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ORIGINAL PAPER

Multilayered catalytic biosensor self-assembled on cyclodextrin-modified surfaces

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Abstract In this paper we describe the deposition of enzyme layers on cyclodextrin-modified surfaces through self-assembly with adamantane-appended alkaline phosphatase using cyclodextrin-capped gold nanoparticles as supramolecular linkers. The system was studied by surface plasmon resonance and electrochemical methods (cyclic voltammetry, impedance spectroscopy). Up to three enzyme layers were formed on the cyclodextrin coated electrodes and the modified surface was used for the electrochemical detection of heavy metals (Cd^{2+}, Ag^{+}) based on the inhibition of enzymatic activity by these metal cations.

Keywords Cyclodextrin · Adamantane · Alkaline phosphatase · Biosensor

Introduction

Interfacial design and functionalization of transducer surfaces plays an important role in the effective and rapid assembly of biosensor devices. A particularly interesting and recently developed approach to construct organized structures on surfaces makes use of supramolecular receptors such as cyclodextrins (CDs) to construct self-assembled nanoarchitectures on 2D and 3D surfaces [1, 2]. However, self-assembly as a nano(bio)technological tool is still in its infancy and a lot of effort is still required in order to make self-assembly a more valuable and versatile instrument in nanoscience [3].

Cyclodextrins (Fig. 1) are among the most studied hosts employed by supramolecular chemists due to their ability to include hydrophobic guests of different nature [4, 5]. The use of host–guest interactions based on cyclodextrin–adamantane pairs for surface modification is becoming an attractive alternative for biosensor construction [6]. The simplest way for CD-based supramolecular immobilization consists in the interaction of adamantane-biomolecule conjugates with a surface modified with a monolayer of perthiolated βCD [7–9]. This method has been employed to immobilize cytochrome c [7] or xantine oxidase [8] on gold electrodes. The fact that the electron-trasfer and catalytic activities of both proteins are retained after electrode modification indicates that the conformation of the modified biomolecules is not affected. In another example, a gold surface was modified with amino-βCD and a second monolayer of carboxylated dextran containing adamantane moieties was associated in a supramolecular manner through inclusion in the βCD cavities. This surface has been used for the attachment of antibodies or β-lactoglobulin [10, 11]. Therefore, CDs could find applications as supports for immobilizing proteins and antibodies using host/guest interactions. In addition, recycling of modified surfaces could also be possible in devices containing complementary pairs of host/guest molecules (i.e., CD/adamantane) due the reversible nature of the supramolecular interactions.

We have recently reported the supramolecular capture of adamantane-modified enzymes on cyclodextrin polymer modified gold electrodes via a layer-by-layer design for the construction of an amperometric hydrogen peroxide biosensor [9]. Successive enzyme layers were attached using

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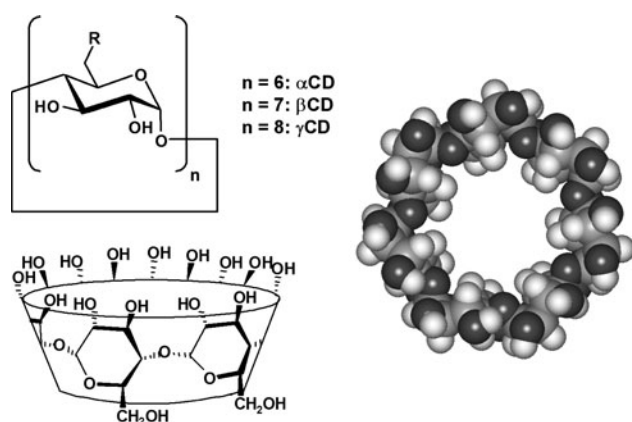

Fig. 1 Structure of cyclodextrins

CD-modified gold nanoparticles (Au-CD) as supramolecular linkers, which due to their spherical shape provide the appropriate directionality to the supramolecular interactions.

In this paper we describe the construction of a multilayered catalytic biosensor based on adamantane-appended alkaline phosphatase self-assembled on cyclodextrin-modified surface (Fig. 2). The system was studied by surface plasmon resonance and electrochemical methods (cyclic voltammetry, impedance spectroscopy) and the modified surface was used for the electrochemical detection of heavy metals (Cd^{2+}, Ag^{+}) based on the inhibition of enzymatic activity by these metal cations.

Materials and methods

Reagents

β-Cyclodextrin was a gift from Roquette (France) and used as received. Adamantane carboxylic acid, potassium hexacyanoferrate, 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC) and Alkaline Phosphatase

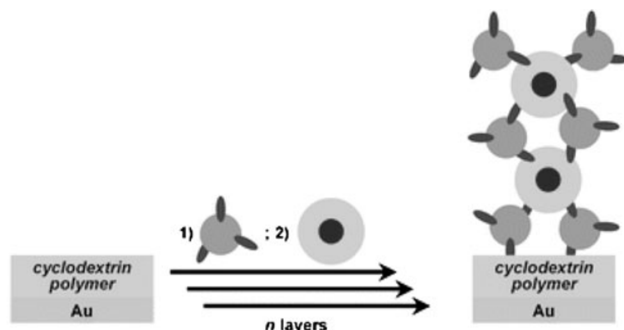

Fig. 2 Multilayered deposition of adamantane-appended alkaline phosphatase self-assembled on cyclodextrin-modified surface: (*1*) ALP–ADA, (*2*) Au-CD

Yellow (pNPP) Liquid Substrate System for ELISA were purchased from Aldrich.

Bovine intestinal alkaline phosphatase (ALP, $M_w =$ 140 kDa, 2.5 DEA units/mg) was purchased from Sigma. One DEA unit is defined as the amount of enzyme that hydrolyzes 1 μmol of p-nitrophenylphosphate to p-nitrophenol in a total reaction volume of 1 mL in 1 min at 37 °C in 1 M diethanolamine-HCl (pH 9.8) with 0.5 mM $MgCl_2$ and 10 mM p-nitrophenylphosphate. [12]. p-Aminophenylphosphate (pAPP) was prepared by catalytic hydrogenation of 4-nitrophenylphosphate as reported by Brewster [13]. Thiolated β-cyclodextrin polymer (CDPSH) and CDPSH-coated gold nanoparticles (Au-CD) with an average diameter of 27 nm were prepared as previously reported [9]. All solutions were prepared with Milli-Q water (Millipore Inc.).

Preparation of adamantane–ALP conjugate (ALP–ADA)

EDC (50 mg) was added to a reaction mixture composed of 10 mg of ALP (2.5 DEA units/mg) and sodium 1-adamantane carboxylate (80 mg) in deoxygenated 50 mM potassium phosphate buffer pH = 6 (5 mL) and the mixture was stirred for 6 h at 4 °C. The solution was then concentrated by centrifugation (8000 rpm for 3 min) using a Microcon 30000 centrifugal filter device (from Milipore) and the filtrated was discarded. The conjugate was collected by inverting the Microcon filter, washed with 500 μL StabilGuard® buffer and kept at 4 °C when not used. This preparation had a protein concentration of 3.2 mg/mL as determined by UV-Vis spectroscopy at 280 nm and a specific activity of 1.5 DEA units/mg after modification.

Surface plasmon resonance (SPR) studies

SPR studies were carried out in a Biacore® 2000 instrument operating a 20 °C. Gold chips from a Biacore SIA kit were firstly cleaned by treatment with ozone using a PSD-UVT cleaning instrument (from Novascan, USA) for 10 min followed by rinsing with ethanol and drying under a filtered Ar stream. The chip was mounted in the Biacore support and a 20 μL/min flow of running buffer (10 mM PBS pH 7.0) was established. After baseline stabilization (~3 h), the flow was lowered to 5 μL/min and 200 μL of CDPSH (5 mg/mL) were injected. The layers of enzyme and nanoparticle were formed by successive injections of ALP–ADA (5 mg/mL) and Au-CD (1 mg/mL). Surface regeneration was carried out using 1 mM sodium adamantanecarboxylate. To calculate the concentration changes on

the sensor surface the equivalence 1 RU = 1 pg/mm^2 was used.

Electrochemical instrumentation

Electrochemical measurements were performed on a PC controlled PGSTAT12 Autolab potentiostat (EcoChemie, The Netherlands) with an built-in frequency response analyzer FRA2 module. Electrochemical impedance measurements were performed using a standard three-electrode configuration (reference electrode, Ag/AgCl(sat.), counter electrode Pt wire) in 1 mM $Fe(CN)_6^{3-/4-}$ in 0.1 M KCl. The impedance spectra were recorded over the frequency range of 10 kHz–0.1 Hz at a bias potential of +0.22 V and AC amplitude of 5 mV. The impedance data were represented in the complex impedance plot (Nyquist plot), and the electrochemical parameters were obtained from simulation using the Autolab FRA software.

Biosensor preparation

The CDPSH modified gold electrode was prepared by immersing a clean gold electrode (A = 0.04 cm^2) into a 5 mg/mL aqueous solution of CDPSH overnight. After rinsing with water, the monolayer was characterized by cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) using 1 mM $Fe(CN)_6^{3-/4-}$ in 0.1 M KCl as an electroactive probe.

The layer-by-layer deposition of ALP–ADA and Au-CD was carried out by overnight incubation of the Au/CDPSH electrode with a 1 mg/mL solution of ALP–ADA in phosphate buffer pH 7.0, followed by washing with buffer and incubation with a 1 mg/mL solution of Au-CD. This process was repeated at least two times. Each enzymatic layer was characterized by CV and EIS using 1 mM $Fe(CN)_6^{3-/4-}$ in 0.1 M KCl as an electroactive probe.

Detection of pAP from ALP catalized pAPP dephosphorylation was carried out using differential pulse voltammetry (DPV) in the interval range of −0.2 to 0.4 V in 10 mM Tris·HCl buffer pH 9.0 containing 1 mM $MgCl_2$. After recording the buffer background signal, a 100 μM aliquot of pAPP in Tris buffer pH 9.0 was added and the DPV response was recorded after 2 min for each formed enzyme layer.

Inhibition studies of ALP activity by metal ions were carried out by recording the DPV response of 100 μM pAPP in phosphate buffer pH 9.0 before (i_0) and after (i_M) the addition of 1–100 μM Cd^{2+} and Ag^+, where i_0 and i_M are the current peak intensities. The percentage of inhibition (%I) was calculated using the equation: $\%I = 100(i_0 - i_M)/i_0$.

Results and discussion

Surface plasmon resonance characterization of multilayer deposition

Surface plasmon resonance (SPR) is an optical technique that enables real-time detection and monitoring of biomolecular events. In this work SPR was used to study the interaction between the thiolated cyclodextrin polymer, adamantane modified ALP and Au-CD. The SPR response for the immobilization of 10 mg/mL polymer on a Biacore Au chip was 570 RU, corresponding to 570 pg/mm^2 and a surface coverage of cyclodextrin polymer of ~3.2 pmol/cm^2, assuming a M_w = 18000.

Successive injections of ALP–ADA and Au-CD on the CDPSH-modified surface provoked a marked increase in the SPR signals, indicating the successful formation of the multilayered structure (Fig. 3). The average increase in surface coverage obtained for the three layers of ALP–ADA was (0.43 ± 0.06) pmol/cm^2, while Au-CD provoked an average coverage increase of (4.2 ± 0.8) pmol/cm^2. ALP is an ovoid shaped protein with overall dimensions 18 nm × 12 nm, which translates into a footprint of

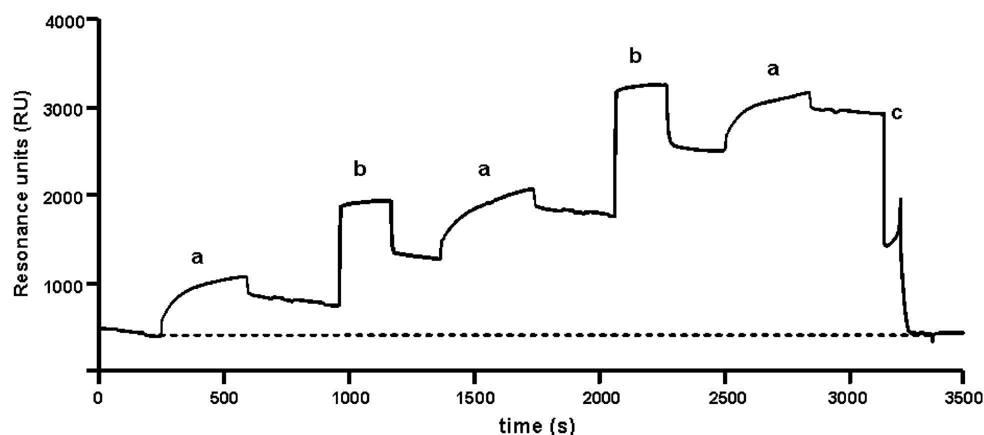

Fig. 3 SPR sensorgram for the sequential deposition of ALP–ADA (*a*) and Au-CD (*b*) on a CDSH-modified surface. Surface regeneration response after the injection of 1 mM sodium adamantane carboxylate (*c*)

$\sim$169 nm^2 [14]. This represents a theoretical surface coverage of 1.1 pmol/cm^2 for a closely packed monolayer of ALP molecules. As can be seen, the experimental coverage observed is lower, which in other leyer-by-layer systems has been ascribed to steric hindrance and random orientation of the deposited molecules [15], although the stepwise deposition process is reproducible as evidenced by the relatively low standard deviation of surface coverage obtained for both, ALP–ADA and Au-CD. If we consider that bovine ALP has 24 possible adamantane attachment points (at lysine units and the amine terminus) [16] and Au-CD have an average of 12 CD units per molecule [9], the observed increase in SPR signal can be attributed to a multipoint host–guest interaction between ALP–ADA and Au-CD. In fact, in a control experiment, injection of 5 mg/mL of native (unmodified) ALP provoked a negligible response variation (ΔRU < 10), corroborating the role of the adamantane units as linking points in the formation of the supramolecular architecture.

The surface can be regenerated by injecting a 1 mM solution of sodium adamantane carboxylate (Fig. 3c), which competes for the CD binding sites thus releasing the modified ALP molecules. This relatively weak adhesion of the functionalized protein is not surprising if we consider that the number of adamantane units in the regenerating agent is 150 times higher than that present in the injected ALP-ADA solution and therefore a strong competition for the CD units can be expected. No decrease in baseline response (corresponding to the CDPSH layer) was observed during this modification–regeneration sequence, indicating that the support CDPSH layer remains stable.

Electrochemical characterization of multilayer deposition

Electrochemical techniques provide a sensitive and accurate method of characterizing interfacial phenomena. This is usually done by studying the variations in the electrochemical behavior exerted by surface modifications on an electroactive probe in solution. Gold electrodes were prepared with increasing numbers of ALP–ADA and CD-Au layers. Figure 4 shows the CV of a $[Fe(CN)_6]^{3-/4-}$ redox probe obtained for the formation of successive layers of ALP–ADA. In CV, an increase of peak-to-peak potential splitting (ΔE_p) associated with a decrease in current intensities are an indication of a blocking effect caused by substance deposition on the electrode surface which inhibits electron transfer from the electroactive probe.

Chemisorption of CDPSH at the gold surface provoked a 15 mV increase in ΔE_p with respect to the bare surface ($\Delta E_p = 80$ mV). In contrast, addition of a first, second and layers of ALP–ADA gave ΔE_p values of 350, 410 and 510 mV, indicating the occurrence of a strong blocking

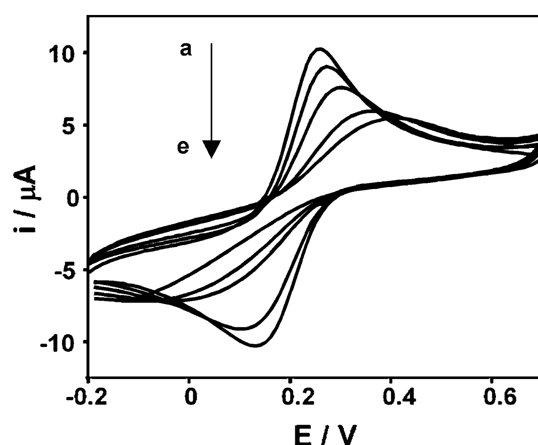

Fig. 4 Cyclic voltammograms (in 1 mM $K_3Fe(CN)_6$ in 0.1 M KCl, scan rate 100 mV/s) obtained at bare gold electrode (*a*), deposition of CDPSH monolayer (*b*) and addition of three successive ALP–ADA layers (*c–e*)

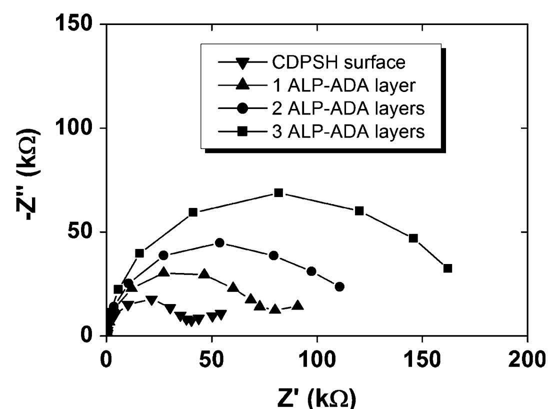

Fig. 5 Complex impedance plots (in 1 mM $K_3Fe(CN)_6$ in 0.1 M KCl) obtained for the sequential deposition of a CDPSH monolayer at gold electrode followed by addition of three successive ALP–ADA layers

effect due to enzyme/nanoparticle deposition, although the current response is not totally suppressed demonstrating that the surface is still permeable to electron transfer.

The electrochemical impedance responses obtained were also consistent with the CV data. In this case, a progressive increase in charge transfer resistance (R_{ct}) of the $[Fe(CN)_6]^{3-/4-}$ redox probe was obtained after each layer deposition with R_{ct} values of 80 kΩ (one layer), 124 kΩ (two layers) and 187 kΩ (three layers) (Fig. 5).

Biosensor fabrication

Once the supramolecular attachment of the different enzyme layers was confirmed, the evaluation of the performance of the biosensor for the detection of pAP was carried out. ALP catalyzes the dephosphorylation of pAPP to pAP. Aminophenols are known to undergo a

two-electron oxidation to quinoneimines and can thus be detected electrochemically. Figure 6 shows the DPV responses of the biosensor modified with an increasing number of enzyme layers to the addition of 100 μM pAPP. As can be seen, the peak intensity increased steadily with the number of enzyme layers. This can be attributed to an increased number of catalytic sites immobilized on t he electrode surface, which provokes a faster catalytic reaction and thus a higher amount of electroactive product can be detected electrochemically. More importantly, in spite of the relative complexity of the formed multilayer structure and the increased R_{ct} values observed in the impedance experiments, the surface is capable of detecting the product of the enzymatic reaction (pAP) even at sub-micromolar levels with a limit of detection of 35 nM, as evidenced by DPV titration experiments in the concentration range 0–100 μM. Negligible performance changes (<7%) were detected for the three layer modified biosensor after 4 weeks of storage at 4 °C in StabilZyme Select® stabilizing buffer.

Certain metals are known to inhibit the activity of ALP through a non-competitive mechanism [17, 18]. Figure 7 shows the amperometric response of the biosensor in absence and in the presence of 10 μM Ag^+ at pH 9. As can be seen, the current response decreases after addition of the metal ion, indicating an inhibition in the activity of immobilized ALP. This property is dependent on the metal concentration, as depicted in Fig. 8 for Ag^+ and Cd^{2+}. It should also be noted that the inhibition effect is observed at a pH value at which both metal cations are expected to exist mainly as hydroxo-complexes. At 100 μM concentration, the biosensor retained only 5 and 12% of its initial response, indicating an effective performance in the studied concentration range. On the other hand, biosensor response was dependant on the number of ALP layers deposited on

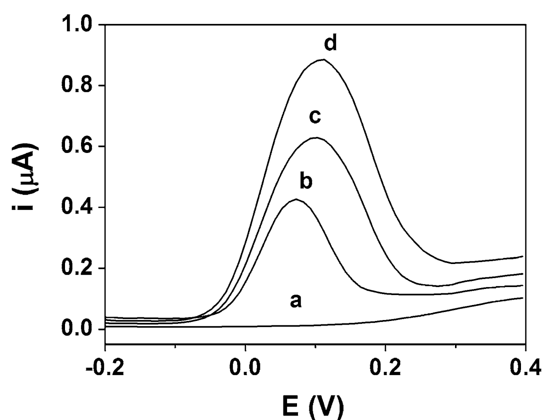

Fig. 6 Differential pulse responses (in 10 mM Tris·HCl buffer pH 9.0 containing 1 mM $MgCl_2$) obtained after CDPSH monolayer deposition (*a*) and addition of three successive ALP–ADA layers (*b*–*d*) in the presence of 100 μM pAPP as substrate

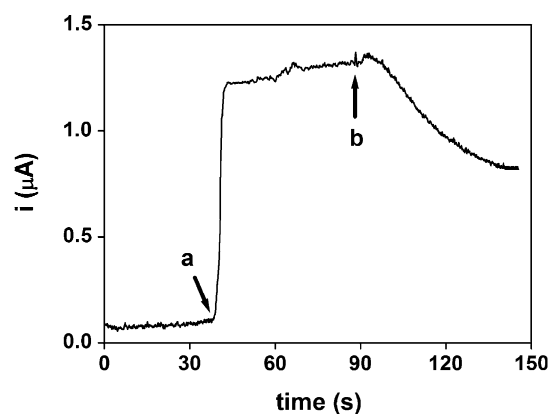

Fig. 7 Amperometric responses of the biosensor after addition of 100 μM pAPP (**a**) and current inhibition in the presence of 10 μM Ag^+ (**b**)

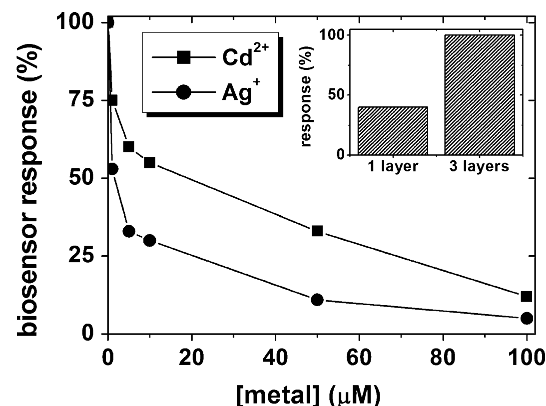

Fig. 8 Variation of biosensor current responses with metal concentration for Cd^{2+} and Ag^+. *Inset*: dependence of biosensor response with the number of enzyme layers

the biosensor surface (Fig. 8, inset). With a simple monolayer the response is ~40% that of the three-layer biosensor, which is explained in terms of the number of enzyme molecules immobilized on the surface. Since heavy metal inhibition to ALP has been associated to non-competitive binding to soft ligands such as thiol groups present in the molecule, the highest inhibition observed for Ag^+ can be explained in terms of its lower hardness degree as compared with Cd^{2+} [19]. This inhibition effect could be used in disposable devices where the irreversibility of this process is not a concern. A detailed study on the analytical performance of the biosensor toward a wide variety of heavy metals is currently underway.

Conclusions

In this paper we have described the construction of a biosensor for heavy metals based on the multilayered

deposition of adamantane-appended alkaline phosphatase self-assembled on cyclodextrin-modified surface. The system was studied by surface plasmon resonance and electrochemical methods and the modified surface was used for the electrochemical detection of heavy metals (Cd^{2+}, Ag^{+}) based on the inhibition of enzymatic activity by these metal cations.

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