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LAYER BY LAYER DEPOSITION OF REDOX POLYMERS/ENZYME ASSEMBLIES ONTO ELECTRODES SURFACES FOR NITRATE ELECTROCHEMICAL SENSING

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Gold electrodes were used as supports for the layer-by-layer deposition of nitrate reductase (NR) and oppositely charged polyviologen derivative (PV) to prepare high-surface area thin films for electroanalysis. Formation of the multilayer films was verified by cyclic voltammetry (CV). The activity of the NR/PV multilayer films was found to be dependent on the amounts of enzyme and mediator in the film, which is determined by the number of enzyme/mediator layers deposited. Films containing NR/PV assemblies showed regularly increasing bioactivity up to eight enzyme/mediator bilayers, with a plateau in activity observed thereafter. This reported method provides a viable approach for the preparation of high-enzyme-content thin films with tailored bioactivity.

INTRODUCTION

During the last two decades, much progress has been made with developing modified electrodes, for instance based on redox and conducting polymers.[1,2] The electrode surface can be deliberately modified by different procedures, such as: adsorption, electroadsorption, electropolymerization, physical coverage and chemical bonding of specific species. An important application of the modified electrodes lies in the design of electrochemical biosensors.[3, 4] A biosensor is a device consisting of a bioactive substance, such as an enzyme, an antibody, a tissue or a microorganism, which can specifically recognize species of interest, in intimate contact with a transducer. The transducer converts the biochemical signal into an electronic signal. In principle, an electrochemical biosensor consists of an electrochemical sensor coated by a thin layer of enzyme. The immobilization of the enzyme on the electrode surface is achieved by different procedures, such as physical entrapment to the electrode surface or to a polymeric support.[5] The successful use of the enzyme electrode depends on the immobilization of the enzyme layer.[6-8] In order to achieve the enzyme immobilization, there has been a growing interest in the entrapment: binding of the enzyme into the negatively charged polyelectrolyte such as Nafion.[9] In this case, the resulting membranes possess high adhesion to the surface of the electrode and low swelling in aqueous media. However, the loose and inactivation of the biocatalyst are the main disadvantages of this immobilization approach.

Layer-by-layer (LbL) modification of electrode surface can be considered an 'on the rise' modification procedure nowadays. The LbL technique of constructing multilayer assemblies by consecutively alternating adsorption of anionic and cationic polyelectrolytes was recently developed.[10-17] This process is based on the electrostatic attraction of oppositely charged species. In an example of layer-by-layer (LBL) assembly, a negatively charged substrate is immersed in a solution of positively charged polymer molecules. The polymer molecules are attracted to the substrate and stay attached even when the substrate is rinsed. The substrate surface now has a positive charge. Then, when the substrate is immersed in a solution of negatively charged polymer molecules, they are

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electrostatically attracted to the surface. Once they are attached, the surface of the growing film is negatively charged again. After the rinsing step, this cycle of immersion steps can be repeated as many times as needed to achieve a film of desired thickness and structure. The LbL self-assembly technique may be an alternative way to create multimaterial composites in a simple fashion.

This paper deals with the elaboration of new electrochemical biosensors based on electrodes modified with spatially organized multilayers. The optimisation of the deposition procedure of nitrate reductase (NR) and oppositely charged polyviologen derivative (PV) to prepare high-surface area thin films for electroanalysis is investigated. The bioactivity of these multilayer films towards nitrate is also investigated.

EXPERIMENTAL

All chemicals: Nitrate reductase (Merck, NR), mercaptoethylsulfonic acid (Merck, MESA), KH_2PO_4 and K_2HPO_4 (Carlo Erba), $NaNO_3$ (Carlo Erba), $CaCl_2$ (Carlo Erba), and H_2SO_4 (Riedel de Häen) were used as received. 2-Pyridin-1-yl-N-[2-(2-pyridin-1-yl-acetylamino)-ethyl]-acetamide (viologen derivative monomer) was synthesized at the Department of Chemistry, University of Turku. De-ionised water (Millipore) was always used to prepare aqueous solutions.

The electrochemical experiments were carried out with an Autolab PGSTAT 12 potentiostat (Ecochemie, Utrecht, The Netherlands) coupled to a PC running GPES software, using a single-compartment, three-electrode cell, at room temperature. A 3-mm diameter Au disk electrode (Metrohm, Herisau, Switzerland) was the working electrode, a saturated mercury-mercuruos electrode was the reference electrode, and a glassy carbon rod (Metrohm) was the auxiliary electrode. All electrode potential values through this paper are expressed versus saturated mercury-mercuros reference electrode. Before each electrochemical test the surface of the working electrode was polished subsequently with 1, 0.3, and 0.05 μm alumina powder to a mirror finish, and rinsed with deionised water. After this mechanical cleaning procedure, the working electrode was sonicated for 7 minutes in an ultrasounds bath. Then the electrode was rinsed with de-ionised water and an electrochemical cleaning procedure, which consists in 200 consecutive scans in 0.5 M H_2SO_4 solution in the potential range from -0.2 to 1.15 V at a scan rate of 100 mV/s, was applied.

Deposition procedure of enzyme/mediator multilayer assemblies

The scheme that has been used for the fabrication of these biosensors is based upon electrostatic attraction between a polycationic redox polymer and a polyanionic enzyme. First, the electrode surface is functionalized with a mercaptoethylsulfonic acid where the thiol end group is chemisorbed to gold surface. The carboxylic acid end group imparts the solution-electrode interface with a net negative charge, depending on the pH experimental working conditions. The polycationic redox polymer (PV) can be afterwards electrostatically bound to the negatively functionalized surface. Subsequent electrostatic binding of anionic enzymes is then possible to the positively charged redox polymer layer. This process is then repeated as desired to deposit multiple layers.

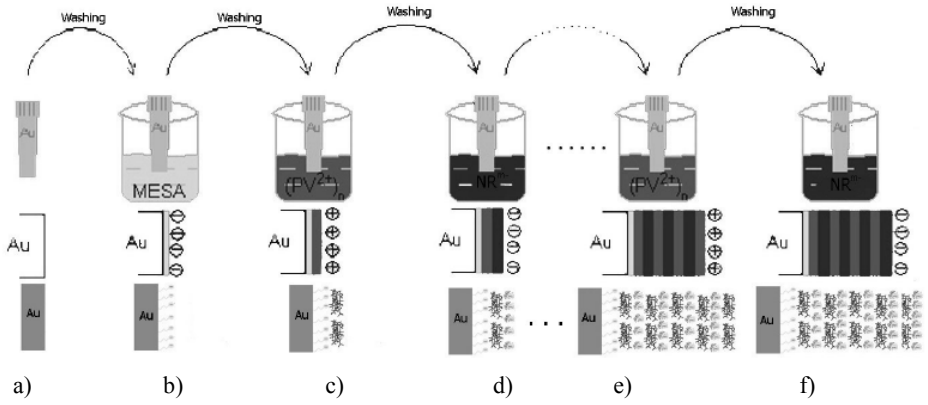
RESULTS AND DISCUSSION

The multilayered enzyme assemblies containing polyviologen derivative mediator were constructed on Au electrodes for the reagentless nitrate biosensing. The multilayer-forming strategy is based on the layer-by-layer procedure, as described in the Experimental section (see Scheme 1). Through alternate deposition of NR and polyviologen derivative, multilayer networks with the desired number of bilayers enzyme/mediator were prepared. In this way, mono- and multilayered NR/PV films on Au electrodes have been deposited.

The resulting modified electrodes were electrochemically characterised in terms of electrochemical redox features, electrocatalytic activity towards nitrate reduction and electrode sensitivity. Figure 1 shows the cyclic voltammograms of both the Au unmodified electrode, Au/MESA/PV and Au/MESA/PV/NR modified electrodes in 0.1M phosphate buffer aqueous solution. The electrode potential was scanned from 0.00 to -0.70 V at a scan rate of $0.1\ V \cdot s^{-1}$.

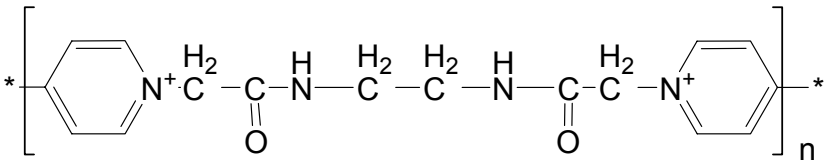
The Au/MESA/PV modified electrode exhibited a pair of redox waves located at -0.40 V, with a small peak separation (ΔE_p) of 38 mV at 100 mV/s, which is typical for the electrochemically active species immobilized on the electrode surface. The full width at half-maximum (ΔE_{fwhh}) of 103 mV was registered, which is close to the ideal value for the immobilized species of 90.6 mV. For the sake of comparison, the voltammogram of the unmodified Au electrode was recorded in the same electrode potential window, when no redox wave is observed. The absence of redox waves for the bare Au electrode ascribes the electrochemical features discussed above to the MESA/PV structure immobilized on Au electrode. Also, the multilayered Au/MESA/PV/NR modified electrode showed surface waves, but the redox peaks were slightly more reduced and separated, which seems to be due to the rather slower charge transfer displayed by the mediator as the successive enzyme layer is deposited. To confirm the

organized formation of a PV/NR structure on the Au surface, the electrochemical characteristics of PV/NR assembly were further investigated. The cyclic voltammograms of Au/MESA/PV/NR modified electrode at different potential sweep rates have been recorded. The registered voltammograms were typical for the surface waves, as evidenced by the small peak separation and the full width at half-maximum of ~100 mV. Also, both the anodic and cathodic peak currents were directly proportional to the potential scan rates in the range of 50-500 mV/s, suggesting facile charge-transfer interactions (see Figure 2).



Steps: a) gold; b) gold surface modified with MESA; c) gold surface modified with MESA and PV; d) gold surface modified with MESA and a bilayer of PV/NR; e) gold surface modified with MESA, three bilayers of PV/NR and another PV layer; f) gold surface modified with MESA and four bilayers of PV/NR.

(A)



(B)

Scheme 1 – (A) Picture of the layer-by-layer procedure for multilayer formation. (B) Structure of the polyviologen derivative.

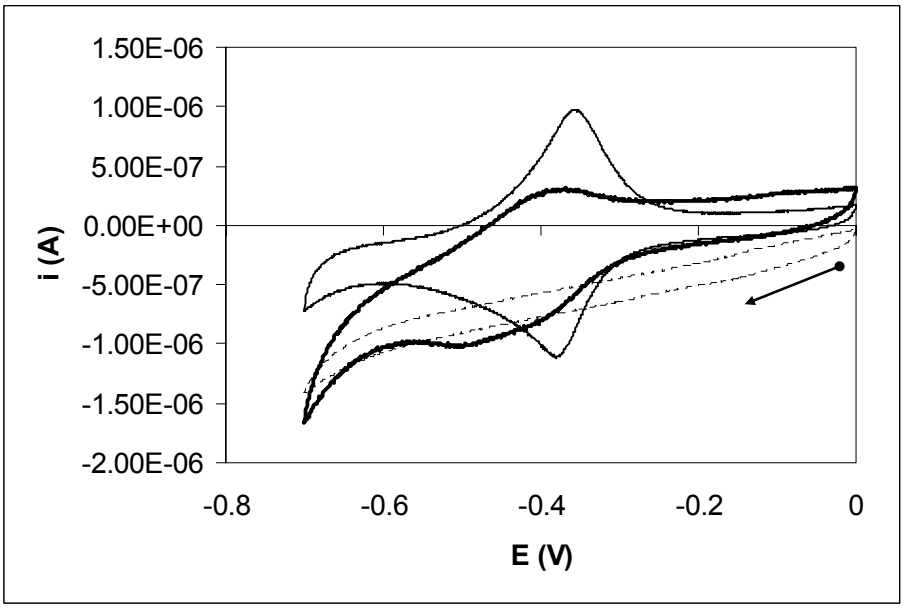


Fig. 1 – Cyclic voltammograms of Au (dotted line), Au/MESA/PV (thin solid line) and Au/MESA/PV/NR (thick solid line) electrodes in 0.1 M phosphate buffer aqueous solution (pH = 7.50). 0.1 V · s^{-1} potential scan rate.

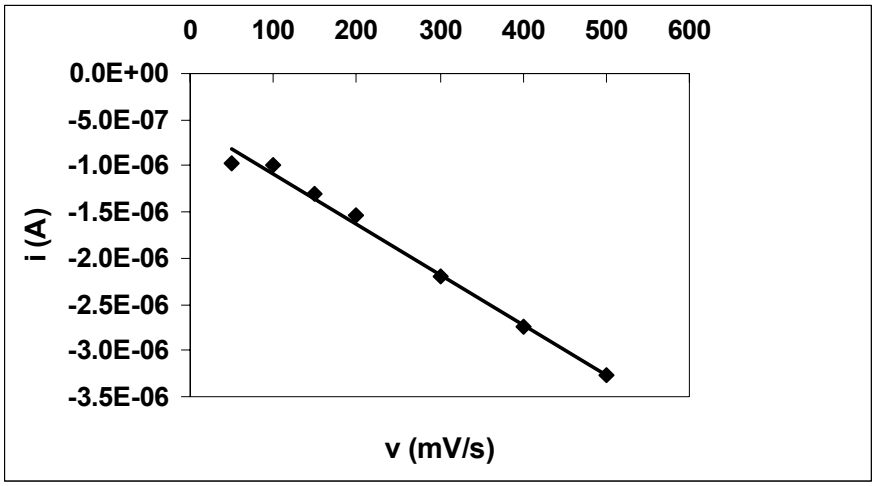


Fig. 2 – Dependence of the cathodic peak current on the potential scan rates for the Au/MESA/PV/NR electrode.

To illustrate the bioelectrocatalytic activity of a monolayered NR/PV assembly, the Au/MESA/PV/NR electrode sensor was used to measure the current-time transients during the reduction of nitrate ion. Figure 3 shows the amperometric response of the Au/MESA/PV/NR electrode to nitrate. Nitrate was injected in the stirred solution resulting in a concentration increment of 0.75 mM and the corresponding current increase was ca. 0.22 μA. As can be seen from the current-time curve, the Au/MESA/PV/NR electrode responded toward the reduction of nitrate, attesting the usefulness of preparing multilayered enzyme/mediator structures on Au electrode surface. However, our attention is focused on the development of new procedures for electrode surface modification. Therefore, our interest is the study of the preparation of multilayered films containing redox mediators and enzymes. The following results are dealing with the electrochemical behaviour of redox mediator/enzyme bilayer pairs.

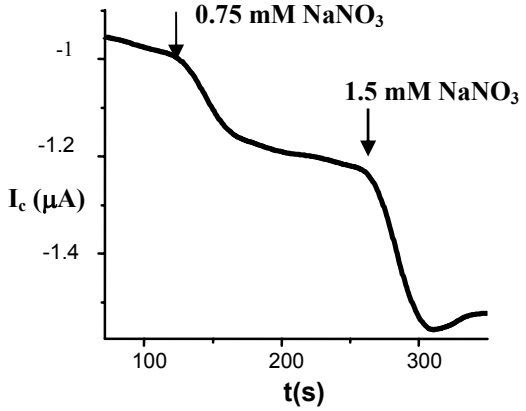


Fig. 3 – Current response to nitrate of a Au/PV/NR electrode at -0.6 V vs. Hg/Hg_2SO_4 in 0.1 M phosphate buffer solution (pH = 7.50). Nitrate was added to the stirred solution in the electrochemical cell at the time points indicated by the arrows.

To confirm the organized formation of a PV/NR multilayered structure on the Au surface and the electrical connectivity between the immobilized polyviologen derivative/enzyme bilayer pairs, the electrochemical characteristics of multilayered assembly were further investigated. The cyclic voltammograms of Au/MESA/$(PV/NR)_9$ modified electrode versus potential sweep rates are shown in Figure 4A. The registered voltammograms were typical for the surface waves, as evidenced by the small peak separation and the full width at half-maximum of ~100 mV. Also, both the anodic and cathodic peak currents were directly proportional to the potential scan rates in the range of 50-500 mV/s, suggesting facile charge-transfer interactions (Figure 4B).

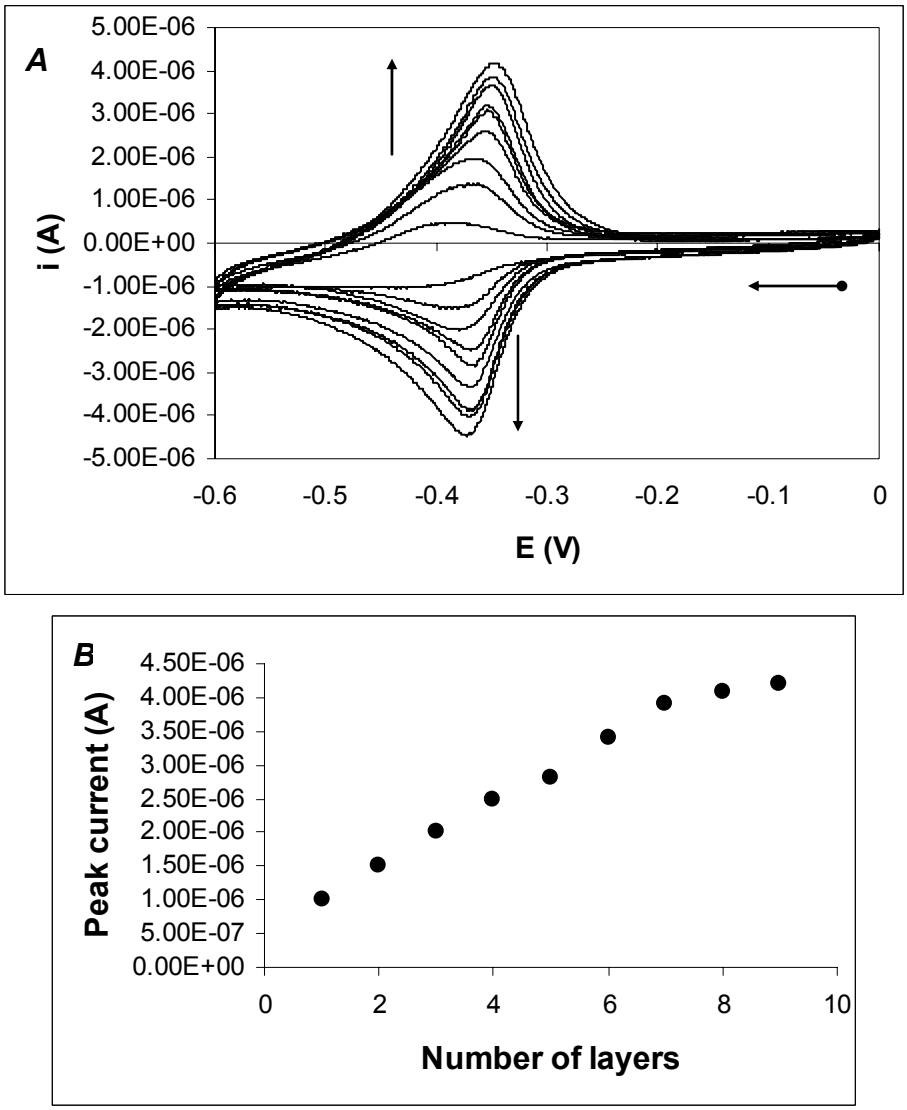


Fig. 4 – Cyclic voltammograms of Au/MESA/$(PV/NR)_9$ electrode in 0.1 M phosphate buffer aqueous solution (pH = 7.50), at 0.1 V · s^{-1} potential scan rate (A), and the dependence of the cathodic peak current on the number of PV/NR pairs layers (B).

To evaluate the analytical performance of the multilayered Au/MESA/$(PV/NR)_9$ electrode, calibration experiments were performed (see Figure 5). The amperometric signals were registered at the working potential of -0.6 V vs. Hg/Hg_2SO_4 in 0.1 M phosphate buffer solution (pH = 7.50) under air at room temperature, both in presence and absence of an activator, that is calcium ion. The cathodic signals were developed in correlation to the nitrate level at low concentration range. Linear ranges of the amperometric signal versus nitrate concentration from 0.75 to 3 mM and 0.75 to 2.25 mM was obtained, in the absence and in the presence of the activator, respectively.

The sensitivity calculated from the linear region of the calibration curve was 0.18 μA mM^{-1} nitrate cm^{-2} in the absence of the activator and increased to 0.23 μA mM^{-1} nitrate cm^{-2} in the presence of the activator, respectively. This sensitivity is similar to the current increase observed for the Au/MESA/PV/NR modified electrode. The deposition of several PV/NR bilayer pairs was intended to produce a modifying layer containing a high redox mediator and enzyme loading. The increase of the number of PV/NR bilayer pairs results in a longer diffusion pathway of the target analyte. This behaviour of the multilayered coating decreases the analytical performances of the biosensor, when compared with other methods for nitrate determination reported in the literature. A large number of methods for the quantification of nitrate have been described in the literature, and most of them are based on techniques such as spectrophotometry, chromatography or electrochemistry. However, the majority of these

procedures involve the stoichiometric conversion of nitrate to nitrite by using various chemical reductors, such as Cu/Cd. Electrochemical methods and the use of nitrate reductase allow the direct quantification of nitrate. For instance, a nitrate-selective coated-wire electrode based on a tetramethyl cyclotetra-decanato-nickel(II) complex exhibits a linear range of 1.0×10^{-5}–1.0 M for nitrate.[18] Low detection limit ($S/N = 3$) value as 1.8 μM and linear calibration curve in the nitrate concentration range from 5 to 100 μM have been also obtained by using nitrate reductase in a flow system.[19] The analytical performances of the Au/MESA/$(PV/NR)_9$ biosensor are not superior to the methods reported in the literature. However, the ability to control the multilayered coating onto the electrode surface should be useful, especially for the electrode containing multiple enzymes with significantly different activities.

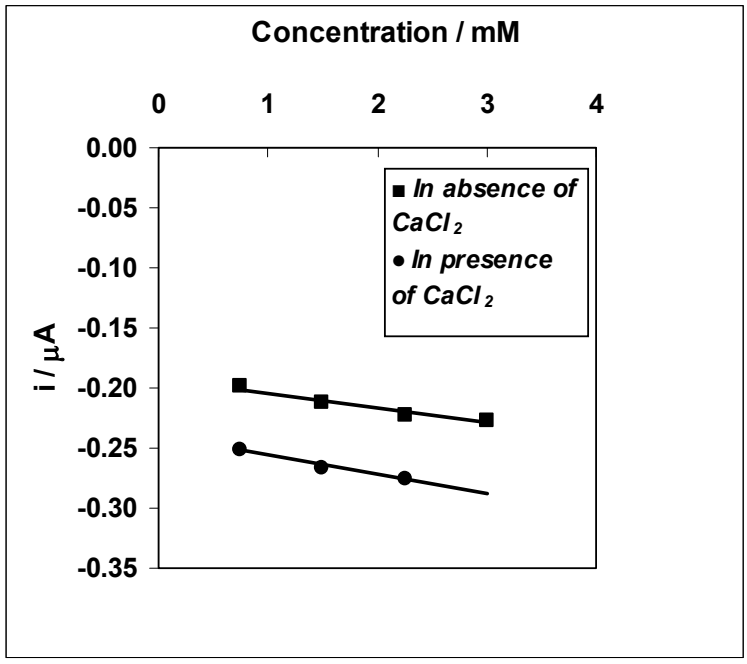


Fig. 5 – Calibration curves for Au/$(PV/NR)_9$ electrode as a function of nitrate concentration in the absence (filled squares) and the presence (filled circles) of $CaCl_2$ at -0.6 V vs. Hg/Hg_2SO_4 in 0.1 M phosphate buffer solution (pH = 7.50).

CONCLUSIONS

In this work, the layer-by-layer deposition of mediator/enzyme bilayer pairs onto electrode surface was investigated. Films containing polyviologen derivative/nitrat reductase assemblies showed regularly increasing bioactivity up to eight enzyme/mediator bilayers, with a plateau in activity observed thereafter. The multilayered electrode showed bioelectrocatalytic activity toward the reduction of nitrate. Linear ranges of the amperometric signal versus nitrate concentration from 0.75 to 3 mM and 0.75 to 2.25 mM was obtained, in the absence and in the presence of the activator, respectively. These results prove the potential of the layer-by-layer deposition procedure to prepare multilayered assemblies onto electrode surfaces for electrochemical sensing.

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