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Affinity assembled multilayers for new dehydrogenase biosensors

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Abstract

We propose a novel approach, which allows the control of the spatial arrangement of redox mediator, coenzyme and enzyme on the electrode at a molecular level, using essentially electrostatic interactions. The first step consists of adsorbing a monolayer of molecules out of a new family of redox mediators, substituted nitrofluorenones. In a second step, a monolayer of calcium cations is immobilized at the interface. It serves as a bridge between the redox mediator and the subsequently adsorbed coenzyme. The weak interaction between a carboxyl group of the redox mediator and the coenzyme's phosphate groups, revealed by QCM measurements, allows the coenzyme to keep its natural activity in the adsorbed state. In the last step, we use the intrinsic affinity of this monolayer of NAD^+ for dehydrogenases to build up a supramolecular sandwich composed of mediator/Ca^{2+}/NAD^+/dehydrogenase. This simple modification procedure, which might constitute a versatile approach for the low cost assembly of well-defined biosensors surfaces, has been successfully applied to the enzymatic detection of glucose, glutamate and alcohol.

Keywords: Modified electrodes; Nitrofluorenones; Electrocatalysis; Self-assembled monolayers; Biosensors

1. Introduction

There continues to be a great deal of interest in developing new biosensors based on NADH dependent enzymes. The performance of such biosensors depends on the preservation of the activity of the enzymes employed. Many efforts have been performed to fix NADH dependent enzymes at the electrode/electrolyte interface but often severe drawbacks like partial enzyme deactivation are observed [1]. We previously described [2,3] a novel approach to build dehydrogenase biosensors, which allows to control the spatial arrangement of redox mediator, coenzyme and enzymes on the electrode surface at the molecular level, essentially using electrostatic interactions. This procedure follows a layer-by-layer strategy that has been also employed by other groups in a similar context but using different ingredients [4–6]. The steps in the construction of our supramolecular sandwich use a calcium bridge between redox mediator and the NAD^+/NADH phosphate groups as well as the natural affinity of dehydrogenase enzymes for NAD^+ [7]. To better understand these interactions at a molecular level, we propose an investigation by quartz crystal microbalance (QCM) measurements. Then, we will finally apply this supramolecular sandwich to 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 from bacillus megaterium (Sigma) had an activity of 50–150 units per milligram.

Solutions were prepared from ultra pure water that had been passed through a purification train (Milli-Q Plus 185, Millipore).

4-Carboxy-2,5,7-trinitro-9-fluorenone was synthesized as described in the literature [8].

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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 ± 2 °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 (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.

Simultaneous 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 commercialised 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 4-Carboxy-2,5,7-trinitro-9-fluorenone 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 nitro-compound 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 two of the three nitro groups into hydroxylamine by choosing the adequate negative potential during the first scan [9]. The transformation of only two out of the three nitro groups has been shown to be the optimal strategy in terms of catalytic activity [10]. 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 free carboxyl substituent of the surface confined molecules is complexed by dipping the electrode in a solution containing 400 mM $CaCl_2$ for 10 min. After 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 will 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.

2.3.3. 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 voltammogramm, characteristic for a clean gold electrode was obtained.

Once cleaned, the modification procedure as described for glassy carbon was used. [9–11].

3. Results and discussion

The cyclic voltammogram in Fig. 1 shows a gold electrode modified with a monolayer of (4-carboxy-2,5,7-trini-

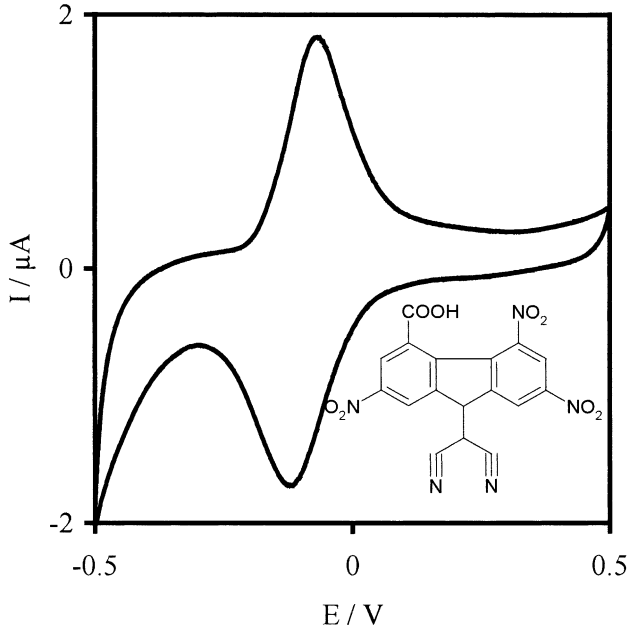

Fig. 1. Cyclic voltammogram of a gold electrode modified with a monolayer of (4-carboxy 2,5,7-trinitro-9-fluorenylidene-malononitrile) (see inset) when two nitro groups are transformed into hydroxylamine [9–11].

tro-9-fluorenylidene-malononitrile) when two of the three nitro groups are transformed into hydroxylamine. The involved NO/NHOH couples at -93 mV are subject to a $2e^-/2\ H^+$ redox process and the obtained monolayer is fairly stable [9,11]. Therefore, it is a good candidate for the catalysis of a two-electron process like the oxidation of NADH. We use the carboxylic group in position 4 to build a well-defined supramolecular sandwich coenzyme/mediator via a Ca^{2+} bridge. To check the formation of the adsorbed layers, we performed QCM measurements. Fig. 2A shows the frequency changes of the gold crystal modified as in Fig. 1 as a function of time when adding sequentially Ca^{2+}, NAD^+ and glucose dehydrogenase. Electrostatic adsorption of calcium cations, coenzyme and enzyme on the layer are shown by the large negative frequency shifts observed by QCM. The adsorption of every component leads to approximately one monolayer compared to theoretical values. The loading of glucose dehydrogenase (GDH) is found to be 1.9×10^{-12} mol cm^{-2} and is close to that calculated for a closed packed monolayer (1.15×10^{-12} mol cm^{-2}), based on the known dimensions of the enzyme ($6.67 \times 12.08 \times 11{,}96$) nm [12]. As can be seen in Fig. 2B, when Ca^{2+} is omitted during the first step, there is no direct adsorption of coenzyme on the electrode surface. Also, there is no unspecific adsorption of enzyme on the mediator modified electrode. 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. This prevents the negatively charged phosphate groups of NAD^+ to approach the surface and furthermore there is also a repulsive electrostatic interaction with the enzyme that globally has a negative surface charge. However, upon addition of Ca^{2+} to the solution in the last step, an important frequency decrease is observed because the cation now serves as a linker between the negatively charged units and attaches the coenzyme and the enzyme to the surface. This frequency change is roughly equal to the sum of the three frequency shifts measured in Fig. 2A, indicating that in the presence of calcium all three components of the multilayer finally undergo an ionic self-assembly, ordering them in the right way. In a control experiment, we were able to show that when adding first Ca^{2+} and then the dehydrogenase no adsorption of the enzyme occurs either. Only after addition of NAD^+ one finally ends up again with an increase in mass. These experiments show that the presence of calcium cations is a necessary but not sufficient condition 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.

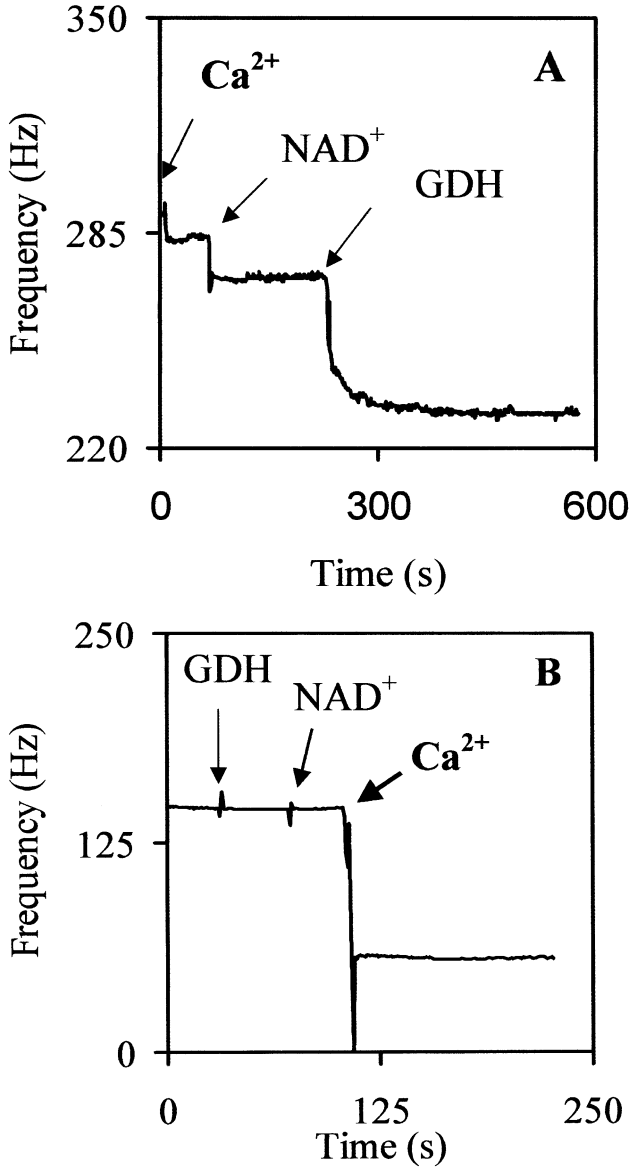

Fig. 2. Quartz crystal microbalance measurements at open circuit versus time. Gold quartz crystal modified as in Fig. 1. (A) Successive addition of 40 mM calcium cations, 1 mM NAD^+ and 100 U/ml glucose dehydrogenase. (B) Successive addition of 1 mM NAD^+, 100 U/ml glucose dehydrogenase and 40 mM calcium cations.

Fig. 3 illustrates the resulting catalytic activity of the four-layer modified electrode. Cycling in pure supporting electrolyte gives the background signal of the redox mediator/Ca^{2+}/NAD^+/GDH assembly (thin line). After addition of 0.5 mM glucose an increase of the oxidation current at the formal potential of the redox mediator is observed (thick line). This indicates that even with only a monolayer of glucose dehydrogenase we are able to detect glucose at a very interesting potential and with a sensitivity of approximately 0.2 μA mM^{-1} cm^{-2}. This value is quite competitive with results known from the literature for other electrode configurations like carbon paste systems [1]. The main intrinsic drawback of the present modification scheme is the limited stability of the assembly. On the one hand when using only non-covalent interactions, it is evident that all components can diffuse back into solution when the electrode is exposed to pure supporting electrolyte. On the other hand, these weak interactions were deliberately chosen in order to leave the enzyme and coenzyme in an environment

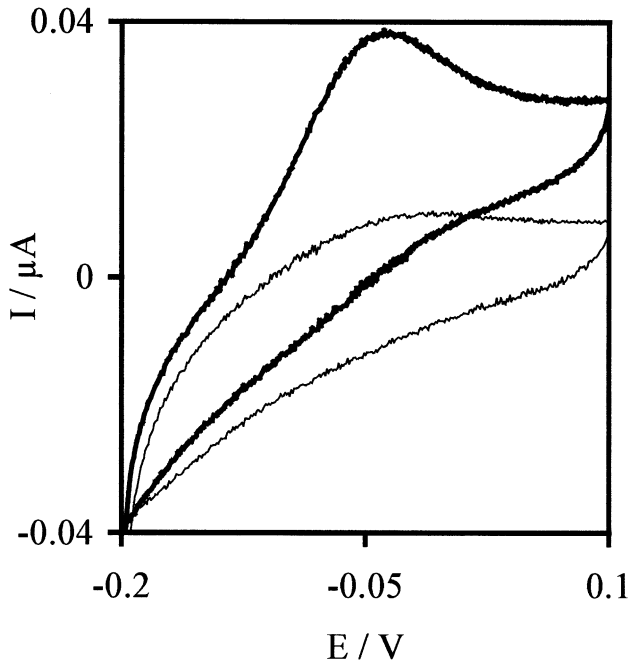

Fig. 3. Detection of glucose with a glassy carbon electrode modified with a (4-carboxy-2,5,7-trinitro-9-fluorenone)/Ca^{2+}/NAD^+/GDH multilayer. The immobilized glucose dehydrogenase is able to convert glucose, resulting in an electrochemical signal. Background current of the modified electrode without glucose (thin line), and with 3.5 mM glucose (thick line).

that is as close as possible to their natural one, so that no or almost no denaturation occurs. In order to circumvent the stability problem, we are testing at the moment electrodes that not only are modified with the mediator/Ca^{2+}/NAD^+/dehydrogenase multilayer but also have a fifth layer of a polyelectrolyte on their surface. This last layer seems to prevent rediffusion of the different ingredients and a stable response could be obtained using rotating disk electrode measurements.

4. Conclusion

Calcium cations induce complexing between the carboxylic group of the redox mediator and the coenzyme's phosphate groups. This interaction, revealed by QCM measurements, allows the coenzyme to keep its natural activity and specificity during the immobilization procedure. Using the natural affinity of this monolayer of NAD^+ for dehydrogenases, we can build a well-defined supramolecular sandwich (mediator/Ca^{2+}/NAD^+/dehydrogenase). This simple modification procedure was successfully applied to different enzymatic systems such as glucose dehydrogenase, glutamate dehydrogenase [2] and alcohol dehydrogenase [3]. It constitutes a versatile approach that, after improvement of the stability by adsorption of a protecting polyelectrolyte layer, might lead to an inexpensive alternative for the construction of well-defined biosensor surfaces.

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