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ORIGINAL PAPER

Electroenzymatic reactions with sorbitol dehydrogenase on gold electrodes

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Abstract Sorbitol dehydrogenase (SDH) originating from recombinant *Escherichia coli* cells is immobilized on gold electrodes. First of all, (4-carboxy-2,5,7-trinitrofluorenyliden)malon-nitrile (CTFM) is adsorbed on the surface as mediator. In a second step, the cofactor β-nicotinamide adenine dinucleotide (NAD^+) is immobilized on the gold electrode. Due to the formation of a complex between the mediator and the cofactor, the electron transfer rate can be enhanced by adding calcium ions to the buffer. The immobilization of NAD^+ and SDH on the surface has been achieved by cross-linking with the glutaraldehyde/bovine serum albumin system. The successful biofunctionalization is monitored by cyclic voltammetry.

Keywords Biofunctionalized electrodes · Electroenzymatics · NAD^+/NADH · Sorbitol dehydrogenase · Glutaraldehyde

Introduction

Electroenzymatic reactions using electrodes biofunctionalized with enzymes are important for analytical and, possibly, also for preparative applications. If β-nicotinamide adenine dinucleotide (NAD^+)-dependent dehydrogenases are used in preparative substrate oxidations, the reduced cofactor has to be continuously re-oxidized. In contrast to well-established nicotinamide adenine dinucleotide hydrogenated (NADH) regeneration systems for biotechnical reduction processes with formate dehydrogenase, the enzymatic regeneration of NAD^+ for biotechnical oxidation process remains expensive and less effective. Several systems have been described using a combination of bacterial NAD(P)H oxidases either with catalases or with dehydrogenases like pyruvate-lactate dehydrogenase, α-ketoglutarate-L-glutamate dehydrogenase or acetaldehyde-ethanol dehydrogenase [1, 2]. Considering the disadvantages of these systems, an electrochemical regeneration of NAD^+ could improve the biotechnical production of enantiomerically pure hydroxyketones as synthons. Furthermore, the electroenzymatic device would benefit from bonding of enzyme and cofactor to the surface of the electrode, which would considerably simplify all subsequent separation steps since only educts and products remain in the reaction solution. Possible applications might be the regioselective oxidation of polyfunctional substrates with dehydrogenases or the kinetic resolution of racemic alcohols [3]. We exemplarily used sorbitol dehydrogenase (SDH, EC 1.1.1.14) that catalyses the oxidation of D-sorbitol in the presence of NAD^+ to fructose under formation of NADH [4]. The latter can be electrochemically re-oxidized. Electrochemistry, i.e., cyclic voltammetry, also allows monitoring the whole process. SDH was used as a model enzyme for typical bacterial short-chain dehydrogenases. The dimeric protein is available as a recombinant enzyme in bulk amounts [4], and its crystal structure has been determined [5], which enables targeted modifications of the biocatalyst. The structural data allocate the NAD^+-binding site close to the surface of the protein [5]. Therefore, it is

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conceivable that the enzyme can bind to a mediator-cofactor complex.

In the last years, various procedures for electrode biofunctionalization have been published [6–16]. Most of them use thiol-modified gold electrodes for the immobilization of enzymes [6, 7] and redox mediators [9–14]. The mediator is necessary for the electron transfer from the enzyme to the electrode surface in case of redox-active biomolecules without internal electron transfer pass way, such that the electron transfer does not directly take place between electrode and enzyme. The mediator can either be present in solution or adsorbed on the surface. The mediator used in this work [(4-carboxy-2,5,7-trinitrofluorenyliden) malon-nitrile (CTFM); see Fig. 1] is a fluorenone derivative with high stability and three nitro groups that can be activated separately by cyclic voltammetry [9]. It is covalently linked to the surface of the electrode via cyano groups, allowing the free carboxylic group of the mediator to form in the presence of Ca^{2+} ions, a complex with the two phosphate groups of the cofactor NAD^+ (Fig. 1) [9]. In this way, only the mediator is covalently linked to the electrode surface, whereas the enzyme–cofactor complex is kept in position only by weak interactions: electrostatic interactions by complexation with bridging calcium and hydrogen bonds between enzyme and cofactor.

The complete electroenzymatic chain is shown schematically in Fig. 2: The redox enzyme (SDH) oxidizes the substrate (D-sorbitol) regioselectively to form the product (fructose) concomitantly with the formation of NADH. The two transferred electrons are taken up by the electrode-linked mediator; thus, NAD^+ is recycled and available for the next oxidation step. In most cases, the cofactor (NAD^+/NADH) is used in solution that, however, requires high over-voltages for its detection [17]. Also unfavourable is, in many cases, the direct immobilization of the enzyme on the electrode surface, as it may result in enzyme denaturation with concurrent loss of activity. Furthermore, since the bonding of the enzyme to an electrode surface takes place at arbitrary positions, it may often lead to a blocking of the active centre if it is oriented to the surface. Therefore, the surface biomodification, shown in Fig. 1 and described in the present work, turns out to be more suited to keep the enzyme accessible to the substrate.

Experimental

Materials

The mediator CTFM was synthesized as described in the literature [18, 19].

The following chemicals were used as received: gold electrodes (NiCr 80/20/Au, A.C.M., France), calcium chloride (Merck), NAD^+ (≥95%, Fluka), NADH (≥98%, Roth), SDH (20 U/ml, Stein et al. [4]), glutaraldehyde (GA, 25 wt.%, Aldrich), bovine serum albumin (BSA, ≥98%, Fluka), D-sorbitol (p.a., Acros Organics), Bis/Tris buffer, pH 8.0, 0.1 M [prepared using Bis/Tris (≥98%, Roth) and HNO_3 to adjust the pH].

The first step is the cleaning of the gold electrodes by cycling in 0.5 M H_2SO_4 for several times. Then, the electrodes are dipped in a solution of 1.2 mM CTFM in 0.1 M Bis/Tris buffer (pH 8.0) for 1 day. The electrodes are rinsed with buffer to remove the excess of the mediator and electrocycled in 0.1 M Bis/Tris buffer (pH 8.0) for the activation of two nitro groups at a potential between −500 and +300 mV (vs Ag/AgCl), 100 mV/s.

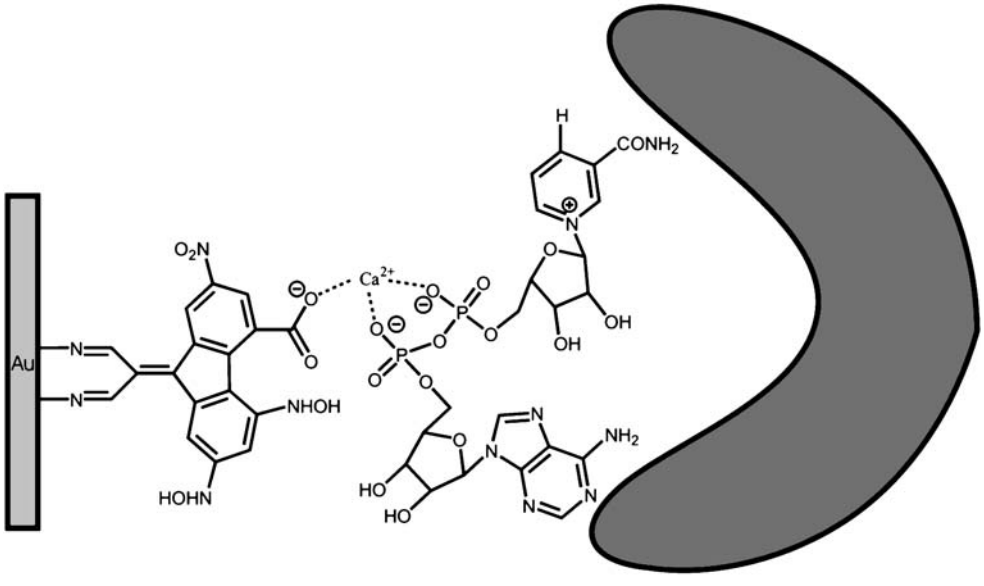

Fig. 1 Principle of immobilization: covalent bonding of the mediator CTFM to the electrode surface, complexation of CTFM and NAD^+ by a Ca^{2+} bridge and bonding between cofactor and enzyme due to their natural affinity

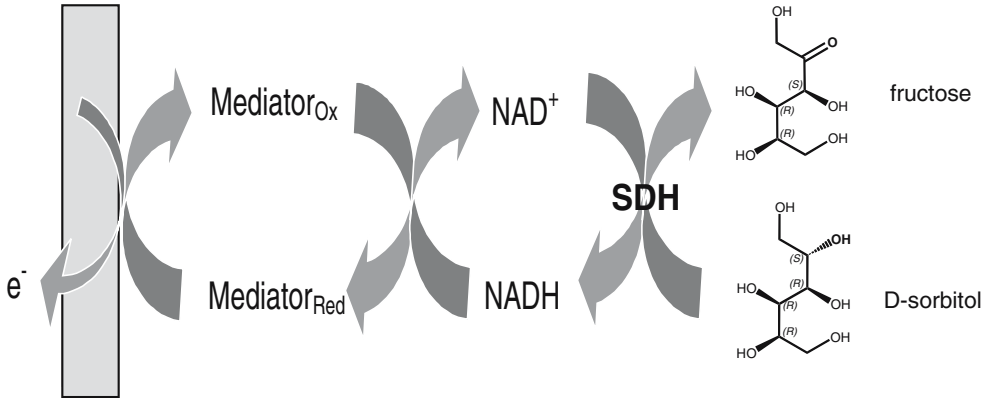

Fig. 2 Scheme of a mediated bioelectrocatalysis: SDH catalyses the oxidation of D-sorbitol to fructose in the presence of NAD^+. To facilitate the electron transfer to the electrode, in addition, a redox mediator is needed

For the immobilization of the cofactor, the mediator-modified electrode is dipped in a solution of 3 mM NADH and 50 mM $CaCl_2$ for 2–3 h. Then, the electrode is rinsed with buffer and assembled into the electrochemical cell. The electrochemical oxidation of the cofactor is determined by cyclic voltammetry in a potential window of −100 to +300 mV (vs Ag/AgCl) with a scan rate of 10 mV/s.

The calcium/cofactor/SDH system was immobilized in the following way: an aliquot of 10 µl from a solution of 120 µl buffer with 3 mM NAD^+ and 50 mM $CaCl_2$, 240 µl SDH, 90 µl 10% BSA and 27 µl 2.5% GA is dropped on the electrode surface covered with the activated mediator and dried at room temperature for several hours. The electrode is rinsed with buffer to remove the residues of GA and BSA, cycled in 0.1 M Bis/Tris buffer at first and then in 10 mM D-sorbitol.

Methods

Escherichia coli BL21(DE3) harboring pASFG3 is grown in 8 l of medium at 37 °C in a 10-l bioreactor (Biostat V; B. Braun Biotech, Melsungen, Germany) with agitation (500 rpm) and aeration (1.6 l/min). The medium contained the following (per liter): 2.5 g of glucose, 17 g of tryptone, 3 g of soybean peptone, 5 g of NaCl and 2.5 g of K_2HPO_4 (pH 7.0). SDH production is initiated by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM when the absorbance at 600 nm is reached a value of about 1.0. After 25 h of growth (22 h after addition of IPTG), cells are harvested by centrifugation for 15 min at 10,000×*g* and 4 °C. SDH purification and activity tests are executed as described previously [4].

Cyclic voltammetry measurements are made with a potentiostat and a cell stand C3 (CV 50-W; Bioanalytical Systems, Indiana, USA) using a platinum wire as counter electrode and Ag/AgCl (1 M KCl) as reference electrode.

The mediator is cycled at potentials between −500 and +300 mV for the activation, starting at +300 mV. The electroenzymatic measurements, on the other hand, are started at the negative potential.

Results and discussion

The expression of SDH in recombinant *E. coli* cells is initiated after 3 h by addition of IPTG. After 25 h of cultivation, 65 g of cells (wet weight) are harvested. The cell-free extract exhibited 28,000 U/l culture, which corresponds to a total yield of 230,000 U from the bioreactor. After purification, a yield of 51% is obtained with a specific SDH activity of 155 U/mg. These values are comparable with earlier data received from cultures grown in smaller volumes [4], demonstrating a successful upgrade of the enzyme production by as much as a factor of 10. The biochemical properties of the recombinant SDH are summarized in Table 1.

Electrode/mediator Figure 3a shows the activation of one nitro group of the immobilized mediator to hydroxylamine in 0.1 M Bis/Tris buffer with a scan rate of 100 mV/s. The cyclic voltammogram for the activation of two nitro groups is shown in Fig. 3b and the conversion of all nitro groups in Fig. 3c. The first cycle shows the activation of the mediator by reduction of the corresponding nitro groups to hydrox-

Table 1 Properties of recombinant SDH from *Rhodobacter sphaeroides* Si4

Properties	
M_r subunit	27,012 Da
Amino acids	256
Active enzyme	Dimer
Crystallization complex	Tetramer
Enzyme family	Short chain DH
Cofactor	NAD^+
NAD^+ binding motif	$G_{12}SARG_{16}IG_{18}$
Metal ion requirement	None

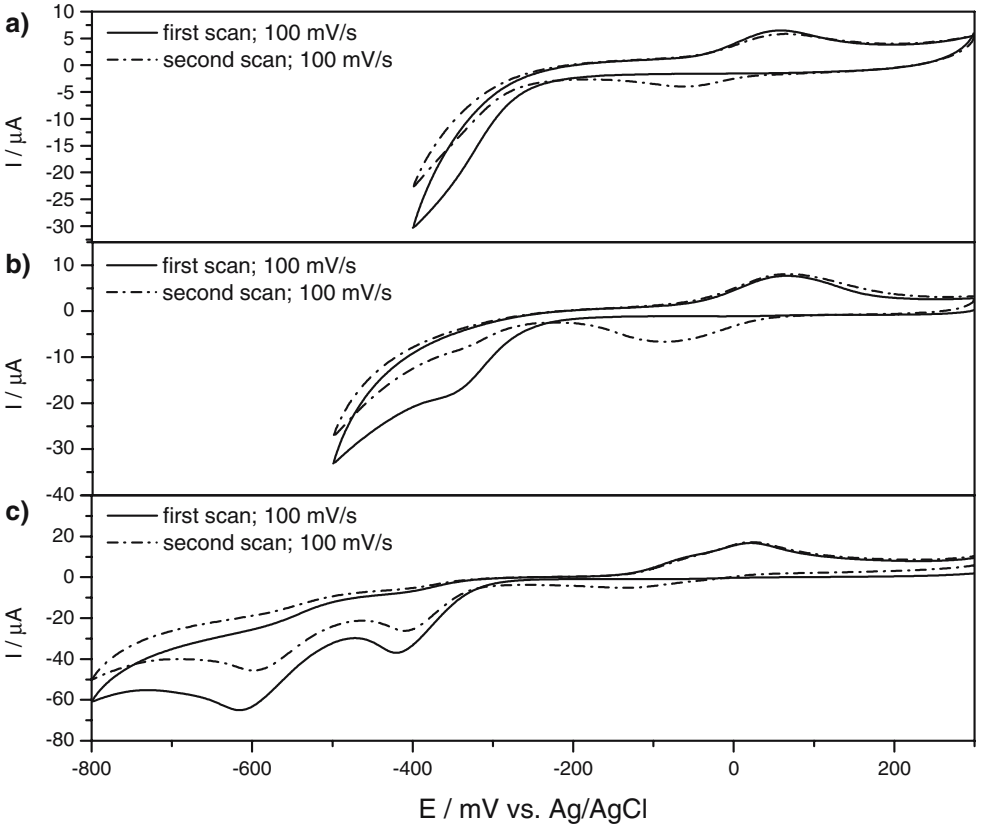

Fig. 3 Activation of the immobilized mediator in 0.1 M Bis/Tris buffer (pH 8.0) at 100 mV/s as observed by cyclic voltammetry. **a** Only one nitro group is transformed into hydroxylamine at potentials between −400 and +300 mV, **b** two of the three nitro groups are reduced between −500 and +300 mV, **c** conversion of all three nitro groups at potentials between −800 and +300 mV

ylamine in all cyclic voltammograms. In the second cycle, we received the reversible redox pair hydroxylamine/nitroso. We observed a displacement of the reduction peak from −57 to −125 mV in dependence on the number of activated nitro groups. The more nitro groups are activated, the more negative is the reduction peak. Two of the three nitro groups are always activated in the following electroenzymatic reactions.

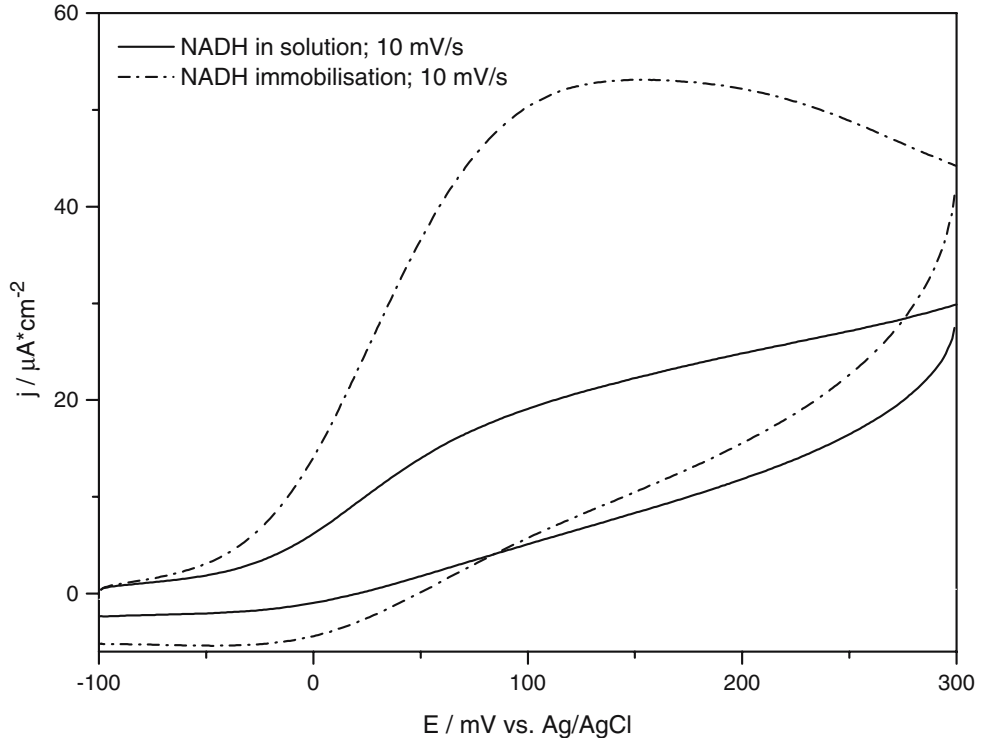

Fig. 4 Comparison of the efficiency of NADH in solution and in immobilized form in 0.1 M Bis/Tris buffer with Ca^{2+} (pH 8.0); 10 mV/s, by using cyclic voltammetry

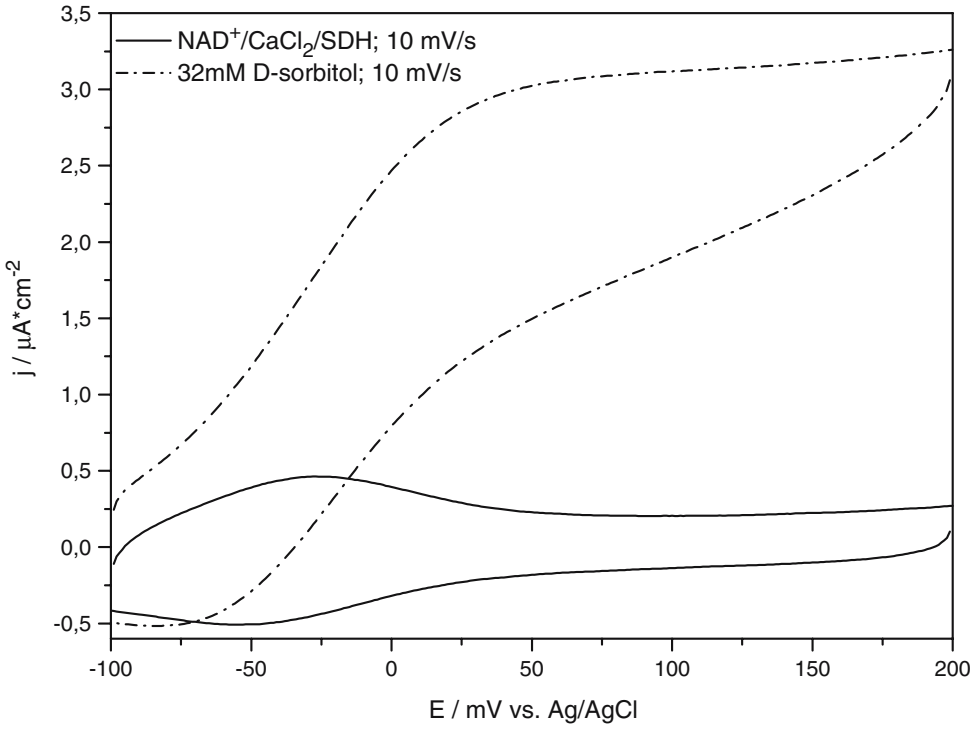

Fig. 5 Electroenzymatic CV measurement (10 mV/s) using a gold electrode coated with the mediator CTFM and a solution containing calcium/cofactor and SDH at 0.1 M Bis/Tris buffer and Ca^{2+} (pH 8.0) without substrate (*thick line*), with 32 mM D-sorbitol as substrate (*dotted line*)

Electrode/mediator/cofactor The regeneration of the cofactor at the electrode coated with the mediator proceeds according to:

$$\begin{aligned} \text{Med} - \text{NO} + \text{NADH} + \text{H}^+ &\rightarrow \text{Med} - \text{NHOH} + \text{NAD}^+ 1 \\ \text{Med} - \text{NHOH} &\rightarrow \text{Med} - \text{NO} + 2\text{H}^+ + 2\text{e}^- \end{aligned} \quad (1)$$

Figure 4 shows cyclovoltammograms of NADH in solution compared to NADH immobilized by means of Ca^{2+} in 0.1 M Bis/Tris buffer at a scan rate of 10 mV/s. The experiment in solution shows an oxidation of NADH to NAD^+ at 90 mV. The immobilization of the cofactor causes a shift of the oxidation peak to a more positive potential at 120 mV due to the formation of a chelate complex between calcium and cofactor (Fig. 2). The measurement of NADH

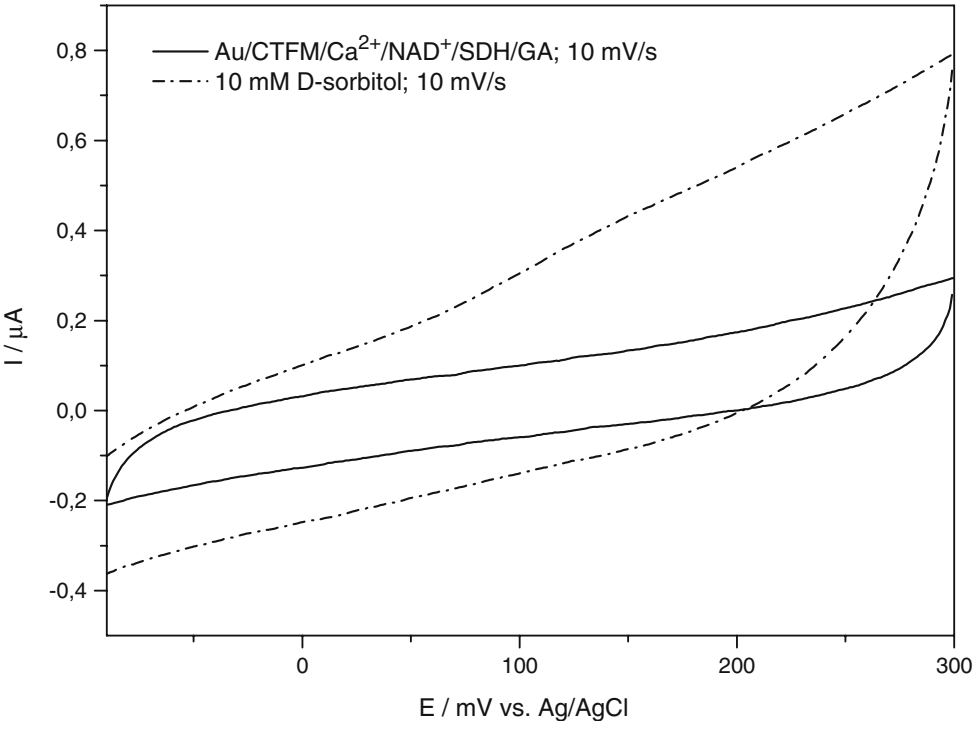

Fig. 6 CV of the immobilized system CTFM/Ca/NAD^+/SDH/BSA/GA at 0.1 M Bis/Tris buffer (pH 8.0), 10 mV/s without substrate (*thick line*), with 10 mM D-sorbitol as substrate (*dotted line*)

without immobilized mediator does not show any oxidation in the applied potential range [17]. In addition, the catalytic current is enhanced compared to the solution. These results are evidence for the adsorption of NADH on the electrode surface.

Electrode/mediator/cofactor/enzyme in solution Figure 5 shows the results of the CV analyses with SDH as enzyme and D-sorbitol as substrate at a scan rate of 10 mV/s in 0.1 M Bis/Tris buffer. Under these conditions, the substrate is regioselectively oxidized to fructose, with simultaneous reduction of the cofactor. The electrochemical re-oxidation of NADH could be determined at a potential of 120 mV as shown in Fig. 5 (dotted line). The current in Fig. 4 is much higher compared to that in Fig. 5 due to the lower concentration of NADH at the beginning of the measurement, which is produced during the experiment. The reference measurements without NAD^+/SDH did not show any unspecific oxidation of the substrate in the applied potential range. Also, the measurement with mediator and NAD^+ does not show any unspecific oxidation. These results prove the successful implementation of the catalytic reaction.

Electrode/mediator/cofactor/enzyme immobilized on the surface The problem in the immobilization of calcium, cofactor and enzyme is the stabilization and the long-term adhesion of the single ingredients on the surface since these interactions are not covalent. So, we have stabilized the gold electrodes coated with a mediator/calcium/cofactor/enzyme system by cross-linking using GA and BSA in analogy to [15, 16]. Figure 6 shows the comparison of a correspondingly modified electrode (1) in buffer only and (2) in a solution of 10 mM substrate. In the first case, a reaction could not be detected due to the absence of a substrate. Hence, the re-oxidation of the cofactor does not take place. After addition of substrate, however, a re-oxidation can be observed with low conversion rates. In this way, the proof-of-concept has been achieved; the optimization of the procedure is in progress now. One reason for the comparatively small conversion rates is the small amount of enzyme we used for the modification. Furthermore, the cross-linking with 2.5% GA could lead to a layer that is too thick, thus impeding the diffusion of the substrate to the active centre of the enzyme through the network of GA and BSA. Also, the immobilization of enzymes with GA could affect the activity substantially. Therefore, the optimal concentrations of GA and BSA have yet to be determined to ensure a sufficient attachment to the electrode and simultaneously a satisfactory activity. In addition, the concentration of NAD^+ has to be varied in order to get better electrochemical signals. Another possibility would be the immobilization in two steps: the cofactor would be immobilized on the mediator-modified electrode first, and subsequently the enzyme/GA/BSA system.

In summary, we have established a complete electroenzymatic chain on a gold electrode involving CTFM as mediator, Ca^{2+} as bridging complex former, NAD^+ as cofactor and SDH as model enzyme. This system allows to regioselectively oxidize D-sorbitol to fructose with simultaneous electrochemical regeneration of the cofactor. Studies on the influence of the restricted mobility on the activity of the different species are in progress now.

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