phthalocyanine mediated SPE did not need any prior functionalization and were alternately immersed in PEI and GOx solution. At neutral pH PEI being positively charged, it allows the formation of LbL nanostructures with negatively charged enzymes.

Glucose oxidase (GOx) is a frequently used enzyme for evaluating newly-developed biosensor architectures, due to its low cost and robustness, together with accurate testing of this LBL biosensor architecture. The sequential assembly of PEI(+)/GOx(-) bilayers was monitored by means of cyclic voltammetry, electrochemical QCM-gravimetry and electrochemical impedance spectroscopy. The influence of each enzymatic layer on the performance of the developed biosensor was analysed by fixed potential amperometric measurements, allowing selection of the biosensor with optimum number of layers, the analytical parameters of which will be presented in detail.

Experimental

Materials and Methods

Cysteamine hydrochloride (Cys) C_2H_7NS, purity 95 % and poly(ethyleneimine) (PEI) 50 % (w/v) in aqueous solution were purchased from Sigma-Aldrich. Sulfuric acid, oxygenated

water and GOx from *Aspergillus niger* (24 U/mg) are from Fluka. Bovine albumin serum (BSA) minimum 98 % and glucose were purchased from Sigma-Aldrich. Aqueous glucose solution is prepared with 24 h prior measurements, in order to obtain the biologically active form (α-D-glucose).

The buffer solution used for all experiments contains a mixture of monosodium phosphate, (NaH_2PO_4) and disodium phosphate, (Na_2HPO_4) at 0.1 M (Riedel-de Haën). Millipore Milli-Q nanopure purified water and analytical reagents were used for the preparation of all solutions. All experiments were performed at room temperature (~24 °C) and all electrodes were kept in buffer solution at ~4 °C in a freezer.

All electrochemical measurements were carried out in a conventional electrochemical cell containing three electrodes. Gold-coated quartz crystals (AuQCM), gold bulk electrodes ans SPE were used as working electrodes for gravimetric and electrochemical studies, with a platinum foil as counter electrode and a saturated calomel electrode (SCE) as reference. The AuQCM electrodes were prepared from AT-cut piezoelectric quartz crystals, 10 MHz central frequency with a geometric area of 0.205 cm^2. The bulk electrode is covered in Teflon, leaving a geometric area of 0.785 mm^2. Regarding the SPE, used only for electrochemical studies, the diameter of the surface area of the working electrode is 4 mm, the auxiliary electrode is a 16×1.5 mm curve line surrounding on two sides the working electrode, and the Ag/AgCl pseudo-reference electrode is a 5×1.5 mm straight line positioned on the third side of the working electrode.

Chronoamperometric and voltammetric measurements were performed by using a computer-controlled μ-Autolab Type II and Autolab potentiostat-galvanostat running with GPES software (Metrohm-Autolab, Utrecht, Netherlands). Electrochemical impedance measurements and gravimetric studies were performed with a Potentiostat / Galvanostat / ZRA (Gamry Instruments, Reference 600) for gold electrodes and Autolab potentiostat-galvanostat for SPE. An rms perturbation of 10 mV was applied over the frequency range 100 kHz–0.1 Hz, with 10 frequency values per frequency decade. The obtained spectra were recorded at different potentials of -0.2 V and 0.0 V vs. SCE for SPE and Au electrodes respectively, and plotted in the form of complex plane diagrams (the so-called Nyquist plots) with an Electrochemical Interface using ZPlot 2.4 and FRA software.

Enzyme Immobilization

The bulk gold electrode was subjected to cyclic scanning in a 0.1 M of sulfuric acid in a potential range from -0.5 V to 1.5 V vs. SCE with a scan rate of 100 mV s^{-1}. When a stable cyclic voltammogram was recorded, the electrode was washed with Milli-Q water and dried

in a N_2 stream. The AuQCM electrodes were first cleaned with piranha solution (3:1 H_2SO_4 95-97 %: 7 M H_2O_2), then washed with Milli-Q water and dried in a N_2 stream. The SPE were used as received.

The clean electrodes were stepwise modified as shown in Fig.1. Enzyme immobilization was performed by immersion, for Au electrodes, or drop-coating procedures using aliquots of appropriate solutions, for SPE, and well-defined nanoscale film structures are built on surfaces by self-assembly. Polyelectrolyte multilayers (PEMs) are formed by sequential deposition of oppositely charged macromolecules via alternate 15 min adsorption from the appropriate solutions, GOx and PEI, which bind to each other by electrostatic interactions.

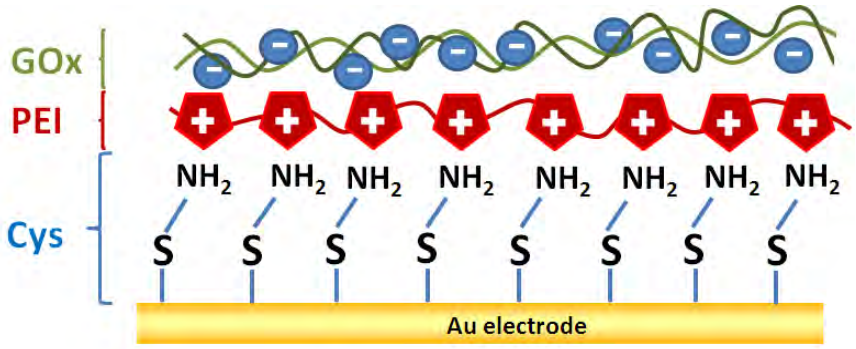

Fig.1: *Schematic representation of mulilayers assembly*: the gold surface was functionalized with amino moieties of Cys, followed by the alternate LbL assembly of PEI and GOx.

For the functionalization of the gold surfaces first a 25 mM aqueous Cys solution was prepared. The electrodes were then immersed in this solution for 2.30 h, washed with Milli-Q water to remove residual molecules and dried under a stream of N_2. For the next 15 min, the electrodes were immersed in PEI solution (1 % w/v dissolved in water), washed and dried under the same conditions. The enzymatic solution contained 1 % GOx and 4 % BSA, and the electrodes were immersed in this solution for 15 min, washed and dried afterwards. The above steps were repeated until the desired number of PEI/GOx bilayers was reached.

The PEI(+)/GOx(-) bilayers on SPE were deposited by drop-coating procedure. PEI and enzymatic solutions were prepared using similar recipes as used for gold electrodes. 2 µl of PEI solution were dropped on modified carbon working electrode for 15 min with subsequent washing using Milli-Q purified water. 2 µl of enzymatic solution were dropped for 15 min and followed by the other washing step.

Results and Discussion

PEI as Multifunctional Agent

Literature shows that PEI has been used in many different applications, together with various materials: for biosensor development, for drug delivery – transfection, membrane formation and much more.

Lakard developed an electrochemical urea biosensor showing that PEI modified electrodes have good reversibility and good stability in time. PEI was electrodeposited onto a Pt electrode. After PEI coating, the electrode was immersed in urease solution overnight. The electrodes showed a good sensitivity due to the reaction between urea solutions and urease fixed on the polymer by physical adsorption. In two weeks, the sensitivity decreased with 50 %, showing a poor long term stability, which may occur due to enzyme leakage from the surface [22]. Another group of researchers coated silicon wafers with a diluted solution of PEI (0.2 % wt/v) obtaining a stable and homogeneous monolayer [21]. Vakurov et al. immobilized acethylcholinesterase onto PEI modified screen-printed carbon electrodes. PEI allowed the enzyme to keep a high activity and stability. The effect of PEI density was tested by immersing the electrodes in PEI solution for three different periods of time: 12 h, 3 and 4 days respectively, concluding that high PEI density results in lower sensitivity of the biosensor. In the same time though, increasing PEI density resulted in an increase of wet and dry stability of the PEI modified electrode, which could be kept for 6 months before enzyme immobilization [27].

Hassler and his group showed that PEI can bind enzymes and cofactors using reversible ionic interactions which allow for the obtained film to be removed and then replaced. PEI was used to couple an electron mediator, cofactor and enzyme to a functionalized gold surface. The obtained biosensor was used for the biocatalytic oxidation of 2-propanol and d-sorbitol, obtaining for both structures sensitivities in the range of 2.5 – 2.9 $\mu A\ cm^{-2}\ mM^{-1}$ [28]. PEI was also used to functionalize ionic liquids (IL). IL units can be covalently modified on PEI, therefor they can be steadily and easily immobilized on glassy-carbon electrode surfaces.

PFIL (polyethylenimine-functionalized ionic liquid) combine the advantages of both functional groups: high ionic conductivity and solvation properties from the IL and good immobilization and film stability from PEI. GOx was immobilized in thin films of PFIL, used to disperse together multi-wall carbon nanotubes (MWCNT) and nanoparticles (AuNPs).

Amperometric measurements showed a linear response for increasing glucose concentrations, ranging between 2-12 mM (R = 0.9991), suitable for practical applications in determining blood sugar concentrations [29].

PEI has the ability to transfect a wide range of eukaryotic cells and is one of the most efficient agents for gene delivery applications [30]. Zinc compounds are used for the treatment of *Helicobacter pylori* infections. Chakraborti et al. prepared ZnO nanoparticles which were followed by PEI capping. They demonstrated that ZnO-PEI NPs are efficiently internalized into H. pylori cells, where they generated considerable amounts of intracellular reactive oxygen species resulting in membrane damage and degradation of their RNA. The advantages of PEI substituted ZnO NPs are, that it disperses better in aqueous media and it lowers the release of Zn^{2+} ions, which are toxic to animal cells [31]. Sun et al. built up polymer dots, produced from an amphiphilic polymer based on PEI, which could easily encapsulate drugs for delivery. Due to the so called “proton sponge effect” of PEI, the PDs loaded with drugs, could easily escape from endosomes / lysosomes, which usually hinder the drug delivery into the cytoplasm [32].

A distinct disadvantage for the use of PEI would be its toxicity towards eukaryotic cells. However, recent studies showed that the use of smaller sized PEI molecules greatly reduces its toxicity [33]. He et al. immobilized GOx and choline oxidase within a PEI-coated silica monolith micro-reactor. Silica-monoliths were loaded with PEI solution, dried, washed and then loaded with enzyme solution and dried. This work shows that PEI molecular weight and concentration can affect the activity of the immobilized enzymes. For high PEI molecular weight (25000) there is a significant reduction in the current response. The best performances were obtained with 10000 MW PEI: the enzymatic activity of the immobilized enzyme, V_{max}, was about 74 times higher than for GOx in solution. Also, immobilized GOx retained 95 % of its initial activity after a continuous run of 1 day, while GOx in solution retained only 60 % of its activity [25].

PEI can also be used as an efficient membrane permeabilizing agent for some bacteria, like Gram-negative bacteria [34]. PEI capping has been demonstrated to improve silver NP colloidal stability and antimicrobial activity [35]. D. Ju and L. Dai used PEI as a stabilizer for the reduction of graphite oxide sheets. The obtained graphene-based material was well dispersed and allowed controllable fabrication of multicomponent hybrid films, using the LbL technique with negatively charged materials [36].

Development and Electrochemical Characterization of the Electrode Interfaces

In this work, mediated carbon-ink screen-printed electrodes and gold electrodes, are used to develop enzyme-based biosensors for the detection of glucose. We have used CoPC mediated carbon-ink electrodes because of low production costs, easy fabrication method and good reproducibility, in comparison to the gold electrodes, which are more expensive.

Carbon-ink is known for its good conducting properties [37] and mediators such as CoPC ease the electron transfer from the surface of the electrode. CoPC is an organic semiconductor with a very high stability [38] and a working potential of 0.1 V, also confirmed by literature [39]. The SPE were prepared accordingly to procedures previously described [40,41]. There is also a disadvantage in the use of redox mediators in conjunction with redox proteins, such as GOx. It has been shown that the selectivity of the biosensor is decreasing, facilitating not only the electron transfer between the electrode and enzyme, but also various interfering reactions [42].

Since PEI doesn't adsorb spontaneously on gold, unlike on the SPE, the Au electrodes were first functionalized with amino moieties by covalent linkage of Cys, for which the Au substrates must be very clean. This allows a good chemisorption of the thiol moietys of Cys to Au, a strong and stable linkage of S-Au being formed [43]. This step allowed the linking of PEI on Au/Cys through hydrogen-bonding.

Mizia M.S. Silva showed that the optimal incubation time for Cys is about 2 h; for lower immersion time, as well as for an immersion time longer than 12 h, the analyical signal was decreasing [4]. After letting Cys interact with the gold surface for 2:30 h, a Cys monolayer was deposited on Au, with free amine groups which easily bound PEI. PEI, being positively charged, allows the electrostatic formation of LbL structures with negatively charged enzyme, GOx. The desired number of PEI(+)/GOx(-) bilayers was obtained by electrostatic interaction between the two substances.

Cyclic Voltammetry

All electrochemical measurements were carried out in a conventional electrochemical cell containing three electrodes using 0.1 M NaPB pH 6.9 as supporting electrolyte with the experimental conditions described in *Materials and Methods* paragraph. The Fig. 2 shows cyclic voltammograms of the step by step assembly of a biosensor by scanning potential after each bilayer deposition, in between 0 and 0.7 V, for bulk gold electrode and between -1.0 and 1.0 V for SPE, at a scan rate of v = 50 mV/s. In fact, this type of measurements offers qualitative information about the overall electrochemical properties of the working electrode.

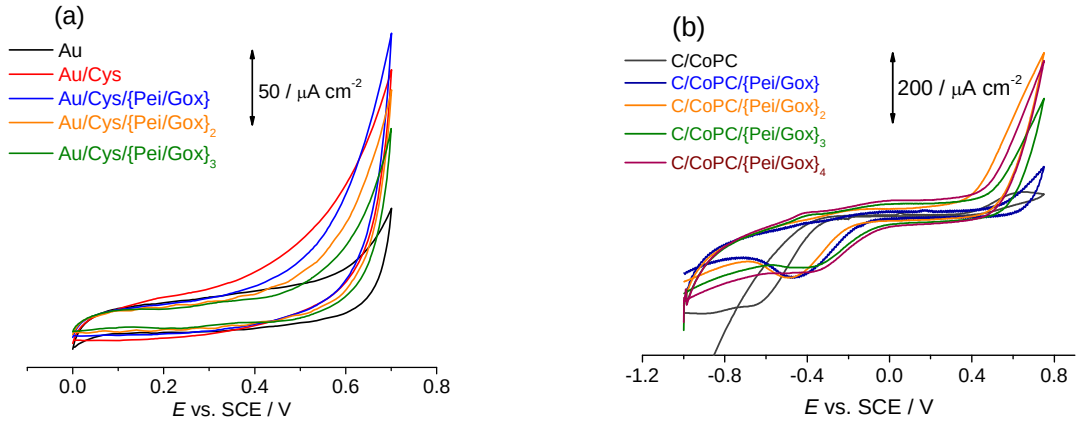

Fig. 2: *Cyclic voltammograms in 0.1 M NaPB pH 6.9 for different numbers of {PEI/GOx} bilayers of* ***(a)*** *bulk Au electrode and* ***(b)*** *SPE*; scan rate 50 mV s^{-1}

The voltammograms shapes obtained for the two electrode substrates are different. This dissimilarity is given first by the electrical properties of electrode material and secondly by the presence/absence of electrochemical mediator. The carbon electrode surface is modified with CoPC and redox peaks, especially reduction waves are present. This peak is shifted towards more positive potentials when the first PEI/GOx bilayer is added. Following the subsequent bilayers deposition, the voltammograms are becoming broader for pozitive potentials, until the third one, after which start to stabilize. All these facts suggest the increasing of multilayer thickness and further bilayer addition will only increase the film thickness, when moderating the electrons transfer through the film.

Regarding the gold bulk electrode, first, it can be noticed that, the respective voltammogram becomes wider once the electrode is functionalized with cysteamine layer, proving its adsorbtion. When functionalized gold surface is used for PEI/GOx bilayers deposition, a similar tendency in the CV shapes as for SPE is observed. An explanation for the behavior of both types of electrodes could be the rather large distance between the third PEI/GOx bilayer and the electrode surface, and the hindered diffusion between the layers.

EIS – Electrochemical Impedance Spectroscopy

The interfacial properties of the electrodes were also investigated by electrochemical impedance spectroscopy (EIS). By comparison with an electrical circuit, the electron transfer resistance depends on the dielectric phenomena which take place at the electrode surface [45]. EIS spectra were recorded in 0.1 M NaPB, pH 6.9 in a conventional electrochemical cell

containing three electrodes with the experimental conditions described in *Materials and Methods* section. An rms perturbation of 10 mV was applied over the frequency range 100 kHz–0.1 Hz, with 10 frequency values per frequency decade. The obtained spectra were recorded at different potentials (-0.2 V vs. SCE for gold electrode and 0.0 V vs. reference for SPE) and imaged as the complex plane diagrams, Nyquist plots, with an Electrochemical Interface using Z-Plot 2.4 and FRA software.

The impedance spectrum obtained by frequency scanning of impedance versus frequency allows the surfaces and interface together with exchange and diffusion processes characterization. AC impedance spectra are presented as a Nyquist plot ($-Z''$, imaginary impedance, versus Z', real impedance). EIS allows an electrical characterization using equivalent circuits as models. A direct correlation has been developed between the response of the real system and an idealized model using resistances and capacitors as components [46]. Equivalent circuits contain usually combinations in series and/or in parallel of resistances (R) and capacitances (C) is used for impedance spectrum fitting. The $-Z''$ value, which corresponds to the highest point of the semicircle on the Nyquist plot is used to characterize the binding events occurring at the electrode surface, along with charge transfer resistance (R_{ct}) and capacitance of double layer (C_{dl}) values, obtained by fitting the spectra with the Randles equivalent circuit, showed in Fig. 3. This consists of a cell resistance, R_Ω (resistance of electrolyte solution and electrical contacts) coupled in series with a parallel combination of an interfacial electron-transfer resistance, R_{ct}, with the capacitance of double layer (C_{dl}). Once molecules are immobilized on electrode surface and an insulating complex is formed, an increase in R_{ct} value can be detected by EIS. Decreasing of R_{ct} values following different molecules deposition shows the improving of interfacial electron-transfer due to conductive immobilized layers. C_{dl} is associated with the capacitance of the capacitor represented by the electrode-electrolyte interface. Capacitors in EIS experiments often do not behave ideally. Instead, they act like a constant phase element, CPE, which has the impedance: $Z_{CPE} = 1/(iw)^n C_{dl}$.

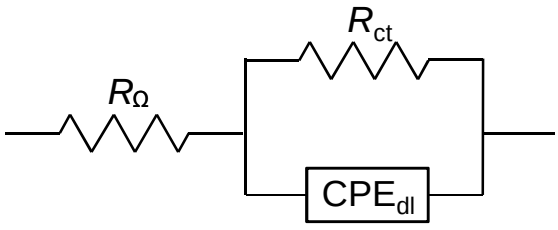

Fig.3: *Equivalent circuit used to fit the spectra, where R_Ω is the resistance of the electrolyte, R_{ct} is the charge transfer resistance and CPE_{dl} is the constant phase element*.

As can be seen in Fig. 4, EIS spectra for both type of electrodes have semicircle shape, which can be fitted by using a typical Randles circuit, to characterize the physicochemical properties of the electrode surfaces and polyelectrolyte multilayers formation, together with exchange and diffusion processes. The spectra were recorded at potential values chosen near redox potentials corresponding to the bare bulk gold electrode (-0.2 V vs. SCE) and CoPC-modified SPE (0.0 V vs. Ag/AgCl).

Cys-functionalized bulk gold electrode exhibited a higher R_{ct} value than for the bare Au electrode, its covalent linkage hindering the electron transfer at the electrode interface. Further PEI/GOx bilayers deposition lead to a continuous decrease of R_{ct} value (Table I and II), showing an improve in the electron transfer through the multilayer film until the PEI/GOx third bilayer. The same behavior can be noticed for SPE modified electrodes, where a significant decrease is observed starting with the first PEI/GOx bilayer deposition, followed by a smaller decrease for the next bilayers until the fourth one, when no improvement in the electron transfer is noticed. We can consider that on gold electrode the Cys layer influences the behavior of the electrode together with the number of PEI/GOx bilayers. Further bilayers deposition in both cases will increase both thickness and R_{ct} and electron transfer will decrease.

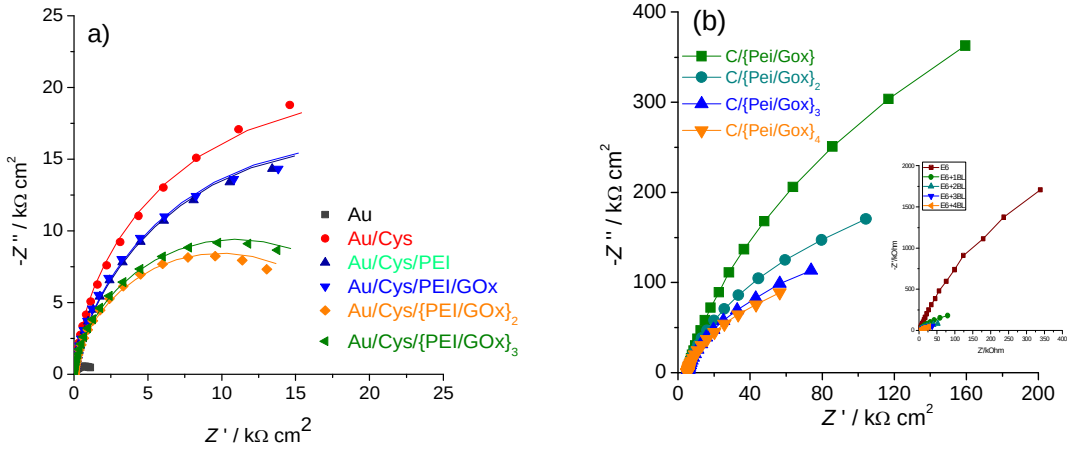

Fig.4: *Nyquist plots in 0.1 M NaPB pH 6.9 for different numbers of {PEI/GOx} bilayers of* ***(a)*** *gold bulk electrode and* ***(b)*** *SPE/{PEI/GOx}$_n$*. Insert shows the plots for SPE (red) in comparison with SPE/{PEI/GOx}$_n$.

Table I: *Equivalent circuit components values* (extracted by fitting the spectra from Fig. 4a; applied potential, -0.2 V vs. SCE).

electrode	R_Ω (Ω)	R_{ct} kΩ cm^2	CPE_{pol} / (mF cm^{-2} s$^{\alpha-1}$)	α
Au	425.9	1.4	126.8	0.85
Au/Cys	430.4	68.0	68.2	0.88
Au/Cys/{PEI_GOx}	423.2	47.2	71.0	0.87
Au/Cys/{PEI_GOx}$_2$	428.2	25.5	71.9	0.86
Au/Cys/{PEI_GOx}$_3$	427.3	21.8	71.2	0.87

Table II: *Equivalent circuit components values* (extracted by fitting the spectra from Fig. 4b; applied potential, 0.0 V vs. SCE).

electrode	R_Ω (Ω)	R_{ct} (kΩ cm^2)	CPE_{pol} (mF cm^{-2} s$^{\alpha-1}$)	α
C-SPE	540	8600	16.8	0.93
C_{PEI_GOx}	580	340.5	14.7	0.93
C_{PEI_GOx}$_2$	590	119.0	27.4	0.92
C_{PEI_GOx}$_3$	600	76.5	40.4	0.92
C_{PEI_GOx}$_4$	600	63.0	52.6	0.92

Gravimetric Study

The formation of the LbL assembly of PEI/GOx, was also monitored with the quarz crystal microbalance (QCM). The QCM is an excellent tool of monitoring adsorption process during the LbL deposition. The frequency variation with time can be used to determine the deposited mass via the relationship described by the Sauerbrey equation [47], for the specific case of rigid films:

$$\Delta f = -\frac{2 f_0^2}{A\sqrt{\mu_q \rho_q}} \Delta m$$

where f_0 is the resonant frequency (Hz), Δf the frequency change (Hz), Δm the mass change (g), A the piezoelectrically active crystal area, ρ_q the density of quartz (g cm^{-3}) and μ_q the shear modulus of quartz for AT-cut crystals (g cm^{-1} s^{-2}). In the specific case of the AuQCM employed in this study, the conversion factor is 226.0 Hz per 1 µg.

The AuQCM cell utilized is a small chamber for 200 µL of liquid. Water was first placed in the chamber in order to allow the stabilization of the crystal resonant frequency in liquid. After this, the AuQCM chamber was emptied by using a micropipette, washed and dried with a nitrogen stream. This step was repeated after each layer deposition. As shown in Fig. 5A, the deposition of first Cys layer lead to a small decrease of *f*, the deposition of PEI/GOx bilayers leading to significant variations of *f*, mostly during GOx deposition. The increase in *f* when changing GOx solution with PEI, is attributed to different solution densities, the PEI solution being less dense than the GOx one. The deposited Cys monolayer was of 200 ng, and of first PEI of 220 ng, respectively. The overall change in frequency was 10.9 kHz, corresponding to a gain in mass of 9.66 µg.

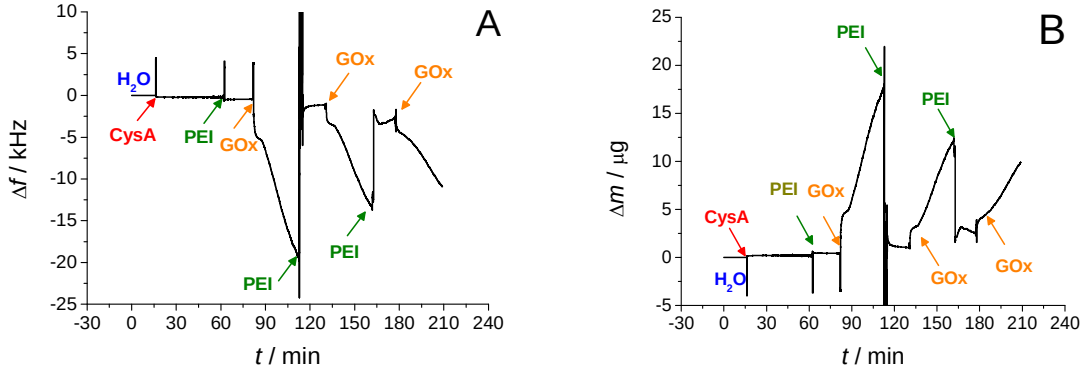

Fig. 5: *Gravimetric measurements with an Au Quartz Crystal (AuQC).* Central frequency 10 MHz, using a AuQC microbalance (AuQCM) : A – frequency shift and B – mass variation during LBL deposition.

Amperometric Detection of Glucose

Fixed potential amperometric technique allows monitoring currents given by the electrochemical reaction of certain species at the surface of the working electrode, at a chosen potential. The current intensity depends directly on the concentration of the analyte. A decrease in current was recorded at Au/Cys/{PEI_GOx}$_n$ following the enzyme substrate injection, so that reduction reactions are recorded at the electrode surface.

The calibration plots corresponding to glucose biosensors containing different numbers of PEI/GOx bilayers are displayed in Fig. 6. As observed, there is a significant increase in sensitivity from the first bilayer Au/Cys/{PEI_GOx} biosensor to the second one,

the third leading to a slight decrease in sensitivity, probably due to diffusion problems in between layers. The highest biosensor sensitivity for the 2-bilayer configuration was of 282.2 nA cm^{-2} mM^{-1}.

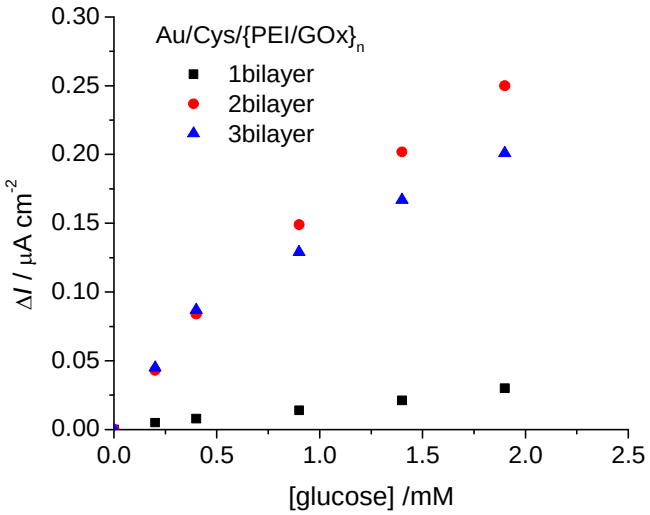

Fig. 6: *Calibration plots of Au/Cys/{PEI_GOx}$_n$ biosensors*; n = 1, 2 and 3 corresponding to chrono-amperograms recorded in 0.1 M NaPB pH 6.9, at -0.35 V vs. SCE.

Conclusions

The Au surface was functionalized with Cys to permit layer-by-layer electrostatic adsorption of PEI(+), as stabilizing agent, and the enzyme GOx(-). It can be stated that:

• EIS results show that the biosensor with 2 and 3 bilayers of {PEI/GOx} exhibits a lower charge transfer resistance than that with one bilayer.

• Gravimetric measurements confirm the deposition of {PEI/GOx} bilayers at Au electrodes, a total mass of 9.66 μg being deposited for 3 bilayers.

• The highest biosensor sensitivity, 282.2 nA cm^{-2} mM^{-1}, was obtained for the 2-bilayer configuration with amperometric measurements. Furthermore, the bilayer deposition did not increase biosensor sensitivity.

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