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Molecular lego for the assembly of biosensing layers☆

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Abstract

We propose a procedure to assemble monolayers of redox mediator, coenzyme, enzyme and stabilizing polyelectrolyte on an electrode surface using essentially electrostatic and complexing interactions. In a first step a monolayer of redox mediator, substituted nitrofluorenones, is adsorbed. In a second step, a layer of calcium cations is immobilized at the interface. It establishes a bridge between the redox mediator and the subsequently adsorbed coenzyme NAD^+. In the next step we use the intrinsic affinity of the NAD^+ monolayer for dehydrogenases to build up a multilayer composed of mediator/Ca^{2+}/NAD^+/dehydrogenase. The so obtained modified electrode can be used as a biosensor. Quartz crystal microbalance measurements allowed us to better understand the different parameters responsible for the adsorption. A more detailed investigation of the system made it possible to finally stabilize the assembly sufficiently by the adsorption of a polyelectrolyte layer in order to perform rotating disk electrode measurements with the whole supramolecular architecture on the electrode surface.

Keywords: Modified electrodes; Nitrofluorenones; Electrocatalysis; Self assembled monolayers; Biosensors; Layer-by-layer assembly

1. Introduction

Nicotinamide adenine dinucleotide (NAD^+/NADH) dependent enzymes are widely studied for the development of reagentless electrochemical biosensors [1]. One key point for a successfully working device is the preservation of the activity of the coenzyme and enzymes employed. This depends among others very much on the strategy used to immobilize these molecules at the electrode surface. Many approaches have been suggested to fix NAD^+-dependent enzymes at the electrode/electrolyte interface but sometimes, severe drawbacks like partial enzyme deactivation are observed [2]. We previously reported on a "soft" approach to assemble the sensing layers [3,4], which allows not only to control the spatial arrangement of redox mediator, coenzyme and enzymes on the electrode surface at the molecular level, but also keeps the enzyme in an environment as close as possible to its natural one. This procedure follows a layer-by-layer strategy that has been also used by other groups in a similar context but with different ingredients [5–9]. The layers are assembled by simple alternate dipping of the electrode in the different solutions. An essential step in the construction of the supramolecular sandwich uses a calcium bridge between substituents of the redox mediator and the NAD^+/NADH phosphate groups [10,11]. After adsorption of the redox mediator, that contains a carboxyl function, the electrode is dipped in an aqueous solution containing calcium ions. They adsorb through electrostatic interactions on the mediator layer. Subsequent dipping of the electrode in a solution of coenzyme leads to its adsorption due to the affinity between the surface confined Ca^{2+} and the phosphate groups of the coenzyme. Furthermore, we take advantage of the natural affinity of de-

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hydrogenases for NAD^+ in order to adsorb a layer of enzyme. In a last step, essentially based on electrostatic interactions, a layer of polyelectrolyte is adsorbed to stabilize the whole assembly. These interactions are studied by quartz crystal microbalance (QCM) measurements and the stability of the layers is tested using rotating disk electrode measurements for the detection of glucose.

2. Experimental

2.1. Materials

Tris, calcium chloride dihydrate and glucose were purchased from Merck and Sigma, respectively, and used as received. Glucose solutions were left 12 h for equilibration before use. Tris buffer was prepared by dissolving the adequate amount of compound and adjusting to pH 8 by addition of HCl. NAD^+ was obtained from Sigma with 99% purity. Glucose dehydrogenase (GDH) from bacillus megaterium (Sigma) had an activity of 50–150 units/mg. Solutions were prepared from ultrapure water that had been passed through a purification train (Milli-Q Plus 185, Millipore). The 4-carboxy-2,5,7-trinitro-9-fluorenone was synthesized as described in the literature [12]. 4-Carboxy-2,5,7-trinitro-9-fluorenylidene-malononitrile has been synthesized by refluxing 0.03 mol 2,5,7-trinitro-9-fluorenone-4-carboxylic acid, 0.06 mol of malonitrile and freshly distilled piperidine, in dry methanol overnight (69% yield). The purity and structure of the final compound has been checked by NMR, IR and thin layer chromatography.

2.2. Apparatus

Cyclic voltammetry (CV) experiments were carried out in a conventional one-compartment cell with an Autolab PGSTAT 10 potentiostat at ambient temperature ($20 \pm 2\,^{\circ}C$) in a solution that had been bubbled with nitrogen for at least 15 min. Commercial glassy carbon electrodes with 3 mm diameter were used as working electrodes (Bioanalytical Systems, BAS). Potentials were measured with respect to a commercial Ag/AgCl reference electrode (BAS) and the counter electrode was a platinum wire. If not otherwise mentioned, scans were started at the positive end of the potential range for studying the adsorbed mediator and at the negative end of the potential range for catalysis experiments. Simultaneously CV and microgravimetric measurements were carried out with a PGSTAT 10 (Autolab) potentiostat using GPES 4.5 software and an electrochemical quartz crystal microbalance, EQCM 5510 commercialized by the Institute of Physical Chemistry (Polish Academy of Sciences, Warsaw). The 14 mm diameter, gold covered and AT cut quartz crystals (5 MHz) were obtained from Omig (Warsaw, Poland). The sensitivity of the mass measurements, calculated from the Sauerbrey equation via silver electrodeposition, was 1 ng Hz^{-1}. Each experiment was performed with a new crystal.

2.3. Procedures

2.3.1. Preparation of a mediator monolayer on glassy carbon

A monolayer of 2,5,7-trinitro-9-fluorenone-4-carboxylic acid was adsorbed on glassy carbon by the following procedure. After polishing the electrode with 0.05 μm Al_2O_3 powder (Buehler) and sonicating it in ultrapure water for 1 min, the catalyst precursor layer is obtained by dipping the electrode for 10 min in a 0.5 mM solution of the nitrocompound in THF. Afterwards, the glassy carbon electrode is rinsed with ultrapure water. This leaves a thin layer of the organic molecule at the interface because THF, which is miscible with water, diffuses into the aqueous phase. Subsequently, the electrode is dipped into the aqueous supporting electrolyte and the catalyst is activated by transforming one, two or three of the nitro groups into hydroxylamine by choosing the adequate negative potential during the first scan [13]. The transformation of two out of the three nitro groups has been shown to be the optimal strategy in terms of catalytic activity [10], but in order to check the changes in Ca^{2+} adsorption we also tested electrodes where only one or all of the nitro groups were reduced. Surface coverages for the modified electrodes were determined by integration of the voltammetric wave and using the geometric area of the electrode.

2.3.2. Preparation of the enzyme modified electrode on glassy carbon electrodes

The monolayer of mediator is fixed as explained above. In a second step the surface confined molecules are complexed by dipping the electrode in a solution containing 400 mM $CaCl_2$ for 10 min. After briefly rinsing in supporting electrolyte the electrode is immersed for another 10 min in a solution with 5 mM NAD^+. As a consequence the calcium ions already bound to the mediator interact with the phosphate groups of the coenzyme and in this way a layer of NAD^+ is attached to the surface. Then, we use the natural affinity of GDH for NAD^+ to bind the dehydrogenase to the surface. The mediator/Ca^{2+}/NAD^+ modified electrode is dipped for 10 min in a buffer solution containing 100 units GDH/ml. The enzyme is now bound to the coenzyme and this complex is attached via the calcium bridge to the immobilized mediator.

2.4. QCM measurements

Prior to each experiment, the gold electrode was cycled between +1 and −0.4 V in 0.5 M H_2SO_4 until a reproducible cyclic voltammogram, characteristic for a clean gold electrode was obtained. Once cleaned, a modification procedure analogue to the one described for glassy carbon was used, but replacing 4-carboxy-2,5,7-trinitro-9-fluorenone by 4-carboxy-2,5,7-trinitro-9-fluorenylidene-malononitrile [14].

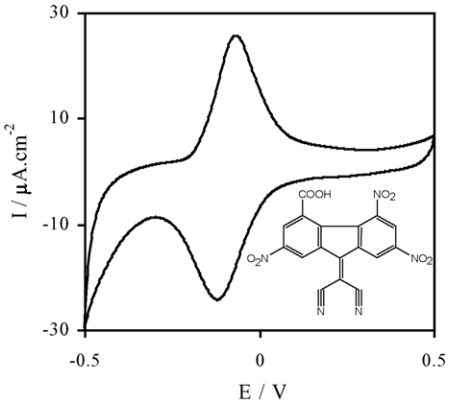

Fig. 1. Cyclic voltammogram of a gold electrode modified with a monolayer of 4-carboxy-2,5,7-trinitro-9-fluorenylidene-malononitrile (see inset) when two nitro groups are transformed into hydroxylamine. 0.1 M Tris buffer, pH 8, 100 mV s^{-1} (from Ref. [4], with permission from the editor).

3. Results and discussion

The cyclic voltammogram in Fig. 1 shows a gold coated quartz crystal electrode modified with a monolayer of 4-carboxy-2,5,7-trinitro-9-fluorenylidene-malononitrile when two of the three nitros groups are transformed into hydroxylamine.

Each of the involved NO/NHOH couples at −93 mV is subject to a $2e^-/2H^+$ redox process and the obtained monolayer is fairly stable. As previously demonstrated the NO/NHOH redox couple is a good candidate for the catalysis of a two-electron process like the oxidation of NADH. We showed in earlier publications that the carboxylic group in position four could be used to build a well-defined mediator/coenzyme sandwich via a Ca^{2+} bridge [11]. To investigate the effect of calcium on the catalyst layer stability we performed both cyclic voltammetry (Fig. 2) and EQCM measurements (Fig. 3). In Fig. 2 a gold electrode is modified with a monolayer of 4-carboxy-2,5,7-trinitro-9-fluorenylidene-malononitrile. Curve a shows a voltammogram that has been recorded after the activation of two nitro groups and cycling until the initial double peak refines into one single peak.

Further cycling in pure Tris buffer pH 8 leads to a gradual loss of mediator (curve b). After 200 scans, only 10% of the initial amount of mediator remains at the electrode surface. This might be explained by the fact that a mutual electrostatic repulsion due to the negatively charged carboxyl group, present in each molecule, favors the desorption especially when scanning to too negative potentials.

This unfavorable interaction is also illustrated by the value of the peak width at half-height, $\Delta E_{p,1/2} \sim 180$ mV, far from the theoretical value of 45.3 mV as expected for ideal Nernstian two-electron two-proton transfer reactions [15]. In presence of 0.1 M calcium cations, however (curve c), we noticed that after 200 scans, 90% of the catalyst remains at the electrode surface. This enhanced stability might be explained by the complexation of the COO^- group with Ca^{2+} leading to a screening of the negative charge and as a result a decrease of the repulsive electrostatic interactions between the molecules of catalysts. The presence of calcium ions most likely also leads to a linking between the mediator molecules in the layer resulting in a significant decrease of the peak width at half-height ($\Delta E_{p,1/2} \sim 70$ mV). In addition, the presence of the positively charged calcium ions and their strong interaction with the carboxyl group decreases the electron density in the mediator molecule and therefore an oxidation of the hydroxylamine group becomes more difficult and on the other hand the reduction of the nitroso group is easier to achieve. As a consequence the redox potential of the mediator molecule shifts to slightly more positive potentials.

The stabilizing interaction between COO^- and Ca^{2+} is also perfectly illustrated in Fig. 3 when using QCM measurements in parallel to the potential cycling. The initial frequency of a non-modified crystal is defined arbitrarily as zero. Adsorption of the mediator leads to a frequency decreases of

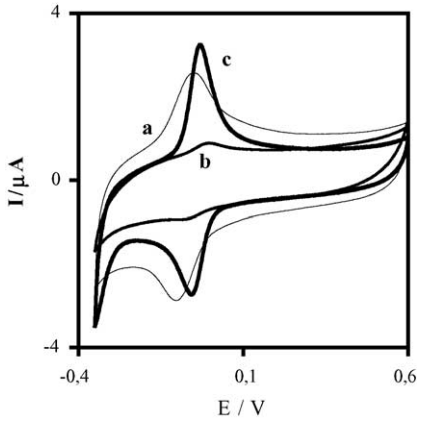

Fig. 2. Cyclic voltammogram of a gold electrode modified with a monolayer of 4-carboxy-2,5,7-trinitro-9-fluorenylidene-malononitrile when two nitro groups are transformed into hydroxylamine: (a), freshly modified; (b), after 200 scans without calcium; (c), after 200 scans in presence of 0.1 M calcium. Tris buffer, pH 8, 100 mV s^{-1}.

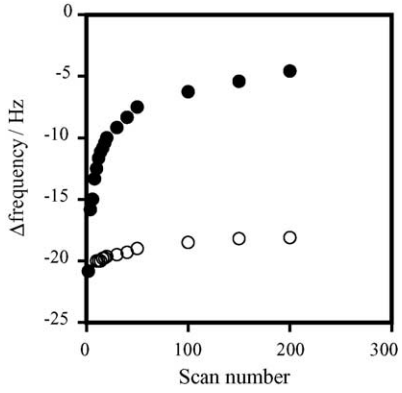

Fig. 3. Quartz crystal microbalance measurements at open circuit vs. the number of scans. Gold quartz crystal modified as in Fig. 1. Frequency changes in the absence of calcium (filled circles), in the presence of 0.1 M calcium (empty circles).

roughly 20 Hz and this value is taken as the starting point of the potential cycling experiment. After every full cycle the new frequency of the crystal is recorded. In the absence of Ca^{2+} the frequency of the mediator modified quartz crystal increases continuously, indicating a gradual mass loss (filled circles). Based on a monolayer surface coverage of 1.3×10^{-10} mol/cm^2 [14] and the molecular weight one can calculate that a complete monolayer should correspond to a mass change of ca. 25 ng.

This means that after 200 cycles approximately 80% of the mediator has desorbed. When calcium ions are present the frequency increase or mass decreases is almost neglectable (empty circles) due to their stabilizing effect.

The specific interaction between mediator and calcium ions can be used to build a well-defined sandwich "catalyst/calcium/catalyst" on the electrode surface. In Fig. 4 a gold electrode was dipped in a 5×10^{-4} M solution of 4-carboxy-2,5,7-trinitro-9-fluorenylidene-malononitrile for 2 min with water, two nitro groups were activated as described before and a CV recorded. The electrode was then dipped for 10 min in a 1 M $CaCl_2$ solution, rinsed 2 min with water and dipped again 10 min in the mediator solution. After rinsing 2 min with water and activating again two nitro groups a new CV was recorded in 0.1 M Tris buffer, pH 8.

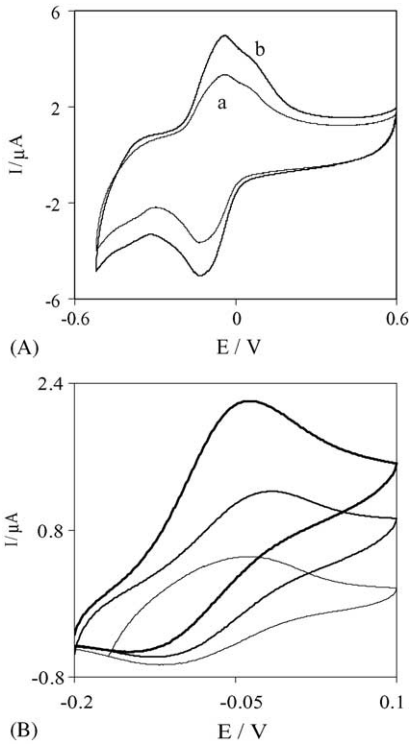

Fig. 4. (A) Adsorption of a mediator bilayer. A gold electrode is dipped in a 5×10^{-4} M solution of 4-carboxy-2,5,7-trinitro-9-fluorenylidene-malononitrile for 10 min. After rinsing for 2 min in water, two nitros groups are activated and then a first cycle in pure supporting electrolyte is performed (a). The electrode is then dipped for 10 min in a 1 M $CaCl_2$ solution, rinsed 2 min in water and dipped again 10 min in the mediator solution. After rinsing 2 min with water and activation of two nitro groups, a new CV (b) is recorded in 0.1 M Tris buffer, pH 8. (B) Catalysis of the electro-oxidation of 0.5 mM of NADH after adsorption of the first mediator monolayer (medium line) and after the adsorption of the second monolayer (thick line). The thin line corresponds to a blank experiment with a bare glassy carbon electrode. Tris buffer, pH 8, 10 mV s^{-1}.

Integration of the oxidation peak of the two voltammograms leads to a surface coverage of 1.4×10^{-10} mol cm^{-2} with respect to the geometric electrode area after the first adsorption step and 3×10^{-10} mol cm^{-2} after the second adsorption. This clearly demonstrates that twice as much catalyst could be adsorbed when building a supramolecular catalyst/Ca^{2+}/catalyst sandwich due to the calcium bridge between the two mediator layers. We then investigated the activity of this modified electrode with respect to the electrooxidation of NADH. As illustrated in Fig. 4(B), upon addition of 0.5 mM NADH, the current reaches 1.1 μA when the electrode is modified with one monolayer of catalyst and 2.2 μA when the electrode is modified with two layers of catalyst. Therefore, it is possible to improve by this simple method the sensitivity by a factor 2 for a given NADH concentration.

Besides the formation of such a mediator double layer the calcium cations can also be used to assemble in a molecular lego like approach a mediator/coenzyme sandwich. In order to check the formation of the adsorbed layers and to elucidate the role of the nitro groups with respect to the complexation, we performed EQCM measurements with a mediator monolayer where all nitro groups were intact (Fig. 5(A)), and a layer where two nitro groups had been activated, that means transformed into hydroxyl amine (Fig. 5(B)). The left column of Fig. 5 shows the frequency change during the successive addition of 40 mM calcium and 1 mM NAD^+ indicated by the arrows, whereas the right column shows the opposite injection sequence. This second experiment allows to check on the one hand, the importance of the electrostatic interaction and on the other hand, whether the observed frequency change is not due to a parasitic effect like a change of viscosity. In the left column electrostatic adsorption of calcium cations and subsequent complexation of NAD^+ is evident from a significant negative frequency shift. In the control experiments (right column) it is obvious that no direct adsorption of coenzyme is possible on the modified electrode surface. This result is not surprising in the sense that the carboxyl groups of the mediator are deprotonated at pH 8 and therefore, the surface is covered with a negatively charged layer. An approach of the negatively charged phosphate groups of NAD^+ to the surface is therefore difficult. However, upon addition of Ca^{2+} to the solution a significant frequency decrease is observed because the cation now serves as a linker between the negatively charged units and allows the coenzyme to adsorb at the surface. More quantitatively one can estimate from these measurements the amount of Ca^{2+} ions and NAD^+.

In the first case with three intact nitro groups rather small frequency changes of 2 Hz for Ca^{2+} and 2 Hz for NAD^+ have been obtained (Fig. 5(A)). When the injection sequence is inverted the global frequency change after the injection of calcium ions corresponds to the sum of the two individual frequency changes (4 Hz, Fig. 5(A), right side). This means

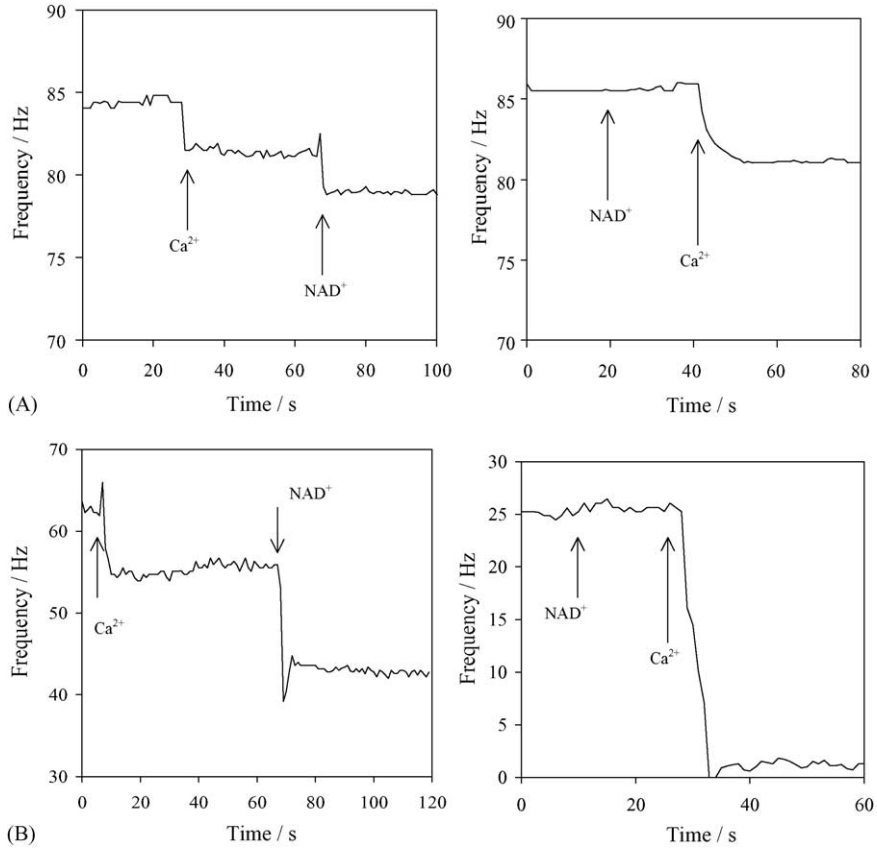

Fig. 5. Quartz crystal microbalance measurements at open circuit vs. time with a mediator modified crystal. The left column shows the successive addition of 40 mM calcium and 1 mM NAD^+ indicated by the arrows, whereas, the right column shows the opposite sequence. (A) Gold quartz crystal modified as in Fig. 1 with all nitro groups intact. (B) Modified gold quartz crystal with two activated nitro groups.

that the final state of the electrode does not depend on the sequence of injection. Evaluating roughly the surface concentration of Ca^{2+} and NAD^+ by taking into account the surface occupied by a hydrated Ca^{2+} ion (7×10^{-15} cm^2) and a NAD^+ molecule (2×10^{-14} cm^2) [16] one has to conclude that in this case a sub-monolayer of both compounds is adsorbed.

However, in the case where two nitro groups are transformed into hydroxylamine the adsorbed amount is more than four times higher and in good agreement with a monolayer coverage. Fig. 5(B) again shows that the sum of the individual frequency changes (8 Hz for Ca^{2+} and 12 Hz for NAD^+ on the left side) fits quite well the overall frequency change when the injection sequence is inverted (right side, 23 Hz). This stronger influence of calcium ions when two hydroxylamine groups are present in the mediator molecule has been previously also demonstrated by NMR experiments [11]. A possible explanation of this effect is that Ca^{2+} not only interacts with the deprotonated carboxyl group as we postulated in earlier publications but also with the –NHOH groups, leading to a dense layer of cations and NAD^+ at the surface.

The final goal of this study being the development of an electrode with a rational surface design leading to an adsorbed mediator/Ca^{2+}/NAD^+/GDH multilayer, we had to add GDH to check the formation of the "molecular lego". Fig. 6 shows the frequency changes of the quartz crystal modified as in Fig. 1 as a function of time when adding sequentially Ca^{2+}, NAD^+ and this time also the enzyme, glucose dehydrogenase. As mentioned above, the electrostatic adsorption of calcium cations and coenzyme are visible by the negative frequency shifts corresponding again to approximately one monolayer compared to theoretical values. The loading of glucose dehy-

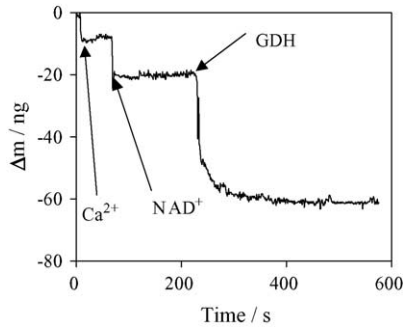

Fig. 6. Quartz crystal microbalance measurements at open circuit vs. time with a crystal modified as in Fig. 1 and activation of two nitro groups. Successive addition of 40 mM calcium cations, 1 mM NAD^+ and 100 U/ml glucose dehydrogenase (from Ref. [4], with permission from the editor).

drogenase is found to be 1.9×10^{-12} mol cm^{-2} and thus, not too far from that calculated for a closed packed monolayer (1.15×10^{-12} mol cm^{-2}), based on the known dimensions of the enzyme (6.67 nm × 12.08 nm × 11.96 nm) [17]. When adding in a control experiment first Ca^{2+} and then the dehydrogenase, or first GDH and then Ca^{2+} no adsorption of the enzyme occurs in either case. Only after addition of NAD^+ one finally ends up again with an increase in mass corresponding to the sum of the individual changes shown in Fig. 6. These experiments demonstrate that every "brick" of the lego is necessary to observe the assembly of the multilayer. All the ingredients have a well-defined structural function in the molecular sandwich and are therefore indispensable for its successful construction.

Using this simple and versatile supramolecular approach we were able to detect in quiescent solution glucose at a sensitivity of 0.2 μA mM^{-1} cm^{-1} and ethanol at a sensitivity of 0.3 μA mM^{-1} cm^{-1}. These values are quite competitive with results known from the literature for other electrode configurations like carbon paste systems. However, intrinsic to its construction principle based on non-covalent interactions, the lifetime of the multilayer in pure supporting electrolyte is very limited, meaning that the components are diffusing back in solution after a few minutes.

In order to circumvent this stability problem a fifth layer, made out of a cationic polyelectrolyte can be added on top of the negatively charged enzyme monolayer. By dipping the electrode for several minutes in an aqueous polylysine solution a layer of the polyelectrolyte is adsorbed by electrostatic interactions (see Scheme 1) and the assembly becomes sufficiently stable to perform rotating disk electrode measurements with the modified electrode.

Fig. 7 shows the electrooxidation of glucose at −50 mV/AgAgCl, using an electrode with (thin line) and without (thick line) the polylysine layer.

As illustrated by the thick line, rotation at 1000 rpm, where the shear stress on the rim of the electrode is 0.1 N m^{-2} [18], the modification layer is mechanically unstable and the supramolecular assembly is stripped immediately by the shear force, leading to no measurable current upon addition of glucose. In the presence of the fifth stabilizing layer, a stable response could be obtained and successive additions

Scheme 1. Representation of the supramolecular structure (catalyst/Ca^{2+}/NAD^+/GDH/polylysyine) at the modified glassy carbon electrode.

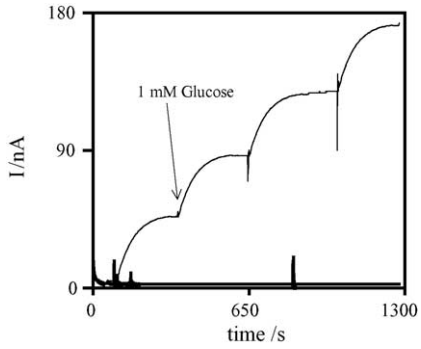

Fig. 7. Detection of glucose in a rotating disk electrode set-up with a glassy carbon electrode modified as in Scheme 1 with (thin line) and without a monolayer of polylysine (thick line). Electrode poised at −50 mV/AgAgCl in a Tris buffer (pH 8), 1000 rpm.

of 1 mM glucose result in a linear increase of the oxidation current (thin line). A sensitivity of 0.67 μA mM^{-1} cm^{-1} was reached, which is close to the values obtained when all the ingredients of the catalytic chain except the mediator are present in solution. The trick we used is that polylysine is adsorbed under slightly acidic conditions (pH 4–5) where the polyelectrolyte is protonated and therefore positively charged and soluble. The measurement itself, however, is performed at pH 8 where the polyelectrolyte is almost completely deprotonated and therefore insoluble. This prevents its back-diffusion into solution and thus, the other ingredients of the catalytic chain are trapped below this last layer, preventing not only their dissociation, but also increasing the sensitivity, the detection limit and the shelf life stability.

4. Conclusion

We could show by QCM measurements that calcium cations are responsible for the adsorption of a NAD^+ coenzyme layer on top of the mediator monolayer. This is not only due to an interaction between Ca^{2+} and the carboxyl group of the mediator but involves also the hydroxylamine substituents. The subsequent adsorption of glucosedehydrogenase due to the natural affinity between enzyme and cofactor leads to a well-defined supramolecular architecture (mediator/Ca^{2+}/NAD^+/GDH) that can be stabilized by adding a last layer of polylysine. The so obtained assembly is stable enough to perform rotating disk electrode measurements, showing a good linear current response with respect to the added glucose concentrations. This simple modification procedure can be easily adapted to different enzymatic systems and constitutes a versatile and inexpensive alternative for the construction of biosensing layers.

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